Formulation of an enzyme cocktail, HoloMix, using cellulolytic and xylanolytic enzyme core-sets for effective degradation of various pre-treated hardwoods

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By

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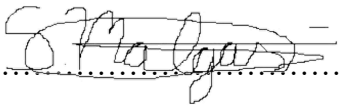
Plagiarism declaration

I, Samkelo Malgas, declare that this thesis is my own, original and unaided work. It is being submitted for the degree of Doctor of Philosophy at Rhodes University. It has not been submitted before, for any degree or examination, at any other university.

Signature 

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Abstract

Currently, there is a growing interest in utilising hardwoods as feedstocks for bioethanol production due to the vast advantages they have over other feedstocks for fermentable sugar production. In this study, two selected hardwoods, *Acacia* and *Populus* spp., were subjected to two pre-treatment processes (Sodium chlorite delignification and Steam explosion) and compared with respect to how these pre-treatments affect their enzymatic saccharification. Hardwoods were selected for this study, because hardwoods are easier to delignify when compared to softwoods, and therefore their polysaccharides are more easily accessible by enzymes for the purpose of producing fermentable sugars. Currently available commercial enzyme mixtures have been developed for optimal hydrolysis of acid-pre-treated corn stover and are therefore not optimal for saccharification of pre-treated hardwoods. In this work, we attempted the empirical design of a hardwood specific enzyme cocktail, HoloMix. Firstly, a cellulolytic core-set, CelMix (in a ratio of Egl 68%: Cel7A 17%: Cel6A 6%: Bgl1 9%), for the optimal release of glucose, and a xylanolytic core-set, XynMix (in a ratio of Xyn2A 60%: XT6 20%: AguA 11%: SXA 9%), for the optimal release of xylose, were formulated using an empirical enzyme ratio approach after biochemically characterising these enzymes. As it is well–known that biomass pre-treatment may result in the generation of compounds that hamper enzymatic hydrolysis and microbial fermentation, the effects of these compounds on CelMix and XynMix were evaluated. Using the optimised CelMix and XynMix cocktails, a HoloMix cocktail was established for optimal reducing sugar, glucose and xylose release from the various pre-treated hardwoods. For delignified biomass, the optimized HoloMix consisted of CelMix to XynMix at 75% to 25% protein loading, while for the untreated and steam exploded biomass the HoloMix consisted of CelMix to XynMix at 93.75% to 6.25% protein loading. Sugar release by the HoloMix at a loading of 27.5 mg protein/g of biomass (or 55 mg protein/g of glucan) after 24 h gave 70-100% sugar yield. Treatment of the hardwoods with a laccase from *Agaricus bisporus*, especially wood biomass with a higher proportion of lignin, significantly improved saccharification by the formulated HoloMix enzyme cocktails. This study provided insights into the enzymatic hydrolysis of various pre-treated hardwood substrates and assessed whether the same lignocellulolytic cocktail can be used to efficiently hydrolyse different hardwood species. The present study also demonstrated that the hydrolysis efficiency of the optimised HoloMix was comparable to (if not better) than commercial enzyme preparations during hardwood biomass saccharification.

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List of units and abbreviations

AA	Auxillary activity
AbLac	*Agaricus bisporus* Laccase
AguA	α-Glucuronidase A
APS	Ammonium Persulphate
Bgl1	β-glucosidase 1
BSA	Bovine Serum Albumin
°C	Degree(s) Celsius
CAZy	Carbohydrate active enzyme database
CBM	Carbohydrate binding domain
CD	Circular dichroism
Cel6A	Cellobiohydrolase II (Non-reducing end cleaving)
Cel7A	Cellobiohydrolase I (Reducing end cleaving)
CrI	Crystallinity Index
µg	Microgram
µL	Microlitre
µM	Micromolar
µmol	Micromole
DNS	Dinitrosalicylic
DS	Degrees of synergy
EC	Enzyme commission number
Egl	Endoglucanase
g	Gram
g	Gravity
GH	Glycoside hydrolase
h	Hour
HPLC	High-performance liquid chromatography

kDa	Kilo Daltons
L	Litre
LiP	Lignin peroxidase
LPMO	Lytic polysaccharide mono-oxygenase
mg	Milligram
min	Minute
mL	Millilitre
mM	Millimolar
MnP	Manganese peroxidases
nm	Nanometer
NREL	National Renewable Energy Laboratory
pI	Isoelectric point
p-NP	*p*-nitrophenyl derivative
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	Scanning electron microscopy
SXA	β-Xylosidase
TEMED	N, N, N', N'-tetramethylethylenediamine
U	Units of enzyme activity
WHC	Water holding capacity
WSC	Water swelling capacity
w	Weight
XRD	X-ray powder diffraction
XT6	β-Xylanase (GH10)
Xyn2A	β-Xylanase (GH11)

List of research outputs

i. Publications in peer reviewed journals and conference proceedings

Malgas, S., Chandra, R., Van Dyk, J.S., Saddler, J.N. and Pletschke, B.I. Formulation of an optimised synergistic enzyme cocktail, HoloMix, for effective degradation of various pre-treated hardwoods. Bioresource Technology 2017; 245, Pages 52-65.

Malgas, S., Thoresen, M., Van Dyk, J.S. and Pletschke, B.I. Time dependence of enzyme synergism during the degradation of model and natural lignocellulosic substrates. Enzyme and Microbial Technology 2017; 103, Pages 1-11.

Pletschke, B., **Malgas, S.,** Bhattacharya, A., Bhattacharya-Shrivastava, A., Clarke, M.D., Mafa, M.S., Morake, S., Thoresen, M. Enzyme synergism: A powerful tool for decreasing enzyme loading for efficient biomass conversion. 24th European Biomass Conference and Exhibition, Amsterdam, Netherlands. ISBN: 978-88-89407-165, Pages 68-82, DOI:10.5071/24thEUBCE2016-1BO.5.4.

ii. Conference presentations

Malgas, S., Chandra, R., Van Dyk, J.S., Saddler, J.N. and Pletschke, B.I. Enzyme cocktail inhibition: Inhibitory effects of individual pre-treatment by-products and wash liquors from hardwoods on HoloMix, a holocellulolytic enzyme core-set. 39th Symposium on Biotechnology for Fuels and Chemicals 2017; Marriot Hotel, San Francisco, California, USA. (Rapid Fire and Poster presentations).

Malgas, S., Chandra, R., Van Dyk, J.S., Saddler, J.N. and Pletschke, B.I. Enzyme synergism between a cellulolytic and a xylanolytic enzyme core-set during the degradation of hardwoods (*Acacia* and Poplar) for the design of enzyme cocktails with reduced enzyme loadings and effective hydrolysis. 39th Symposium on Biotechnology for Fuels and Chemicals 2017; Marriot Hotel, San Francisco, California, USA. (Oral presentation).

Thoresen, M., **Malgas, S.,** Chandra, R., Van Dyk, J.S., Saddler, J.N. and Pletschke, B.I. The effect of pre-treatment on enzyme synergism between various lignocellulolytic enzymes on hardwoods - a comparative evaluation of *Populus, Eucalyptus* and *Acacia* spp. as potential feedstocks for sugar release. 38th Symposium on Biotechnology for Fuels and Chemicals 2016; Hilton Camden Yards, Baltimore, MD, USA. (Oral presentation).

Malgas, S., Chandra, R., Van Dyk, J.S., Saddler, J.N. and Pletschke, B.I. Enzyme synergism between a cellulolytic and a xylanolytic enzyme core-set during the degradation of untreated, delignified and steam exploded Poplar for fermentable sugar production. 27th Annual CATSA Conference 2016; Champaigne Sports Resort, Drakensburg, Kwazulu Natal, South Africa. (Oral presentation).

iii. Anticipated publications

Malgas, S. and Pletschke. The effect of an oligosaccharide reducing-end xylanase, Rex8A, on the synergistic degradation of hetero-xylan backbones by xylanolytic enzymes. In preparation for submission to Enzyme and Microbial Technology.

Thoresen, M., **Malgas, S.** and Pletschke, B.I. Revisiting the mechanism of cellulase action: Not all endo- to exo-cellulase interactions are synergistic. In preparation for submission to Journal of Molecular Catalysis: Enzymatic B.

Malgas, S., Mafa, M.S., Mathibe, B.N. and Pletschke. Unravelling hetero-synergism between various GH family xylanases and debranching enzymes during hetero-xylan degradation. In preparation for submission to Enzyme and Microbial Technology.

Dedication and Acknowledgements

To my late grandmother, Ester Nosapho Malgas, I dedicate my work to in a special way. Because of her love and support in my life, I have been shaped to the person that I am today because of her.

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Chapter 1: General Introduction and Literature review

1.1. Introduction

Lignocellulose is the most abundant biomass in the world. It consists of lignin, which forms a protective barrier around the holocellulose component, which is a combination of cellulose, pectin and hemicellulose in the plant biomass (Beukes & Pletschke 2011). Currently, numerous lignocellulosic feedstocks are considered for biofuel and other value-added chemical production; these include agricultural residues (e.g. corn stover and wheat straw), herbaceous energy crops (e.g. switchgrass and *Miscanthus*), short rotation forestry crops (e.g. hybrid poplar and willow) and cellulosic portions of municipal waste (Saini et al. 2014). Its abundance and high polysaccharide content makes lignocellulosic biomass a promising feedstock for the production of biofuels and several commercial cellulosic ethanol facilities have already begun operating (Malgas et al. 2017).

Since the 1980's, enzymatic hydrolysis of plant polysaccharides emerged as the most prominent technology for the conversion of biomass into monomeric sugars for subsequent fermentation into bioethanol (Van Dyk & Pletschke 2012; Yang et al. 2011). The degradation of the polysaccharides into their respective monomeric sugars within the lignocellulosic biomass is achieved biologically through the synergistic use of a mixture of glycoside hydrolases (GHs), lignin modifying enzymes and other accessory enzymes (Singhania et al. 2013; Yang et al. 2011). The synergy between enzymes facilitates an enhanced hydrolytic activity, where the resulting activity is greater than the theoretical sum of the individual enzyme activities. The released sugar monomers are subsequently fermented into bioethanol by yeasts, e.g. *Saccharomyces cerevisiae*.

1.2. Composition of lignocellulose

Lignocellulose is a hetero-polymeric material consisting of lignin, which forms a protective barrier around the holocellulose component made up of cellulose and hemicellulose (Beukes & Pletschke 2011; Yang et al. 2011; Zhang & Lynd 2004). According to Yamabhai and co-workers, different lignocellulosic feedstocks have a varying composition of macromolecules, but the major components are on average of the following order: glucan > lignin > xylan > mannan > arabinan > galactan (Yamabhai et al. 2014). Interactions between the various plant polysaccharides have an effect on the overall hydrolysis of the cell wall by hydrolytic enzymes.

1.2.1. **Cellulose**

Cellulose makes up the major component of plant biomass (35-50%) and has been considered as the most abundant carbon resource on earth (Lynd et al. 2002; Zhang & Lynd 2004). Cellulose is a homo-polysaccharide made up of glucose residues connected via β-1,4-glycosidic bonds (Teeri 1997). The polymer chains pack tightly together in a parallel arrangement through the stabilisation of strong intra and inter-molecular hydrogen bonds to form elementary crystalline structures (Kim et al. 2014). Although cellulose is generally considered to be largely crystalline in nature, it is also composed of less ordered regions that are classified as amorphous (non-crystalline) and para-crystalline regions (Park et al. 2010). Depending on the source of cellulose, the degree of crystallinity can range between 50 and 90% (Ju et al. 2015; Hall et al. 2010). Attractive sources (containing high content) of cellulose include and are not limited to wood, crop residues, and bacterial cellulose.

1.2.2. **Hemicellulose**

Hemicellulose refers to a group of linear and branched heterogeneous polysaccharides composed of pentoses (xylose, arabinose), hexoses (mannose, glucose, galactose), and sugar acids (Saha 2003). Hemicelluloses are named according to the major monosaccharide found in their backbones and most of these are linked together by β-1,4-glycosidic bonds (Saha 2003; Van Zyl et al. 2010). The distribution of hemicellulose in softwoods (gymnosperms) and hardwoods (angiosperms) varies greatly (Moreira & Filho 2008; Yamabhai et al. 2014). Hardwoods and grasses comprise of xylans as the major hemicellulosic component, whereas softwoods, plant seeds and fruits are comprised of mannans as more prominent types of hemicelluloses (Moreira & Filho 2008; Van Zyl et al. 2010; Yamabhai et al. 2014).

1.2.2.1. **Xylans**

Xylan, the most common hemicellulose, comprises approximately 40% of the hemicellulose content in plants and is considered to be the second most abundant polysaccharide in nature, after cellulose (Subramaniyan & Prema 2002). Xylan is a hetero-polysaccharide, primarily made up of xylose residues that are connected via β-1,4-glycosidic bonds (Subramaniyan & Prema 2002). Xylan is closely associated with cellulose and lignin through the formation of covalent and non-covalent linkages, thereby providing a rigid and protective structure for the plant cell wall (Van Dyk & Pletschke 2012). The backbone chain is substituted with different side chains, such as glucuronopyranosyl, 4-O-methyl-D-glucuronopyranosyl, α-L-arabinofuranosyl, acetyl, feruloyl and *p*-coumaroyl residues to varying degrees (Pinto et al.

2005). Xylan is present in many different plant species, however its abundance and composition may vary between plants (Kormelink & Voragen 1993; York & O'Neill 2008). Xylan is most prominent in hardwoods (beechwood and willow), contributing to approximately 15-30% of the total dry weight and is found to be less dominant in softwoods (spruce, pine, Douglas-fir), contributing to approximately only 7-12% of the total dry weight (Haltrich et al. 1996; Van Dyk & Pletschke 2012). In hardwoods, every tenth xylose residue is substituted with an α-1,2 linked 4-O-methylglucuronic acid residue, whereas this substitution occurs on every twentieth xylose residue in softwoods (Pinto et al. 2005; Huisman et al. 2000). Hardwood xylose residues are also substituted with acetyl groups at positions C-2 and/or C-3 (Busse-Wicher et al. 2014; Kormelink & Voragen 1993).

1.2.2.2. **Mannans**

Mannans and hetero-mannans are hemicelluloses predominantly found in softwoods, in seed endosperms and in vacuoles of a wide variety of plants as a non-starch reserve polysaccharide, and as a constituent of glycoproteins in yeast cell walls (Van Zyl et al. 2010; Yamabhai et al. 2014). Mannans are classified into four subfamilies: linear mannan, glucomannan, galactomannan, and galactoglucomannan (Moreira & Filho 2008). The backbones of mannans are divided into two groups depending on whether the β-1,4-linked backbone contains only mannose residues (called mannans) or a combination of both mannose and glucose residues (called glucomannans). In addition, the mannan and glucomannan backbones can be substituted with side chains of α-1,6-linked galactose residues to give rise to galactomannan and galactoglucomannan, respectively (Malgas et al. 2015). Furthermore, acetylation of the C-2 or C-3 positions in mannose residues in mannans can take place (Dhawan & Kaur 2007).

1.2.3. **Lignin**

Lignin is an aromatic polymer consisting of phenylpropane units produced through oxidative coupling and is covalently connected to hemicellulose residues via ferulates (Várnai et al. 2011; Yang et al. 2011). In cell walls, lignin acts as an amorphous aromatic-rich barrier to microbial degradation (Van Zyl et al. 2010). The three monomers that constitute lignin are the phenylpropanoids *p*-hydroxyphenyl, guaiacyl and syringyl, respectively (Adapa et al. 2009; Palmqvist & Hahn-Hägerdal 2000a). According to Nakagame and co-workers, the structure and content of lignin differs according to its plant origins (Nakagame et al. 2011). Softwood lignin is composed mainly of guaiacyl units with small quantities of *p*-hydroxyphenyl units, while hardwoods are composed of both guaiacyl and syringyl units with small quantities of *p*-

hydroxyphenyl units (Nakagame et al. 2011; Adapa et al. 2009; Palmqvist & Hahn-Hägerdal 2000b). Softwoods generally contain more lignin than hardwoods and other agricultural residues (Palmqvist & Hahn-Hägerdal 2000a; Palmqvist & Hahn-Hägerdal 2000b).

1.2.4. **Pectin**

Pectin is said to be the most complex polysaccharide known in nature (Van Dyk et al. 2013). Pectin plays important roles in the formation of higher plant cell walls, influencing ion transport in the cell wall and activating plant defence responses (Voragen et al. 2009). Three different types of pectin are found in plants, namely: homogalacturonan (HG) , rhamnogalacturonan I (RGI) and rhamnogalacturonan II (RGII) (Van Dyk et al. 2013). According to Van Dyk et al. (2013), HG is the major component in pectin and can constitute up to 60% of the pectin, although the exact content of HG may differ depending on the source. HG has an α-1,4-galacturonic acid backbone with different degrees of methyl esterification at C-6 and/or acetylation at the O-2 or O-3 sites on the sugar residue. RGI has a backbone composed of alternating rhamnose and galacturonic acid repeating units (Van Dyk et al. 2013). RGII, on the other hand, has a homogalacturonan backbone with clusters of four different side chains, such as apiose, aceric acid, 3-deoxy-lyxo-2-heptulosaric acid (DHA), and 3-deoxy-manno-2-octulosonic acid (KDO) (Voragen et al. 2009).

1.3. **Uses of lignocellulosic feedstocks**

The fact that lignocellulosic biomass is a renewable resource, abundant, and environmentally friendly makes it an appropriate candidate for producing a number of value added products (Gao et al. 2011; Beukes & Pletschke 2011). These include and are not limited to: pectin (gelling agent, thickener and stabiliser in foods), dietary fibre, smart polymers, lycopene (antioxidant), phenolics (antimicrobial and anticancer properties), D-limonene (flavour and fragrance additive in perfumes, soaps and foods), citric acid and biofuels such as bioethanol (Van Dyk et al. 2013).

1.3.1. **Motivation for the use of hardwoods as a feedstock for biofuel production**

From a biochemical point of view, wood is composed of about 50% cellulose, 25% lignin and 25% hemicelluloses, with traces of pectin and proteins, impregnated with phenolics (Déjardin et al. 2010). Hardwoods are the type of wood chosen in this study; the reason for this is because hardwoods are easier to delignify when compared to softwoods (Hu et al. 2015; Munoz et al. 2007), and therefore their polysaccharides are more easily available to enzymes for the purpose

of producing fermentable sugars. Two hardwoods have been selected for the purpose of this study, *Populus* spp. and *Acacia* spp, and a detailed review on them is provided below. Table 1.1 given below shows the general chemical composition of *Populus* spp. and *Acacia* spp. wood samples.

According to Folch and Ferrer (2015), poplar (*Populus* spp.) is well known for its large biomass production, its ability to adapt to different environments, its ability to integrate and synergise with agriculture and its high energy potential (Folch & Ferrer 2015). For energy production purposes, short rotation coppice periods of about 2-5 years are applied as this period can produce sufficient biomass (Rosso et al. 2013). Poplar species and hybrids have cellulose contents ranging from ~42 to 49%, hemicellulose from 16 to 23%, and total lignin contents from 21 to 29% for dried material (dry-weight). These characteristics makes poplar a good feedstock for biofuel production (Kenney et al. 1990; Kačík et al. 2012; Gierlinger et al. 2008).

Acacia is a leguminous, fast-growing, woody, multinational genus distributed in the Australia-Pacific region, throughout the south of Asia, Africa and in North and South America. Most species are distributed primarily in the dry tropics, and several Australian *Acacia* species have become highly invasive weeds around the world (Ferreira et al. 2011; Rosso et al. 2013). One of the beneficial ways of controlling the distribution of this invasive plant species may be in its use as a feedstock for bioethanol production. According to Dahl et al. (2001) and De Neergaard et al. (2005), South Africa suffers from two invasive, alien wattle species, *Acacia dealbata* (Silver Wattle) and *Acacia mearnsii* (Black Wattle), and they have widespread distribution in the foothills of the Drakensberg Escarpment of the Eastern Cape and KwaZulu-Natal provinces (Dahl et al. 2001; De Neergaard et al. 2005).

It is reported that Black wattle is among the top 10 invading species in South Africa, growing wild on 2,500,000 ha, while Silver wattle occurs on the top 25 list (De Neergaard et al. 2005). Using the *Acacia* spp. as a feedstock for the production of biofuels would be advantageous to the South African perspective, as removal of these invasive plants from the environment would assist in the conservation and recovery of the threatened native plants. These plants are also fast growing with ample biomass (Dahl et al. 2001) which can serve as feedstocks for biofuels production. This would then lead to a more balanced natural plant biodiversity since removal of these invasive plants would allow the native plants to get re-established into the environment.

Table 1.1. The chemical composition of *Acacia* spp. and *Populus* spp. biomass (dry weight). "Nd" = not determined and "-" = not detected.

Wood sample	Glucan (%)	Xylan (%)	Arabinan (%)	Mannan (%)	Galactan (%)	Lignin (%)	Acetyl (%)	Extractives (%)	Ash (%)	References
Acacia dealbata	50.5	19.3	0.19	1.8	0.60	20.1	4.4	3.1	1.1	(Munoz et al. 2007)
	42.4	16.4	0.29	-	-	19.3	3.90	5.85	0.50	(Yáñez et al. 2009a; Yáñez et al. 2009b)
	42.37	16.18	0.23	-	-	18.90	3.90	5.85	0.50	
Acacia mangium	45.3	14	-	-	-	Nd	-	-	-	Willfor et al., 2005
Hybrid poplar (*P. alba* x *tremula*; P717)	50-54	14.5-18.2	0.3-0.5	0.6-1.9	0.3-1.1	19.5-22.4	-	-	-	(Skyba et al. 2013)
Hybrid poplar (*P. nigra* x *maximowiczii*)	43.8	14.9	0.61	3.9	1	29.1	3.6	3.6	1.1	(Balan et al. 2009)
Poplar	84 (Holocellulose)					-	-	6	-	(Kenney et al. 1990)
Poplar clones	43.5-47.6	35.6-36.9 (Hemicellulose)				17.7-21.9	-	1.5-5.4	-	(Kačík et al. 2012)

1.4. **Pre-treatment of lignocellulosic biomass**

Even though the use of lignocellulosic biomass for biofuel production can apparently address the issue of replacing petroleum-based fuels, its degradation presents several obstacles which are mostly due to plant recalcitrance (Saini et al. 2014). Himmel et al. (2007) ascribed plant recalcitrance to plant biomass evolving complex structural and chemical mechanisms for resisting assault on its structural sugars from microbes and animals (Himmel et al. 2007). Zhang and Lynd (2004) have indicated that, since enzymatic hydrolysis of native lignocellulose usually results in solubilisation of less than 20% of the original glucan, some form of pretreatment to increase amenability to enzymatic hydrolysis is included in most process concepts for biological conversion of lignocellulosic biomass to value added products (Zhang & Lynd 2004). Current pre-treatment technologies attempt to increase the accessibility of cellulose while either removing hemicellulose (steam explosion, dilute acid pre-treatment, cellulose solvent and organic solvent based lignocellulose fractionation) or altering their structure to reduce competitive inhibition (alkaline methods, Ammonia Fibre Expansion or AFEX, etc.). Figure 1.1 below illustrates general structural changes lignocellulosic biomass undergoes upon pre-treatment.

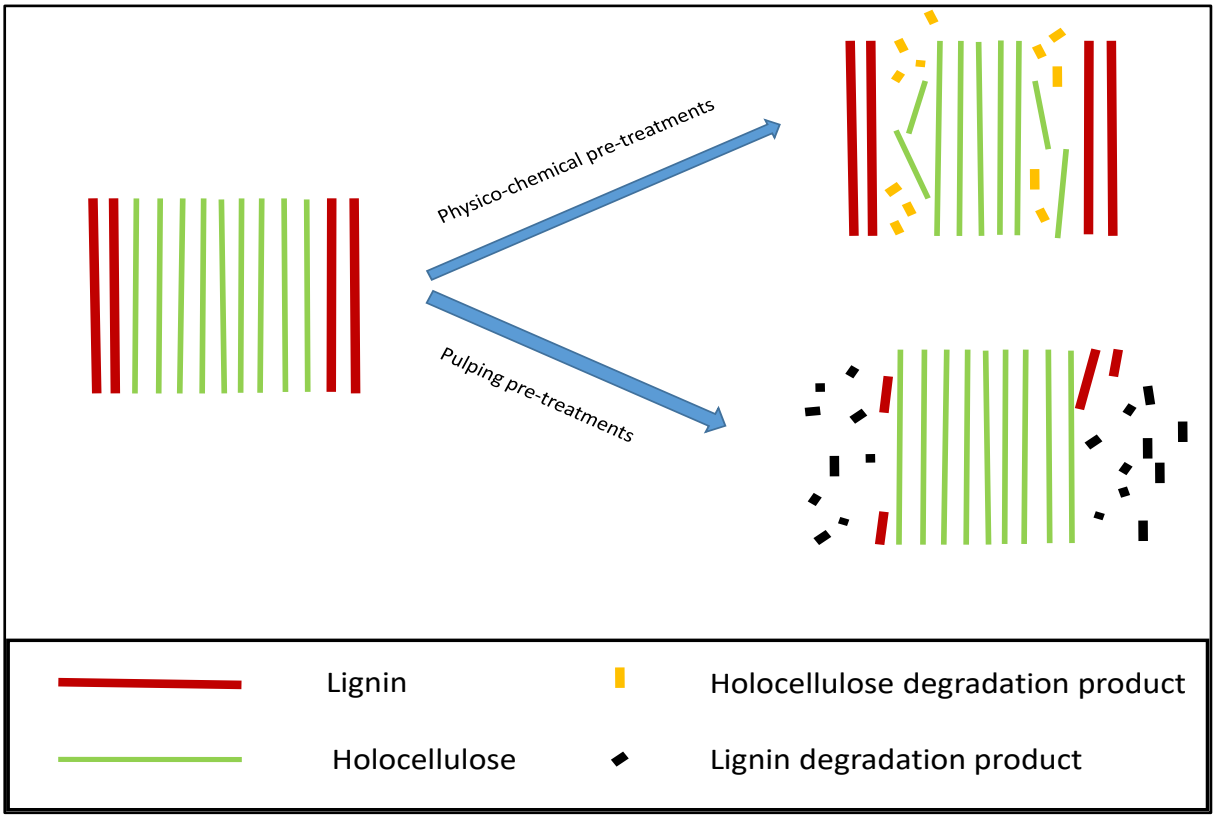

Figure 1.1. Lignocellulosic biomass structural disruption upon pre-treatment.

Steam explosion and dilute acid pre-treatment both produce a liquor stream containing partially hydrolysed hemicellulose and a pre-treated solid with an approximate cellulose: hemicellulose ratio of 12:1 (Rodríguez-Zúñiga et al. 2015; Rastogi & Shrivastava 2017). Steam explosion pre-treatment involves rapidly heating lignocellulose under high pressure steam in order to degrade the hemicellulose in biomass followed by biomass explosion resulting in a rapid decrease in pressure in the reactor (Alvira et al. 2010; Hendriks & Zeeman 2009). The generation of some toxic compounds, that could affect the following hydrolysis and fermentation steps, is the major drawback of steam explosion pre-treatment (Alvira et al. 2010). In addition to the above mentioned drawbacks, in thermochemical pre-treatments such as steam explosion and hydrothermal pre-treatment, high energy input is required, thus making these processes economically unattractive (Rodríguez-Zúñiga et al. 2015; Guilherme et al. 2015; Alvira et al. 2010). To avoid or at least minimize hemicellulosic sugar loss during thermochemical pre-treatment, the use of mild severity conditions has often been used (Bura et al. 2009).

AFEX and other chemical methods (Organosolv and Sodium chlorite/acetic acid), on the other hand, retain most of the hemicellulose in the pre-treated solid, reducing cellulose crystallinity and reducing the recalcitrance of lignin to enzymatic degradation, while yielding a cellulose: hemicellulose ratio near that of untreated biomass (8:5) (Balan et al. 2009; Zhao et al. 2009; Ahlgren & Goring 1971; Koo et al. 2012). The use of chemical pre-treatments is commonly implemented in the paper and pulping industry. Its main aim is to remove lignin from the fibrous holocellulose (Siqueira et al. 2013), which leads to improvements in paper brightness (low kappa numbers). Alkaline pre-treatment of biomass leads to solvation and saphonication, causing a swollen state of the biomass thus making it more accessible for enzymes (Hendriks & Zeeman 2009). Among chemical methods, alkaline peroxide processes are effective for both delignification and removal of hemicelluloses, but their use is limited due to extensive degradation of cellulose caused by the peroxide radical (Hubbell & Ragauskas 2010; Beukes & Pletschke 2011). Due to this, acid-chlorite is more favourable for efficient delignification without modifying the holocellulose content. However, it is noteworthy to mention that although acid-chlorite primarily acts mainly on lignin in the biomass, it can also affect the polysaccharides (especially near a neutral pH). As a result, acetic acid is generally added to the delignification procedure to reduce the pH (Hubbell & Ragauskas 2010). Oxidative pre-treatments such as acid-chlorite achieve delignification via several reactions such as electrophilic substitution, displacement of side chains, cleavage of alkyl aryl ether linkages or

the oxidative cleavage of aromatic nuclei. A high risk associated with oxidative methods is the formation of inhibitors. As lignin is oxidized, soluble aromatic compounds are generally formed (Hendriks & Zeeman 2009; Ahlgren & Goring 1971; Kumar et al. 2013; Yu et al. 2011).

As mentioned previously, biomass pre-treatment can lead to the generation of undesired by-products. The soluble enzyme inhibitors that can be found after biomass pre-treatment include sugars (monomers, oligomers), furan derivatives (hydroxymethyl furfural, furfural), organic acids (acetic, formic, levulinic acids), and lignin derivatives (poly- and mono-phenolic compounds such as ferulic acid, *p*-coumaric acid, vanillin, vanillic acid and gallic acid) (Kim et al. 2011; Morrison et al. 2011). Currently, studies are focussed on understanding the chemical modifications of woody biomass during the application of various pre-treatment processes and the release of chemical by-products. Such studies are important as they could provide insights into the selection of an appropriate pre-treatment method to overcome biomass recalcitrance and enzyme (or fermentative microbe) inhibition.

1.4.1. Enzyme inhibition by pre-treatment degradation products

Lignin-derived and plant-derived phenolic compounds are reported to be among the major pre-treatment degradation compounds which elicit inhibitory effects during enzymatic saccharification of lignocellulosic biomass (Pletschke et al. 2016). Investigations into the mechanism of cellulase inhibition by phenolic compounds revealed that most of these compounds display reversible and non-competitive inhibition on the enzymes (Li et al. 2014; Ximenes et al. 2010). Studies have further revealed that functional groups on the phenolic compounds play a crucial role in the inhibition of enzymes (Malgas et al. 2016; Pan 2008)

It is also noteworthy to mention that enzymes may differ with regards to chemical tolerance, which may occur as a result of the microorganisms from which the particular enzyme is derived. For instance, Ximenes and co-workers have demonstrated that phenolics inhibit β-glucosidase from *T. reesei* about twice as much as that from *A. niger* (Ximenes et al. 2010, 2011). In our research group, we have also demonstrated that mannanolytic enzymes (mannanases, mannosidases and galactosidases) from different sources and glycoside hydrolase families interact differently with pre-treatment degradation products (Malgas et al. 2016). A better understanding of the interactions between pre-treatment by-products such as phenolics or lignin and individual enzymes will contribute to the rational design of better enzymatic consortium (that which would be composed of resistant pre-treatment by-products enzymes) to effectively break down the lignocellulose.

1.5. **Enzymes required to degrade lignocellulose**

Due to the complexity of lignocellulosic biomass, various enzymes are required to degrade the different polysaccharides and polymers in the biomass into their respective monomers.

1.5.1. **Cellulases**

Due to the recalcitrance of the cellulose structure, cellulases with synergistic functions are required to degrade cellulose (Kim et al. 2014). The three classes of cellulases that are necessary for this enzymatic degradation system include endoglucanases (EG, EC 3.2.1.4), cellobiohydrolases (CBHs, EC 3.2.1.91 and EC 3.2.1.176) and β-glucosidases (BGLs EC 3.2.1.21) (Kim et al. 2014). Endoglucanases cleave the glycosidic bonds within the cellulose chain (amorphous region), thereby providing cellobiohydrolases with more accessible sites to act upon (Ganner et al. 2012). Cellobiohydrolases have been classified as exo-acting enzymes and are considered to be the key enzymes necessary for cellulose degradation (Boisset et al. 2000). They act in a processive manner on the free ends of the polymer (crystalline region), liberating cellobiose from the cellulose chain (Maki et al. 2013). They have been classified into two different classes (CBHI and CBHII) as they have different specificities for opposite sides of the cellulose chain (reducing and non-reducing ends) (Wei et al. 2014; Zhang & Viikari 2012). The β-glucosidases complete the hydrolysis reaction by converting cellobiose to glucose (Kostylev & Wilson 2012).

1.5.2. **Accessory enzymes required for cellulose degradation**

While the consensus model for cellulose degradation states that the key enzymes required for efficient degradation are cellobiohydrolases, endoglucanases and β-glucosidases, studies have shown that synergistic proteins such as carbohydrate binding modules (CBMs), expansins, and lytic polysaccharide monooxygenases (LPMOs) greatly enhance the activity of hydrolytic enzymes (Arfi et al. 2014; Horn et al. 2012). These proteins are known to make the substrate more accessible to cellulases, ultimately leading to lower production costs by potentially reducing the enzyme loading required for hydrolysis (Kim et al. 2014).

1.5.2.1. **Carbohydrate binding modules**

Cellulases are often linked to non-catalytic modular accessory domains, commonly referred to as CBMs (Shoseyov et al. 2006). They are known to dramatically boost cellulase activity by increasing the adsorption of cellulases onto the polysaccharide surface (Kim et al. 2014). By

increasing cellulase to substrate binding, CBMs play a crucial role in achieving complete degradation of the crystalline cellulose.

1.5.2.2. **Expansins and swollenins**

The recalcitrant structure of plant cell walls impedes enzymatic hydrolysis, therefore novel plant loosening proteins such as expansins and expansin-like proteins (swollenins) have gained significant attention in enzymatic hydrolysis (Arantes & Saddler 2010). Through a non-hydrolytic process, these proteins are able to enlarge cell walls by breaking the hydrogen bonds between polysaccharides (Suwannarangsee et al. 2012; Kim et al. 2014; Gourlay et al. 2015). Consequently, microfibrils within the structure are loosened, making the substrate more accessible for the cellulases to act upon, thus leading to more efficient cellulose degradation (Arantes & Saddler 2010; Suwannarangsee et al. 2012).

1.5.2.3. **Lytic polysaccharide monooxygenases**

Recently, novel enzymes, known as lytic polysaccharide monooxygenases (LPMOs) or Auxillary activity proteins (AA9s) have been discovered and hold major potential for future biomass degradation applications (Arfi et al. 2014; Horn et al. 2012). These enzymes enhance cellulase activity by cleaving glycosidic bonds of polysaccharides in the crystalline chain, consequently resulting in complete cellulose degradation (Kim et al. 2014; Müller et al. 2015; Cragg et al. 2015).

1.5.3. **Hemicellulases**

Hemicellulose has a varied composition and therefore a large number of diverse enzymes are required to hydrolyse it effectively. Total biodegradation of hemicellulose requires several xylanolytic, mannanolytic, and accessory enzymes. These hemicellulolytic enzymes are generally divided into two groups: depolymerising enzymes which cleave the backbone of hemicelluloses and side-chain cleaving enzymes which remove substituents which may pose steric hindrances to the backbone depolymerising enzymes (Moreira & Filho 2008; Van Dyk & Pletschke 2012).

1.5.3.1. **Xylanases**

To accomplish efficient and complete xylan hydrolysis, the synergistic action of various xylanases is required (Biely et al. 2016). The two key xylanases required for this degradation process are endo-1,4-β-D-xylanases (EC 3.2.1.8) and 1,4-β-D-xylosidases (EC 3.2.1.37) (Van

Dyk & Pletschke 2012). Endo-1,4-*β*-D-xylanases are responsible for cleaving internal 1,4 *β* bonds in the xylan backbone into shorter oligosaccharides, whereas 1,4-*β*-D- xylosidases initiate their mode of action from the non-reducing end of the chain, hydrolysing xylo-oligosaccharides to D-xylose (Van Dyk & Pletschke 2012). In addition, the activities of accessory enzymes are required to liberate the substituents from the xylan backbone (Haltrich et al. 1996). These enzymes are α-arabinofuranosidases, α-D-glucuronidases (EC 3.2.1.131) and phenolic esterases (EC 3.1.1.73) (Polizeli et al. 2005). Arabinofuranosidases remove arabinose residues from the backbone (Subramaniyan & Prema 2002). The α-D-glucuronidases (EC 3.2.1.131) are known to catalyse the hydrolysis of the α 1,2-linkages between xylose and D-glucuronic acid residues (Subramaniyan & Prema 2002). Phenolic esterases include acetyl xylan esterases (EC 3.1.1.6), feruloyl acid esterases (EC 3.1.1.73) and *p*-coumaroyl esterases (EC 3.1.1.73) (Subramaniyan & Prema 2002). Acetyl xylan esterases release acetylated substituents at the C-2 and/or C-3 positions of the xylan backbone, feruloyl acid esterases cleave the linkages between ferulic acids and arabinose residues and *p*-coumaroyl esterases cleave the bonds between *p*-coumaric acid and arabinose residues, at the O5 position in the xylan backbone (Subramaniyan & Prema 2002; Nkhata et al. 2017). Recently discovered exo-xylanase enzymes (oligosaccharide reducing-end xylanase [Rex], EC. 3.2.1.156) show a different mode of action when compared to β-xylanases and β-xylosidases, and are strictly found in the GH8 class of enzymes, according to the CAZy database. Rex enzymes hydrolyse the xylan back-bone from the reducing ends producing xylo-oligomers (Juturu & Wu 2012; Lagaert et al. 2007; Honda & Kitaoka 2004).

1.5.3.2. **Mannanases**

The hydrolysis of mannans and hetero-mannans requires a concerted action of various mannanolytic enzymes due to the complexity of these structures. The enzymes required to hydrolyse mannan and hetero-mannan backbones include β-mannanases (EC 3.2.1.78), β-mannosidases (EC 3.2.1.25) and β-glucosidases (EC 3.2.1.21). Mannanases randomly cleave mannan and hetero-mannan backbones to release short β-1,4-manno-oligomers and create new chain ends (Shallom & Shoham 2003). The short β-1,4-manno-oligomers are subsequently hydrolysed by mannosidases and glucosidases from the non-reducing ends into mannose and glucose, respectively (Moreira & Filho 2008). The enzymes required to hydrolyse the side groups of hetero-mannans are α-galactosidases (EC 3.2.1.22) and acetyl-mannan esterases (EC 3.1.1.6) (Moreira & Filho 2008; Van Zyl et al. 2010; Yamabhai et al. 2014). Galactosidases, de-branching enzymes, remove the α-1,6-linked galactopyranosyl substituents attached to

galactomannan and galactoglucomannan backbones, whereas acetyl mannan esterases release the acetyl groups from galactoglucomannan (Moreira & Filho 2008; Van Zyl et al. 2010). Over the past decade, a new mannanolytic enzyme class has been discovered and suggested to be implicated in the efficient degradation of the mannan backbone, this enzyme class being the endo-processive mannobiohydrolases (EC 3.2.1.100). Endo-processive mannobiohydrolases hydrolyse mannosidic linkages in mannans, to remove successive mannobiose residues from the non-reducing chain ends (Cartmell et al. 2008; Bågenholm et al. 2016).

1.5.4. Ligninolytic enzymes

Degradation of lignin also requires an arsenal of ligninolytic enzymes, such as manganese peroxidase (MnP) (EC 1.11.1.13), lignin peroxidase (LiP) (EC 1.11.1.14) and laccase (EC 1.10.3.2), which have the ability to catalyse the oxidation of phenolic compounds (Howard et al. 2003; Kolb et al. 2012; Heinzkill et al. 1998; Dashtban et al. 2010). Adding lignin degrading enzymes such as LiP and MnP with cellulases and hemicellulases results in enzyme inhibition due to the release of monomeric phenols (Tabka et al. 2006). On the other hand, adding laccases which are copper containing helps to polymerise monomeric phenols produced during pre-treatment into polymeric lignin and as a result these enzymes act as detoxifying agents (Chen et al. 2012; Jurado et al. 2009).

1.5.5. Pectinases

The hydrolysis of pectin into its constituents requires four types of pectinolytic enzyme activities: i) protopectinases (EC 3.2.1.99) which are responsible for converting insoluble protopectin into soluble pectin; ii) pectinesterases (EC 3.1.1.11) which are subsequently responsible for the removal of methoxyl esters, resulting in polygalacturonic acid and methanol; iii) polygalacturonases (endo-polygalacturonases, EC 3.2.1.15 and exo-galacturonases, EC 3.2.1.67) which are responsible for hydrolysing the poly-galacturonic acid into monogalacturonic acid and iv) pectate lyases (endo, EC 4.2.2.2 and exo EC 4.2.2.9) that depolymerize pectic substances by a trans-eliminative split at C-4 (Dhillon et al. 2013; Parmar & Rupasinghe 2013; Concha & Zúñiga 2012; Nahar & Pryor 2013).

As mentioned before, the complexity of lignocellulose necessitates the use of various enzymes to degrade the different polysaccharides into their respective monomers (See Figure 1.2).

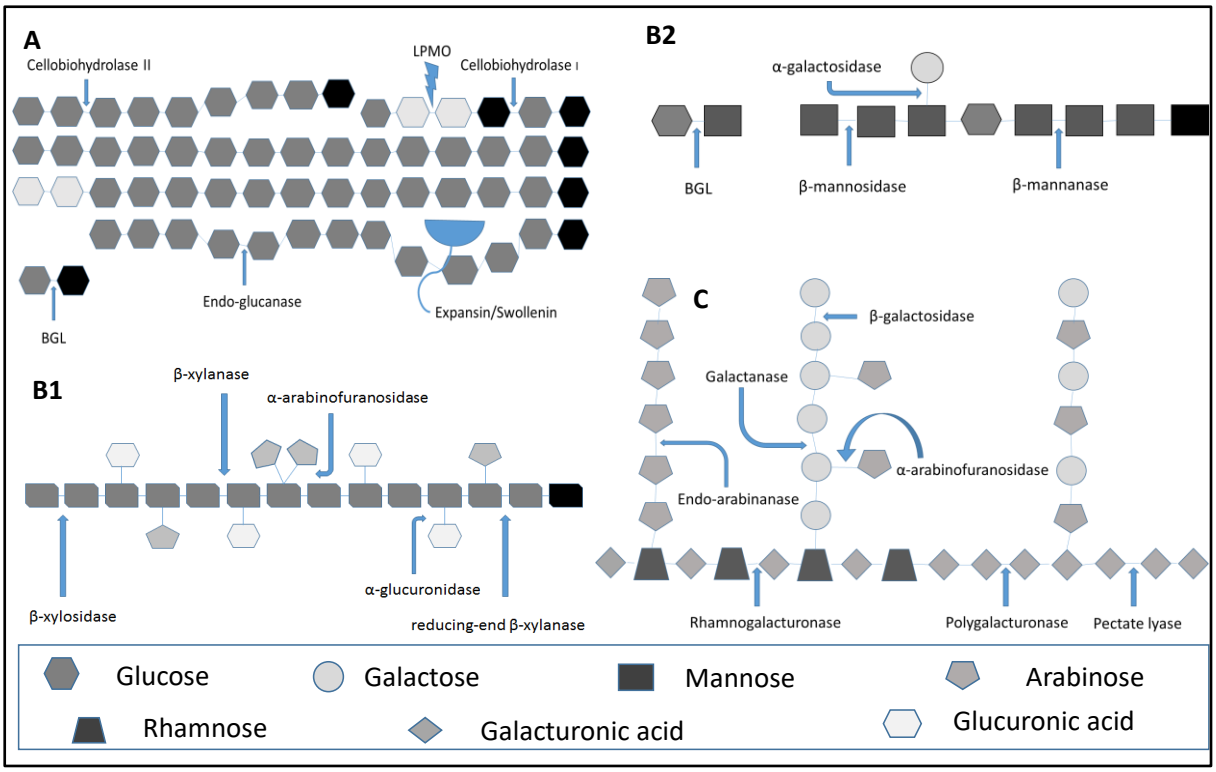

Figure 1.2. Schematic representation of plant cell wall polysaccharides and selected corresponding polysaccharide-degrading enzymes. Enzymatic degradation of: (A) Cellulose; (B1) glucuronoarabinoxylan; (B2) galactoglucomannan; and (C) pectin/arabinogalactan. Black squares represent reducing ends, while the arrows represent the points of cleavage by the various enzymes (Adapted from Malgas et al., 2017).

1.6. Enzyme synergism during lignocellulose degradation

Currently available lignocellulolytic enzyme commercial preparations mostly come from fungi such as *Trichoderma reesei* and *Aspergillus niger*, these organisms generally produce copious amounts of various lignocellulolytic enzymes. However, proteomic and enzyme assay analysis of these fungal secretome based preparations has shown that β-glucosidase and hemicellulolytic activity is low among these preparations, thus low saccharification yields are realized by these preparations (Malgas et al. 2017; Nieves et al. 1998; Singhania et al. 2013). Studies have suggested that this challenge can be overcome by numerous approaches such as heterologous gene expression in the fungus or co-cultivation of the fungus with microbes that produce high levels of the lacking enzyme (Liu et al. 2013).

Banerjee and co-workers, on the other hand, suggested using pure enzymes to make a core enzyme set, which hydrolyses most of the holocellulose (Banerjee et al. 2010). This set would be selected based on enzyme characterization and enzyme synergism data on the particular

biomass of interest. For further enzyme preparation enhancement, Banerjee and co-workers suggested that the core set would then be supplemented with accessory enzymes, depending on the process design, such as the type of substrate and pre-treatment. On the other hand, other studies have suggested that extensive optimization may not be necessary as addition of many accessory enzymes may not have a significant effect on the sugar yield (Sills & Gossett 2011). These improvements with regards to saccharification yields by the core enzyme set Banerjee and co-workers refer to are due to enzyme synergism.

Enzyme synergism may be influenced by several factors, including the reaction time, the characteristics and concentration of the substrate as well as the characteristics and concentration of the enzymes in the reaction mixture (Woodward et al. 1988; Van Dyk & Pletschke 2012). An extensive review on the effect of time on enzyme synergism during the degradation of lignocellulosic biomass is provided by Malgas et al. (2017).

1.6.1. **Types of enzyme synergism**

Depending on how the enzymes are applied (simultaneously, sequentially or successively) to hydrolyse lignocellulosic biomass, the synergism displayed between the enzymes may vary substantially. Simultaneous synergy is defined as the cooperation that occurs when enzymes are combined at the same time to hydrolyse biomass, while sequential synergy refers to cooperation between enzymes when one enzyme is incubated first and is heat-killed/denatured prior to the addition of the second enzyme (Van Dyk & Pletschke 2012). Successive synergy is similar to sequential synergy, however, the denaturation step is omitted before the addition of the second enzymes (Pletschke et al. 2016). Studies have shown that synergy is not straightforward and is highly dependent on the characteristics of the enzyme and the substrate evaluated (Pletschke et al. 2016).

With respect to hemicellulases, two types of synergies have been observed, known as homeosynergy and heterosynergy (Moreira and Filho, 2008). According to Moreira and Filho (2008) and Van Zyl et al. (2010), homeosynergy is defined as cooperability between two main-chain-cleaving enzymes (for example, β-mannanase and β-mannosidase) or two side-chain-cleaving enzymes (for example α-galactosidase and acetyl mannan esterase), while heterosynergy is the synergistic interaction between a side-chain-cleaving and a main-chain-cleaving enzyme (for example β-mannanase and α-galactosidase).

1.6.2. **Enzyme synergism during the hydrolysis of hardwoods**

Over the past few years, xylanases have become attractive for the saccharification process of lignocellulosic biomass, since the hydrolysis of xylan by these enzymes has been demonstrated to assist in the hydrolysis of cellulose to obtain fermentable sugars with potential use in the production of value added products and biofuels (Van Dyk & Pletschke 2012). Not only are xylanases attractive with regards to improving cellulose degradation to glucose, but their ability to degrade xylan into xylose is profitable when assessing at the possibility to ferment both glucose and xylose into ethanol (Kim et al. 2013; Watanabe et al. 2015; Fenske et al. 1998). In literature, synergism between cellulases and xylanases during the degradation of lignocellulosic biomass (derived from agricultural residues and perennial grasses) is the best studied, such as that reported during the degradation of sugarcane bagasse (Gottschalk et al. 2010; Li et al. 2014; Kim et al. 2017), wheat straw (García-Aparicio et al. 2007; Agrawal et al. 2015) and corn stover (Murashima et al. 2003; Song et al. 2016; Zhang & Viikari 2014). This literature review focuses solely on studies which demonstrated synergism between enzymes during the degradation of various hardwood substrates, and listed below is a list of such studies which were conducted:

- Balan and co-workers (2009) conducted experiments whereby they evaluated different enzyme loadings; low enzyme loading and high enzyme loading, during the degradation of AFEX treated Poplar. In some experiments, either 31.3 mg (low) or 125 mg (high) per gram of glucan of Multifect xylanase enzyme was also supplemented. Combinations with a ratio of 2:1 Spezyme CP cellulase to Multifect xylanase at low protein loading rendered glucan saccharification yields comparable to those rendered by Spezyme CP cellulase alone at high protein loading (Balan et al. 2009).
- Supplementing a cellulase mixture (10 FPU and 20 CBU/g cellulose for Spezyme CP and β-glucosidase, respectively) with Multifect xylanase (0.06 g of protein/g of cellulose), the conversion increased by 22 and 32%, for cellulose to glucose and for xylan to xylose conversion, respectively, during the degradation of steam exploded poplar (Bura et al. 2009).
- With Spezyme CP cellulase, at a loading of 120 mg of cellulase plus β-glucosidase/g glucan or cellulase to xylanase mixture (cellulase at 14.5 mg and a xylanase at 84.2 mg/g glucan), saccharification of untreated and pre-treated corn stover and poplar was evaluated. Higher saccharification was reported when xylanase was supplemented to

the cellulase, Spezyme CP (Kumar & Wyman 2009a; Kumar & Wyman 2009b; Kumar & Wyman 2009c).

- Hu and co-workers evaluated the effect of adding 5 mg of xylanase (either GH10 or GH11 endo-xylanase)/g cellulose to 15 mg of Celluclast 1.5L mono-component (Cel7A)/g cellulose during steam exploded poplar degradation (Hu et al. 2013). The addition of GH11 endo-xylanase resulted in similar hydrolysis improvements (about 20%) on poplar steam pre-treated at high and low severities despite the almost 2-fold higher xylan content in the 180^{o}C pre-treated substrate as compared to the 200^{o}C pre-treated substrate. In contrast, GH10 endo-xylanase addition improved the Cel7A catalytic activity 4-fold when applied to the 180^{o}C pre-treated substrate (95%) compared to the 200^{o}C pre-treated substrate (24%) (Hu et al. 2013).
- Hu and co-workers also reported that only an amount as small as 5% protein dosage of xylanase in a cellulase: xylanase: AA9 combination was required for efficient degradation of steam pre-treated Poplar (Hu et al. 2015).

From all these studies it has become apparent that xylanases improve the access of cellulases to the substrate granted these substrates display xylan content−thus the reported synergism between these two groups of enzymes. Interestingly, the type of xylanase (GH family) appears to have an effect on the synergistic associations between it and cellulases, as reported by Hu and co-workers (2015) in their study. From the same study by Hu and co-workers, it also appears that the percentage composition of xylan and its organisation within the biomass may have an impact on the synergism between xylanases and cellulases during saccharification.

It is noteworthy to mention that literature reports enzyme synergism data during lignocellulose degradation in terms of "synergism models" (Pletschke et al. 2016). Current enzyme synergism models proposed for the degradation of lignocellulosic biomass can be divided into two: (i) Intra-polymer synergism models, those that unravel patterns of synergistic associations between enzymes during the degradation of one polysaccharide i.e. cellulose or hemicellulose (Malgas et al. 2017; Pletschke et al. 2016), and (ii) Inter-polymer synergism models, those which elucidate the order of interactions between enzymes that target different polysaccharides during the degradation of complete lignocellulose, biomass with all polysaccharides (i.e. cellulose and hemicellulose) interacting and associated/interwoven with each other (Malgas et al. 2017; Pletschke et al. 2016). In this study, both intra-polymer (Chapter 4) and inter-polymer synergism (Chapter 7) between enzymes will be evaluated in order to formulate an efficient enzyme cocktail for the degradation of various pre-treated hardwoods.

Chapter 2: Problem statement and Hypothesis

2.1. Problem identification

Hardwoods are regarded as an abundant renewable resource, which can be used as a feedstock for the production of value-added chemicals and biofuels via enzymatic conversion of polysaccharides into fermentable sugars. As already mentioned, hardwoods have many advantages as biofuel feedstocks, including their short rotation coppice periods accompanied by large biomass production (Rosso et al. 2013), their ability to adapt to different environments (Folch & Ferrer 2015) and their ease to delignify during pre-treatment compared to other feedstocks such as softwoods (Munoz et al. 2007; Zhao et al. 2014; Hu et al. 2015). However, the use of enzymes as biocatalysts in this field has been a concern due to the following problems:

- The cost of enzymes required for the hydrolysis of lignocellulosic biomass and the low enzyme activities on lignocellulosic biomass are bottlenecks for the commercialization of value-added product production from lignocellulosic biomass.
- Currently available commercial enzyme preparations are optimized for saccharification of agricultural grass residues and not hardwoods.

2.2. Hypothesis

A universal optimized synergistic enzyme cocktail, HoloMix, can effectively degrade various pre-treated hardwoods at a higher efficiency compared to currently available commercial enzyme cocktails for fermentable sugar production−thus decreasing the cost of value-added product production associated with enzymes.

2.3. Aims and Objectives

- Characterisation of cellulolytic and xylanolytic enzymes (physico-chemical properties, molecular properties, substrate specificity and product inhibition profiles);
- To establish synergistic associations between various cellulolytic and xylanolytic enzymes during the degradation of cellulose and xylan, respectively.
- Pre-treatment of hardwoods by sodium chlorite/acetic acid delignification and steam explosion, respectively;
- Compositional, morphological and chemical analysis of untreated and pre-treated hardwoods.

- To determine the inhibitory effects of pre-treatment by-products and additives on the optimised enzyme cocktails, CelMix and XynMix.
- To establish synergistic associations between CelMix and XynMix (HoloMix formulation) during the degradation of various pre-treated hardwoods.
- To compare the hydrolytic efficiency of the optimised cocktail, HoloMix, to commercially available enzyme cocktails during the degradation of various pre-treated hardwoods.

2.4. **Overview of Thesis**

The various holocellulolytic enzymes, both cellulolytic and xylanolytic, used in this study were characterised in terms of their physico-chemical properties, substrate specificities and product inhibition profiles as described in Chapter 3. The hypothesis that not only enzyme classifications (EC affiliations) of hydrolytic enzymes affect their synergy, but also their GH families and substrate specificities, was explored and developed in Chapter 4 during the formulation of the cellulolytic and xylanolytic enzyme cocktails. Pre-treatment of hardwoods by sodium chlorite/acetic acid delignification and steam explosion was conducted in Chapter 5. It was demonstrated that xylan may be more responsible for biomass recalcitrance than lignin, as the delignified biomass exhibited lower water holding capacities compared to those exhibited by steam exploded biomass. Chapter 6 of the thesis dealt with determining which potential products (released from leading pre-treatment technologies) would pose inhibitory effects on the activities of the optimised cellulolytic and xylanolytic core-enzyme sets during hardwood biomass degradation. The chapter also evaluated the use of additives, proteins and surfactants, for alleviating inhibitory effects of the pre-treatment by-products on the activities of the optimised core-enzyme sets during hardwood biomass degradation. Chapter 7 of the thesis dealt with optimizing a synergistic holocellulolytic binary cocktail using CelMix and XynMix (optimized in Chapter 4) during the hydrolysis of sodium chlorite/acetic acid delignified and steam exploded hardwoods. Chapter 8 of the thesis provides a general discussion, and highlights the conclusions and future perspectives regarding the work conducted in this study.

Chapter 3: Characterisation of various Glycoside Hydrolase (GH) family lignocellulolytic enzymes

3.1. Introduction

Wood is composed of about 50 % cellulose, 25 % lignin and 25 % hemicellulose (in the form of glucuronoxylan in hardwoods), with traces of pectin and proteins (Zhou et al. 2015; Willför et al. 2005; Moxley & Zhang 2007). This then necessitates the use of a consortium of lignocellulolytic enzymes, which include cellulolytic, xylanolytic and ligninolytic enzymes, to hydrolyse hardwoods into fermentable sugars that can be utilized for the production of value added products such as organic acids, alcohols, lipids, amino acids and vitamins (Kačík et al. 2012; Kaida et al. 2009; Verma & Dwivedi 2014).

The cellulolytic and xylanolytic enzymes are commonly known as glycoside hydrolases, classified into EC 3.2.1, these being enzymes responsible for hydrolysing glycosidic bonds in carbohydrates (www.cazy.org). It has been observed that organisms can host numerous closely related enzymes (same EC classification/enzyme activity, but different GH families), and this likely reflecting the evolutionary pressure to accommodate the subtle differences in the structure of the cell wall polysaccharides that occur both between species and within one organism in a way that reflects tissue differentiation (Tailford et al. 2009). A list of the major cellulolytic and xylanolytic enzymes required to degrade hardwoods into fermentable sugars are mentioned below.

The three classes of cellulases that are necessary for this enzymatic degradation system include endoglucanases (EGs, EC 3.2.1.4), cellobiohydrolases (CBHs, [CBHII] EC 3.2.1.91 and [CBHI] EC 3.2.1.176) and β-glucosidases (BGLs, EC 3.2.1.21) (Kim et al. 2014). EGs randomly cleave cellulose chains (amorphous regions) resulting in a decrease in the length of the cellulose chains (Ganner et al. 2012). CBHs, on the other hand, remove cellobiose residues from the resulting shortened cellulose chains from both reducing and non-reducing ends (Kostylev & Wilson 2012; Kostylev & Wilson 2014). Finally, BGLs cleave cellobiose into glucose and are said to prevent end product inhibition of upstream enzymes (such as CBHs) (Xiao et al. 2004).

The complete degradation of xylans requires the synergistic action of a consortium of glycoside hydrolase enzymes, including endo-xylanase (EC 3.2.1.8), β-xylosidase (EC 3.2.1.37), α-glucuronidase (EC 3.2.1.131) and α-arabinofuranosidase (EC 3.2.1.55), and accessory

enzymes, such as acetyl xylan esterase, ferulic acid esterase, and *p*-coumaric acid esterase (Biely et al. 2016). Endo-xylanases are responsible for randomly cleaving the xylan backbone into xylo-oligosaccharides in an endo-acting manner (Teplitsky et al. 2004; Rosa et al. 2013). Arabinose residues on the xylan backbone are then removed by α-arabinofuranosidases (Polizeli et al. 2005). The α-glucuronidases act on xylo-oligosaccharides which carry uronic acids on the non-reducing ends of xylopyranoside residues (Rosa et al. 2013; Jia et al. 2014). To complete xylan degradation, β-xylosidases are required, which cleave xylose residues from the non-reducing end of xylans and xylo-oligosaccharides in an exo-acting manner (Jordan & Li 2007).

In the present study, various GH lignocellulolytic enzymes were subjected to detailed comparative biochemical characterization, and analysed for substrate specificities and also product inhibition profiles.

3.2. **Aims and objectives**

3.2.1. **Aims**

To conduct comparative biochemical characterisation and substrate specificity studies on the lignocellulolytic (cellulo- and xylano-lytic) enzymes

3.2.2. **Objectives**

- To conduct SDS-PAGE and *in silico* characterisation of the lignocellulolytic enzymes (ProtParam);
- To determine the protein concentration of the lignocellulolytic enzyme preparations;
- To determine substrate specificities of the lignocellulolytic enzymes;
- To determine temperature optima of the lignocellulolytic enzymes;
- To determine temperature stability of the lignocellulolytic enzymes;
- To determine pH optima of the lignocellulolytic enzymes;
- To determine product inhibition parameters of the lignocellulolytic enzymes.

3.3. Materials and Methods

3.3.1. Materials

A xylosidase from *Selenomonas ruminantium* (SXA), a *Trichoderma longibrachiatum* cellobiohydrolase I (Cel7A, GH7), a microbial cellobiohydrolase II (Cel6A, GH6), a glucuronidase (AguA, GH67) from *Geobacillus stearothermophilus* and a *Geobacillus stearothermophilus* xylanase (XT6, GH10) were all purchased from Megazyme™. A glucosidase (Novozyme 188, Bgl1), endo-glucanase mixture from *Aspergillus niger* (EglA, EglB, EglC; GHs 12, 5 and 74) and xylanase from *Trichoderma viride* (Xyn2A, GH11) were all purchased from Sigma Aldrich. All the substrates (CMC, Avicel, *p*NPG, *p*NPX, *p*NPA, *p*NPC, tamarind xyloglucan, konjac glucomannan and RBBR-Xylan) were purchased from Sigma Aldrich, except for AZCL-Avicel, beechwood xylan, arabinoxylan (Wheat-flour), aldouronic acids mixture and 1,4-β-D-cellotetraitol which were purchased from Megazyme™.

3.3.2. SDS-PAGE and *In silico* characterisation of the GH lignocellulolytic enzymes

All the lignocellulolytic enzymes were diluted and then mixed in a 4 to 1 ratio with sodium dodecyl sulphate (SDS) sample buffer (50 mM Tris HCl (pH 6.8); 40% (w/v) glycerol; 3% (w/v) SDS; 0.14% (w/v) bromophenol blue; 5% (w/v) β-mercaptoethanol). The sample volumes of these mixtures were made up to 25 µL (with 0.1 mg/mL final protein concentration) and placed at 100°C for 3 min (Labnet AccuBlock™ digital dry bath). Approximately 16 µL of each protein sample was added to each well and separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970). A discontinuous gel composed of 10% resolving and 4% stacking gels was used. The 4% stacking gel consisted of the following: 6.1 mL of dH_2O, 1.3 mL of 30% (w/v) degassed acrylamide/Bis; 2.5 mL of 0.5 M Tris-HCl pH 6.8 and 0.1 mL of 10% (w/v) SDS; while the 10% resolving gel consisted of the following: 6.1 ml of dH_2O, 3.3 mL of 30% (w/v) degassed acrylamide/Bis; 2.5 mL of 1.5 M Tris-HCl pH 8.8 and 0.1 mL of 10% (w/v) SDS. In each case, the gel solutions were polymerized with the addition of 100 µL of 10% (w/v) ammonium persulphate (APS) and 50 µL of pure N,N,N',N'-tetramethylethylenediamine (TEMED). The polymerizing gel solutions were immediately poured to set between glass setting plates. Upon polymerization, the gel was placed in a Mini-Protein® 3 cell tank with SDS running buffer (25 mM Tris base; 192 mM glycine; 1% (w/v) SDS). A constant voltage of 150 V (Bio-Rad Power Pac™ Basic) was applied until the dye front had migrated to within 5 mm of the gel bottom. The gel was then removed from the glass setting plates and placed in a sealed plastic container for staining to be conducted. The staining procedure was conducted with Coomassie staining solution (0.1%

(w/v) Coomassie Brilliant Blue G250; 20% (w/v) methanol and 15% (w/v) glacial acetic acid) for 20 minutes. The gels were then destained with destain solution (45% (w/v) methanol and 10% glacial acetic acid). A gel imaging system (Uvitech – Uvipro chemi) was used to photograph the gel. With the confirmed presence of a visualized protein, the samples were subsequently measured to quantify the protein concentration.

From the Carbohydrate-Active Enzyme (CAZy) database (www.cazy.org), sequences of the lignocellulolytic enzymes were retrieved. Computation of various physical and chemical parameters of the given proteins were conducted using the ProtParam tool in the ExPasy Bioinformatics Resource Portal (Gasteiger et al. 2005).

3.3.3. **Protein concentration estimation**

The Bradford method was used to determine the protein concentration of the lignocellulolytic enzyme preparations (Bradford 1976). A standard curve for protein concentrations was constructed using bovine serum albumin (BSA) as the standard. For enzyme sample analysis, appropriate dilutions of solutions were made to produce protein content readings within the standard curve reading region. About 25 µL of each enzyme sample was added to 230 µL of Bradford's reagent. Sodium-citrate buffer (50 mM, pH 5.0) was used as a blank for each set of samples. After 10 minutes of shaking at room temperature, the absorbance values of the samples were measured at 595 nm with a Power Wavex Spectroquant using KC Junior software.

Where the bicinchoninic acid (BCA) method was used, the Working Reagent (WR) was prepared by mixing 50 parts of BCA Reagent A with 1 part of BCA Reagent B (50:1, Reagent A:B). Bovine serum albumin (BSA) was used as the standard (0.1 to 1 mg/mL). For enzyme sample analysis, appropriate dilutions of solutions were made to produce protein content within the standard curve reading region. About 25 µL of each enzyme sample was added to 230 µL of WR. The mixtures were then loaded into microtiter plates which were covered with foil and then incubated at 37^{o}C for 30 minutes before reading at 562 nm.

3.3.4. **Activity assays**

3.3.4.1. **Polymeric and oligomeric substrate based assays**

Where polymeric substrates were used, the total solution volume used in the standard activity assay was 400 µL. The reaction mixture was composed of 100 µL enzyme volume and 300 µL of 1.33% (w/v) substrate volume. A buffer control was used and acted as a blank for each sample set. Substrate and enzyme controls were also used whereby either the enzyme or the

substrate of the mixture volume was replaced by buffer to make up the respective volume. All reaction mixtures were kept at 37°C until the addition of enzyme and were assayed for 60 minutes at 37°C with tumbling at 25 rpm (Labnet Revolver™).

The assays were then centrifuged at 16, 060 *x g* (desktop centrifuge-Heraeus biofuge pico) for 5 min to pellet out insoluble substrates. A sample of 150 µL of prepared supernatant containing the unknown reducing sugar concentration was added to a sterile eppendorf tube with 300 µL DNS solution (1% (w/v) NaOH; 1 % (w/v) dinitrosalicylic acid; 20 % (w/v) sodium potassium tartrate; 0.2% (w/v) phenol and 0.05% (w/v) sodium metabisulphite). The samples were then heated to 100°C for 7 minutes (Labnet AccuBlock™ digital dry bath). Samples were then cooled on ice for a further 7 minutes. From this, 250 µL of solution supernatant was added to 96 well plates and the absorbance at 540 nm was measured (Power Wavex Spectroquant on KC Junior software). An appropriate regression equation from the established reducing sugar (either glucose or xylose) standard curve was used to convert absorbance values into reducing sugar concentrations.

Enzyme activity was measured in units (U), where 1 unit was defined as the amount of enzyme releasing 1 µmol of reducing sugar per minute under the specified assay conditions.

3.3.4.2. **Azo-dyed/chromogenic polymeric substrate based assays**

Where Azo-substrates were used, 0.8 mL of 95% (v/v) ethanol was added to a 0.2 mL reaction, reactions were kept for 5 minutes at room temperature then centrifuged at 16, 060 *x g* (desktop centrifuge-Heraeus biofuge pico) for 5 min to pellet out insoluble substrates. From this, 250 µL of solution supernatant was added to 96 well plates and the absorbance at 590 nm measured (Power Wavex Spectroquant on KC Junior software). An appropriate regression equation from the established Remazol Brilliant Blue R (RBBR) standard curve was used to convert absorbance values into relative reducing sugar concentrations. Enzyme activity was measured in units (U), where 1 unit was defined as the amount of enzyme releasing 1 µmol of reducing sugar per minute.

3.3.4.3. **Soluble chromogenic substrate based assays**

Where *p*NP-based substrates were used, the assay reaction mixture was in a ratio of 1:9 with 25 µL of appropriately diluted enzyme: 250 µL of 2.25 mM *p*NP-substrate in buffer. The reaction was conducted at 37°C for 15 minutes and terminated by the addition of 250 µL of 2 M sodium carbonate. The released *p*-nitrophenyl product was measured at 405 nm with a Power Wavex Spectroquant on Kc Junior software. *p*-Nitrophenol was used as a suitable standard.

Enzyme activity was measured in units (U), where 1 unit was defined as the amount of enzyme releasing 1 µmol of *p*-nitrophenol per minute

3.3.5. **Temperature optimum and thermo-stability determination**

The temperature optima of the lignocellulolytic enzymes were determined using a range of temperatures (30-90^{o}C) (Labnet AccuBlock™ digital dry bath) using a 50 mM sodium citrate buffer at pH 5.0. The activities of the backbone cleaving (endoglucanase and xylanases) and processive enzymes (CBH I) were determined using the DNS method. The activities of the side-chain cleaving glucuronidase and exo-enzymes (glucosidase and xylosidase) were determined using *p*NP substrates.

Thermal stabilities of the lignocellulolytic enzymes were also investigated over time at 37 and 50^{o}C using a 50 mM sodium citrate buffer at pH 5.0. Each enzyme was incubated at these different temperatures for selected time periods (0, 1, 3, 6, 12 and 24 hours) and the residual activity of each enzyme was determined as mentioned in the temperature optima studies.

3.3.6. **pH optimum determination**

The optimal pH for each lignocellulolytic enzyme was determined over a range of pH (pH 3.0-9.0) using 50 mM citrate-phosphate (McIlvaine) (pH 3.0-8.0) and 50 mM Tris-HCl buffer (pH 8.0-9.0). The enzyme assays were performed in triplicate at 37^{o}C (Labnet AccuBlock™ digital dry bath) using the protocols described in the sections above.

3.3.7. **Enzyme substrate specificity determination**

The substrate specificities of the lignocellulolytic enzymes were determined under standard assay conditions (pH 5.0 and 37^{o}C) as described in Section 3.3.4 with 2 mM *p*-nitrophenyl derivatives (*p*NPA, *p*NPX, *p*NPG, and *p*NPC), or 1% (w/v) beechwood xylan, wheat-flour arabinoxylan, arabinogalactan, konjac glucomannan, tamarind xyloglucan, aldouronic acids mixture, 1,4-β-D-cellotetraitol and/or 2% (w/v) CMC-Na and Avicel.

3.3.8. **Product inhibition studies**

Inhibition of the lignocellulolytic enzymes by the hydrolysis sugar products, glucose and xylose, was conducted at a concentration range of 2-100 mM for each sugar. The inhibition assays were set up as described in sections 3.3.4.2 and 3.3.4.3, with the substitution of buffer with the appropriate amount of sugar to reach the desired concentration (mM) in the particular reaction.

3.3.9. **Data analysis**

One way analysis of variance (ANOVA) was used to analyse the activity of the enzymes. All pairwise comparison procedures were based at 95% confidence level (p=0.05) using data analysis in Microsoft® Excel. Error bars represent standard deviations of triplicate values.

3.4. **Results**

3.4.1. ***In silico* characterisation and SDS-PAGE analysis of holocellulolytic enzymes**

In silico characterization of various physical and chemical parameters using sequences for the given proteins was conducted using the ProtParam tool in the ExPasy Bioinformatics Resource Portal (Gasteiger et al. 2005). Table 3.1 below displays the results obtained from sequence analysis of each lignocellulolytic enzyme using ProtParam.

Table 3.1. Data obtained from Uniprot, Protein Data Bank, Megazyme and ProtParam for the lignocellulolytic enzymes used in this study. "-" unknown.

Name (PDB ID/Uniprot ID/Supplier)	Sequence length (aa)	Mass (kDa)	pI
Cellobiohydrolases			
Cel7A (Megazyme)	-	65	-
Cel6A (Megazyme)	-	42.3	4.7
Endoglucanases			
EglA (PDB 1KS4/5)	239	25.9	4.49
EglB (O74706)	331	36.5	4.36
EglC (Q8TFP1)	857	90.3	4.31
Xylosidase			
SXA (O52575)	538	61.1	5.4
Xylanases			
XT6 (PDB 1HIZ)	379	43.8	6.84
Xyn2A (Q7M519)	190	20.7	8.75
Glucosidase			
Novozyme 188, Bgl1 (Q9P8F4)	860	93.3	4.7
Glucuronidase			
AguA (PDB 1K9D)	679	78.5	5.47

Any annotated post-translational modifications were not taken into account

A majority of the enzymes selected for this study either had their 3D structures solved and available in the RCSB Protein Data Bank (PDB) (http://www.rcsb.org/) or had been biochemically characterized before with their sequence data available in Uniprot (http://www.uniprot.org/) – except for Cel6A, Cel7A and XT6.

The purity and molecular weights of the commercial cellulolytic and xylanolytic enzymes used in this study were assessed using SDS-PAGE. Upon observation, a majority of these commercial proteins were found to be mostly single bands, confirming their purity, except for Bgl1, Egl and XT6, which presented bands other than the anticipated ones (see Figure 3.1).

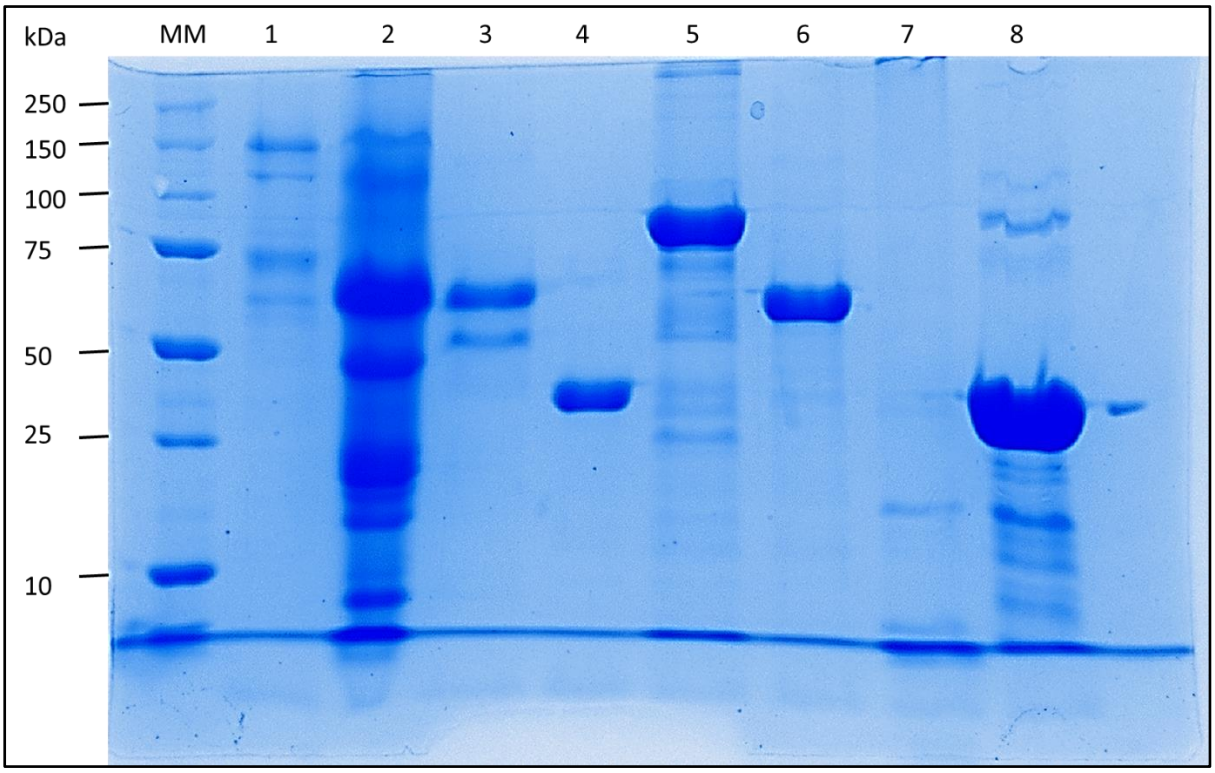

Figure 3.1. SDS-PAGE analysis of commercial lignocellulolytic enzymes. Lane MM: Molecular marker (BIO-RAD Precision Plus Protein Standards 1610363); Lane 1: Bgl1; Lane 2: Egl; Lane 3: Cel7A; Lane 4: Cel6A and Lane 5: AguA; Lane 6: SXA; Lane 7: Xyn2A and Lane 8: XT6.

As estimated from SDS-PAGE using a calibration curve derived with BIO-RAD Precision Plus Protein Standards 1610363 (Appendix B. 4), the molecular masses of the commercial holocellulolytic enzymes were estimated to be: 73 kDa for *A. niger* Bgl1, 63.1 kDa for *T. longibranchiatum* Cel7A, 41.6 kDa for the microbial Cel6A, 20.1 kDa for *T. viride* Xyn2A, and for *G. stearothermophilus* T6 AguA and XT6, the molecular masses were 79.4 kDa and 39.8 kDa, respectively. For *A. niger* endoglucanase (Egl), three prominent bands at 26, 48 and 64 kDa were observed, corresponding to EglA, EglB and EglC, respectively. The *G.*

stearothermophilus T6 xylosidase, SXA, displayed a molecular mass of 61.9 kDa. It is noteworthy to mention that the sizes of the proteins as determined by SDS may be different from those estimated by Protparam as any annotated post-translational modifications on the proteins were not taken into account by the tool.

3.4.2. **Protein content estimation**

The protein concentrations were determined using the Bradford protein assay (Bradford 1976). The protein concentration of each lignocellulolytic enzyme is expressed in Table 3.2 below.

Table 3.2. Determination of the protein concentrations of the commercial lignocellulolytic GH enzymes (Bradford assay).

Lignocellulolytic enzyme	Protein concentration (mg/mL)
XT6	20.65
Xyn2A	17.5*
SXA	5.73
Cel7A	3.18
Cel6A	4.00
Egl	2.70
Bgl1	57.10
AguA	12

*Where * represents protein content of the sample estimated using the BCA Method*

Using the Bradford method, the protein concentration of each lignocellulolytic enzyme was shown to be similar to the amount reported by the suppliers in their product specification sheets, except for Xyn2A which appeared to be less than what was reported by the suppliers (15 mg/mL protein) and the BCA method had to be used to determine the protein content.

3.4.3. **Temperature optimum and thermo-stability determination**

Temperature optimum evaluation of each lignocellulolytic enzyme was assessed using 50 mM sodium citrate buffer (pH 5.0). Figures illustrating the various optima and stability temperature results for each the lignocellulolytic enzymes are shown in Fig 3.2 and Fig 3.3, respectively.

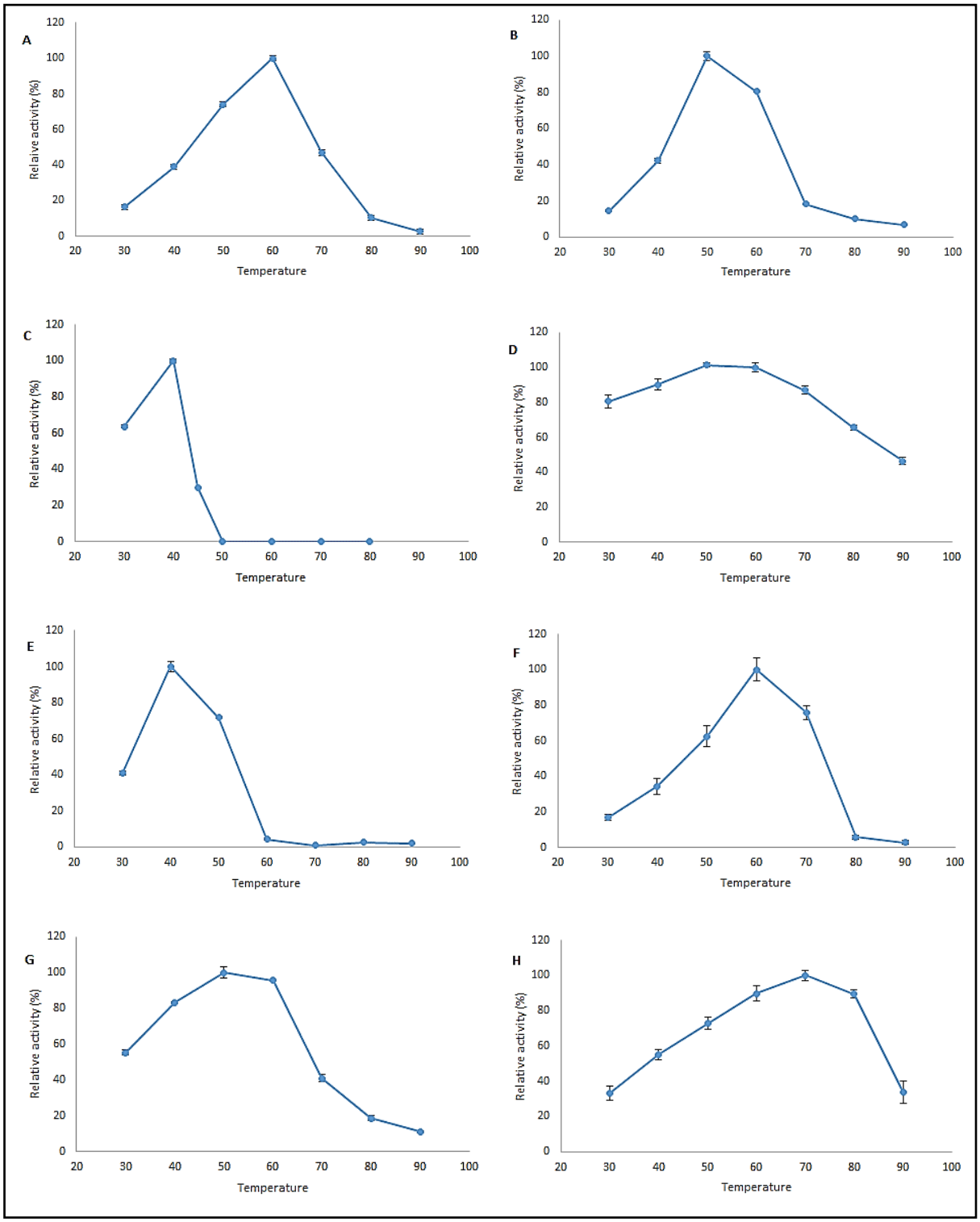

Figure 3.2. Temperature optima profiles of the lignocellulolytic enzymes; (A) Bgl1, (B) Cel7A, (C) Cel6A, (D) Egl, (E) SXA, (F) AguA, (G) Xyn2A and (H) XT6, determined using a 50 mM sodium citrate buffer (pH 5.0).

Among the enzymes assessed, Cel6A and SXA demonstrated the lowest temperature optimum of 40^{o}C. Cel7A, Egl and Xyn2A displayed optimal activity at a temperature of 50^{o}C, while

Bgl1 and AguA exhibited temperature optima values of 60°C. Among the enzymes, only XT6 exhibited a temperature optimum of 70°C, which was the highest optimum. Temperature stability studies for each enzyme were conducted and their results are shown in Fig 3.3 below.

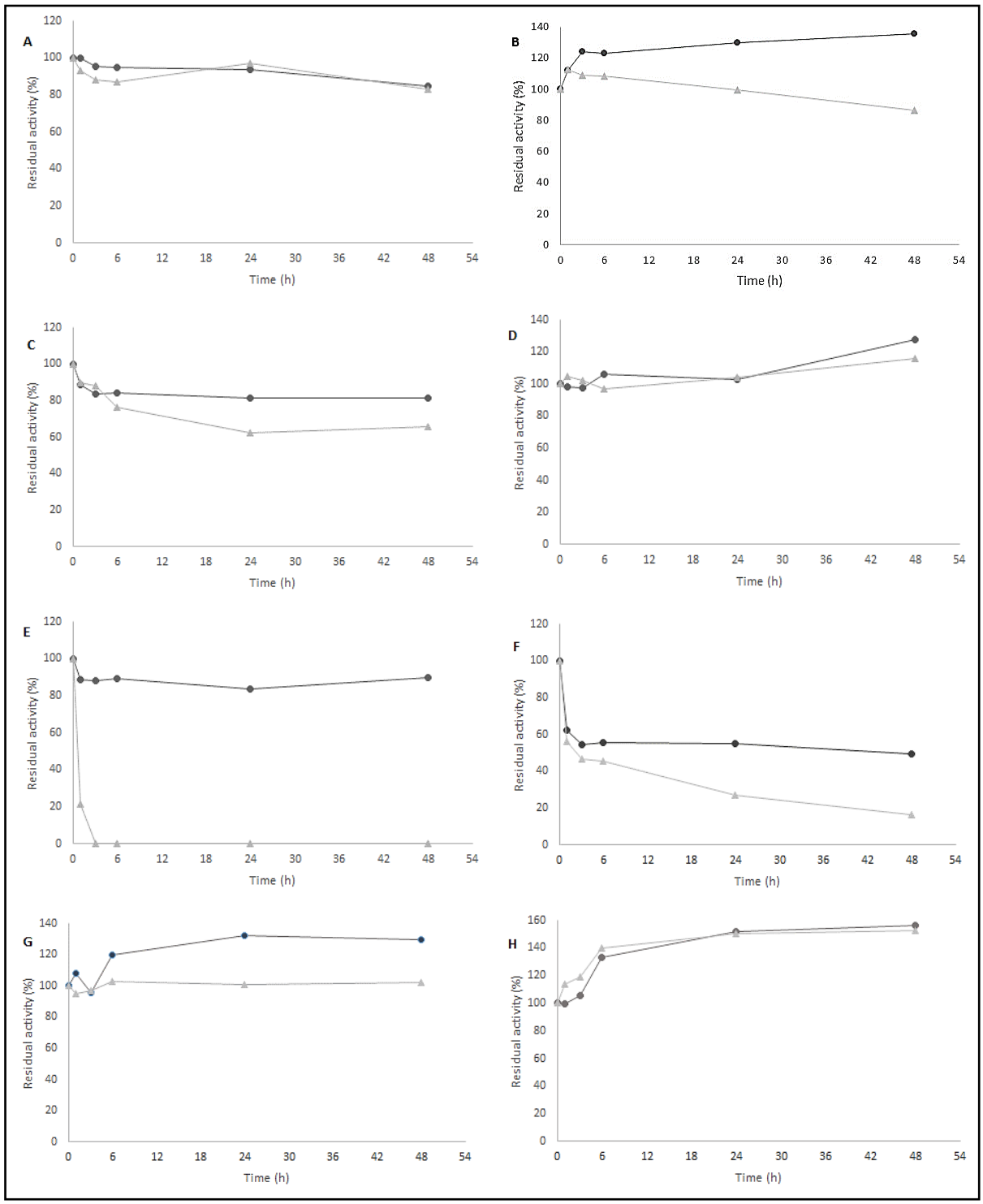

Figure 3.3. Temperature stability profiles of the lignocellulolytic enzymes; (A) Bgl1, (B) Cel7A, (C) Cel6A, (D) Egl, (E) SXA, (F) AguA, (G) Xyn2A and (H) XT6, determined using 50 mM sodium citrate buffer (pH 5.0) at 37 (●) and 50°C (Δ) over a 48 hour period.

Among the enzymes assessed, SXA demonstrated the least temperature stability at 50^{o}C, with all of its activity being completely abolished after 3 h incubation at this temperature. The enzyme was highly stable at 37^{o}C with retention of over 80% of its activity up to 48 h. Cel6A was more stable at 37^{o}C, compared to 50^{o}C, with activity retention of over 80% and 60%, respectively, up to 48 h. Cel7A and Egl retained 100% of their activities at both 37 and 50^{o}C up to 48 h. Cel6A was also fairly stable with over 60% of its residual activity retained after over 48 h at both 37 and 50^{o}C. AguA, in contrast, exhibited low thermostability among the CAZymes assessed, displaying 50% residual activity at 37^{o}C and 20% residual activity at 50^{o}C, respectively. Xyn2A displayed activation of activity when incubated at 37^{o}C, while XT6 displayed activation at both 37 and 50^{o}C up to 48 h. Bgl1 on the other hand was fairly stable at both 37 and 50^{o}C with retention of over 80% of its activity up to 48 h.

3.4.4. **pH optimum determination**

The pH optimum of each the lignocellulolytic enzymes was evaluated using sodium citrate/phosphate buffer in the pH range of 3 to 9 (Fig 3.4). The reactions were conducted at 37^{o}C using the standard activity assay parameters described in Sections 3.3.4 and 3.3.6.

The various cellulolytic (Bgl1, Cel6A, Cel7A and EglA) and xylanolytic enzymes (AguA, SXA, Xyn2A and XT6) selected for use in this study were evaluated with regards to their pH optima profiles, and they exhibited pH optima around values in the range of pH 4.0 to 7.0 (see Fig 3.4 below). Bgl1, Cel7A and Cel6A particularly demonstrated pH optima of 4.0, while EglA, Xyn2A and XT6 demonstrated pH optima values of 6.0, while SXA and AguA separately displayed pH optima values of 5.0 and 7.0, respectively.

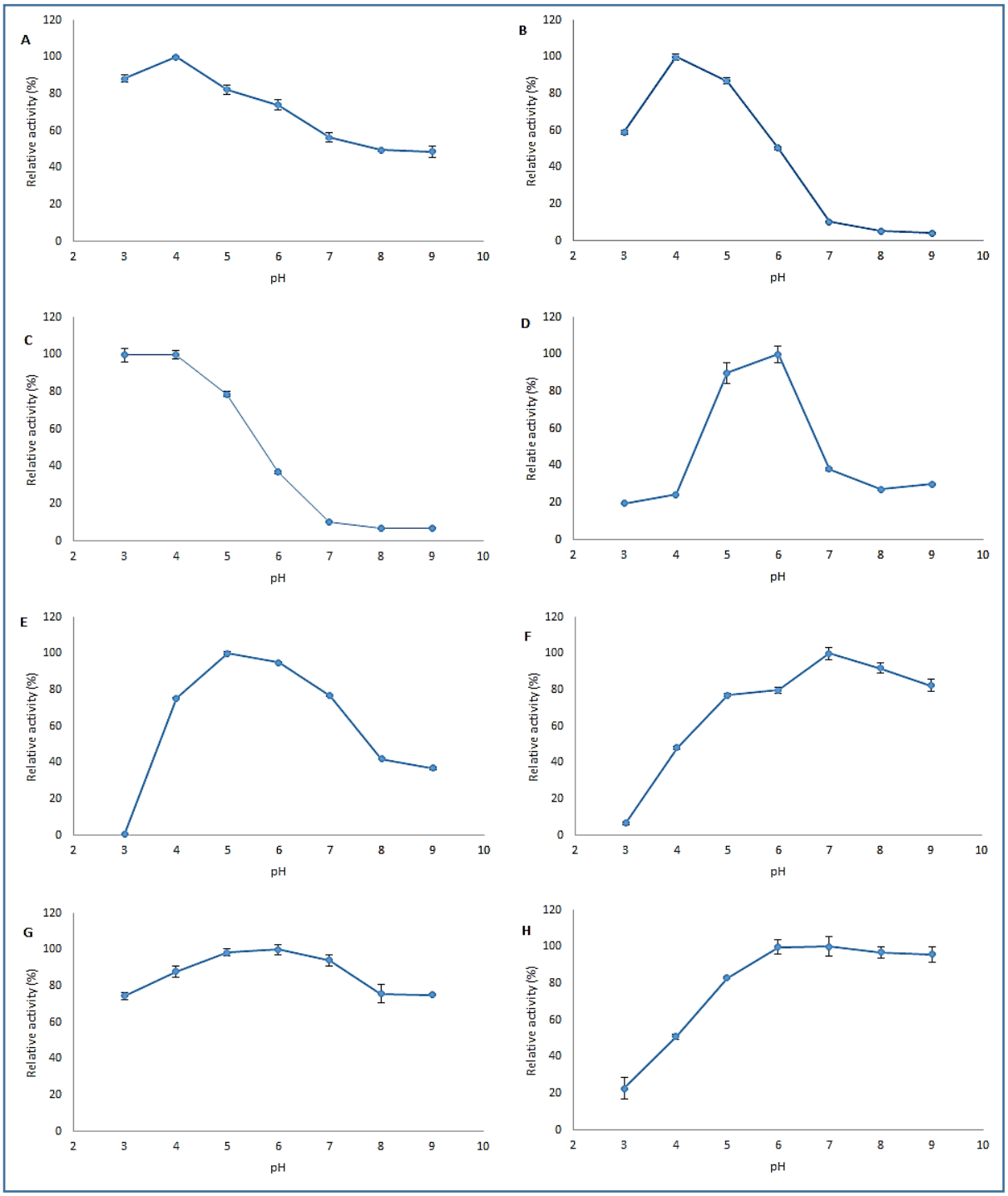

Figure 3.4. pH optima profiles of the lignocellulolytic enzymes; (A) Bgl1, (B) Cel7A, (C) Cel6A, (D) Egl, (E) SXA, (F) AguA, (G) Xyn2A and (H) XT6, determined at 37°C with 50 mM McIlvaine buffer (pH 3-8) and 50 mM Tris-HCl buffer (pH 8-9).

Generally, all the lignocellulolytic enzymes evaluated in this study retained over 80% of their activity in a pH range of 4.0 to 6.0.

3.4.5. **Enzyme substrate specificity determination**

The enzymes were tested for their specific activities on different substrates at 37^{o}C using 50 mM sodium citrate buffer at pH 5.0 (Table 3.3). AguA was the only enzyme that showed significant activity towards aldouronic acids for α-glucuronidase activity. The enzyme also showed appreciable activity on arabinoxylan and beechwood glucuronoxylan; we propose that the activity on arabinoxylan demonstrated by the enzyme could be due to its partial arabinofuranosidase activity (2 U/mg) which was shown on *p*NPA. Bgl1 showed the highest activity on *p*NPG (53.2 U/mg), with minimal to no activity on polymeric substrates, and was therefore assumed to be exhibiting β-glucosidase-like catalytic properties. As expected, the xylosidase, SXA, showed the highest activity (155.6 U/mg) on *p*NPX. The enzyme also showed appreciable activity on arabinogalactan and we therefore propose that the activity on arabinogalactan demonstrated by the enzyme could be due to its partial arabinofuranosidase activity (19.8 U/mg) which was shown on *p*NPA.

As expected, Cel7A was the most active enzyme (1.76 U/mg) on *p*NPC, while Cel6A showed its highest activity (0.5 U/mg) on cellotetraitol. The endo-glucanase mixture, Egl, showed very high activity on beechwood xylan, CMC, glucomannan and xyloglucan. This suggested that Egl had a broad catalytic versatility on lignocellulosic polysaccharides. As expected, both family 10 and 11 endo-xylanases, Xyn2A and XT6, were able to effectively hydrolyze the xylan substrate, beechwood glucuronoxylan, while the other enzymes showed very low or undetectable levels of activity on this substrate. Xyn2A showed substantially higher hydrolytic activity (258.4 U/mg) on the beechwood xylan substrate than did XT6 (141.8 U/mg).

Table 3.3. Specific activities (U/mg protein) of the various GH lignocellulolytic enzymes on various defined lignocellulosic substrates. "Nd" = not detected.

Lignocellulosic substrate	**Putative activity**	**Lignocellulolytic enzyme assessed**							
		AguA	**Bgl1**	**SXA**	**Cel7A**	**Cel6A**	**Egl**	**Xyn2A**	**XT6**
*p*NPA	Arabinofuranosidase	2	1.1	19.8	0.1	0.1	0.2	2.1	2.1
*p*NPC	Cellobiohydrolase I	0.2	0.3	0.7	1.76	Nd	0.4	0.5	3.1
*p*NPG	Glucosidase	0.2	53.2	0.7	Nd	Nd	4.9	0.5	0.3
*p*NPX	Xylosidase	0.2	0.4	155.6	Nd	Nd	0.1	0.4	0.4
Aldouronic acids	Glucuronidase	5.7	Nd	Nd	Nd	Nd	Nd	Nd	Nd
Arabinogalactan	Galactanase	0.1	1.55	6.64	0.37	0.4	11.7	0	0
Avicel	Total Cellulase	0.16	0	0	0.26	0.1	11.3	0	0
Beechwood xylan	Endo-xylanase	3.79	0	3.9	0.61	0.1	28.6	258.4	141.8
Cellotetraitol	Cellobiohydrolase II	Nd	Nd	Nd	Nd	0.5	Nd	Nd	Nd
CMC-Na	Endo-glucanase	0.28	0	0.4	0.7	0.1	36.3	0.5	0
Glucomannan	Endo-mannanase	0	0	0	0.4	0.3	32.8	0	0
Arabinoxylan	Endo-xylanase	7.1	0	6.65	1	0.1	32.8	502.1	273.6
Xyloglucan	Xyloglucanase	1.1	0	0.86	1	Nd	39.1	0	14.2

3.4.6. **Product inhibition studies**

Inhibition of the various GH lignocellulolytic enzymes by D-glucose and D-xylose was conducted over a concentration range of 0-100 mM for each of the sugar products. The figure below (Fig 3.5) indicates the tolerance levels of each enzyme to the two sugar products.

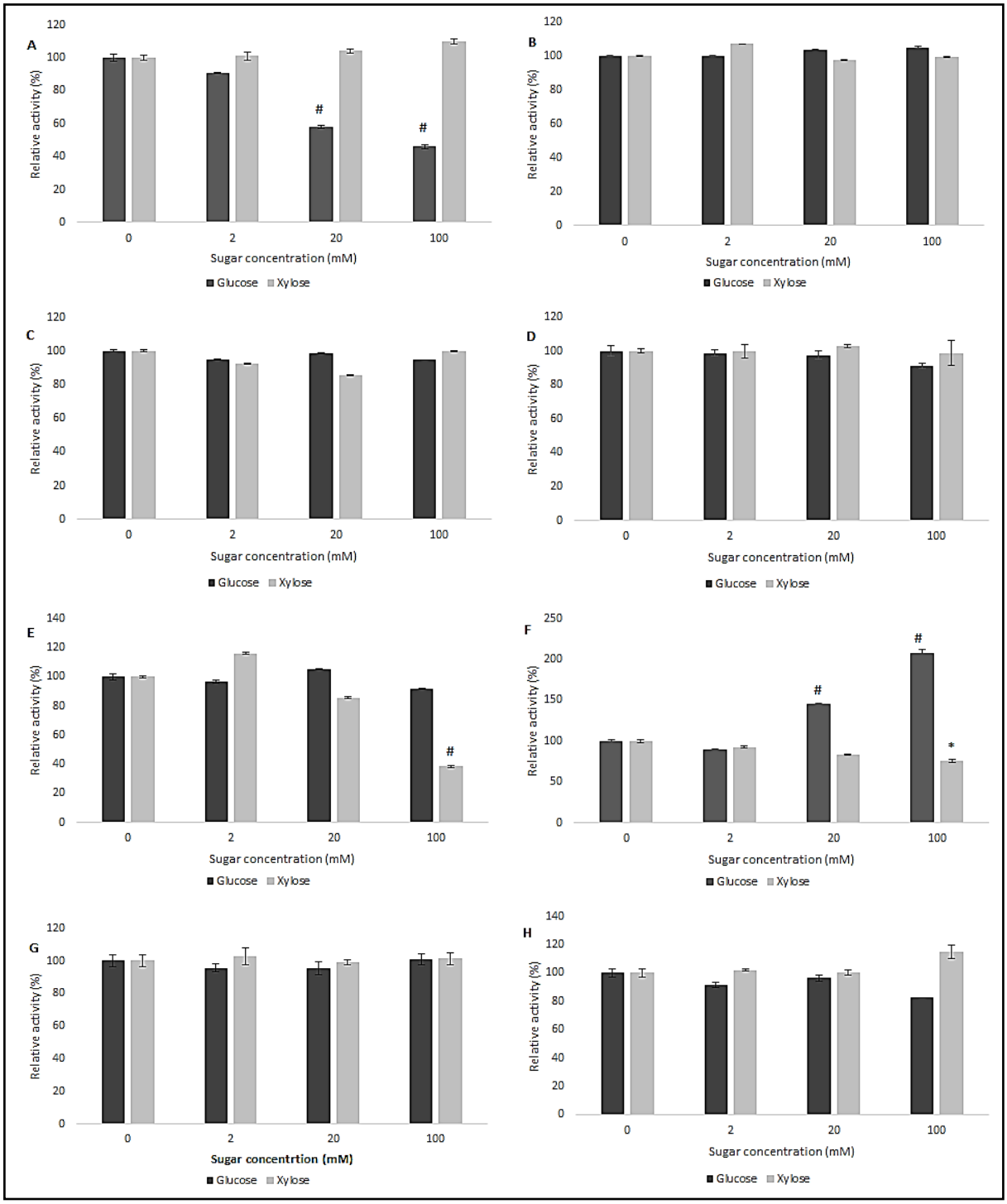

Figure 3.5. Tolerance of the lignocellulolytic enzymes; (A) Bgl1, (B) Cel7A, (C) Cel6A, (D) Egl, (E) SXA, (F) AguA, (G) Xyn2A and (H) XT6, to glucose and xylose at various concentrations (0-100 mM). ANOVA analysis of inhibition or activation of enzymes by the different sugars was compared to controls containing 0 mM sugar, test keys; * (p value<0.05) and # (p value<0.01).

3.5. **Discussion**

3.5.1. **SDS-PAGE and *in silico* characterisation of holocellulolytic enzymes**

Based on SDS-PAGE analysis, Bgl1 has a molecular mass of 73 kDa – this size is similar to that of the same enzyme purified from a commercial preparation, SPP188 (75 kDa), by Chauve et al. (2010) and that expressed in *A. niger* (60 kDa) by Farinas and co-workers (Chauve et al. 2010; Farinas et al. 2010). In our study, the endoglucanase preparation showed three distinct bands which we anticipated to belong to the three endoglucanase isoforms; EglA, EglB and EglC expressed by *A.niger*. A 26 kDa protein was reported in this study and we suggest that this is EglA which Quay et al. (2011) reported to be 30 kDa and Dobrev and Zhekova (2012) to be between 26.9-28.8 kDa (Quay et al. 2011; Dobrev & Zhekova 2012). For EglB, the molecular mass (48 kDa) reported in this study was similar to that reported for the same enzyme (51 kDa) by other researchers (Li et al. 2012). We suspect the 64 kDa band on the endoglucanase preparation reported in this work to be the xyloglucan-specific endo-β-1,4-glucanase, EglC, which is reported to be 90 kDa when fused with a CBD (Hasper et al. 2002).

In our study, a molecular mass of 63.1 kDa was reported for *T. longibranchiatum* Cel7A. This is in agreement to a size (67 kDa) reported for its homologs originating from *T. viride* T100-14 Cel7A (Zhou et al. 2008), *Talaromyces emersonii* (Voutilainen et al. 2010) and *T. reesei* (Wei et al. 2014). The reported Cel6A molecular mass (45 kDa) in our study was similar to that reported for *T. viride* T100-14 Cel6A (Zhou et al. 2008). Our Cel6A also had similar molecular weight and pI values to those reported for *Chrysosporium lucknowense* CBHII (42 kDa and pI 4.2) (Bukhtojarov et al. 2004).

The *G. stearothermophilus* T6 glucuronidase, AguA, exhibited a molecular mass of 79.4 kDa for its homodimers, and this was comparable to molecular masses (77.3 kDa and 75 kDa) of other GH67 glucuronidase homodimers from *Paenibacillus curdlanolyticus* B-6 (Septiningrum et al. 2015) and from *Pseudomonas cellulosa* (Nurizzo et al. 2002), respectively. The xylosidase, SXA, exhibited a molecular mass of 61.9 kDa, which was similar to the value (61.1 kDa) reported in previous work on the same enzyme (Jordan & Li 2007). The pI and molecular mass of Xyn2A (pI 8.75 and 20.1 kDa) reported in our study coincided with those reported by Ujiie et al. (1991) (pI 9.3 and 22 kDa) for the same enzyme. Similarly, Silva et al. (2015) reported XylII from *Trichoderma inhamatum* to have molecular mass of 21 kDa (Silva et al. 2015). The molecular mass of the xylanase, XT6 (39.8 kDa), reported in this study was similar to the molecular mass (43 kDa) reported previously for the same enzyme (Teplitsky et al. 2004).

3.5.2 **Physico-chemical properties of holocellulolytic enzymes**

The temperature optima of the *A. niger* Bgl1 used in this study was similar to that (60°C) exhibited by the same enzyme in a report by Chauve and co-workers (Chauve et al. 2010). Our Bgl1 was more thermo-stable than the solid-state fermentation expressed and purified version reported by Junior et al. (2014). Possible reasons for lowered stability of their Bgl1 could be due to the fact that, upon purification, the protein concentration was lowered and did not contain other proteins and preservatives which may have been found in the Bgl1 containing Novozyme 188 commercial preparation (Junior et al. 2014). Contrary to other findings for Cel6A homologs, the microbial Cel6A characterized in this study exhibited pH and temperature optima values of pH 4.0 and 40^{o}C, while Bukhtojarov and co-workers reported pH and temperature optima values of 4.8 and 60^{o}C, respectively, for *Chrysosporium lucknowense* Cel6A (Bukhtojarov et al. 2004).

Similar to our *T. longibranchiatum* Cel7A, Cel7A from *Humicola grisea* var. *thermoidea* exhibited pH and temperature optima of 5.0 and 60^{o}C, respectively (Takashima et al. 1999), while Cel7A from *T. emersonii* displayed a temperature optimum value 60-65^{o}C (Voutilainen et al. 2010). The temperature optimum (50-60^{o}C) exhibited by Egl preparation used in our study coincided well with the optimal temperature for EglA (50-65^{o}C) reported by Quay et al. (2011) and Dobrev and Zhekova (2012), respectively, and EglB (60^{o}C) reported by Li et al. (2012). In this study, Egl displayed a pH optimum of 6.0, and this was similar to that of a recombinant EglB reported by Li et al. (2012). Similar to our *A. niger* derived endoglucanase preparation, a wild-type strain of *A. niger* expressed an endoglucanase which retained 100% of its activity after 24 h at 37^{o}C. However, only about 55% activity was retained at 50^{o}C for the same duration (Florencio et al. 2016).

The xylosidase, SXA, lost about 40% of its activity after incubation for 60 min at either 37 or 50^{o}C. Interestingly, Jordan et al. reported a loss in SXA activity after 60 min when the enzyme was incubated at temperatures beyond 50^{o}C. With respect to its pH profile, SXA exhibits a favourable pH optima (nearly constant *k*cat between pH 4.5 and 6.0) (Jordan et al. 2007; Jordan & Li 2007). In this study, AguA exhibited a temperature and pH optima of 60^{o}C and pH 7.0– similarly, researchers reported on the same enzyme exhibiting a temperature optimum of 65^{o}C (Teplitsky et al. 2004; Golan et al. 2004). Similar to our AguA from *G. stearothermophilus* T6 reported in this study, Agu115A from *Amphibacillus xylanus* exhibited a temperature and pH optima of 55^{o}C and pH 7.0 (Yan et al. 2017).

The pH and temperature optima of the *T. viride* Xyn2A used in this study (pH 6.0 and 50^{o}C) were similar to those reported by Ujiie et al (1991) for the same enzyme from the same species and for an isoform from *T. harzianum* (Ujiie et al. 1991). Interestingly, Xyn2A was highly thermostable with over 90% activity retention after 24 hours at both 37 and 50^{o}C – more so than XylI and XylII which retained only 80% activity at 40^{o}C and less than 30% at 50^{o}C, respectively (Silva et al. 2015). Similar trends with respect to pH and temperature optima profiles were observed for XT6 in this study (which was most active at pH 6.0 and 70^{o}C), compared to that reported by Khasin and co-workers, which was most active in the neutral pH range, between pH 6.5 and 7.0 and at 75^{o}C (Khasin et al. 1993). Exposure of XT6 for 24 h at 50^{o}C did not inactivate the enzyme. Similarly, Khasin et al. (1993) reported that exposure of the enzyme for more than 10 h at 65°C did not affect its activity.

With respect to reaction parameters, the majority of the lignocellulolytic enzymes evaluated in this study generally retained fairly appreciable activity at pH 5.0 and 50^{o}C, except for SXA and AguA, which demonstrated low thermo-stability at this temperature. These are the standard saccharification conditions applied in industry (Van Dyk & Pletschke 2012).

3.5.3. **Enzyme substrate specificities of holocellulolytic enzymes**

Similar to Egl evaluated in this study, the highest activity by EglB was detected on barley β-glucan, followed by CMC, while laminarin, xylan from beechwood, chitin, and salicin were slightly degraded by the enzyme (Li et al. 2012). On the other hand, EglA was reported to exhibit its highest activity only on barley β-glucan and CMC (Quay et al. 2011). The high xyloglucanase activity exhibited by the endoglucanase preparation used in this study confirmed the presence of the of the xyloglucan-specific endo-β-1,4-glucanase, EglC, as reported by Hasper and others (Hasper et al. 2002).

In our study, Cel7A exhibited activity on Avicel (1.76 U/mg), negligible activity against CMC and was inactive on *p*NPG. As expected, Cel7A was the most active enzyme on *p*NPC. The results were similar to what was found by Tuohy et al. (2002), who determined and reported that *T. emersonii* Cel7A was inactive against CMC and *p*NPG (Tuohy et al. 2002). Zhou et al. (2008) also reported that the Cel7A enzyme produced by *T. viride* T100-14 also showed minimal to no activity on CMC and *p*NPG (Zhou et al. 2008). The microbial cellobiohydrolase II, Cel6A, showed the highest activity on the oligosaccharide, cellotetraitol (0.5 U/mg), and negligible activity (~0.1 U/mg) on the amorphous and crystalline cellulose substrates, CMC and Avicel, respectively. Since hydrolysis of crystalline cellulose (such as Avicel) is an

identified characteristic of cellobiohydrolases (Bukhtojarov et al. 2004; den Haan et al. 2013), the low activity of our Cel6A on Avicel was rather unexpected. However, the lowered activity of Cel6A on CMC was anticipated as cellobiohydrolases prefer crystalline cellulose. Interestingly, the microbial Cel6A used in this study did not display activity on *p*NPC while the *T. viride* T 100-14 Cel6A exhibited 5.7 U/mg on the same substrate (Zhou et al. 2008).

In our study, a mixture of aldotriouronic, aldotetraouronic and aldopentaouronic acids (2:2:1) was used to assess AguA activity. However, it was reported that glucuronidases display similar catalytic efficiencies on aldouronic acids with a DP length of 3-5 (Nagy et al. 2002; Nurizzo et al. 2002). The enzyme, AguA, also showed appreciable activity on beechwood glucuronoxylan. Interestingly, Nagy et al. (2002) and Nurizzo et al. (2002) noted that the enzyme does not release 4-*O*-methyl-glucuronic acid from glucuronoxylan. This observation may be due to the fact that AguA is reported to release methyl glucuronic acid only from xylo-oligosaccharides, where the methyl glucuronate is bound to the non-reducing end of the oligosaccharide (Rosa et al. 2013). Jordan and others similarly reported that SXA was 10-fold more active on *p*NPX compared to *p*NPA (Jordan & Li 2007).

In summary, all the evaluated lignocellulolytic enzymes generally exhibited their expected activities. With the diversity of activities demonstrated by these enzymes on various polysaccharides and their oligomers, we were hopeful that synergistic associations could be established between these enzymes during complex lignocellulosic biomass degradation.

3.5.4. **Product inhibition of holocellulolytic enzymes**

Many studies have demonstrated that product inhibition of lignocellulolytic enzymes during saccharification is one of the important causes of decreased hydrolysis rate during this process (Murphy et al. 2013). Here, we evaluated each individual enzyme instead of mixed enzyme systems as this approach provides a guide to allow quantification of which sugar may inhibit which enzyme and by how much.

Bgl1 was only inhibited in the presence of glucose in a concentration dependent fashion and not by xylose. Similarly, Xiao et al. (2004) demonstrated that a glucosidase (Novozym 188®) was not inhibited by xylose, however, glucose significantly inhibited this enzyme (Xiao et al. 2004). Glucose did not elicit SXA inhibition; however, SXA was inhibited by the presence of xylose in a concentration dependent manner. Watanabe and co-workers have also similarly demonstrated (with a *Corynebacterium glutamicum* β-xylosidase) that these enzymes are generally inhibited by xylose and not glucose (Watanabe et al. 2015). The cellobiohydrolases,

endoglucanases and xylanases were not significantly inhibited by the monosaccharides, glucose and xylose, at all concentrations evaluated. Previous studies have reported on these enzymes being highly tolerant to such products (Hsieh et al. 2014), with others reporting IC_{50} values ≥200 mM for cellobiohydrolases and endoglucanases (Teugjas & Väljamäe 2013), while others reported enzyme activation by these sugars (Paul & Varma 1990). According to our knowledge, there are no reports in literature with regards to α-glucuronidase inhibition studies using sugar monomers. For the first time we have reported on the inhibition of α-glucuronidase, AguA, by xylose at high concentrations and its concentration dependent activation in the presence of glucose. Several studies have reported on the activation of GH enzymes by sugar monomers, such as the activation of *Cellulomonas mixtus* β-mannosidase (Man5A, GH5) in the presence of mannose (Malgas et al. 2016).

Our results show that the presence of monosaccharides does not affect exo- and endo-acting enzymes equally, with the former (Bgl1 and SXA) being more susceptible to inhibition. It is likely that the mechanisms of sugar product inhibition depend on both the characteristics of the specific sugar and the structures of the different enzymes. As reported by other researchers such as Chauve and co-workers (2010), glucose inhibition can have a deleterious effect in a bioethanol production process, in which high sugar and accordingly high ethanol concentrations are expected. This therefore calls for an investigation into strategies to mitigate the effect of product inhibition on enzymes in order for high saccharification yields to be realised. With AguA displaying catalytic activation in the presence of glucose, prospecting for novel enzymes displaying such features in order to be added in enzyme cocktails may be the next step required to effectively break down lignocellulose, thus leading to the realisation of cost effective production of biofuels from lignocellulosic biomass.

3.5.5. **Summary of physico-chemical properties of holocellulolytic enzymes**

In summary, the results obtained from the biochemical characterisation of the cellulolytic and xylanolytic enzymes indicated the following and are illustrated in Table 3.4 below:

Table 3.4. Comparison of the properties of the cellulolytic and xylanolytic enzymes used in this study

Enzyme	Mw (kDa)	pH_{opt}[a]	T_{opt}[b] (°C)	Thermal stability τ [c]	GH Family
AguA	78.5	7	60	>3h, 50°C	67
Bgl1	93.3	4	60	>24 h, 50°C	3
Cel6A	42.3	3-4	50	>24 h, 50°C	6
Cel7A	65	4	50	>24 h, 50°C	7
Egl	26, 36.5, 90.3	6	50-60	>24 h, 50°C	5, 12, 74
SXA	61.1	5	40	>1h, 50°C	43
Xyn2A	20.7	6	50	>24 h, 50°C	11
XT6	43.8	6	70	>24 h, 50°C	10

[a] pH_{opt}: optimum pH. [b] T_{opt}: optimum temperature. τ [c]: thermal stability half-life.

The glycoside hydrolases displayed maximal activity in the pH and temperature ranges 4-6 and 50-60°C, with a majority of these enzymes displaying half-lives which were greater than 24 hours at 50°C.

3.6. **Conclusions**

A consortium of cellulolytic (Bgl1, Cel6A, Cel7A and EglA) and xylanolytic (AguA, SXA, Xyn2A and XT6) enzymes required for efficient degradation of cellulose and glucuronoxylan, respectively, were successfully characterised with respect to both their physico-chemical characteristics and substrate specificities. Based on their biochemical properties, all the assessed enzymes appear to perform reasonably well at operating conditions of pH 5.0 and 50^{o}C, except for Cel6A which lost activity above 40^{o}C. The characterization of each holocellulolytic enzyme also led to an improved understanding of the optimal conditions of its activity and substrate specificities. Numerous enzymes appeared not to exhibit cross activity with others on defined substrates, except for Xyn2A and XT6. This is preferable to avoid substrate competition during biomass degradation by the consortium of hydrolytic enzymes. This data facilitated a better understanding and interpretation of the synergy and interactions between these enzymes on model (Avicel and beechwood xylan) and complex (Acacia and Poplar) substrates in the following chapters.

Chapter 4: Evaluation of synergism between various cellulolytic and hemicellulolytic enzymes on model substrates

4.1. Introduction

Lignocellulosic biomass is a complex network of polymers making up the cell walls of plants. Thus it requires various enzymes to degrade the different polysaccharides into their respective monomeric sugar residues. Glycoside hydrolases form a core part of this arsenal of enzymes required for lignocellulose degradation, and are secreted by a variety of organisms, including wood-degrading fungi, higher eukaryotes and bacteria, for polysaccharide utilisation. These glycoside hydrolases (GHs) are diverse in nature and include cellulose, xylan, mannan, pectin, chitin, chitosan and starch degrading enzymes. For ease in classification, glycoside hydrolases are grouped into clans based on active site architecture, and these are further sub-classified into families based on amino acid sequence, structural and mechanistic similarity in the Carbohydrate-Active Enzyme database (CAZy) (http://www.cazy.org).

In this section, we shall discuss intramolecular synergism models reported during the degradation of various individual lignocellulosic components such as cellulose and hemicellulose, in an attempt to build an overall scenario of synergistic degradation of lignocellulosic biomass. With regards to enzymatic cellulose hydrolysis, it's been reported that endo-cellulases (endoglucanases) attack the amorphous regions within cellulose, creating new and more chain ends for exo-cellulases (cellobiohydrolases) to attack. In turn, exo-cellulases expose new amorphous regions within the bulk substrate and this then necessitates re-application of endo-cellulase activity. In addition, cellobiohydrolase I (CBHI) starts catalysis at the reducing end of cellulose chains, whereas CBHII initiates activity on the non-reducing, opposite chain ends of cellulose (Kostylev & Wilson 2012). Others have proposed a synergistic enzymatic hydrolysis model whereby endoglucanase, and cellobiohydrolases I and II, are present in the cellulolytic enzyme mixture. Here, it is proposed that this enzymatic mixture attacks reducing and non-reducing ends of cellulose chains, as well as amorphous regions within cellulose (Ganner et al. 2012).

With respect to glucuronoxylan degradation, synergism between main-chain cleaving enzymes, xylanases and xylosidases, has been reported (Yang et al. 2014; Gomes et al. 1993). Here, the proposed synergism model is that the small fragment products of xylanase, such as xylo-oligosaccharides with non-reducing ends, are preferred substrates of a xylosidase. It appears, in highly decorated xylans, as if the presence of α-glucuronidase would assist by

removing substituents that would have hindered the xylanase from cleaving the xylan backbone (Rosa et al. 2013). The action of the xylanase is then required to produce short decorated oligomers which are more attractive substrates for the α-glucuronidase compared to decorated xylan polymers. We propose that these steps occur simultaneously and are interdependent of each other during xylan depolymerisation (Pletschke et al. 2016).

Prior knowledge of "proposed" synergy models during the degradation of a particular lignocellulosic substrate and between enzymes is very important information to have as it is useful when selecting which enzymes to use (i.e. which sugars and linkages do the enzymes recognize) and in which order to use (i.e. *sequential* or *simultaneous*) those enzymes so that optimal degradation of that particular substrate can be achieved, with lowered protein loadings as the desired outcome (Pletschke et al. 2016). In the present study, various GH family cellulolytic and xylanolytic enzymes characterised in Chapter 3 were assessed for their synergistic interactions during Avicel and beechwood xylan degradation, respectively.

4.2. **Aims and Objectives**

4.2.1. **Aims**

To conduct synergy studies on Avicel and beechwood xylan degradation using cellulolytic and hemicellulolytic enzymes from various EC classifications and GH families.

4.2.2. **Objectives**

- To conduct binary and ternary simultaneous synergy studies between various cellulolytic and hemicellulolytic enzymes during the hydrolysis of Avicel and beechwood xylan, respectively;
- To quantify both reducing sugar (using DNS method) and monosaccharides (using Megazyme sugar detection kits) release from the synergy studies;
- To establish synergistic interactions between the lignocellulolytic enzymes using the data from the synergy studies;
- To compare the efficiency of applying the cocktails sequentially versus simultaneously during the degradation of the model substrates.

4.3. Materials and Methods

4.3.1. Synergy studies

In this chapter we conducted synergy studies on Avicel and beechwood xylan using cellulolytic and hemicellulolytic enzymes, respectively, in order to improve lignocellulosic biomass degradation.

4.3.1.1. Cellulolytic enzyme synergy studies

The experiments were carried out in triplicate at an Avicel loading of 2% (w/v) in 50 mM sodium citrate buffer (pH 5.0) in a 400 µL total volume at 50°C, mixing at 25 rpm (Labnet Revolver™) for up to 24 h. Unless otherwise specified, enzyme loadings for all enzyme benchmarks and for all enzyme combination experiments were kept constant at 1.25 mg/g Avicel. The first binary enzyme combination run was between Cel7A and Cel6A, the optimal combination with respect to reducing sugar and glucose release was selected as the "core CBH set" (CBHs) for further studies. The final binary study conducted was between the core CBH set, CBHs, and the endo-glucanase, Egl. The optimal combination with respect to reducing sugar and glucose release was selected as the "core cellulolytic set" (CelMix) and kept for further studies. The hydrolysis was then terminated by boiling for 5 minutes at 100°C to inactivate the enzymes. Hydrolysis controls included substrate (without the enzyme) and enzyme controls (without substrate). The samples were stored at 4°C for further analysis. Analysis of reducing sugar and glucose release was conducted according to the methods described in Section 3.3.4.1 of Chapter 3. Enzyme activity was measured in units (U), where 1 unit was defined as the amount of enzyme releasing 1 µmol of reducing sugar per hour. Glucose was used as the sugar standard.

4.3.1.2. Xylanolytic enzyme synergy studies

Enzyme mixtures were used in various binary combinations using 1% (w/v) beechwood xylan (50 mM citrate buffer, pH 5.0) as the substrate. The assays were setup as previously mentioned in Sections 4.3.1.1; however, they were only ran for 12 h with an enzyme loading of 0.25 mg/g beechwood xylan. The first binary enzyme combination run was between Xyn2A and XT6, the optimal combination with respect to reducing sugar and xylose release was selected as the "core xylanase set" (Xyns) for further studies. The final binary study conducted was between the core xylanase set, XynMix, and the α-glucuronidase, AguA. The optimal combination with respect to reducing sugar, glucuronic acid and xylose release was selected as the "core xylanolytic set" (XynMix) and kept for further studies. The hydrolysis was then terminated by boiling for 5 minutes at 100°C to inactivate the enzymes. Hydrolysis controls included substrate controls

(without the enzyme) and enzyme controls (without substrate). The samples were stored at 4^{o}C for further analysis. Analysis of reducing sugar release was conducted according to the methods described in Section 3.3.4.1 of Chapter 3. Enzyme activity was measured in units (U), where 1 unit was defined as the amount of enzyme releasing 1 μmol of reducing sugar per minute. Xylose was used as the sugar standard.

4.3.1.3. **Simultaneous versus sequential application of CelMix and XynMix components**

The optimal cellulolytic and hemicellulolytic sets, CelMix and XynMix, respectively found to synergistically degrade Avicel and beechwood xylan in Sections 4.3.1.1 and 4.3.1.2, were used in this section. Hydrolysis was carried out for 24 h and 12 h for Avicel and beechwood xylan, respectively, and divided into three groups. One group had the whole synergistic enzyme Mix (either CelMix or XynMix) running for the designated time simultaneously and was then boiled for 5 min to heat denature the enzyme, and was then processed for analysis, as described previously. To the other two groups, the first mono-enzyme component of the Mix or the second one was added first, and then the reaction mixtures ran for 24 h in Avicel hydrolysis and 12 h for beechwood xylan hydrolysis. The reactions were then boiled for 5 min to heat denature the enzyme mixture, cooled down to 50^{o}C, the missing component of the enzyme Mix added and ran for a further duration equal to the first (either 12 or 24 h). The reaction was boiled again for 5 min to denature the enzyme mixture and was then processed for analysis.

4.3.2. **Estimation of sugar monomers (glucose and xylose)**

Quantification of glucose in the hydrolysates was performed by the glucose oxidase/peroxidase (GOPOD) method (K-GLUC, Megazyme, Ireland). Then xylose quantification in the hydrolysates was performed enzymatically (K-XYLOSE, Megazyme), with the incubation time (20 min) twice the manufacturer's recommended time, as this gave more reproducible results with the microtiter plate format.

4.3.3. **Data analysis**

One way analysis of variance (ANOVA) was used for evaluation of significant increases exhibited by the enzyme combinations considered with respect to reducing sugar and monosaccharide release, compared to those released by the most active individual enzyme or Mix. All pairwise comparison procedures were based at 95% confidence level (p=0.05) and conducted using the Data analysis feature in Microsoft® Excel.

4.4. Results

The interaction that occurs between two or more hydrolytic enzymes, producing a total effect greater than the sum of the effects of the individual enzymes, is used to define the term "enzyme synergy". Synergistic interactions between various cellulases and between various xylanases with respect to Avicel and beechwood xylan degradation were evaluated in this chapter.

4.4.1. Cellulolytic enzyme synergy studies

Numerous studies have evaluated synergism between cellulolytic enzymes during the degradation of cellulose, particularly: i) exo–exo synergism, whereby CBHs degrade cellulose at opposite cellulose fibril ends and ii) endo-exo synergism between EGs and CBHs (Ganner et al. 2012; Igarashi et al. 2009). In this study, enzyme synergism between the cellulolytic enzymes, CBHI (Cel7A), CBHII (Cel6A) and EG (Egl) was assessed using Avicel as a substrate. These studies were conducted so that a "cellulolytic core set" required to efficiently degrade cellulose into glucose could be formulated. The figure below (Fig 4.1A) illustrates the binary enzyme combination between Cel7A and Cel6A selected as the "core CBH set" (CBHs). The second binary study conducted was between the core CBH set and the endo-glucanase, Egl, used to formulate a "core cellulolytic set", CelMix (Fig 4.1B). In all the considered synergy studies, Novozyme 188 glucosidase, Bgl1, was added at 10% of the total protein loading in the hydrolysis reaction in order to prevent end product inhibition of upstream enzymes (such as CBHs) by cellobiose.

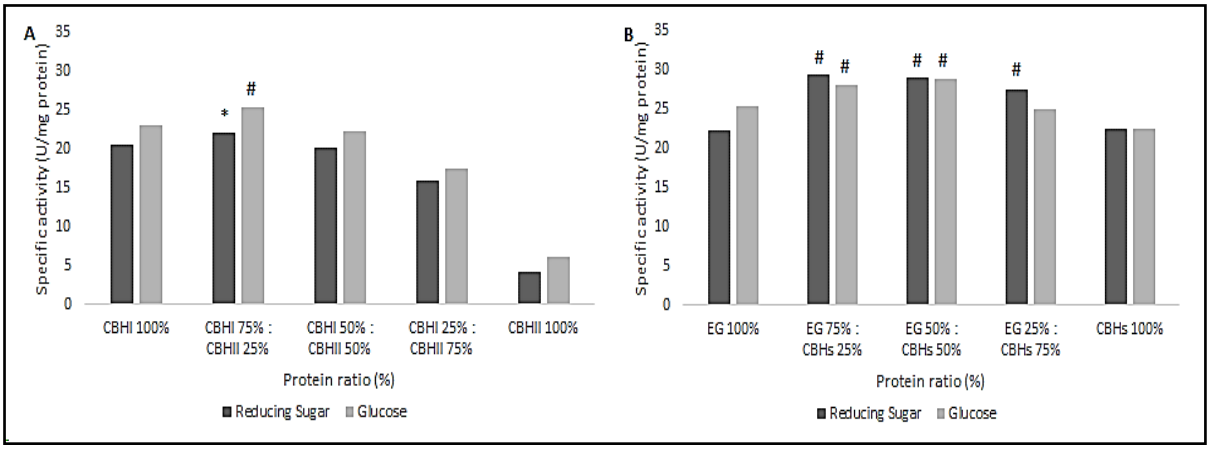

Figure 4.1. Glucose and reducing sugar released from Avicel by the various combinations of enzymes, (A) CBHI and CBHII, and (B) Most synergistic CBHI: CBHII set (CBHs) and Egl (EG). ANOVA analysis for improvement of hydrolysis with respect to reducing sugar and glucose release by the enzyme combinations compared to 100% enzyme protein loading of the most active enzyme, keys: * (p value<0.05) and # (p value<0.01). Values are represented as mean values ±SD (n=3).

With respect to exo-exo synergism, only 75% Cel7A to 25% Cel6A released reducing sugar and glucose (25.38 U/mg protein) that was statistically significantly higher than that released by Cel7A alone at 100% protein loading (23.06 U/mg protein) (Fig 4.1A). Among the two CBHs, Cel7A was more active compared to Cel6A, thus increasing the protein ratio of Cel7A in the combined protein loadings which led to an increase in activity up until the activity was greater than that of 100% Cel7A protein loading. Also, the partial endo-glucanase activity (0.7 U/mg protein) of Cel7A could have produced new chain ends for both CBHs to act upon, thus the improvement with respect to cellulose degradation observed. With respect to endo-exo synergism, all EG to CBHs combinations considered released reducing sugar and glucose (≈29 U/mg protein) that was statistically significantly higher than that released by either CBHs or EG alone at 100% protein loading (≈25 U/mg protein).

The optimized CBHs and CelMix combinations, CBHI to CBHII at 75% to 25% and EG to CBHs at 75% to 25%, were subjected to sequential synergism studies to assesse the mechanism of synergism between these enzymes during the degradation of cellulose (Fig 4.2).

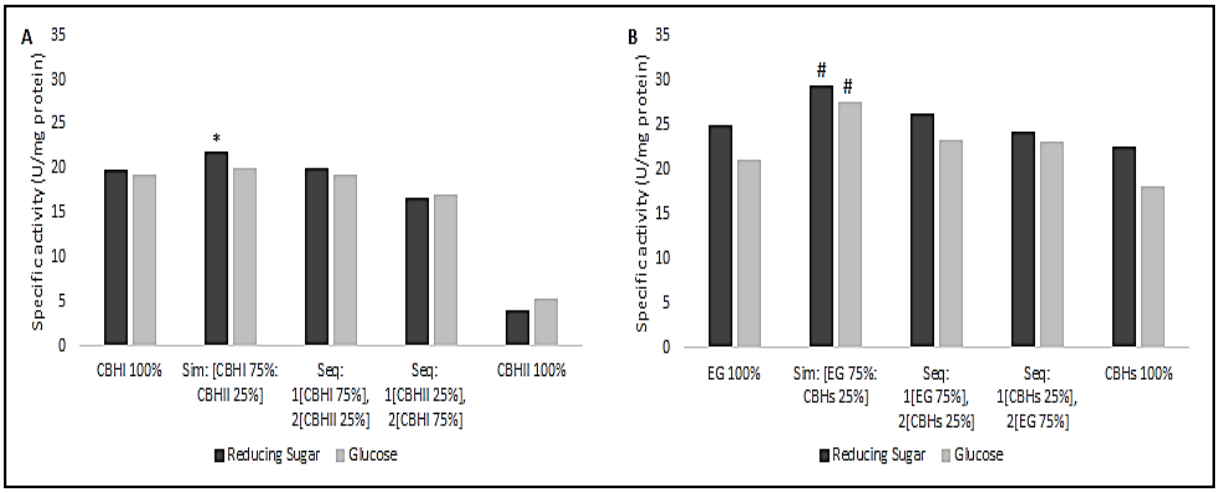

Figure 4.2. Glucose and reducing sugar released during sequential and simultaneous application of the various combinations of enzymes, (A) Cel7A (CBHI) at 75% and Cel6A (CBHII) at 25%, and (B) Cel6A: Cel7A set (CBHs) at 25% and Egl (EG) at 75% protein loading on Avicel hydrolysis. ANOVA analysis for improvement of hydrolysis with respect to reducing sugar and glucose release by the enzyme combinations compared to 100% enzyme protein loading, keys: * (p value<0.05) and # (p value<0.01). Values are represented as mean values ±SD (n=3).

Based on the sequential exo-exo synergism studies, pre-incubation of the Avicel cellulose biomass with Cel6A followed by Cel7A (16.64 U/mg protein) was less synergistic to when the biomass was pre-incubated with Cel7A and then followed by Cel6A (19.87 U/mg protein).

Moreover, sequential application of the cellobiohydrolases mixture with any of the two approaches was found to be less synergistic compared to their simultaneous application (21.87 U/mg protein) during Avicel degradation. With respect to sequential endo-exo synergism studies, pre-incubation of the Avicel cellulose biomass with the endo-glucanase, Egl, followed by the CBHs mixture (26.12 U/mg protein) was more synergistic to when the biomass was pre-incubated with the CBHs mixture followed by Egl (24.09 U/mg protein). Moreover, sequential application of the cellobiohydrolases mixture and Egl with any of the two approaches was found to be less synergistic, compared to their simultaneous application (29.02 U/mg protein) during Avicel degradation.

4.4.2. **Xylanolytic enzyme synergy studies**

O-acetyl-(4-O-methylglucurono)-xylans are the main hemicelluloses present in traditional industrial hardwoods, with their content varying between 15 and 30% (wood weight basis) (Pinto et al. 2005). Complete degradation of these xylans requires the synergistic action of a consortium of glycoside hydrolase enzymes, including endo-xylanase, β-xylosidase and α-glucuronidase, and accessory enzymes, such as acetyl xylan esterase, ferulic acid esterase, and *p*-coumaric acid esterase. Various studies have evaluated heterosynergistic associations between glucuronidase and xylanase during the hydrolysis of glucurono-(arabino)-xylans (Jia et al. 2014; Rosa et al. 2013; Cobucci-Ponzano et al. 2015). In the current study, enzyme synergism between xylanolytic enzymes, Xyn2A, XT6 and AguA was assessed using beechwood xylan as a substrate.

These studies were conducted so that a "xylanolytic core set" required to efficiently degrade xylan into xylose could be formulated. The figure below (Fig 4.3A) illustrates the binary enzyme combination between Xyn2A and XT6 selected as the "core Xyn set" (Xyns). The second binary study conducted was between the core Xyn set, Xyns, and the α-glucuronidase, AguA, used to formulate a "core xylanolytic set", XynMix (Fig 4.3B). In all the considered synergy studies, the xylosidase, SXA, was added at 10% of the total protein loading in the hydrolysis reaction to prevent end product inhibition of upstream enzymes by xylobiose.

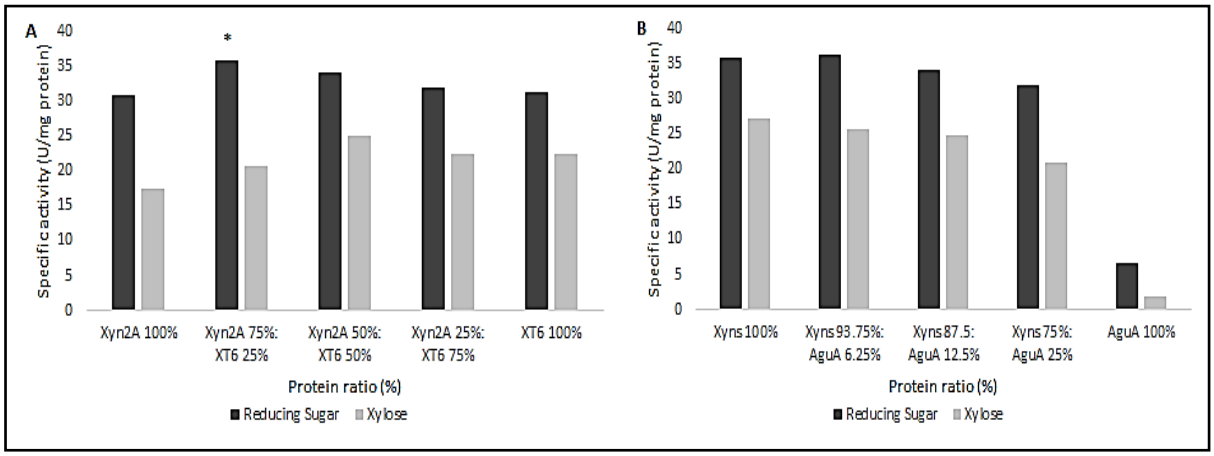

Figure 4.3. Xylose and reducing sugar released from beechwood xylan by the various combinations of enzymes, (A) Xyn2A and XT6, and (B) Most synergistic Xyn2A: XT6 set (Xyns) and AguA. ANOVA analysis for improvement of hydrolysis with respect to reducing sugar and glucose release by the enzyme combinations compared to 100% enzyme protein loading, keys: * (p value<0.05) and # (p value<0.01). Values are represented as mean values ±SD (n=3).

With respect to endo-endo synergism, 75% Xyn2A to 25% XT6 released significantly higher reducing sugar (36 U/mg protein) to that released by either XT6 or Xyn2A alone (31 U/mg protein) – and xylose (21 U/mg protein) similar to that released by XT6 alone (22 U/mg protein) at 100% protein loading (Fig 4.3). Among the two xylanases, XT6 (22 U/mg protein) was more active–compared to Xyn2A (17 U/mg protein) with respect to xylose release, and similar with respect to reducing sugar release. However, increasing the protein ratio of Xyn2A in the combined protein loadings led to an increase in activity.

Endo-debranching-enzyme synergism was also assessed using the endo-xylanases combination, Xyns, and the glucuronidase, AguA. All the endo-xylanases to AguA combinations used in this work did not release statistically significantly higher reducing sugars than those released by the endo-xylanases combination, Xyns. However, the combination Xyns at 75% to AguA at 25% (36.3 U/mg protein) liberated reducing sugar content comparable to that released by Xyns alone at 100% dosage (35 U/mg protein) (Fig 4.3B). This combination, Xyns: AguA at 75:25%, released a significantly higher amount of glucuronic acid (1.80 U/mg protein) compared to that released by AguA alone (1.56 U/mg protein) at 100% protein loading during beechwood xylan degradation (data not shown).

The optimized Xyns and XynMix combinations, Xyn2A to XT6 at 75% to 25% and AguA to Xyns at 25% to 75%, were subjected to sequential synergism studies to assess the mechanism of synergism between these enzymes during the degradation of beechwood glucuronoxylan.

From the sequential endo-endo synergism studies, pre-incubation of the beechwood xylan with Xyn2A followed by XT6 (293.66 U/mg protein) released reducing content similar to that released by a simultaneous application of the two enzymes (288.91 U/mg protein) (Fig 4.4A). However, sequential application of the Xyns mixture whereby XT6 was loaded first followed by Xyn2A yielded a slightly higher reducing sugar release (308.05 U/mg protein). With respect to endo-debranching-enzyme sequential synergism studies, simultaneous application of the enzymes or pre-incubation with either Xyns or AguA and then followed by AguA or Xyns released statistically significantly similar reducing sugar yields.

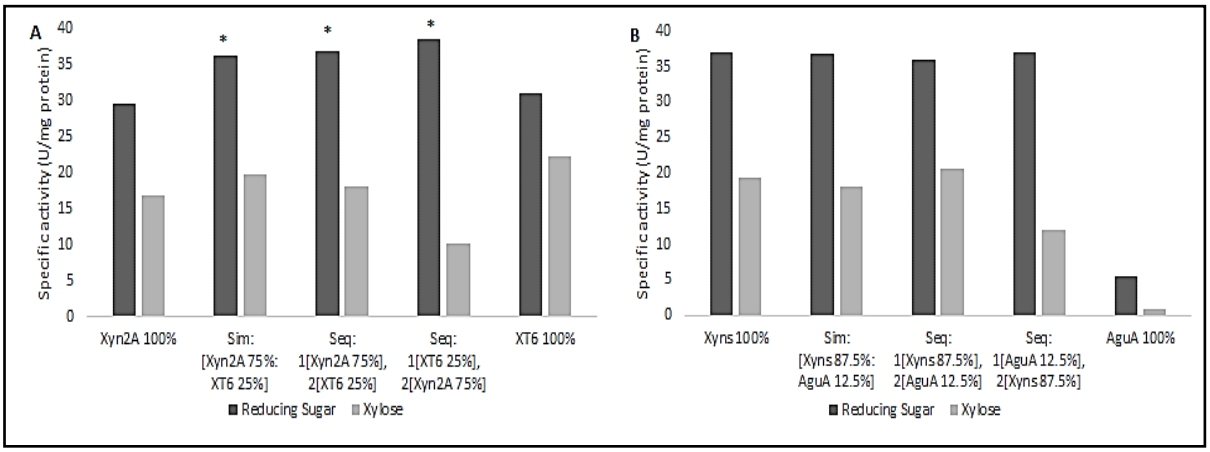

Figure 4.4. Xylose and reducing sugar released during sequential and simultaneous application by various combinations of enzymes, (A) Xyn2A at 75% and XT6 at 25%, and (B) Xyn2A: XT6 set (Xyns) at 75% and AguA (EG) at 25% protein loading on beechwood xylan hydrolysis. ANOVA analysis for improvement of hydrolysis with respect to reducing sugar and glucose release by the enzyme combinations compared to 100% enzyme protein loading, keys: * (p value<0.05) and # (p value<0.01). Values are represented as mean values ±SD (n=3).

4.5. Discussion

In this chapter, we elucidated intramolecular synergism mechanisms between cellulolytic enzymes and between xylanolytic enzymes during the degradation of individual lignocellulosic components (such as cellulose and glucuronoxylan-based hemicellulose, respectively), in an attempt to gain an overall understanding of synergistic degradation of lignocellulosic biomass by lignocellulolytic GH enzymes.

4.5.1. Cellulolytic enzyme synergism

The Classical Exo-cellulase-exo-cellulase model of synergistic enzymatic hydrolysis of cellulose was proposed by Fagerstam and Pettersson in 1980 (Väljamäe et al. 1998). Here, it was proposed that cellobiohydrolase I (CBHI) starts catalysis at the reducing end of cellulose chains, whereas CBHII initiates activity on the non-reducing, opposite chain ends of cellulose (see Figure 1.2 in Chapter 1). There have been further reports which affirmed the Classical Exo-cellulase-exo-cellulase model of synergistic enzymatic hydrolysis of cellulose by others such as Hoshino et al. and Väljamäe et al. (Hoshino et al. 1997; Väljamäe et al. 1998). In our work, it was demonstrated that this synergism occurs between a CBHI (Cel7A) from *Trichoderma* sp. and a CBHII (Cel6A) from an unknown microbial organism during the degradation of Avicel cellulose. The two enzymes were mostly synergistic when the CBHI to CBHII ratio was 75%:25% protein loading. This combination was contrary to that reported by Boisset et al. (2000), whereby Cel6A (CBHII) was at a higher proportion to that of Cel7B (CBHI) in the combination, at 62.5% to 37.5%, respectively. In our study it was observed that CBHI (Cel7A) was more active than CBHII (Cel6A) and also postulated that the partial endo-glucanase activity (0.7 U/mg protein) of CBHI could have produced new chain ends for both CBHs to act upon, thus the improvement with respect to cellulose degradation when there was a higher proportion of this enzyme in the protein loadings during synergy studies. Previous researchers have also reported that CBHI is more processive and active compared to CBHII. Moreover, our results showed reversed roles of the two exo-enzymes compared to the roles reported by Ganner and others, who proposed that CBHII does more than just attack from the non-reducing end, and also exhibited EG activity, as it displayed activity on the amorphous regions (Ganner et al. 2012).

According to Wood and McCrae, the Classical endo-cellulase-exo-cellulase(s) model involves endo-cellulases (endo-glucanases) which attack the amorphous regions within cellulose, creating new and more chain ends for exo-cellulases (cellobiohydrolases I and or II) to attack

(Wood & McCrae 1979). In turn, exo-cellulases expose new amorphous regions within the bulk substrate and this then necessitates re-application of endo-cellulase activity. Data attesting to this interdependence between endo-glucanases and cellobiohydrolases during cellulose degradation has been reported by others (Kostylev & Wilson 2014). In our study, it was found that the enzyme combination of 25% CBHs: 75% EG released the highest quantity of reducing sugars and glucose. In contrast to our observations, a study reported by Woodward et al. (1988), determined the best enzyme combinations for Avicel hydrolysis to be 50% CBHI to 50% EGII; 97.4% CBHs to 2.6% EG and 67% CBHs to 33% EGII, respectively (Woodward et al. 1988). Our EG was a commercial *Aspergillus niger* preparation which exhibited partial cellobiohydrolase I activity (0.4 U/mg protein). We therefore propose that this could have somehow reduced the necessity of a higher proportion of CBHs in the combinations mentioned above for optimal hydrolysis of Avicel cellulose.

4.5.2. **Xylanolytic enzyme synergism**

Generally, GH10 xylanases are reported to attack the glycosidic linkage next to a single or double substituted xylose toward the non-reducing end and require two unsubstituted xylose residues between branched residues, while GH11 xylanases are said to only hydrolyse xylosidic bonds where the two corresponding xylose moieties in subsites (−1) and (+1) are not branched (Mendis & Simsek 2015; Paës et al. 2012). Rosa et al. (2013) have also proposed that the two GH family endo-xylanases (GH10 and 11) produce different types of oligosaccharides as hydrolysis products, as a result of differences in cleavage specificities of the enzymes, with GH10 xylanases generally releasing oligomers in which the sugar at the non-reducing end is decorated with methyl glucuronate, while those from family 11 liberate oligomers with internal glucuronate substituents during glucuronoxylan degradation (Rosa et al. 2013). In this study, we have, for the first time, evaluated endo-endo synergism between a GH10 xylanase, XT6, and a GH11 xylanase, Xyn2A, during beechwood glucuronoxylan degradation. Whether these binary enzyme combinations have synergistic effects on beechwood xylan degradation was determined by *simultaneous* or *sequential* addition. All the considered combinations of the binary xylanase mixture showed no synergistic interactions during beechwood xylan degradation. When the combination 75% Xyn2A to 25% XT6 was applied sequentially with XT6 activity first followed by Xyn2A, a slightly higher reducing sugar content was liberated compared to that liberated by the simultaneous application of this very same combination or to that by Xyn2A alone at 100% protein loading. We postulate that the GH10 XT6 will act on the less decorated regions of beechwood xylan and produce oligomers, while leaving the decorated

polymers and oligomers to be acted upon by the GH11 Xyn2A. However, reversing this sequential application of these enzymes leads to less synergism as XT6 will act on both undecorated and decorated regions of xylan, producing decorated oligomers which Xyn2A cannot act upon due to its specificity for undecorated xylans.

Finally, synergism between the xylanases and a glucuronidase, AguA, during xylan degradation was also evaluated. All combinations of sequential or simultaneous enzyme additions of AguA and Xyns had no significant synergistic effects on beechwood xylan degradation based on reducing sugar liberation. However, addition of AguA in the enzyme combination led to a release of glucuronic acid (data not shown). According to Ebringerová (2006), 4-O-methyl-D-glucurono-D-xylan (MGX) represents more than 90% of the hemicellulose component in hardwoods with an average methyl-glucuronic acid: xylose ratio of 1:10 (Ebringerová 2006). In our study, based on the sequential enzyme addition studies, pre-treating beechwood xylan with AguA did not improve Xyns activity. This shows that the low content of 4-O-methyl-D-glucuronic acid (10%) is negligible enough not to cause steric hindrance on the xylanases when they are acting on the xylan polymers. Glucuronoxylan degradation by the concerted action of xylanases and glucuronidases has been evaluated previously (Jia et al. 2014; Rosa et al. 2013; Cobucci-Ponzano et al. 2015)–from these studies it has been demonstrated that the addition of the side chain cleaving enzyme, glucuronidase, did not significantly improve the release of reducing sugars from these xylan substrates.

4.6. Conclusions

In the quest for lignocellulosic biomass conversion into biofuels and other commodity chemicals, efficient saccharification of the biomass is the major bottleneck (Zhao et al. 2012). Therefore, elucidation and improvement of synergistic interactions between lignocellulolytic enzymes to improve lignocellulose saccharification and lowering protein loading (thus lowering biomass conversion costs) has been of interest over the past few decades (Ganner et al. 2012). In this study, the synergistic effects between various enzyme combinations were investigated for the degradation of cellulosic and hemicellulosic fractions of lignocellulosic biomass. The cellulolytic and xylanolytic cocktails consisting of (Egl 75%: Cel7A 18.75%: Cel6A 6.25%) and (Xyn2A 65.63: XT6 21.87%: AguA 12.5%), respectively, were optimized for cellulose and beechwood glucuronoxylan degradation, and then selected for an investigation that studied synergy between these two binary enzyme preparations on the hydrolysis of native/untreated and sodium chlorite/acetic acid delignified Acacia and Poplar wood, respectively–see Chapter 7.

Chapter 5: Pre-treatment and characterization of hardwoods

5.1. Introduction

The enzymatic degradation of lignocellulosic biomass for biofuel production presents several obstacles which are mostly due to plant recalcitrance (Zhang & Lynd 2004; Ding et al. 2012). Since enzymatic hydrolysis of native lignocellulose usually results in saccharification yields less than 20% of the original glucan, some form of pretreatment to increase amenability to enzymatic hydrolysis is required and, as a result, included in most process concepts for the biological conversion of lignocellulose (Zhang & Lynd 2004). Generally, the term pre-treatment refers to the disruption of the carbohydrate-lignin shield surrounding the holocellulose (cellulose and hemicellulose matrix), which will increase the surface area of the cellulose and hemicellulose accessible to enzyme degradation (Lynd et al. 2002).

The main goal of various pre-treatment technologies is an attempt to increase the accessibility of cellulose. Some pre-treatments achieve this by removing hemicellulose from the biomass (steam explosion and dilute acid). Other pre-treatments achieve this by de-lignification of the biomass (pulping methods). While other methods achieve cellulose accessibility by removing certain components and/or by altering biomass structure to reduce competitive inhibition and/or blockage of enzymes from accessing cellulose (alkaline methods and AFEX). Pre-treatments such as steam explosion and dilute acid generally yield a liquor stream containing partially hydrolysed hemicellulose and a pre-treated solid with an approximate cellulose: hemicellulose ratio of 12:1, with a relatively higher lignin content than the untreated biomass (Heise et al. 2017; Balan et al. 2009). On the other hand, AFEX and other chemical pre-treatments (Organosolv and sodium chlorite/acetic acid) generally retain most of the hemicellulose in the pre-treated solid, reduce cellulose crystallinity and lignin content, while yielding a cellulose: hemicellulose ratio similar to that of untreated biomass (8:5) (Balan et al. 2009; Shao et al. 2010; Dien et al. 2008; Koo et al. 2012; Ahlgren & Goring 1971).

Since numerous studies have demonstrated that removal of lignin in lignocellulosic biomass enhances holocellulose hydrolysability, sodium chlorite-acetic acid (SC/AA) was chosen as a form of pre-treatment in this study. Furthermore, we want to understand the complex structure of various hardwoods' holocellulosic biomass and the impact of these biomass features on the ability of the GH enzymes to hydrolyse them. The SC/AA method, originally known as the Wise method, is said to be an oxidative treatment that can selectively remove up to 60% lignin from lignocellulosic biomass (Siqueira et al. 2013). However, when more lignin is removed,

the treatment also tends to partially degrade the polysaccharide fraction as well. In addition, it can also affect cellulose reactivity through oxidation and structural changes (Jungnikl et al. 2008; Kumar et al. 2013). Steam explosion (SE), which is a physico-chemical process that results in partial hemicellulose hydrolysis and solubilisation, and lignin redistribution in biomass (Alvira et al. 2010; Leskinen et al. 2017), was chosen as the second form of hardwood pre-treatment used in this study. The main mechanism of this process is high pressure steam which enters the fibers and then is rapidly released from the closed pore in the form of air to cause flash cooling, which afterwards causes mechanical fracture on the fibers (Chen et al. 2017; Hu et al. 2008).

To understand the chemical modifications of the woody biomass during the application of a pre-treatment process and how the wood's structural and morphological characteristics change after the pre-treatment provides insightful information into the selection of an appropriate pre-treatment method to overcome biomass recalcitrance in addition to a method that will allow the utilisation of all lignocellulosic sugars rather than the currently harvested cellulosic sugars after conventional treatments such as steam pre-treatment.

5.2. **Aims and Objectives**

5.2.1. **Aims**

To subject *Acacia* and Poplar hardwoods to sodium chlorite/acetic acid and steam explosion pre-treatment, and to characterize both the untreated and the pre-treated hardwoods, respectively.

5.2.2. **Objectives**

- To pre-treat the hardwood substrates using sodium chlorite/acetic acid delignification and steam explosion;
- To determine the composition of untreated and pre-treated hardwoods;
- To visually illustrate differences between untreated and pre-treated hardwoods substrates using microscopic techniques;
- To determine the functional groups in untreated and pre-treated hardwoods using Fourier-transform infrared spectroscopy (FTIR);
- To determine the water retention values of untreated and pre-treated hardwoods;
- To determine the crystallinity of untreated and pre-treated hardwoods using X-ray diffraction (XRD).

5.3. **Materials and Methods**

5.3.1. **Materials**

About 20 kg of *Populus* spp. and 20 kg of *Acacia* spp. wood stems (without the barks) were processed into wood chips and kindly provided by Mondi Limited, Durban, South Africa.

5.3.2. **Pre-treatment of hardwood substrates**

The *Populus* spp. and *Acacia* spp. wood chips were laid in shelves inside an oven and dry heated at 80^{o}C for about 18 hours or up until no further change in the mass of the wood chip fractions was apparent. The dried wood chips were then cooled down, placed in air-tight, sealed bags and kept in the dark until further processing.

Steam pre-treatment of the hardwoods was conducted in a 2 L StakeTech III steam gun (Stake Technologies, Norvall, ON, Canada) in the Forest Products Biotechnology/Bioenergy Laboratory at the University of British Columbia. Briefly, wood chips from poplar and *Acacia* (DW; dry weight of 300 g) were impregnated overnight with 3% (w/w) SO_2 in sealable plastic bags. The bags were then opened and left for 60 min in a fume hood to release the unabsorbed SO_2. A batch of biomass (50 g) was loaded in the steam gun and the conditions used for pre-treatment were 5 min residence time at 210^{o}C. Once the desired residence time and temperature had been reached, the steam pressure was transiently discharged from the steam gun.

Before the delignification procedure could be conducted, the hardwood chips of both *Acacia* and Poplar wood were passed trice through a TRIMTECH garden vacuum blower (with the vacuum/mulch speed set at 6). The mulched wood chips were then crushed twice for up to 2 minutes each run into fine residues using a WARING Commercial Blender CAC90, with the spin speed set to High). The fine residues produced were then treated as the untreated/natural hardwood samples for the sodium chlorite/acetic acid delignification procedure.

A total of 10 grams of each hardwood substrate was treated using sodium chlorite/acetic acid for up to 4 hours according to modified protocols by Kumar et al. (2013) and Siqueira et al. (2013). For each gram of hardwood, 0.3 g of sodium chlorite, 0.1 mL of anhydrous acetic acid and 32 mL of distilled water were added. The flasks used for the treatment were incubated in a hot water bath at 70^{o}C and the treatment was conducted in duplicate. The slurry was shaken every 15-20 minutes during the incubation process. After 2 hours of incubation, the flasks were withdrawn from the bath, and the same amount of sodium chlorite and acetic acid was added and incubated for a further 2 hours. Finally, the samples were removed from the bath, allowed

to cool down to room temperature, filtered using a cheese cloth and washed with a litre of deionized water.

5.3.3. **Microscopic analysis of hardwood substrates**

Various microscopic techniques were used to assess the impact of the steam pre-treatment and sodium chlorite/acetic acid pre-treatment on *Acacia* and Poplar hardwood substrate morphology and lignin content.

5.3.3.1. **Scanning electron microscopy (SEM)**

For scanning electron microscopy (SEM), the untreated and pre-treated hardwood substrates were mounted on a metal stub, dried using the critical point-drying process and coated with a thin layer of gold prior to SEM analysis (Ma et al. 2011).

5.3.3.2. **Light microscopy (Wiesner histochemical assay for lignin)**

Prior to use, two parts of 5% (w/v) phloroglucinol prepared in 95% (v/v) ethanol were mixed with one part of concentrated HCl and then applied to the untreated and the pre-treated hardwood substrates (Pomar et al. 2002). The samples were then incubated at room temperature for about 3 minutes. The red colour produced by the presence of lignin from the staining procedure was visualized using an Olympus BX40 light microscope and photographed using an Olympus DP72 digital camera.

5.3.4. **FTIR spectroscopic analysis of hardwoods**

A Spectrum 100 FT-IR spectrometer system (Perkin Elmer, Wellesley, MA) was used to characterize the untreated and pre-treated hardwood samples. Each sample was pressed uniformly and tightly against the sample spotting surface using a spring-loaded anvil. FT-IR spectra were obtained by averaging 4 scans from 4000 to 650 cm^{-1}. Baseline and ATR corrections for penetration depth and frequency variations were carried out using the Spectrum One software supplied with the equipment.

5.3.5. **Hardwoods composition analysis**

The various hardwood substrates were characterized using a modified sulphuric acid method (National Renewable Energy Laboratory-NREL) by the Department of Wood Science, Stellenbosch University, South Africa (Sluiter et al. 2010). The samples (untreated and pre-treated hardwoods) were extracted using sequential washes with hexane, acetone and ethanol. The remaining material was hydrolysed with 72% (v/v) sulphuric acid. During this process, the samples were incubated at 30^{o}C for an hour with frequent mixing using glass rods. After this, the samples were transferred into Schott bottles and the concentration of the sulphuric acid

diluted down to 3% (v/v) by adding deionized water, and autoclaved for an hour to remove the sugar from the samples. After the hydrolysis the fractions were filtered to remove the insoluble lignin from the sugar solutions. The insoluble fraction was kept in an oven at 50°C for 48 hours or until no further change in the mass of the fraction was apparent. The acid soluble lignin present in the sugar solutions was detected by UV-Vis spectroscopy (Hyman 2008).

5.3.6. **Determination of Water Holding Capacity (WHC) and Water Swelling Capacity (WSC) of hardwoods**

WHC and WSC parameters of the hardwood samples were measured using modified protocols by Brachet et al. (2015) and Ramasamy et al. (2015), respectively. To measure WHC, in Eppendorf tubes, about 30 mg of each hardwood material was mixed with 1.5 mL of distilled water; this corresponded to a biomass loading of 2% (w/v) (Brachet et al. 2015; Ramasamy et al. 2015). After 1 h incubation on a Labnet Revolver™ (25 rpm, at 37°C), the mixture was centrifuged (16060 *x g* for 10 min at room temperature). The supernatant was then removed using a pipette with a tip muffled with cotton wool to prevent suction of micro-particulate biomass before weighing the hydrated material. The hydrated hardwood material was then left in a lab fume hood to evaporate excess water for 10 min before WHC measurement. WHC was expressed as mL of H_2O retained per g of biomass.

To measure WSC, 1.5 mL of distilled water was added to 30 mg of hardwood material in a burette. The volume of dry hardwood material sample was measured before adding the water (T0) and then after 60 min of adding the water. SC was expressed as a real volume of water adsorbed at T60 as mL of H_2O absorbed per g of hardwood biomass. All WHC and WSC measurements were performed in triplicate.

5.3.7. **XRD analysis of hardwood samples**

5.3.7.1. **Biomass crystallinity determination**

Crystallinity of the hardwood samples was determined by X-ray diffraction using Cu K radiation (1.5405 Å, nickel filter) on a Bruker D8® Discover equipped with a proportional counter. The samples were scanned from 2θ of 10 to 40° with a step size of 0.02°. The determination time was 0.02° per second. The crystallinity index (CrI) was then defined as follows:

$$CrI = [(I_{002}-I_{am})/I_{002}]*100$$

Where I_{002} and I_{am} are the intensity of diffraction at 2θ 22.6° and 18.9°, respectively.

5.3.7.2. **Apparent Crystallinity Size (ACS) determination**

Apparent crystallinity size (ACS) was estimated through the use of the Scherrer equation:

$$ACS = (0.89*\lambda)/(\beta*Cos\theta)$$

Where, λ, is the wavelength of incident X-ray (1.5405 Å); θ, the diffraction angle corresponding to 002 crystal plane and β, the half-value width of peak angle of 002 crystal plane.

5.4. **Results**

5.4.1. **Pre-treatment of hardwood substrates**

This study made use of sodium chlorite/acetic acid delignification and steam explosion pre-treatments. Figure 5.1 displays the untreated and pre-treated hardwood biomass.

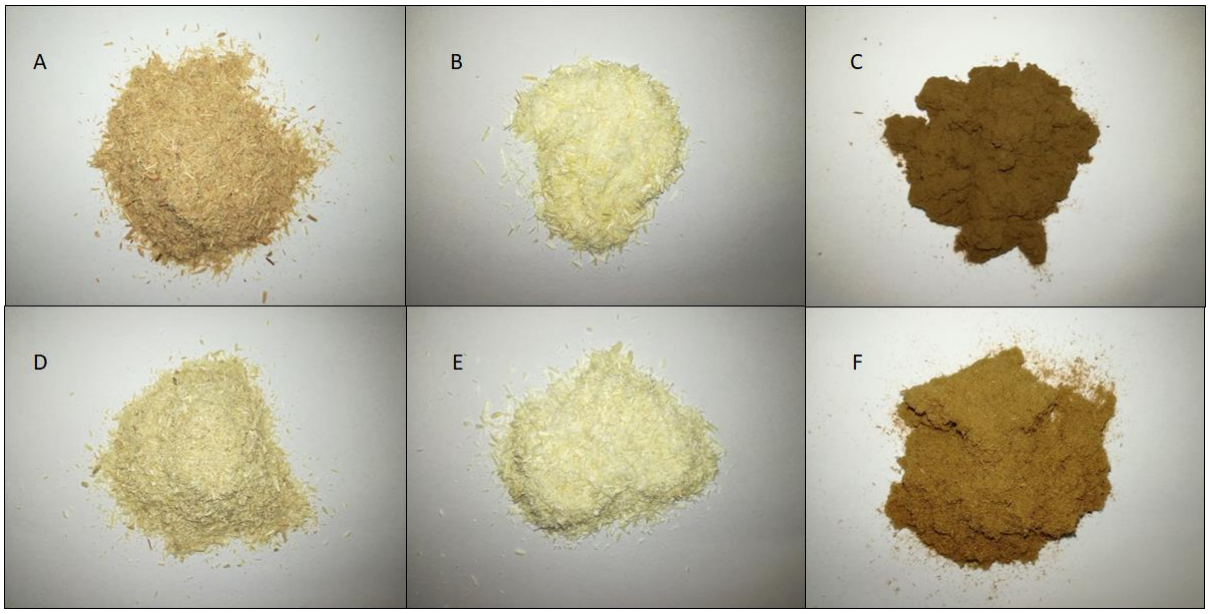

Figure 5.1. An illustration of the observable differences between (A) untreated *Acacia*, (B) delignified *Acacia* and (C) steam exploded *Acacia*, and those between (D) untreated Poplar, (E) delignified Poplar and (F) steam exploded Poplar.

From Figure 5.1, it can be observed that the untreated (Figs 5.1A and 5.1D) and steam exploded hardwood biomass (Figs 5.1C and 5.1F) had a light and dark brown hue attributable to the presence of lignin, while the delignified biomass exhibited an off white colour attributable to a high polysaccharide content and the removal of lignin (Figs 5.1B and 5.1E).

5.4.2. **Microscopic analysis of hardwood substrates**

5.4.2.1. **Scanning electron microscopy (SEM) of hardwoods**

The untreated and pre-treated hardwood biomass samples were analysed under various magnifications using SEM (see Figure 5.2).

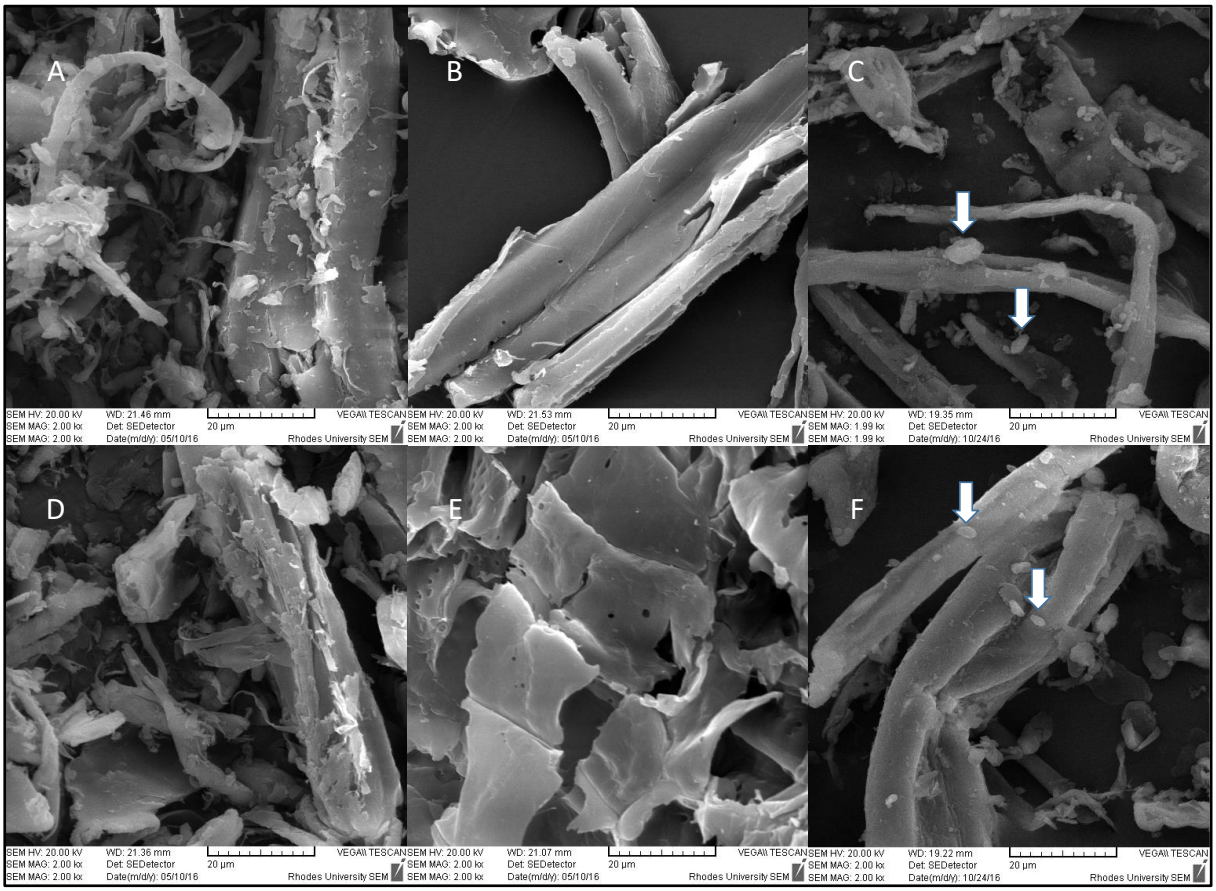

Figure 5.2. SEM analysis of (A) untreated *Acacia*, (B) delignified *Acacia*, (C) steam exploded *Acacia*, (D) untreated Poplar, (E) delignified Poplar and (F) steam exploded Poplar biomass at 2kx magnification.

Based on the SEM images shown in Figure 5.2 above, both untreated hardwoods appeared to possess fluffy matrices which appear to be coating the fibrils (Figs. 5.2A and 5.2D), while upon sodium chlorite/acetic acid delignification, the hardwoods exhibited smooth and bare fibrils that were without any matrices coating them (Figs. 5.2B and 5.2E). The delignified hardwood fibrils also exhibited pores on their surfaces when viewed at high magnification (Figure not shown). Interestingly, upon steam explosion, the hardwoods possessed defined separate/loose fibrils coated by droplet-like particles (see white arrows in Figs. 5.2C and 5.2F). In summary, there were prominent morphological changes in the biomass structure due to delignification and steam explosion, respectively.

5.4.2.2. **Light microscopy of hardwoods**

To examine lignin distribution in the untreated and delignified hardwoods, the biomass was stained with the lignin-staining dye phloroglucinol (Weisner staining) to show lignin (see Figure 5.3 below).

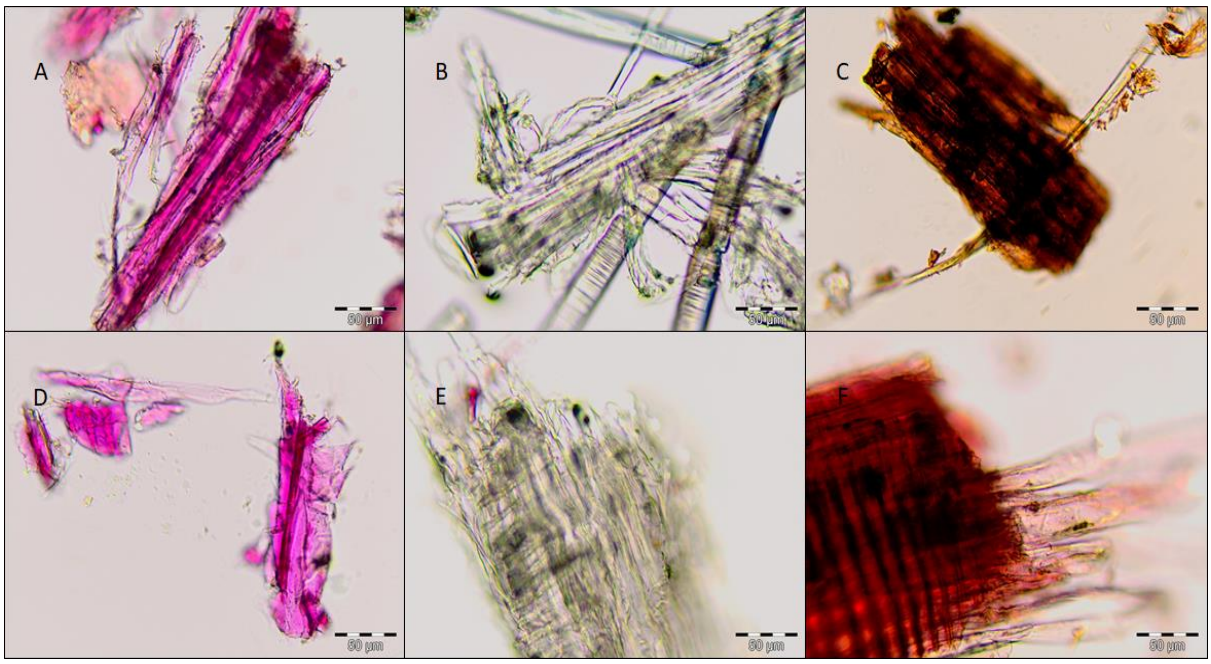

Figure 5.3. Histochemical localization of cinnamyl lignin moieties in (A) untreated *Acacia*, (B) delignified *Acacia*, (C) steam exploded *Acacia*, (D) untreated Poplar, (E) delignified Poplar and (F) steam exploded Poplar by the Weisner method (Phloroglucinol-HCl). Bars: 50 µm, 100 x mag.

Examination of the hardwoods after Weisner staining showed that untreated *Acacia* and Poplar samples were stained deep magenta (Figs. 5.3A and 5.3D), whereas the delignified biomass was barely stained by the dye (Figs. 5.3B and 5.3E), showing that a substantial amount of lignin was removed by this pre-treatment procedure. Steam exploded hardwood biomass, on the other hand, exhibited a deep maroon/burgundy hue upon staining with phloroglucinol showing that the biomass still contained lignin.

5.4.3. **FTIR spectroscopic analysis of hardwoods**

The FT-IR spectra of untreated, sodium chlorite acetic acid delignified and steam hardwoods (*Acacia* and *Populus* spp.) are presented in Figure 5.4 to show the abundant functional groups in each type of biomass.

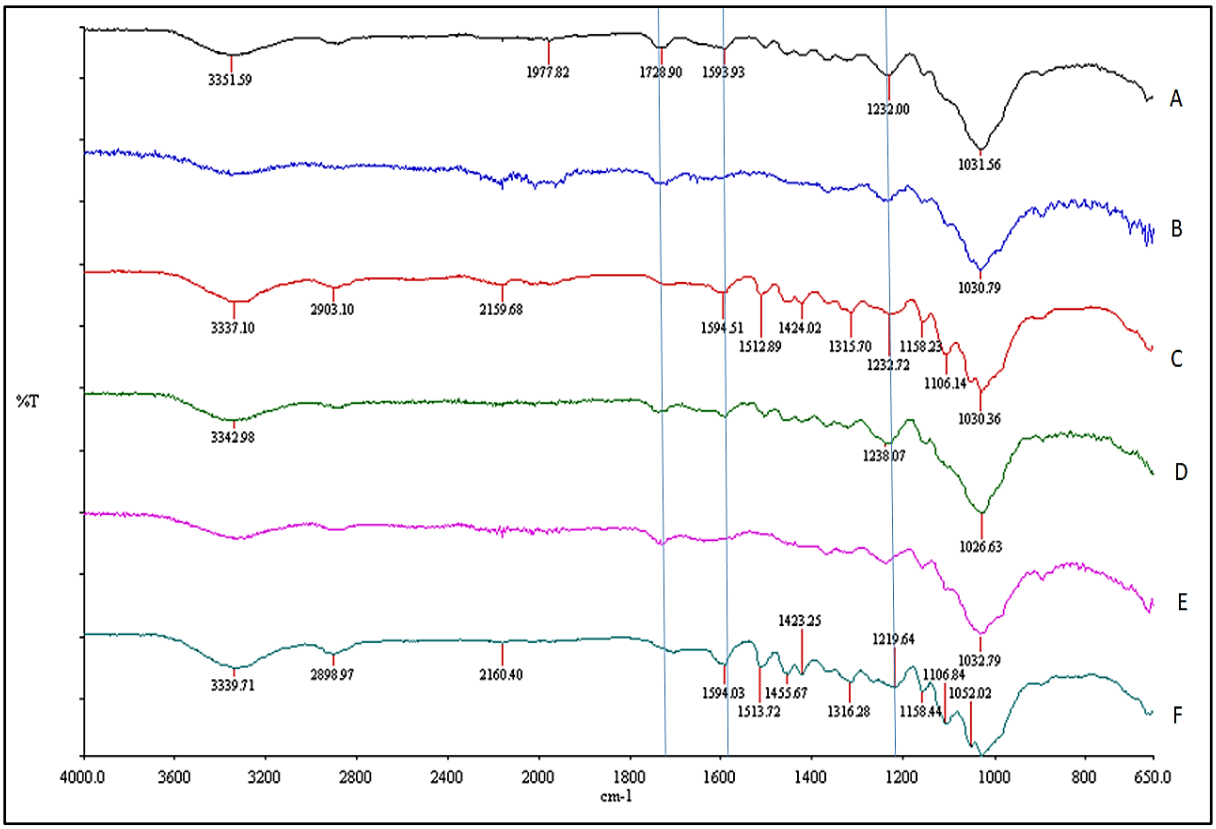

Figure 5.4. FT-IR spectrum of (A) untreated *Acacia*, (B) delignified *Acacia*, (C) steam exploded *Acacia*, (D) untreated Poplar, (E) delignified Poplar and (F) steam exploded Poplar biomass in the frequency range of 650-4000 cm^{-1}.

In general, peaks representing the lignin content (1234-1594 cm^{-1}) (assigned to aromatic skeletal vibrations and other lignin related bands) of delignified hardwood samples (Figs 5.4B and 5.4E) were of lower intensity than those of the starting untreated material and/or steam exploded material. This indicated the removal of lignin by this pre-treatment, while the proportion of carbohydrates retained in the solids were consistent before and after delignification. Steam exploded biomass (Figs 5.4C and 5.4F) on the other hand showed an intensification of peaks in the 1234-1594 cm^{-1} region, this showing an increase in lignin proportion in this biomass compared to untreated and the delignified material (Figs 5.4A and 5.4D).

5.4.4. **Biomass composition analysis**

In order to determine the efficiency and the effect of the sodium chlorite/acetic acid delignification and steam explosion pre-treatments on the hardwoods, the determination of the chemical composition of the different samples was essential (Table 5.1).

Table 5.1. Chemical composition of hardwoods before and after pre-treatment (dry weight basis).

Biomass	Total lignin (%)	Glucan (%)	Xylan (%)	Arabinan (%)
Untreated *Acacia*	22.38	48.75	23.88	Nd
Delignified *Acacia*	11.61	47.85	24.71	Nd
Steam treated *Acacia*	28.51	44.74	6.61	Nd
Untreated Poplar	28.74	41.46	14.84	Nd
Delignified Poplar	12.15	50.34	22.13	Nd
Steam treated Poplar	36.56	44.19	6.11	Nd

Where "Nd" = not determined

When sodium chlorite delignification was carried out, the acid insoluble/Klason lignin content decreased, with a concomitant increase in the xylan content for both Acacia and Poplar biomass. However, with steam explosion, an opposite effect was observed, with an increase in lignin content and a decrease in xylan content in the two hardwoods (Table 5.1).

5.4.5. **Water holding capacity of hardwoods**

According to Ramaswamy et al. (2013), the water holding capacity (WHC) is defined as the amount of water held (in mL) per g dry matter of sample, while according to Brachet et al. (2015), water swelling capacity (WSC) is a measure of potential volume occupancy of water within a given amount of biomass (also expressed as mL per g of dry biomass). Results from WHC and WSC measurements (Table 5.2) showed that Poplar materials (both untreated and pre-treated) exhibited higher values compared to *Acacia* materials. For Poplar, the delignified material showed significantly higher WHC and WSC values compared to the untreated biomass, while for *Acacia*, similar WHC and WSC values were reported for the delignified and the untreated biomasses. Steam exploded material, for both *Acacia* and Poplar, exhibited the highest WHC and WSC values, compared to both untreated and delignified materials. WHC of steam exploded hardwoods is probably higher than those of untreated and delignified hardwoods (intact fibrils) as a result of the fibril splitting/separation caused by the steam pre-treatment which possibly allows for more up-take of water.

Table 5.2. Swelling capacity (WSC) and water-holding capacity (WHC) of the hardwoods.

Biomass	**WSC (mL/g)**	**WHC (mL/g)**
Untreated *Acacia*	1.44±0.2	3.86 ±0.1
Delignified *Acacia*	1.55±0.4	3.73 ±0.2
Steam exploded *Acacia*	11.21±4.2	6.44±1.4
Untreated Poplar	4.67±1.2	6.3 ±0.2
Delignified Poplar	8.0±0.6	8.93 ±0.3
Steam exploded Poplar	19.62±5.4	9.38±1.0

5.4.6. **XRD analysis of hardwoods samples**

Here, the crystallinity index (CrI) of the various pre-treated hardwoods was estimated using the peak height method as described by Segal et al. (1959), while the apparent crystallite size (ACS) was estimated through the use of the Scherrer equation (see Table 5.3).

Table 5.3. Crystallinity indexes and apparent crystallite sizes of the pre-treated hardwoods.

Biomass	**Crystallinity index (CrI) (%)**	**Apparent crystallite size (ACS) (Å)**
Untreated *Acacia*	55.76	18.8
Delignified *Acacia*	73.19	24.2
Steam exploded *Acacia*	82.46	33.9
Untreated Poplar	62.14	18.8
Delignified Poplar	70.04	24.2
Steam exploded Poplar	78.22	33.9

For both Acacia and Poplar, it was evident that pre-treatment of the biomass led to greater exposure of cellulose fibres as characterized by the increase with regards to both CrI and ACS values (see Table 5.3).

5.5. Discussion

Future production of fuels and platform chemicals is likely to depend on plant biomass, which is the most abundant bio-resource to consider as a feedstock for bio-refineries (Jönsson & Martín 2016). However, the plant biomass cannot be utilized as is for biochemical conversion and requires a form of pre-treatment for disrupting the inter-component associations between the main constituents of the plant cell wall and removal thereof in other instances (Jönsson & Martín 2016).

5.5.1. Visual inspection of hardwoods

With respect to visualization with a naked, unaided eye, untreated and steam exploded biomass normally displays a dark brown colour, but after delignification the biomass usually exhibits an off-white colour (see Figure 5.1). This is usually used as visual evidence to assume that the majority of the lignin was removed from the substrate. Similar results (with respect to visual changes on substrates subsequent to bleaching) were obtained for the delignification of lignin coated cellulose by sodium chlorite/acetic acid delignification in a study conducted by Hubbell and Ragauskas (Hubbell & Ragauskas 2010). Auxenfans et al. (2017) also reported on the particle colour of biomass (i.e. Poplar) becoming browner with increasing combined severity factors during steam explosion, revealing the relatively large presence of residual lignin (Auxenfans et al. 2017).

5.5.2. SEM of hardwoods

With respect to SEM analysis, the micrographs exhibited a change in surface morphology of the two hardwoods, *Acacia* and Poplar, after pre-treatment, with the fibre surfaces becoming smoother and porous after delignification of both woods. However, visualization of the delignified woods at low magnification demonstrated subtle differences between the two woods. At low magnification, the delignified Poplar appeared to be composed of smooth flakes, while delignified *Acacia* was composed of smooth and compact fibrils. Similar to our findings for delignified Poplar, Sewsynker-Sukai and Kana (2017) reported on delignified corn cob exhibiting distorted, porous and disintegrated structures with many cracks on the surface of the biomass (Sewsynker-Sukai & Gueguim Kana 2017). This indicated that SC/AA pre-treatment possibly disrupts the micro-structure and increases the surface area of pre-treated Poplar biomass.

With steam explosion, on the other hand, the biomass fibrils were separate/loose, possibly accompanied with a larger surface area and greater enzymatic accessibility. The biomass fibres

were also coated by droplet-like particles which we supposed to be lignin condensation droplets after the high temperature experienced during steam pre-treatment. Others have reported native Poplar samples exhibiting a rigid, compact fibrillary morphology with thick-walled fibre cells and fibres constituted by parallel stripes, while steam exploded pre-treated samples presented a more disorganized morphology characterized by the separation and greater exposure of fibres as well as loosening of the fibrous network, which may be due to the solubilisation of cell wall components (Auxenfans et al. 2017). Interestingly, contrary to our findings and those by Auxenfans et al. (2017), for Pine wood, it was reported that condensed-like structures or droplets were present upon steam pre-treatment without explosion, while pre-treatment with explosion showed a different structure (Pielhop et al. 2016). In summary, there were prominent morphological changes in the biomass structure due to the two diverse pre-treatment methods – delignification and steam explosion.

5.5.3. **FTIR analysis of hardwoods**

The structural changes in the two hardwoods by the SC/AA and steam explosion pre-treatments were investigated by FTIR analysis. FTIR spectra of the untreated and pre-treated woods are presented in Figure 5.4. With respect to cellulose characteristics analysis using FTIR, two bands are of prime importance; the band at 1430 cm^{-1} assigned to the presence of cellulose I and the band at 896 cm^{-1} assigned to cellulose II (Noori & Karimi 2016); these absorbance peaks were observable on all the hardwood biomass substrates assessed in this study. After the SC/AA pre-treatment, absorbance decreased for acetyl groups (1733 cm^{-1}) in *Acacia* wood, while it remained constant in Poplar wood (1730 cm^{-1}) (Noori & Karimi 2016). After the SC/AA pre-treatment, hardwood sample delignification was demonstrated by the disappearance in absorbance for aromatic skeletal vibrations (1422 cm^{-1} and 1505 cm^{-1}) and stretching of the aromatic benzene ring in lignin (1594 cm^{-1}), and decreases for aromatic C-H and C=O stretches at 1160 cm^{-1} and 1230 cm^{-1}, respectively. This was similar to reports by Jungnikl et al. (2008) reporting on data obtained on SC/AA delignification of softwood samples and that by Fuelbiol et al. (2015) on the impact of glycerol organosolv pre-treatment on the chemistry of wheat straw. On the other hand, steam explosion of hardwood samples led to an enhancement of the absorbance for aromatic skeletal vibrations (1422 cm^{-1} and 1505 cm^{-1}) and stretching of aromatic benzene ring in lignin (1594 cm^{-1}), and for aromatic C-H and C=O stretches at 1160 cm^{-1} and 1230 cm^{-1}, respectively (Jungnikl et al. 2008; Fuelbiol et al. 2015). This demonstrated that the proportion of lignin in the composition of the steam exploded hardwoods was higher than that in untreated and delignified samples.

5.5.4. **Compositional analysis of hardwoods**

The untreated, steam exploded and delignified wood samples in this study were extracted and dried. Following this, hydrolysis with 72% (v/v) sulphuric acid was conducted for compositional analysis. It is noteworthy to mention that the mass balance of the components for the biomass (as reported in Table 5.1) is approximately 85%, this falling below the general value of 95% for compositional analysis. This can be explained to be as a result of omitting the percentage composition of other wood components such as mannan, galactan, acetyl groups and extractives which constitute about 8–11% of the wood mass for both *Acacia* (Munoz et al. 2007; Willfor et al. 2005) and Poplar (Balan et al., 2009; Hu et al. 2015, 2013). Based on Table 5.1, the composition of the untreated, steam exploded and delignified wood samples was different, with the acid insoluble/Klason lignin content having decreased, with a concomitant increase in the xylan content for both *Acacia* and Poplar after delignification, while steam explosion led to an increase in Klason lignin and a decrease in xylan content. The glucan content of Poplar also increased after the pre-treatment from 41% to 50%. However, the glucan content of *Acacia* did not demonstrate an increase after pre-treatment but remained between 47-49%.

The composition analysis data obtained in this study for both untreated and delignified Poplar was similar to that reported by Kumar et al. (2013), who reported glucan, xylan and Klason lignin contents of 42.4%, 14.2% and 26.7% for untreated Poplar, and 55.6%, 17.1% and 1.7% for delignified Poplar, respectively (Kumar et al. 2013). Currently, no composition analysis data is available for *A. mearnsii* (the *Acacia* species used in this study), however there is data available for *A. dealbata* in literature, and this was used for comparison. The composition analysis data obtained in this study for both untreated and delignified *A. mearnsii* was similar to that reported by Ferreira et al. (2011) and Muñoz et al. (2007) for untreated *A. dealbata* with the following composition: cellulose, 43.1-50.5%; xylan, ≈19%; acid insoluble lignin, 20.7%; and acid soluble lignin, 5.2% (Ferreira et al. 2011; Munoz et al. 2007). The holocellulose content reported for the two hardwoods used in this study (*Acacia* and Poplar) is similar to that of other well studied hardwoods used in ethanol production (Berlin et al. 2006; Skyba et al. 2013). We anticipate the lower lignin and a higher quantity of sugar relative to percentage of dry mass of delignified hardwoods than that of the untreated hardwoods will facilitate higher enzyme accessibility and activity on the delignified biomass.

The composition analysis data obtained in this study for both untreated and steam exploded Poplar resembled that reported by Meng et al. (2013), with steam pre-treatment leading to a

higher glucan content, (50.3 to 61.4%), while the xylan content decreased from 11.5% to around 6% (Meng et al. 2013). Similarly, Auxenfans and co-workers reported that hardwood lignin (27.8 to 28.8%) and cellulose (from 43.2 to 59.4%) content increased upon steam explosion, while xylan content decreased (from 20.2 to 6.4%) (Auxenfans et al. 2017). Also, similar to our findings for steam exploded hardwoods, Chandra et al. (2016) reported that the xylan content decreased (from 16 to 4%), while lignin and glucan content increased (from 28 to 36% and from 46 to 54% for lignin and cellulose, respectively) upon steam pre-treatment of Poplar (Chandra et al. 2016).

5.5.5. **Water retention values of hardwoods**

According to Karimi and Taherzadeh (2016), lignocellulosic biomass has external and internal surfaces, where the total accessible surface area is the sum of these areas (Karimi & Taherzadeh 2016). The external surface depends on the size and shape, and its area can be increased by size reduction, i. e. a typical physical pre-treatment of the lignocelluloses can achieve this. On the other hand, the internal surfaces of lignocellulose depend on the pore sizes and distributions. According to Noori and Karimi (2016), water swelling capacity (WSC) is used as an indication of the accessible interior surface area and the suitability of the lignocelluloses to enzymatic hydrolysis (Noori & Karimi 2016). This is based on the assumption that if water cannot enter the pores in lignocellulosic biomass, then certainly no enzyme can enter those particular pores either.

In our data, Poplar wood samples showed higher WSC values compared to *Acacia* wood samples. For *Acacia*, a slightly higher WSC value was observed for the native wood compared to the delignified wood. Linking SEM analysis with WSC values for Acacia wood samples, the surface of sodium chlorite delignified sample was much smoother and fibrillar in nature, while the native biomass surface was rough and bulky. It is possible that the structure of the biomass fibers collapsed after delignification and limited biomass porosity, due to the removal of lignin and amorphous polysaccharide regions (demonstrated by an increase in insoluble reducing ends). Similarly, Yu et al. (2011) reported a decrease in pore volume and smaller accessible pore volume by ozone delignification which was indicative of structural collapse of fibers (Yu et al. 2011). On the other hand, Poplar showed an inverse trend, with the delignified wood showing a higher WSC value compared to the untreated wood. It is noteworthy to mention that the holocellulose content of the delignified Poplar wood (72%) was higher than that of the native/untreated wood (57%), especially due to an increase in the proportion of xylan content. We assume this to be the reason for the higher WSC value reported for the delignified biomass.

Similarly, Ju et al. (2013) also reported that a higher xylan content in biomass increased swelling, while lignin decreased the WSC value of biomass (Ju et al. 2013). Linking SEM analysis with WSC values for Poplar wood samples, massive fragmentation and pores were observed on the surface of the sodium chlorite delignified sample, which might be an indication of the space generated through lignin removal; this was similar to data for delignified hardwood and softwood pulps in a study conducted by Yu et al. (2011).

With respect to steam explosion, the procedure decreased the proportion of holocellulose content of the hardwoods while increasing that of lignin (see Table 5.1). Interestingly, this led to an increase in WSC of hardwoods (see Table 5.2). Chandra et al. (2009) also showed that higher severities of steam pre-treatment lead to a decrease in holocellulose content, while increasing WSC of the biomass and this was accompanied by an improvement in biomass hydrolysability (Chandra et al. 2009). Based on SEM, steam exploded hardwoods in this study have loose fibres compared to untreated and delignified biomass, and this could be the reason why this biomass takes up higher volumes of water and swells up more upon suspension in water as seen by the high WSC values.

5.5.6. **XRD analysis of hardwoods**

From our XRD analysis of the pre-treated hardwoods, it was evident that there was a direct relationship between CrI and ACS (see Table 5.3). Similarly, Li and Renneckar (2011) showed that CrI and ACS of wood pulps were directly proportional to each other, with wood pulps exhibiting high CrI values also having large ACS values (Li & Renneckar 2011). The untreated *Acacia* wood (*A. mangium*) used in this study exhibited a CrI of 55.75%. This value was similar to that reported for untreated *A. confusa* (CrI 59.1%) (Yeh et al. 2014). The steam exploded Poplar used in this study exhibited a CrI of 78.22%, which was considerably lower than the value reported by Hu and co-workers (CrI 62%) for the same biomass (Hu et al. 2015). It is noteworthy to mention that we used a different method for calculating CrI to that used by Hu et al. (2015), whereby CrI was calculated as to be 100*(crystalline area/total area), in which the total area is the crystalline area plus amorphous area. Our untreated Poplar exhibited a higher CrI value (62.14%) compared to that reported for raw yellow Poplar (49.9%) (Lee et al. 2017).

From our data, the untreated hardwoods contained a higher proportion of non-cellulosic content (lignin and xylan) compared to that of pre-treated biomass (delignified and steam exploded). It was also noted that the recorded CrI and ACS values for the biomasses were inversely

proportional to the non-cellulosic content found in these samples. Similarly, Yeh and co-workers (2014) reported on this and attributed the increase of the biomass CrI to the removal of amorphous hemicelluloses and partial removal of lignin, and amorphous regions of native cellulose during the pre-treatment process. These would therefore relatively increase the crystalline portions of the pre-treated residue (Yeh et al. 2014). Thygesen and co-workers also showed that higher cellulosic contents in biomass corresponded to higher CrI values (Thygesen et al. 2005). Also, similar to our findings, Makoto and co-workers reported higher CrI values for delignified *Miscanthus sinensis* samples (46-56) compared to untreated *M. sinensis* samples (26-54) (Makoto et al. 2008).

After dissolution of the amorphous fraction of the cellulose due to the pre-treatment, the obtained particles are termed microcrystals or crystallites. Yu et al. (2011) have also reported that lignin removal had an influence on the crystallinity index of woody biomass, here the crystallinity index increased with decreased lignin content due to the delignification procedures employed (Yu et al. 2011). An increase in the degree of crystallinity with delignification, due to the increase of glucan content in the pre-treated solid fraction of corn stover, was similarly reported by Kim and Holtzapple (Kim & Holtzapple 2006). We propose that this cleavage of amorphous regions in delignified biomass may cause the delignified biomass to behave differently to the untreated biomass when digested using enzymes. The higher crystallinity will favour the action of processive enzymes while obviating the action of backbone cleaving enzymes which prefer amorphous, higher polymers (Karimi & Taherzadeh 2016).

As much as both lignin and xylan have a direct effect on the CrI values of biomass, xylan appeared to decrease the crystallinity of biomass more than lignin, as delignified biomass (high xylan and low lignin content) showed lower CrI values than steam exploded biomass (high lignin and low xylan content) (see Table 5.3). The range of ACS (18-34 Å) of the hardwoods evaluated in this study was similar to that reported by others for hardwood pulps (Cao & Tan 2005; Li & Renneckar 2011; Long et al. 2017). For delignified hardwoods, ACS values increased from 18.8 Å for untreated biomass to 24.2 Å for delignified biomass, however similar WSC values were obtained for both types of woods. As reported by Sun et al. (2014), delignification leads to increased crystallite size which causes microfibrils to coalesce by expelling any biopolymer or solvent (Sun et al. 2014). This explains why our delignified biomass SEM micrographs showed smooth bundles of microfibrils (with a high extent of hornification) with similar WSC values to those of untreated biomass. Sun et al. (2014) further argue that cellulose microfibril coalescence would mainly be reflected in a decrease of

accessible cellulose surfaces, enlargement of lateral fibril aggregation dimensions (LFADs), and an increase in cellulose crystallinity (Sun et al. 2014). Based on high WSC and CrI/ACS values, steam exploded biomass appears to potentially have high accessibility as high WSC implies that the glucan to lignin network is not tight and compacted as the biomass can take up a large amount of water, while high CrI values imply that the cellulose surface is highly exposed.

5.6. **Conclusions**

This chapter described and discussed the pre-treatment of hardwoods, *Acacia* and Poplar, by steam explosion and sodium chlorite/acetic acid (SC/AA) delignification. The effects of the pre-treatments on the hardwoods, such as impacts on the composition and structure of the biomass, were evaluated. The above mentioned biomass parameters were assessed as the effectiveness of enzymatic hydrolysis of the biomass material is critically dependent upon them. Similar to that reported by Sun et al. (2014) on dilute acid pre-treatment of Poplar, our results suggested the increased cellulose accessibility on steam exploded hardwoods was mainly due to hemicellulose removal, lignin-hemicellulose phase separation and/or lignin redistribution. The following chapters discuss the synergistic enzymatic degradation of native/untreated and pre-treated hardwood biomass and identifies the effects pre-treatments have on the combinations of enzymes required to efficiently hydrolyse these hardwood substrates.

Chapter 6: Evaluation of inhibitory effects of wash liquors from pre-treated hardwoods on CelMix and XynMix

6.1. Introduction

During biofuel production, plant biomasses are first subjected to pre-treatment processes in order to open up cell walls and adapt them accessible to enzymes for subsequent fermentation and bioconversion (Zhang & Lynd 2004). Diverse phenolic compounds are formed as residues of lignin degradation during these wood and plant residue pre-treatment processes for hydrolysate production and wood pulping (Guo et al. 2013; Jönsson et al. 1998; Le Bourvellec et al. 2011). The phenolic compounds identified in lignocellulosic hydrolysates upon lignin fragmentation by pre-treatment technologies are inclusive of simple phenolics (gallate, vanillin, ferulic acid, syringaldehyde and conifer alcohol, etc.), non-phenolic aromatics (benzoic acid and cinnamic acid, etc.) and oligomeric phenolics (ellagic acid, epicatechin and tannic acid, etc.) (Li et al. 2014). Polysaccharide (cellulose, hemicellulose and pectin) degradation during pre-treatments generally liberates sugars (monomers and oligomers), furan derivatives (hydroxymethyl furfural and furfural) through dehydration of sugars, and organic acids (acetic, formic and levulinic acids) through further degradation of furans and from acetyl groups on hemicellulosic polysaccharides (Zhai et al. 2016).

The composition and concentration of the different by-product compounds formed during biomass pre-treatment varies, and depends on both the plant source and the pre-treatment method employed (Jönsson & Martín 2016). Acids, furans (furfural and 5-HMF) and phenols have recently been reported as dominant inhibitors in acid pre-treated and steam explosion pre-treated biomass liquors (Jönsson & Martín 2016). On the other hand, acids and phenolic compounds are reported to be mainly produced after alkali pre-treatment of biomass (Jönsson & Martín 2016). It has also been reported that harsh pre-treatment conditions, such as high chemical doses and/or cooking conditions, can lead to lignin condensation/re-precipitation during the cooling process of the pre-treated slurry which can then lead to negative impacts on enzymatic hydrolysis of the biomass (Chen et al. 2017).

In literature, inhibition of lignocellulose-derived by-products, particularly phenolic mono-lignols, on fermentative strains have been widely investigated, whereas only a few reports are available on their inhibition effects on biomass saccharification by enzymes. A few studies have investigated the effect of soluble inhibitors on cellulases (Pham et al. 2010; Kim et al. 2011; Ximenes et al. 2010; Ximenes et al. 2011) and xylanases (Morrison et al. 2011; Silva et

al. 2015; González-Bautista et al. 2017), while only one study clearly reported on the inhibition of mannanases (Malgas et al. 2016). Previous studies have demonstrated that phenolic hydroxyl groups are crucial functional groups in the inhibitory effects of lignin and mono-lignols (Malgas et al. 2016; Nakagame et al. 2011; Pan 2008). Studies have also reported on the existence of interactions between phenolics and enzymes that lead to complexes, resulting in enzymes precipitation during biomass degradation (Haslam 1974; Kim et al. 2011; Tejirian & Xu 2011). Malgas and co-workers further suggested that protein-to-phenolic complex precipitation might not be the only mode of enzyme inactivation. Only a minimal amount of the phenolics is required to physically form a complex with the proteins in order to precipitate them out of solution. Inhibition at higher phenolic concentrations could be due to saturation of the enzyme active site and micro-environment with the phenolics, leading to competition with the enzyme's substrate (Malgas et al. 2016).

To block this non-productive adsorption of hydrolytic enzymes to lignin during lignocellulosic biomass degradation, additives such as exogenous proteins (bovine serum albumin (BSA), peptides and soybean protein), and also surfactants such as Tween 20 and Tween 80, Triton X100, polyethylene glycol (PEG), polyvinylpyrrolidone (PVP) and lignosulfonates have been used. They were found to be effective at improving the enzymatic hydrolysis of pre-treated lignocellulosic biomass (Akimkulova et al. 2016; Kumar & Wyman 2009c; Obeng et al. 2017; Tejirian & Xu 2011). The major explanations for the positive effects of additives on cellulases are as follows: (1) the interaction between the additives and lignin possibly reduces non-productive adsorption of enzymes on lignin and/or steric hindrances to enzymes which shield the enzymes away from the substrate, (2) additives protect enzymes from denaturation by heat, solvents and shear force and (3) additives possibly alter the biomass structure thus increasing cellulose accessibility (Chen et al. 2017; Li et al. 2015; Obeng et al. 2017).

Therefore, it is of great interest to conduct research into elucidating the mode of inhibition of pre-treatment by-products on enzymes during biomass saccharification for cellulosic ethanol production, as this could provide theoretical support to reduce the inhibition effects in hydrolysates to enhance ethanol yield from lignocellulosic biomass. In addition, we explored the use of biological (e.g. bovine serum albumin and laccases) and chemical (e.g. surfactants) additives to improve the performance of enzymes during pre-treated biomass saccharification in the presence of pre-treatment degradation products.

6.2. **Aims and Objectives**

6.2.1. **Aims**

To determine the individual inhibitory effects of known pre-treatment by-products and the inhibitory effect of pre-treatment by-products liquor obtained from hardwoods pre-treated by various technologies on the cellulolytic (CelMix) and xylanolytic (XynMix) core-components of the HoloMix. To determine the effect of surfactant addition on the activities of the HoloMix core-components in the presence of liquors from hardwoods pre-treated by various technologies.

6.2.2. **Objectives**

- To conduct CelMix and XynMix inhibition studies using individual known pre-treatment by-products;
- To identity and quantify by-products from hardwoods pre-treated by various technologies;
- To conduct CelMix and XynMix inhibition studies using wash liquors from hardwoods pre-treated by various pre-treatment technologies;
- To evaluate the effect of surfactants and proteins on improving HoloMix activity in the presence of liquors from hardwoods pre-treated by various technologies;
- To evaluate the mode of action of AbLac on improving HoloMix activity in the presence of liquors from steam exploded hardwoods.

6.3. Materials and Methods

6.3.1. Pre-treatment by-product inhibition assays

Enzyme activity assays were carried out with each individual inhibitor (0.5-2 mg/mL) and activity assays for CelMix and XynMix were set up as described in Section 3.3.4.1 using Avicel and beechwood xylan as substrates, respectively. The inhibitors assessed were: acetic acid, formic acid, kraft lignin, levulinic acid, furfural, hydroxymethylfurfural, *p*-coumaric acid, vanillin, gallic acid and vanillic acid. Rates of substrate hydrolysis in the presence and absence of added inhibitors were then compared.

6.3.2. Preparation of pre-treatment by-product liquors from pre-treated hardwoods and inhibition studies

6.3.2.1. Preparation of pre-treatment by-products liquors from hardwoods treated by various pre-treatment technologies

Hardwoods (*Acacia* and Poplar) that were: (1) untreated, (2) delignified and (3) steam exploded were used to obtain pre-treated biomass wash streams. About 20 mL of 2.66 % (w/v) of each pre-treated hardwood was prepared using 50 mM sodium citrate buffer (pH 5.0) in 50 mL Schott bottles. The pre-treated hardwood slurries were mixed using a magnetic stirrer for 24 hours. After this, the slurries were transferred into Beckman centrifuge tubes (50 mL) and centrifuged at 15, 316 x *g* (Beckman Avanti centrifuge) for 10 minutes at 4°C. The pH of the washes was measured (before and after 24 hours) using a pH meter (PHS-3BW BANTE instruments) and compared to that of sodium citrate buffer (pH 5.0). Finally, the supernatant was kept at 4^{o}C until further studies were performed.

6.3.2.2. Analysis of the contents of liquors from hardwoods pre-treated by various pre-treatment technologies

The total amount of phenolics in the various substrate liquors was determined using a modified Folin–Ciocalteu method described by Anesini and co-workers (Anesini et al. 2008). Here, 10 µL of pre-treatment liquor, 20 µL of Folin reagent and 200 µL of distilled water were added to a 96 well plate. After 3 minutes had lapsed, 50 µL of 2 M sodium carbonate was added to the mixture. A blank was also prepared in the same manner except 10 µL of distilled water was used as a sample. Finally, the plates were incubated at 40^{o}C for 30 minutes before reading the absorbance at 765 nm.

Individual phenolic compounds and furans in the various pre-treated hardwood liquors were analysed using a reverse phase C18 column. The HPLC system consisted of a Symmetry®-

C18 column (39×150 mm, 5 µm particle size, Waters Corp., Milford, MA) with analysis being performed on a Shimadzu HPLC system (Shimadzu Corp, Japan) with a SPD-20AV detector and controlled using LabSolutions software. Ambient conditions were used for analysis. The mobile phase A was 0.01 M phosphoric acid and the mobile phase B was HPLC grade methanol. The isocratic mobile phase consisted of A: 70% and B: 30% and was run for 30 min at a flow rate of 1.0 mL/min. The injected sample size was 20 µL and the effluent's UV absorption was monitored at 280 nm. Standards for the C18 column included the following compounds: furfural, hydroxymethylfurfural, gallic acid, vanillin, vanillic acid, *p*-coumaric acid and guaiacol (all from Sigma–Aldrich).

Individual organic acids (acetic and formic acid) and monosaccharides (glucose and xylose) in the various pre-treated hardwood wash liquors were analysed using Megazyme kits as stipulated for the microtiter assays in the manufacturer's guide. For acetic acid, the kit K-ACET was used, while for formic acid, the kit K-FORM was used. Quantitative analysis of glucose concentrations was performed by the glucose oxidase/peroxidase (GOPOD) method (K-GLUC), while the K-XYLOSE kit was used for determining xylose concentration in the wash liquors.

6.3.2.3. **Enzyme inhibition studies using wash liquors from hardwoods pre-treated by various pre-treatment technologies**

Activity of the HoloMix components, CelMix and XynMix, was then assessed in the presence of these washes as described previously. Reactions set up included a positive control (containing enzyme with substrate prepared in citrate buffer), assay (containing enzyme with substrate prepared using a wash from a particular pre-treated substrate), substrate controls prepared in 50 mM citrate buffer (pH 5.0) and another prepared using wash streams from pre-treated biomass (containing only the substrate without the enzyme) and enzyme controls (containing only the enzyme without the substrate). In the assays, the wash streams contained a final concentration of pre-treatment soluble compounds corresponding to those that would be liberated if the pre-treated biomass were loaded at 2% (w/v) substrate loading.

6.3.3. **Evaluation of the effect of additives on HoloMix activity in the presence of liquors of hardwoods pre-treated by various technologies**

Improvement in the activity of the HoloMix components; CelMix and XynMix, by additives (Tween 20, Tween 80, Triton X, PEG 2000 and SDS) and proteins (*Agaricus bisporus* laccase, AbLac, and BSA) at 2.5 mg/mL was then assessed in the presence of an inhibitor cocktail (most inhibitory pre-treated biomass wash liquor). The laccase, AbLac, was assessed at a loading of 0.25 mg/mL in the reaction. Reactions set up included a positive control (containing enzyme with substrate in the presence of an inhibitor cocktail, here pure citrate buffer was used), assay (containing enzyme with substrate in the presence of both individual surfactants and the inhibitor cocktail), substrate controls prepared in citrate buffer and another prepared which included the inhibitor cocktail (containing only the substrate without the enzyme) and enzyme controls (containing only the enzyme without the substrate).

6.3.4. **Data analysis**

One way analysis of variance (ANOVA) was used to analyse abolition or activation of the activity of the enzymes by the inhibitors or additives, respectively. All pairwise comparison procedures were based at 95% confidence level (p=0.05) using data analysis in Microsoft® Excel. Error bars represent standard deviations.

6.4. Results

6.4.1. Analysis of the contents of liquors from hardwoods pre-treated by various pre-treatment technologies

Pre-treated biomass hydrolysates contain a broad variety of phenolic compounds (Jönsson et al. 2013; Cavka et al. 2014) that act synergistically as inhibitors. In this work, the quantities of these compounds contained in wash liquors from hardwoods pre-treated by two different methods were quantified. Table 6.1 shows the amounts of pre-treatment by-products released by the pre-treated hardwoods.

Steam exploded *Acacia* and Poplar, as well as untreated *Acacia*, contained the highest content of total soluble phenolics and formic acid. Interestingly, the steam exploded biomasses were the only samples with wash liquors containing *p*-coumaric acid and vanillic acid. These substrates also contained the highest xylose content. None of the biomass wash liquors contained free glucose, acetic acid, guaiacol or vanillin.

Only about 0.1 mg/g biomass of furfural was found in all the pre-treated hardwood samples, except for untreated *Acacia*, which did not generate detectable levels of furfural. Hydroxymethylfurfural was found in very high levels in delignified and steam exploded Poplar, while it was found in small amounts in untreated *Acacia*. Only delignified *Acacia* and untreated Poplar samples did not contain any detectable levels of gallic acid, while the other biomass liquors contained detectable levels of gallic acid.

Table 6.1. Chemical composition of liquors obtained from hardwoods pre-treated by various technologies (mg compound/g biomass).

Compound (mg/g biomass)	Untreated Acacia	Untreated Poplar	Delignified Acacia	Delignified Poplar	Steam exploded Acacia	Steam exploded Poplar
Acetic acid[a]	Nd	Nd	Nd	Nd	Nd	Nd
Formic acid[a]	1.9	Nd	Nd	1.5	5.3	5.5
Gallic acid[b]	0.1	Nd	Nd	0.1	0.1	0.4
Guaiacol[b]	Nd	Nd	Nd	Nd	Nd	Nd
Furfural[b]	Nd	0.1	0.1	0.2	0.1	0.1
Hydroxymethylfurfural[b]	0.4	Nd	Nd	2.0	Nd	1.2
p-Coumaric acid[b]	Nd	Nd	Nd	Nd	0.2	0.2
Vanillic acid[b]	Nd	Nd	Nd	Nd	0.3	0.3
Vanillin[b]	Nd	Nd	Nd	Nd	Nd	Nd
Total phenolics[c]	5.49	Nd	Nd	Nd	6.48	7.98
Glucose[a]	Nd	Nd	Nd	Nd	Nd	Nd
Xylose[a]	1.5	0.1	0.4	0.6	1.9	2.5

[a]-Determined by Megazyme kits, [b]-Determined by HPLC and [c]-Determined by the Folin-Ciocalteu method. Where "Nd" = not determined.

6.4.2. **Inhibitory effect of enzyme by pre-treatment degradation products**

The inhibitory effects of lignocellulose degradation products on the holocellulolytic enzyme core-sets, CelMix and XynMix, were evaluated over a concentration range of 0.5 to 2 mg/mL of each compound. Figures 6.1 below illustrates the tolerance levels of the CelMix and XynMix enzyme cocktails to the various carbohydrate-derived pre-treatment by-products.

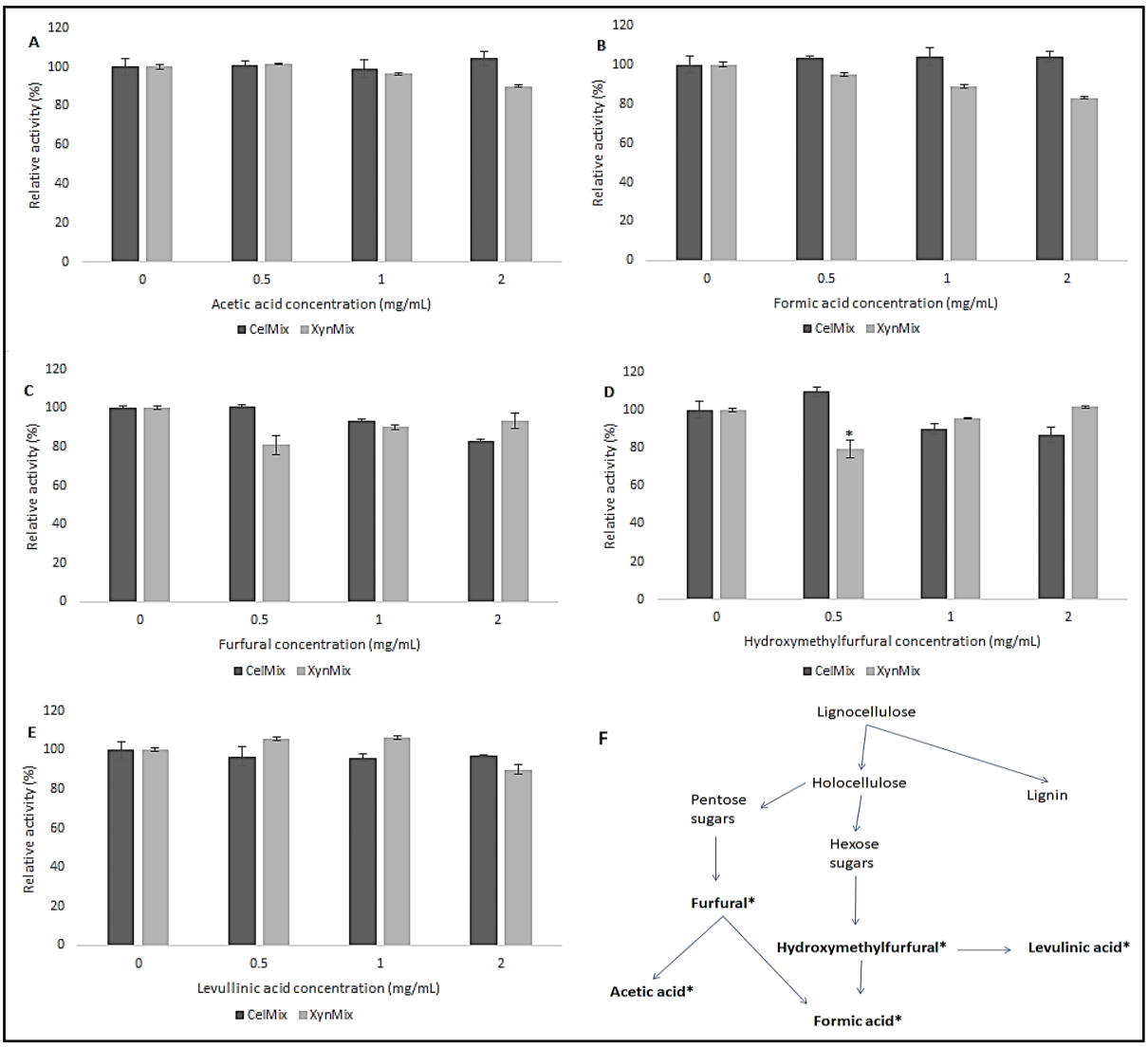

Figure 6.1. Inhibition of CelMix and XynMix by (A) acetic acid, (B) formic acid, (C) furfural, (D) hydroxymethylfurfural and (E) levulinic acid at 0.5, 1 and 2 mg/mL. Values are represented as mean values ±SD (n=4). ANOVA analysis of inhibition or activation of enzymes by the different sugar degradation products was compared to controls containing no degradation products, test keys; * (p value<0.05) and # (p value<0.01). (F) An illustration of how these sugar lignocellulose degradation products are formed during the pre-treatment of biomass.

From the polysaccharide degradation by-products inhibition assays, none of the enzyme cocktails, CelMix and XynMix, were significantly inhibited in the presence of these by-

products, except for XynMix which was inhibited by hydroxymethylfurfural only at low concentrations (0.05 mg/mL). The figure below displays the inhibitory effects of the pre-treatment lignin degradation by-products (gallic acid, low sulfonated kraft lignin, *p*-coumaric acid, vanillic acid and vanillin) on the activity of cellulolytic and xylanolytic cocktails, CelMix and XynMix, respectively (Fig. 6.2).

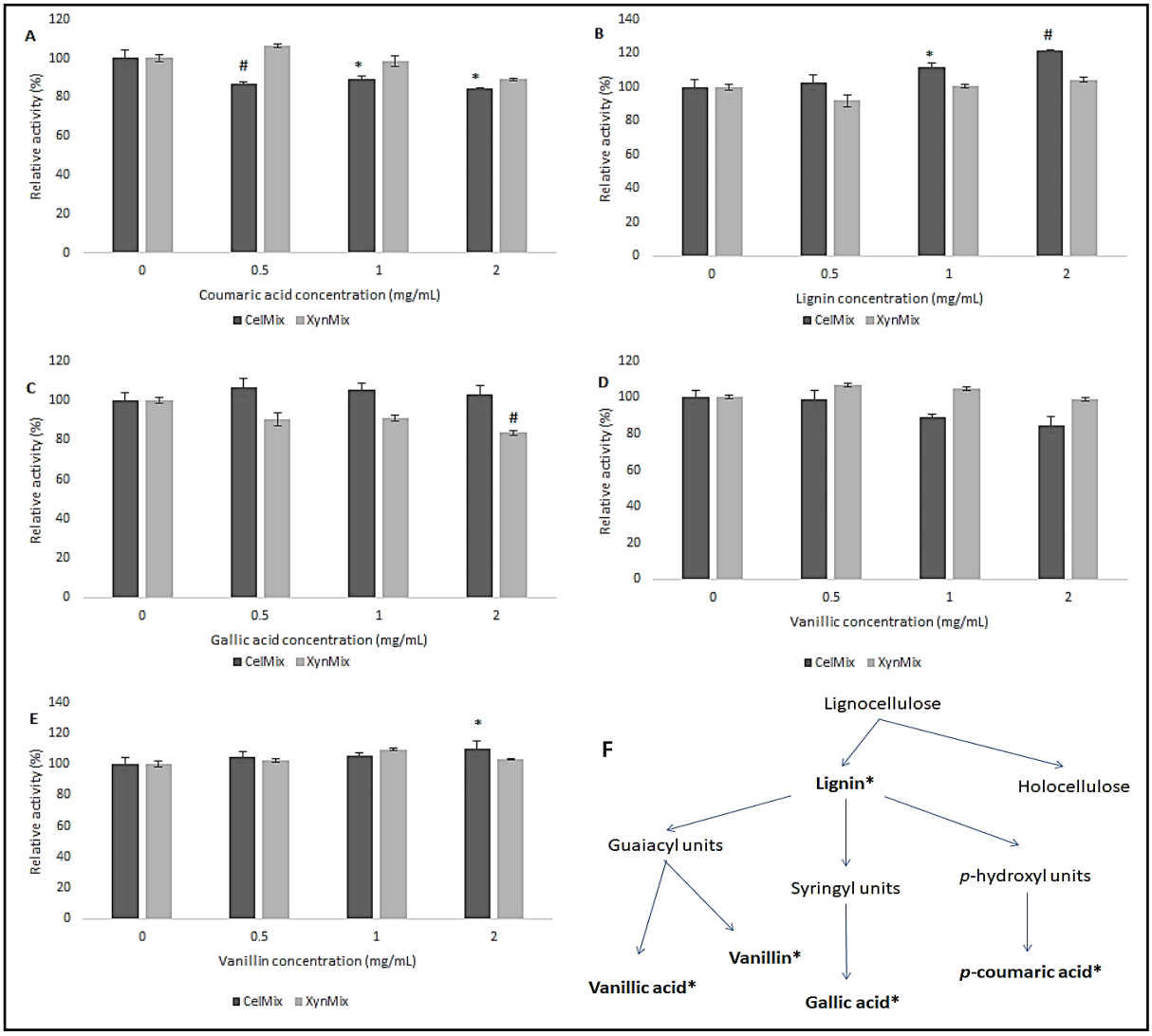

Figure 6.2. Inhibition of CelMix and XynMix by (A) *p*-coumaric acid, (B) lignin, (C) gallic acid, (D) vanillic acid and (E) vanillin at 0.5, 1 and 2 mg/mL. Values are represented as mean values ±SD (n=4). ANOVA analysis of inhibition or activation of enzymes by the different lignin degradation products was compared to controls containing no degradation products, test keys; * (p value<0.05) and # (p value<0.01). (F) An illustration of how these lignin lignocellulose degradation products are formed during the pre-treatment of biomass.

From the lignin degradation by-products inhibition assays, CelMix was only significantly inhibited by *p*-coumaric acid in a dose dependent fashion, while it was significantly activated

by kraft lignin and vanillin in a dose dependent fashion as well. XynMix on the other hand was only inhibited by gallic acid.

6.4.3. Enzyme inhibition studies using wash liquors from hardwoods pre-treated by various pre-treatment technologies

The possible inhibitory effects of the wash liquors from hardwoods pre-treated by various technologies on CelMix and XynMix enzyme preparations were assessed during the hydrolysis of Avicel and beechwood xylan, respectively (Figure 6.3).

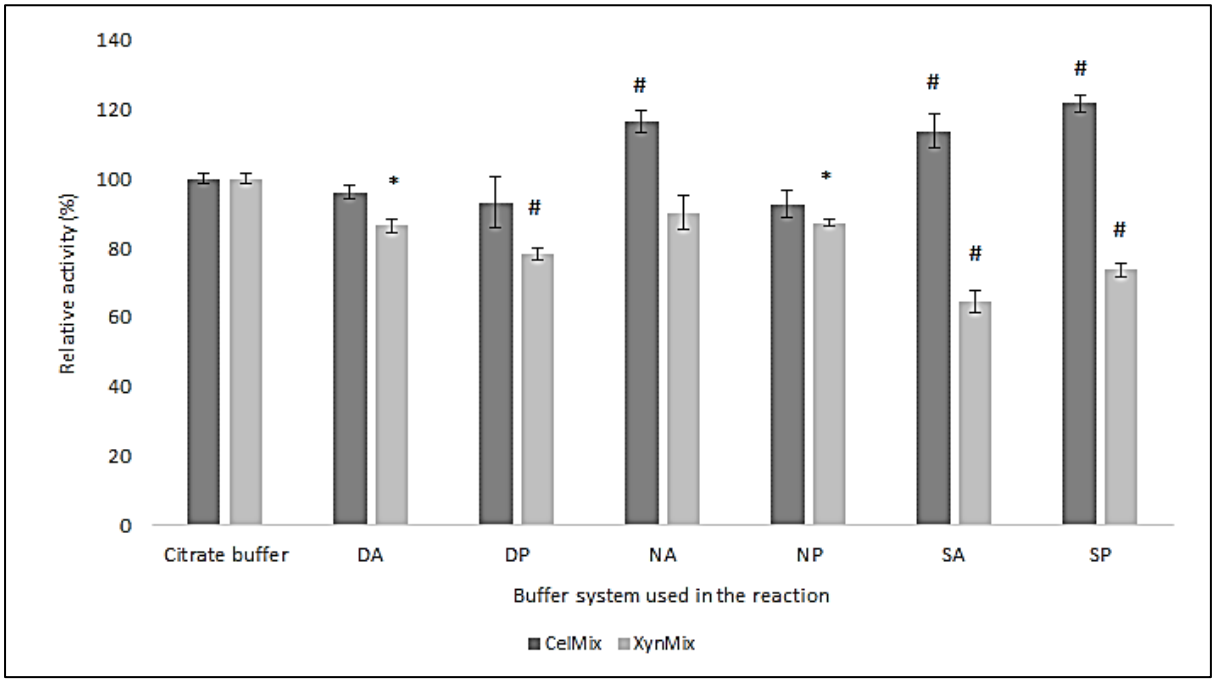

Figure 6.3. Inhibition of CelMix and XynMix by citrate buffer wash liquors of hardwoods pre-treated by various pre-treatment technologies. The washes used were from: delignified *Acacia* (DA) and Poplar (DP), native/untreated *Acacia* (NA) and Poplar (NA), and steam exploded *Acacia* (SA) and Poplar (SP). ANOVA analysis for inhibition or activation of CelMix and XynMix in a buffer system of pre-treated substrate wash compared to these cocktails in a 50 mM citrate (pH 5.0) buffer system, keys: * (p value < 0.05) and # (p value < 0.01). Values are represented as mean values ±SD (n = 4).

CelMix was significantly activated by the 50 mM citrate buffer (pH 5.0) wash liquors containing pre-treatment by-products from hardwoods as the buffering medium in the reaction, compared to when pure citrate buffer was used, except for those wash liquors derived from delignified hardwoods. On the other hand, XynMix was highly inhibited in the presence of all the wash liquors in the reaction medium compared to when citrate buffer was used, especially those liquors extracted from steam exploded hardwoods.

6.4.4. Evaluation of the effects of additives on XynMix activity in the presence of a liquor from steam exploded Acacia

The direct effect of each additive/protein on xylanolytic activity by XynMix was determined relative to controls containing no pre-treatment wash liquor from steam exploded *Acacia* (no inhibition) and that containing the liquor (inhibition). Due to XynMix being the only enzyme cocktail significantly inhibited by pre-treatment by-products (while CelMix was activated by these compounds), CelMix was excluded in the study for evaluating the effects of additives on enzyme cocktails in the presence of pre-treatment liquors. Figure 6.4 below illustrates the results obtained from the study.

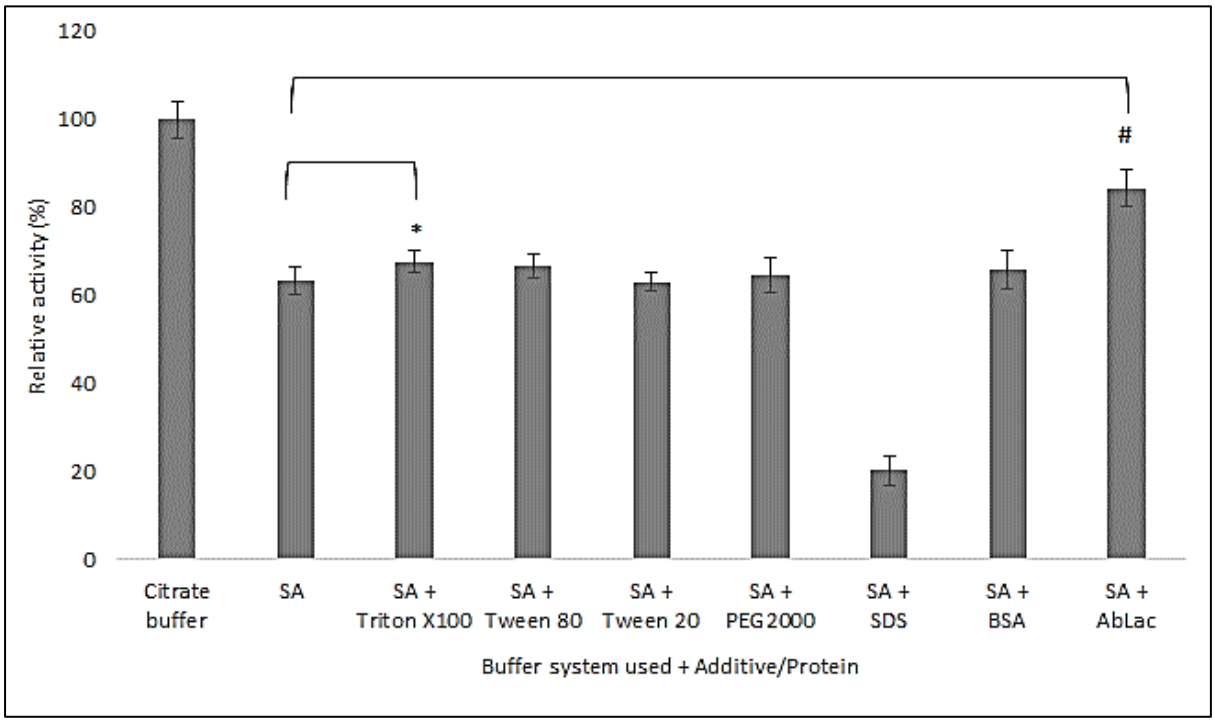

Figure 6.4. Prevention of the inhibition of XynMix by a citrate buffer wash liquor from steam exploded Acacia (SA). ANOVA analysis for activation of XynMix in a buffer system of pre-treated substrate wash compared to the cocktail in the same buffer system, but in the presence of additives, keys: * (p value < 0.05) and # (p value < 0.01). Values are represented as mean values ±SD (n = 4).

Only Triton X100 and the laccase, AbLac, could significantly alleviate the inhibitory effects of the wash liquor from steam exploded *Acacia* (SA) on the activity of XynMix (see brackets illustrating this in Fig 6.4). SDS on the other hand led to a further decrease in the activity of XynMix during beechwood xylan degradation in the presence of the wash liquor. Tween 20 and 80, PEG 2000 and BSA had no effect on the activity of XynMix in the presence of the wash liquor from steam exploded *Acacia*.

Following evaluation of the direct effect of each additive/protein on xylanolytic activity by XynMix, the total phenolic content of the additive (AbLac) that was successful was evaluated using the Folin's method as described in Section 6.3.2.2, see Figure 6.5 below.

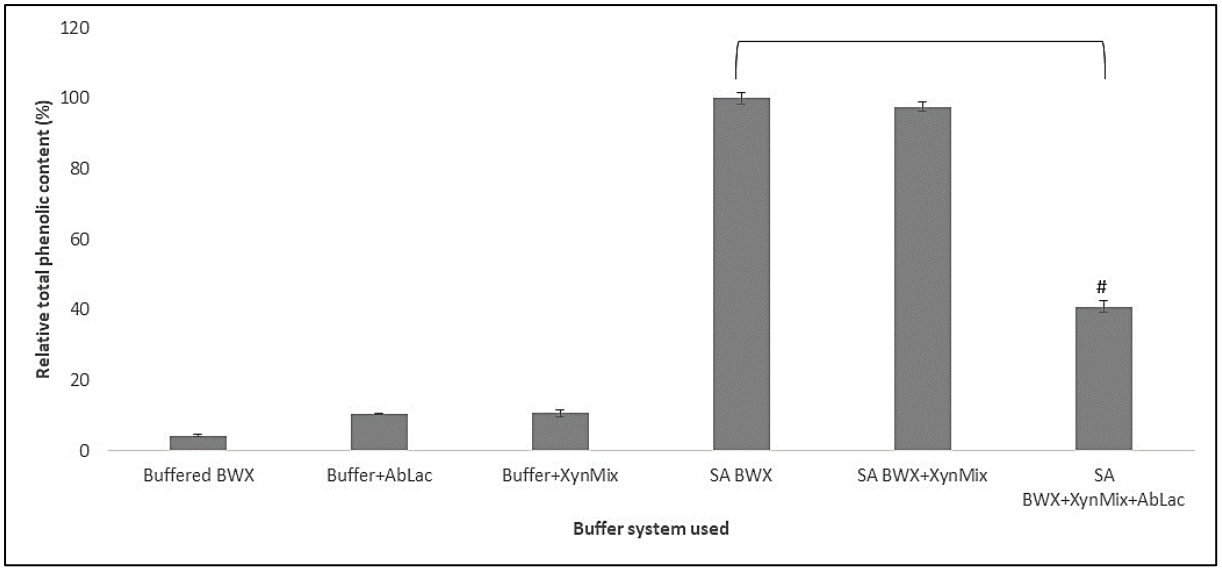

Figure 6.5. Total phenolic content in the beechwood hydrolysate (BWX) buffered by steam exploded *Acacia* wash liquor (SA) in the absence and presence of laccase, AbLac. ANOVA analysis for activation of XynMix in a buffer system (50 mm citrate, pH 5.0) of pre-treated substrate wash compared to the cocktail in the same buffer system, but in the presence of additives, keys: * (p value < 0.05) and # (p value < 0.01). Values are represented as mean values ±SD (n = 4).

The addition of AbLac during the degradation of beechwood xylan in the presence of the wash liquor from steam exploded *Acacia* by XynMix led to over 50% reduction of total phenolics in the hydrolysate (this reduction was significant with over 99% confidence) (see bracket illustrating this in Fig 6.5).

6.5. Discussion

6.5.1. Chemical composition of liquors from hardwoods pre-treated by various technologies

Analysis of the wash liquors from hardwoods, *Acacia* and Poplar, subjected to different pre-treatment technologies revealed that these led to the release of different pre-treatment soluble products (see Table 6.1). Steam exploded biomasses contained the highest contents of soluble pre-treatment by-products including formic acid and xylose which are derived from xylan solubilisation during the steam pre-treatment process. Similar to our findings, Zhai and co-workers detected high levels of hemicellulose derived sugars, such as xylose, and phenolics, while small amounts of sugar degradation products, such as 5-hydroxymethylfurfural (HMF), furfural, and some acetic acid in the water-soluble fraction of steam exploded Poplar (Zhai et al. 2016). A study by Fenske and co-workers showed that diluted acid pre-treatment of Poplar at 10% (w/v) solid level generates 0.16 g/L syringyl, 0.08 g/L guaiacyl, 0.01 g/L hydroxyphenyl phenolics and 2.9 g/L acetate (Fenske et al. 1998). Similarly, Du and co-workers evaluated eight different pre-treatments (ranging from lime, ammonia, wet oxidation, to acid-based processes) of Poplar and produced between 14-120 mg/L acetic acid, 41-210 mg/L formic acid, 2.4-7.1 mg/L vanillin, 0.16-190 mg/L for HMF and furfural, and 2.3-8.7 mg/L vanillic acid, respectively, of pre-treatment by-products in the process (Du et al. 2010). Interestingly, untreated *Acacia* wash liquors contained about 5.49 mg total phenolics/g biomass. However, HPLC analysis detected only gallic acid at about 0.1 mg/g biomass – it is likely that the majority of phenolics detected by the Folin method were oligomeric and not monomeric thus HPLC could not detect their presence. All the pre-treatment wash liquors (untreated Poplar, and delignified Acacia and Poplar) which had no detectable levels of total phenolics by the Folin method also consistently contained no mono-phenolics as reported by HPLC – except for delignified Poplar wash liquor which had traces (0.1 mg/g biomass) of gallic acid.

6.5.2. Pre-treatment by-products inhibition assays

In general, the data for individual inhibitor compound effects on both CelMix and XynMix activity showed that an increase in the inhibitor concentration from 0.5 to 2 mg/mL led to increased inhibition, see Figures 6.1 and 6.2. However, XynMix inhibition by both furfural (Fig 6.1C) and hydroxymethylfurfural (Fig 6.1D) appeared not to be consistent with this trend, but were rather inhibitory at lower concentrations compared to higher concentrations. It is noteworthy to mention that the pH of the reaction medium at 0.5 to 2 mg/mL of each of the

pre-treatment by-products was always within 0.5 pH units to that of citrate buffer used as the positive control (pH 5.0), suggesting that the inhibition caused by the compounds studied was due to the physical interaction between enzyme and compound, rather than due to changes in pH. The general lack of concentration dependent inhibition indicated that there was no feasibility in preventing enzyme inhibition by either reducing the concentration of phenolic compounds with an appropriate detoxification procedure or by increasing protein loadings. The latter approach to combat enzyme inactivation has been proposed previously in literature. However, increasing enzyme loadings to prevent enzyme inactivation by inhibitors leads to lowered specific reaction rates (Cantarella et al. 2014; Kim et al. 2011).

The presence of vanillin exhibited no inhibitory effect on XynMix activity (Fig 6.2). Kaya and co-workers also demonstrated that vanillic acid enhanced xylanase activity in the presence of increasing vanillic acid up to concentrations of 0.05% (w/v) (Kaya et al. 2000). Upon CD spectroscopy analysis, Kaya and co-workers observed a change in the secondary structure of the enzyme in the presence of vanillic acid and attributed this to the observed enzyme activation. The inhibition of XynMix by the pre-treatment by-products emphasises the importance of investigating the effects of these compounds on holocellulolytic enzymes and the development of methods to mitigate their effects for more efficient enzymatic hydrolysis of lignocellulose.

Kraft lignin and vanillin significantly activated CelMix activity in a concentration dependent fashion. This finding was contrary to what was reported for a commercial *Trichoderma reesei* cellulase preparation, which was inhibited by up to 10% in the presence of 5 mg/mL of vanillin (Li et al. 2014). When 0.5 to 2 mg/mL of *p*-coumaric acid was present, the hydrolysis of Avicel by CelMix experienced a ~20% loss in the extent of hydrolysis, indicating an inhibition from the mono-lignol (Fig 6.2A). Our results showed that the significant inhibition of enzyme activity (both xylanolytic and cellulolytic) was mostly due to the presence of monomeric residues such as mono-aromatic phenolics, and to a lesser extent, by polymeric lignin compounds such as low sulfonated kraft lignin (see Fig 6.1 and Fig 6.2). This finding was contrary to that reported in previous studies which showed strong inhibition of enzyme activity due to polymeric lignin residues and, to a lesser extent, by monomeric lignin compounds (Mhlongo et al. 2015; Tejirian & Xu 2011).

6.5.3. **Enzyme inhibition studies using wash liquors from hardwoods pre-treated by various pre-treatment technologies**

The liquors from all the pre-treated biomasses were inhibitory to the xylanolytic cocktail, XynMix (Figure 6.3). Interestingly, the biomass liquors which contained the highest content of soluble pre-treatment by-products (untreated *Acacia* and steam exploded *Acacia* and Poplar) (see Table 6.1 for liquor contents and their quantities) activated CelMix activity during cellulose degradation, while they drastically inhibited XynMix (see Figure 6.3). Based on Table 6.1, the concentrations and composition of pre-treatment by-products in the various technologies pre-treated liquors were quite diverse, possibly due to the variety and complexity of the pre-treatments conducted. It is possible that the inhibitory effects or activating effects in the case of CelMix, are due to additive or synergistic effects by the multiple kinds of inhibitors found within these liquors. Contrary to our findings on CelMix activation by lignocellulosic biomass water extractives, a study by Gamble and colleagues showed that water extracts from flax stems containing mixtures of phenolic compounds that appear to be linked to sugars or hydroxy acids through glycosidic linkages and flavonoids served as inhibitors for various polysaccharide-degrading enzymes, cellulases and pectinase (Gamble et al. 2000). Zhai and co-workers also showed both Celluclast 1.5L and CTec3 were inhibited to some extent by compounds present in the water-soluble fraction of steam pre-treated poplar (Zhai et al. 2016). Similar to our findings, recently, Smith and Huijgen demonstrated that water-soluble extractives from wheat straw could enhance the enzymatic hydrolysis of wheat straw pulp obtained by Organosolv, dilute acid and alkaline pre-treatment. These researchers had previously shown that the promotional effect of water-soluble extractives on enzymatic hydrolysis of wheat was also possible using wash liquors from biomass pre-treated by a low-temperature acetone-based Organosolv process (Smit & Huijgen 2017).

6.5.4. **Evaluation of the effect of additives on XynMix activity in the presence of a liquor from steam exploded Acacia**

In many studies, it has been reported that additives such as PEG are capable of reducing the inhibitory effect of phenolics on enzymes (Akimkulova et al. 2016; Obeng et al. 2017). It has been proposed that these additives (such as PEG) bind to lignins via hydrophobic interactions and hydrogen bonding (Qin et al. 2016), sometimes form micelles to separate enzymes from inhibitors (González-Bautista et al. 2017) and this leads to the reduction of unproductive binding of enzymes onto lignin. In our study, the use of Tween 20 and 80, and also PEG 2000, had no effect on the inhibitory effects of steam exploded *Acacia* liquor on XynMix activity

(see Fig 6.4). Qin and co-workers similarly demonstrated that these additives, PEG and BSA, have no major impact on alleviating the inhibition of cellulases by soluble inhibitors (Qin et al. 2016). Our work and that conducted by Qin et al. (2016) demonstrated that the inhibition mechanism of phenolics was different from insoluble lignin. The use of SDS as an additive to prevent the inhibitory effect of steam exploded *Acacia* wash liquor led to more inhibition. This finding was rather unexpected as the chemical modification of reactive functional groups on enzymes by SDS was expected to increase the ratio of negative to positive surface charges on the enzymes' surfaces and due to negative charge of aromatic carbonyls contained in the liquor, the repulsive interactions between the two should increase and thus the hydrolytic efficiency.

Laccases are multicopper-containing oxidases with phenoloxidase activity, which catalyse the oxidation of phenols with molecular oxygen as the electron acceptor that is reduced to water generating unstable phenoxyl radicals that lead to the formation of higher molecular phenolic polymers and aromatic compounds (Jönsson et al. 1998; Jurado et al. 2009). Laccase supplementation to enzymatic hydrolysis has resulted in controversial findings. In our work, the addition of AbLac led to the enhancement of xylose recoveries when XynMix was used to carry out beechwood glucuronoxylan hydrolysis. Interestingly, AbLac addition led to lowered total phenolics in the wash liquor from steam exploded *Acacia* (see Fig 6.5). We believe the laccase, AbLac, may be coupling the mono-aromatic phenolics in the liquor into oligomers – thus lowering the amounts of total phenolics that can be determined by the Folin method. Similarly, Jönsson and co-workers showed that treatment of the hydrolysate (from willow pre-treated with steam and SO_2) with a laccase led to the removal of mono-aromatic phenolic compounds present in the hydrolysate (Jönsson et al. 1998). Recently, Jurado and co-workers also reported on laccase treatment performed on steam-exploded wheat straw which led to the removal of about 75% of the phenolic compounds (Jurado et al. 2009). Implications of our findings may be that oligomeric phenolics are less inhibitory to xylanolytic enzymes than monomeric phenolics. However, further studies are required to confirm this hypothesis.

6.6. **Conclusions**

From our study it was elucidated that the inhibition of enzyme cocktails, CelMix and XynMix, relies on the particular relationship which occurs between the specific cocktail and pre-treatment by-product(s). In general, CelMix was activated by some of the individual by-products and a majority of the pre-treated biomass wash liquors, except for those obtained from delignified hardwoods, while XynMix was inhibited by HMF, gallic acid and all of the wash liquors. From our study, it also appeared that the inhibition effect exerted by the individual pre-treatment by-products was dependent on the concentration of these compounds. This implies that the inhibitory effect of these pre-treatment by-products can be limited by modifying their concentration or alternatively, enzymes can be loaded at higher loadings. Furthermore, this study provides insight into the impact of individual inhibitors on hydrolytic enzymes, which in future will inform the appropriate pre-treatment strategies for different substrates. We also demonstrated that lignin-blocking agents such as surfactants and BSA have no effect on the inhibitory effect of soluble by-products such as phenolics, indicating that their inhibition of enzymes occurs via a different mechanism to that of insoluble lignin.

Chapter 7: Evaluation of synergism between CelMix and XynMix during the degradation of hardwoods

7.1. Introduction

Lignocellulose is the most abundant biomass in the world. It consists of lignin, which forms a protective barrier around the holocellulose component, which is a combination of cellulose, pectin and hemicellulose in the plant biomass (Beukes & Pletschke 2011; Rastogi & Shrivastava 2017). The fact that lignocellulosic biomass is a renewable resource, abundant, and environmentally friendly makes it an appropriate candidate for replacing fossil fuels (Beukes & Pletschke 2011; Rae et al. 2017). To unlock some of the potential value-added products (or precursors thereof) from the lignocellulosic biomass, pre-treatment and/ or enzyme hydrolysis is required (Parmar & Rupasinghe 2012; Rae et al. 2017). From a biochemical point of view, wood is composed of about 50% cellulose, 25% lignin and 25% hemicellulose, with traces of pectins and proteins (Chen et al. 2017; Déjardin et al. 2010). Hardwoods were chosen for this study (as opposed to softwoods), as the lignin monomers of hardwoods are composed of both guaiacyl and syringyl units (easy to delignify), while those of softwood lignin are mainly guaiacol units (difficult to delignify) (Munoz et al. 2007; Zeng et al. 2014). Their polysaccharides are, therefore, more easily accessible to enzymes for the purpose of producing fermentable sugars after pre-treatment. Two hardwoods were selected for the purpose of this study, *Populus* spp. and *Acacia* spp.

Upon pre-treatment, SC/AA leaves a higher proportion of the hemicellulose still associated with the cellulose-rich water insoluble fraction while selectively removing lignin to a high extent (Siqueira et al. 2013). However, it has been reported that increased removal of lignin tends to partially degrade the polysaccharide fraction as well, particularly above 60% lignin removal (Ahlgren and Goring, 1971). In addition, the cellulose reactivity can also be affected through oxidation and structural changes upon SC/AA pre-treatment (Jungnikl et al. 2008; Kumar et al. 2013).

The second method selected in this study was steam explosion (SE), a physico-chemical process that results in hemicellulose solubilisation, producing a glucan and lignin rich residue (Alvira et al. 2010; Hendriks & Zeeman 2009; Leskinen et al. 2017). Steam explosion produces a liquor stream containing partially hydrolysed hemicellulose and a pre-treated solid with an approximate cellulose: hemicellulose ratio of 12:1. These two different pre-treatment methods (delignification and hemicellulose retention versus hemicellulose solubilisation and lignin

redistribution) were selected so that we could understand the complex structure of various hardwoods' holocellulosic biomass and the impact of these biomass features on their hydrolysability by glycoside hydrolase (GH) enzymes.

7.2. **Aims and Objectives**

7.2.1. **Aims**

This study attempted to understand if the same enzyme cocktail could be used to degrade various hardwoods and how chemical modifications of the woody biomass during the application of various pre-treatment processes affect the synergistic associations between CelMix and XynMix which were optimized in Chapter 4.

7.2.2. **Objectives**

- To obtain the core-holocellulolytic enzyme set, HoloMix, by conducting binary simultaneous synergy studies between cellulolytic and hemicellulolytic enzymes during the hydrolysis of the hardwoods;
- To compare simultaneous and sequential application of the HoloMix to elucidate its mechanism of action;
- To quantify both reducing sugar (DNS method) and monosaccharides (Megazyme sugar detection kits) release from the synergy studies;
- To compare HoloMix hydrolytic efficiency to those of commercial enzyme preparations during the degradation of hardwoods;
- To evaluate the effect of HoloMix loading on the yield of hardwood saccharification;
- To evaluate the effect of hardwood biomass pre-treatment with AbLac on the performance of HoloMix;
- To evaluate the enhancement of HoloMix efficiency by the addition of an accessory enzyme mixture during hardwood degradation.

7.3. Materials and Methods

7.3.1. Optimization of HoloMix during hardwood degradation

Binary combinations of CelMix and XynMix were assessed for their synergistic interactions during the degradation of various untreated and sodium chlorite delignified hardwoods. The experiments were carried out in triplicate at a hardwood biomass loading of 2% (w/v) in 50 mM sodium citrate buffer (pH 5.0) in a 400 µL total volume at 50°C, mixing at 25 rpm (Labnet Revolver™) for up to 24 h. Total protein loadings for all individual enzyme core-set benchmarks (at 100% protein loading) and for all enzyme core-sets combinations experiments were kept constant at 1.25 mg/g of untreated or pre-treated hardwood. The optimal combination with respect to reducing sugar, glucose and xylose release was chosen as the "core holocellulolytic/glycosyl hydrolase set" (HoloMix) and kept for further studies. Where two combinations released equal amounts of reducing sugar, the combination that released the highest proportion of monosaccharides (glucose or xylose) was preferred.

The optimal holocellulolytic/glycosyl hydrolase set, HoloMix, was used to synergistically degrade natural/untreated, delignified, and steam exploded *Acacia* and Poplar wood substrates, respectively, for 24 h. Enzyme activity was measured in units (U), where 1 unit was defined as the amount of enzyme releasing 1 µmol of reducing sugar per hour. Glucose was used as the sugar standard.

7.3.2. Comparison of simultaneous and sequential application of the HoloMix during hardwood degradation

The optimal holocellulolytic set(s), HoloMix, found to synergistically degrade untreated, steam exploded and delignified *Acacia* and Poplar wood substrates in Section 7.3.1 were used in this section. Sequential enzyme addition assays were conducted under the same conditions used for the simultaneous synergy assays. Enzymes and substrate were incubated for 24 h and divided into three groups. One group had the whole HoloMix running for 24 h and was then boiled for 5 min to denature the enzyme, and was then processed for analysis, as described previously (Simultaneously applied HoloMix). To the other two groups, either CelMix or XynMix was added first and the reaction mixtures ran for 24 h. The reactions were then boiled for 5 min to denature the enzyme mixture, cooled down to 50°C, the missing component of the HoloMix added and ran for a further 24 h. The reaction was boiled again for 5 min to denature the enzyme mixture and then processed for analysis.

7.3.3. **Comparison of HoloMix hydrolytic efficiency to commercial enzyme preparations during the degradation of hardwoods**

The hydrolysis of hardwoods was evaluated with their respective optimized HoloMix enzyme core sets and commercial enzyme preparations (Celluclast 1.5L, Cellic CTec2, Primafast 200 and Viscozyme L) at a constant protein dosage of 1.375 mg protein per g of untreated or pre-treated hardwood, following the procedure outlined in Section 7.3.1.

7.3.4. **Effect of enzyme loading on the hydrolysis yields of HoloMix on hardwoods**

The hydrolysis of hardwoods was evaluated with their respective optimized HoloMix enzyme core sets at a protein dosage range of 1.375 to 41.25 mg protein per g of untreated or pre-treated hardwood, following the procedure outlined in Section 7.3.1.

7.3.5. **Effect of hardwood biomass pre-treatment with AbLac on HoloMix performance**

The substrate, 2,6-dimethoxyphenol (ε_{405} = 49 600 M^{-1} cm^{1}) was used to assay laccase activity and the enzyme unit (U) was defined as the amount of enzyme required to oxidise 1 µmol of 2,6-dimethoxyphenol per min. Sequential AbLac and HoloMix treatment of the hardwoods was conducted as follows: hardwood biomass was treated with 5.375 U of AbLac per gram of biomass for 24 h at a solids loading of 2% (w/v) at 50^{o}C; the system was then boiled for 5 min to denature laccase and terminate the reaction. After cooling to 50^{o}C, the hardwoods were then treated with HoloMix at 1.25 mg protein per g of hardwood biomass for 24 h.

7.3.6. **Effect of the addition of an accessory enzyme mixture on HoloMix efficiency during hardwood degradation**

An accessory enzyme cocktail, AccMix, consisting of β-glucosidase (Bgl1), β-xylosidase (SXA), *Bifidobacterium* sp. α-arabinofuranosidase (Araf43), α-glucuronidase (AguA) and *Orpinomyces sp.* acetyl xylan-esterase (AxeA) at a ratio of 1:1:1:1:1 was formulated. Following this, replacement of 13.75 mg protein from 41.25 mg protein/g biomass of the HoloMix by AccMix or XynMix or AbLac or a 1:1 AccMix: XynMix/AbLac and or 1:1 XynMix: AbLac mixture was conducted during the degradation of untreated *Acacia*, delignified *Acacia* and delignified Poplar following the procedure outlined in Section 7.3.1. These biomasses were selected as 100% saccharification yields could not be obtained with the optimised HoloMix.

7.3.7. **Estimation of sugar monomers (glucose and xylose)**

The quantitative analysis of glucose concentration in the synergism assay hydrolysates was performed by the glucose oxidase/peroxidase (GOPOD) method (K-GLUC, Megazyme,

Ireland). Quantitative analysis of xylose concentration in the synergism assay hydrolysates was then performed enzymatically (K-XYLOSE, Megazyme) with the incubation time (20 min) twice the manufacturer's recommended time, as this gave more reproducible results with the microtiter plate format.

7.3.8. **Yield calculations**

For yields from enzymatic hydrolysis, the amount of sugar monomers (glucose and xylose) in solution following enzymatic hydrolysis was compared to the glucan and xylan remaining in the solids after each hardwood biomass pre-treatment.

7.3.9. **Data analysis**

One way analysis of variance (ANOVA) was used for the evaluation of significant increases exhibited by the binary enzyme cocktail combinations (considered with respect to reducing sugar and monosaccharide release), compared to those released by 100% protein loading of the most active single enzyme cocktail. All pairwise comparison procedures were based at 95% confidence level (p=0.05) and conducted using the Data analysis feature in Microsoft® Excel.

7.4. Results

7.4.1. Enzyme synergy

Enzyme synergism between the cellulolytic enzyme core set, CelMix and xylanolytic enzyme core set, XynMix was evaluated during the degradation of the untreated, steam exploded and delignified *Acacia* and Poplar wood substrates, respectively.

7.4.1.1. Holocellulolytic enzyme synergy studies on hardwoods

The optimal synergistic combination between CelMix and XynMix was determined by measuring the reducing sugar and monomeric sugars released during hydrolysis of *Acacia* and Poplar pre-treated by various technologies (Fig 7.1).

With respect to untreated *Acacia* degradation, the supplementation of CelMix with XynMix, significantly improved xylose release from the biomass at all ratios considered. However, with respect to high reducing sugar release, minimal supplementation with XynMix improved reducing sugar release, with CelMix to XynMix at 93.75% to 6.25% showing the highest reducing sugar release (Fig 7.1A). On the other hand, supplementation of CelMix with XynMix, during Poplar degradation, at all considered ratios did not improve xylose release and also led to a lower reducing sugar release compared to when CelMix is used alone at 100% protein dosage (Fig 7.1D). With respect to delignified *Acacia* and Poplar degradation, the supplementation of the cellulase optimized mixture, CelMix, with the optimized xylanolytic mixture, XynMix, significantly improved both reducing sugar and xylose release from the biomass at all ratios considered for xylose release–and only at 75%:25% of CelMix to XynMix loading for reducing sugar release (Figs 7.1B and 7.1E). With the CelMix to XynMix combinations, it is worth mentioning that the reducing sugar content liberated by these enzymes on the delignified hardwoods was solely in the form of glucose and xylose, with no significant traces of oligomers as determined by DNS method (see Figs 7.1B and 7.1E).

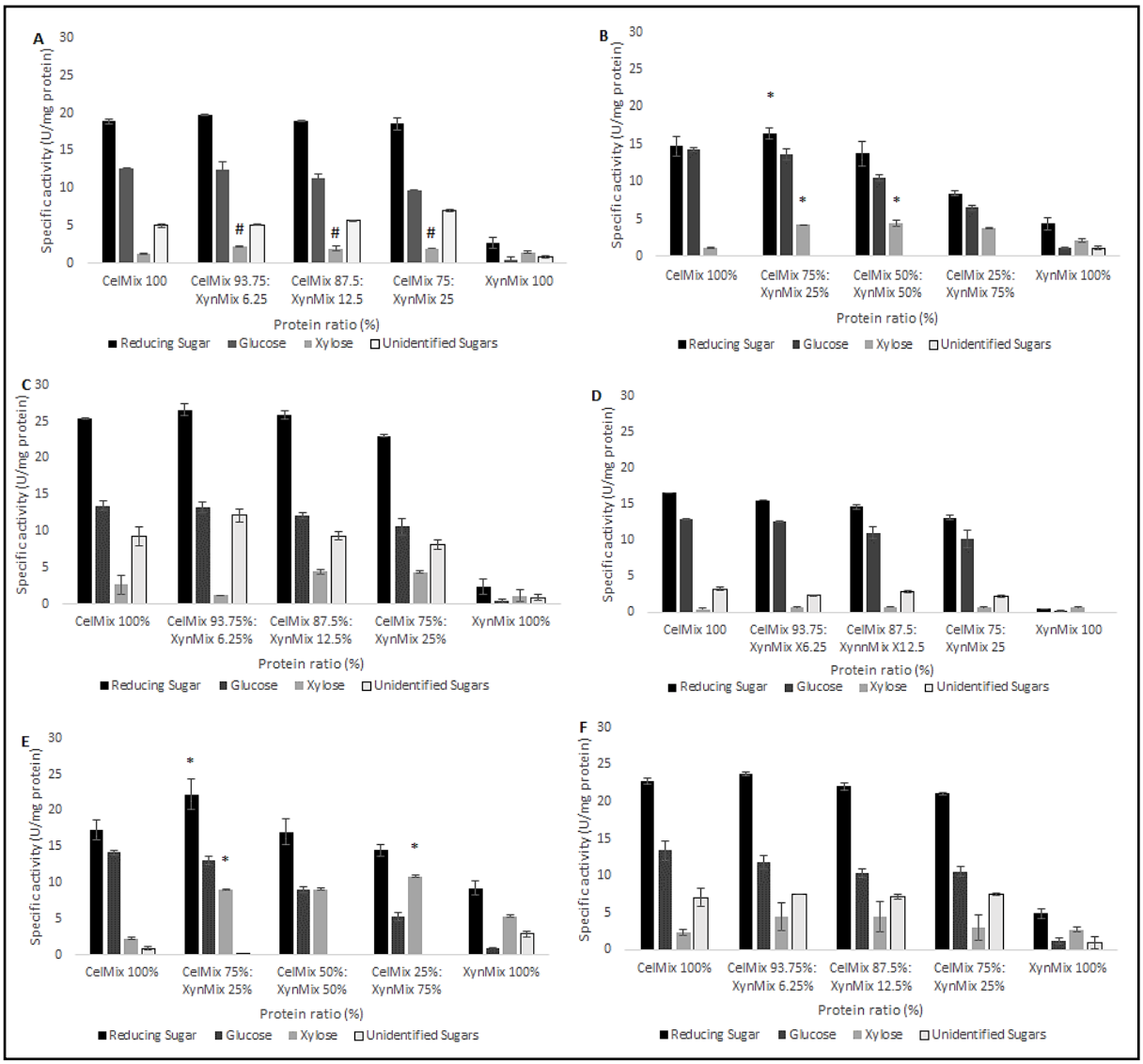

Figure 7.1. Glucose, xylose and reducing sugar released on (A) untreated *Acacia*, (B) delignified *Acacia*, (C) steam exploded *Acacia*, (D) untreated Poplar, (E) delignified Poplar and (F) steam exploded Poplar woods by the various combinations of CelMix and XynMix. ANOVA analysis for improvement of hydrolysis with respect to reducing sugar, xylose and glucose released by the enzyme combinations compared to 100% enzyme protein loading, keys: * (p value<0.05) and # (p value<0.01). Values are represented as mean values ±SD (n=3).

7.4.1.2. HoloMix sequential vs simultaneous enzyme synergy studies on hardwoods

The optimized HoloMix combination on delignified *Acacia* and Poplar, CelMix to XynMix at 75% to 25%, was subjected to sequential synergism studies to assess the mechanism of synergism between these enzymes during the degradation of holocellulose from the delignified hardwoods (Fig 7.2).

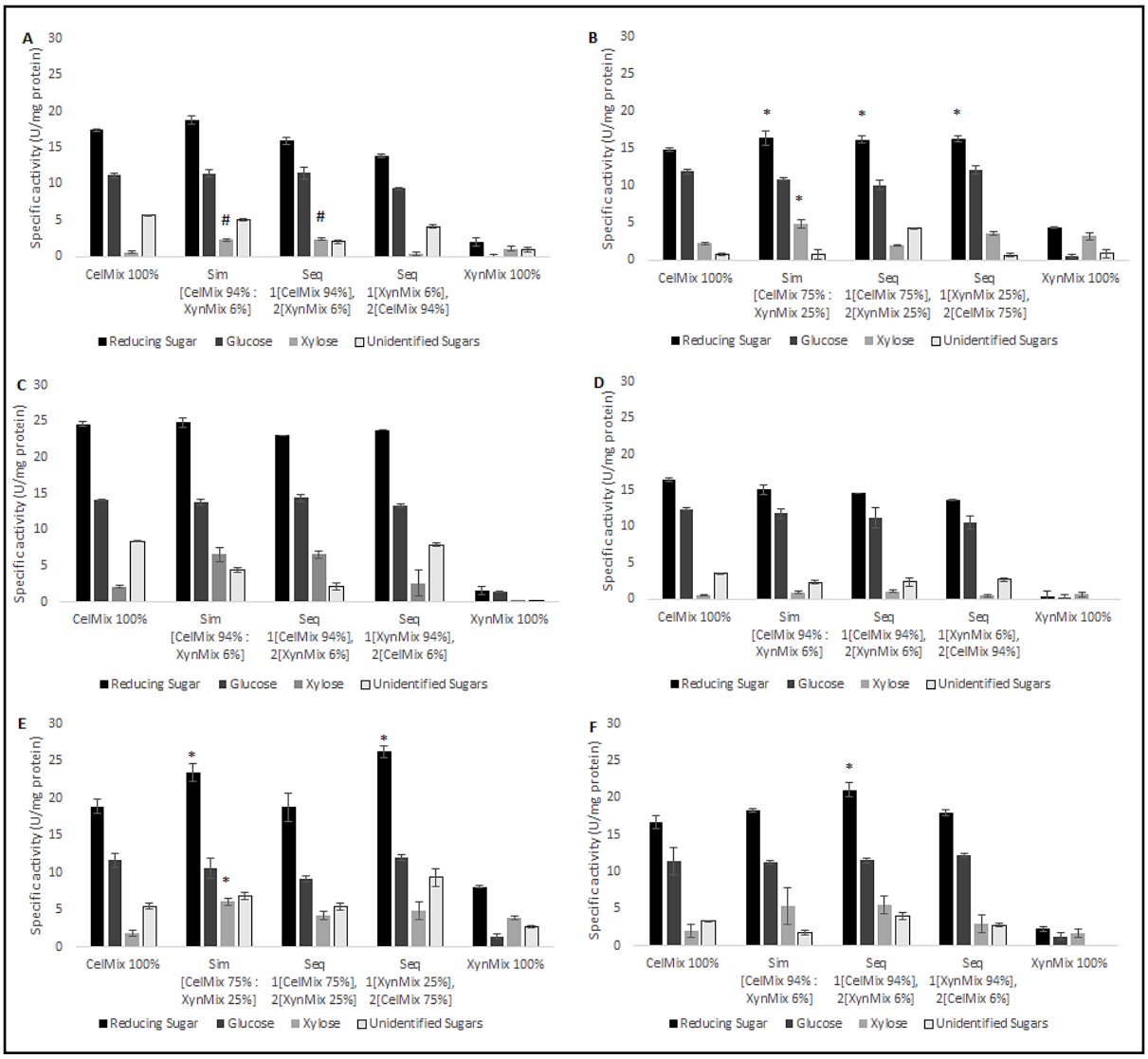

Figure 7.2. Glucose, xylose , reducing sugar released from sequential and simultaneous application of the optimized CelMix to XynMix combination on (A) untreated *Acacia*, (B) delignified *Acacia*, (C) steam exploded *Acacia*, (D) untreated Poplar, (E) delignified Poplar and (F) steam exploded Poplar woods. ANOVA analysis for improvement of hydrolysis with respect to reducing sugar and glucose release by the enzyme combinations compared to 100% enzyme protein loading, keys: * (p value<0.05) and # (p value<0.01). Values are represented as mean values ±SD (n=3).

During untreated *Acacia* degradation, simultaneous application of the CelMix to XynMix combination proved more synergistic and released a higher reducing sugar content (17.4 U/mg protein) compared to sequential application of this combination (Fig 7.2A). On the other hand, with respect to native Poplar degradation by CelMix to XynMix combination, there was no observable difference with respect to reducing sugar (≈15 U/mg protein) and monomeric sugar (glucose and xylose) release when the combination was applied sequentially compared to when it was applied simultaneously (Fig 7.1D). Sequential application of CelMix to XynMix

whereby XynMix is used to pre-treat the biomass then followed by CelMix released a higher amount of reducing sugar (26.3 U/mg protein) compared to the simultaneous application (23.5 U/mg protein) of these enzymes during delignified Poplar degradation (Fig 7.2E), while no observable differences were observed between the two approaches during delignified *Acacia* degradation (Fig 7.2B). Interestingly, with respect to steam exploded *Acacia* degradation by the CelMix to XynMix combination, there was no observable difference with respect to reducing sugar and monomeric sugar (glucose and xylose) release when the combination was applied sequentially to when it was applied simultaneously (Fig 7.2C), while sequential application on steam exploded Poplar, with CelMix first followed by XynMix, led to higher reducing sugar release compared to the other approaches (Fig 7.2F).

7.4.2. **Comparison of HoloMix hydrolytic efficiency to commercial enzyme preparations during the degradation of hardwoods**

To compare HoloMix performance to that of commercial enzymes (Celluclast 1.5 L, Cellic CTec2, Primafast 200 and Viscozyme L) on hardwood biomass under identical conditions, all enzymes were evaluated with various pre-treated *Acacia* and Poplar woods using the same protein loading (1.375 mg protein/g biomass) (Fig. 7.3).

The optimized HoloMix core sets, CelMix to XynMix at a ratio of 75 to 25% for delignified hardwoods and at 93.75 to 6.25% for untreated and steam exploded hardwoods, generally out-performed Celluclast 1.5L (except during delignified Poplar degradation), Primafast 200 and Viscozyme L (Figs. 7.3A-F). On the other hand, Cellic CTec2 significantly out-performed the optimized HoloMix enzyme core sets during delignified and steam exploded hardwoods hydrolysis (except for steam exploded Poplar, where the specific activities were comparable) (Figs. 7.3C, 7.3D and 7.3E), while the HoloMix out-performed Cellic CTec2 during the hydrolysis of untreated hardwoods (Figs. 7.3A and 7.3B).

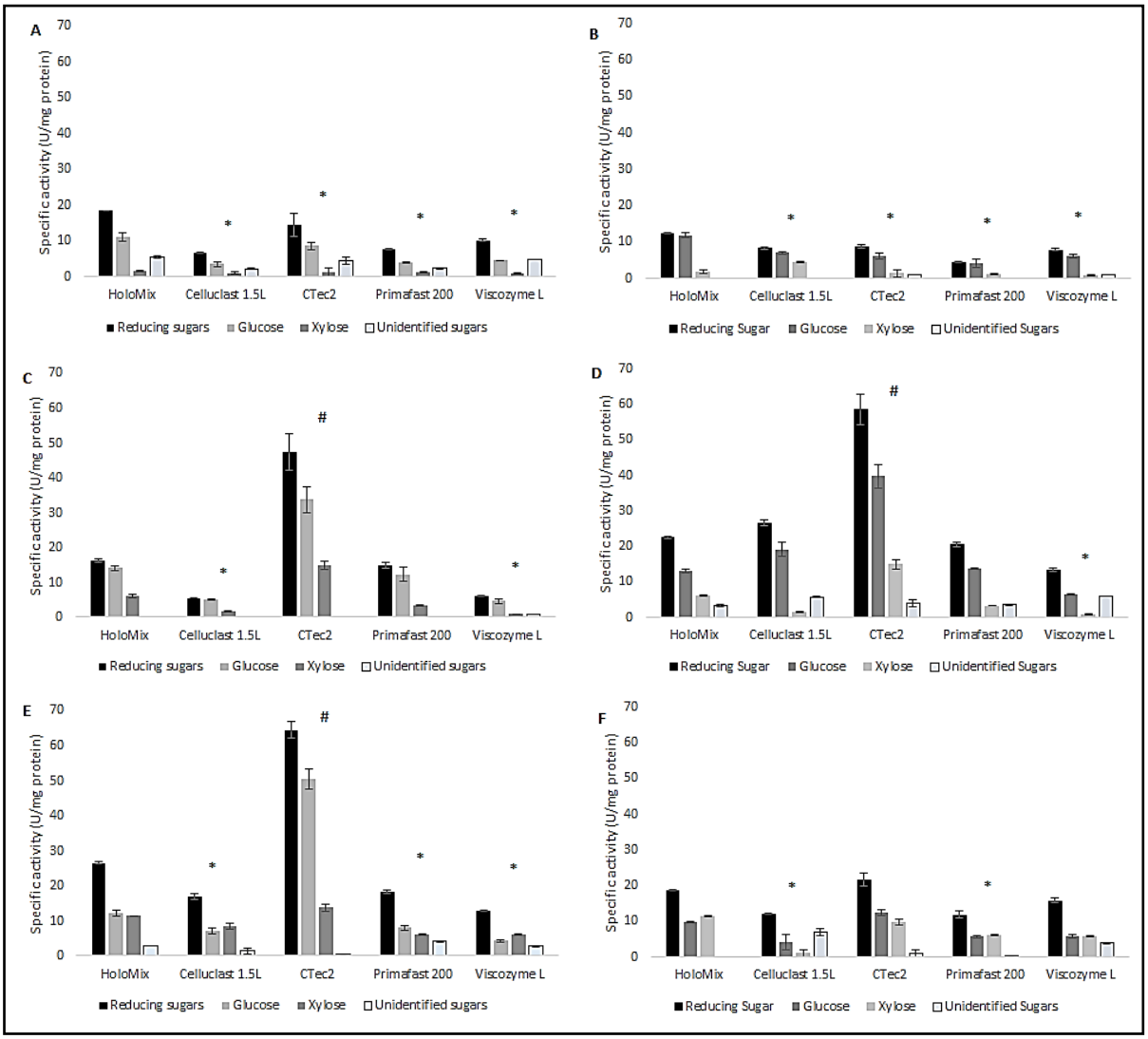

Figure 7.3. Enzymatic hydrolysis of various pre-treated hardwoods [untreated (A) *Acacia* and (B) Poplar, delignified (C) *Acacia* and (D) Poplar, and steam exploded (E) *Acacia* and (F) Poplar] with different commercial cellulase preparations. The optimized HoloMix core sets, CelMix to XynMix at 75 to 25% for delignified hardwoods and at 93.75 to 6.25% for untreated and steam exploded hardwoods, compared against Celluclast 1.5L, Cellic CTec2, Primafast 200 and Viscozyme L. All enzymes were evaluated at same protein loading (1.375 mg protein per g biomass) for 24 h. ANOVA analysis of commercial cocktail significantly more active than HoloMix (#) and HoloMix significantly more active than a commercial cocktail (*) (p value<0.05).

7.4.3. Effect of enzyme loading on the hydrolysis yields of HoloMix on hardwoods

After optimising the ratios of CelMix to XynMix required for efficient degradation of the various hardwoods, we then determined the protein loading of HoloMix required to reach maximal saccharification of the substrates (Figure 7.4).

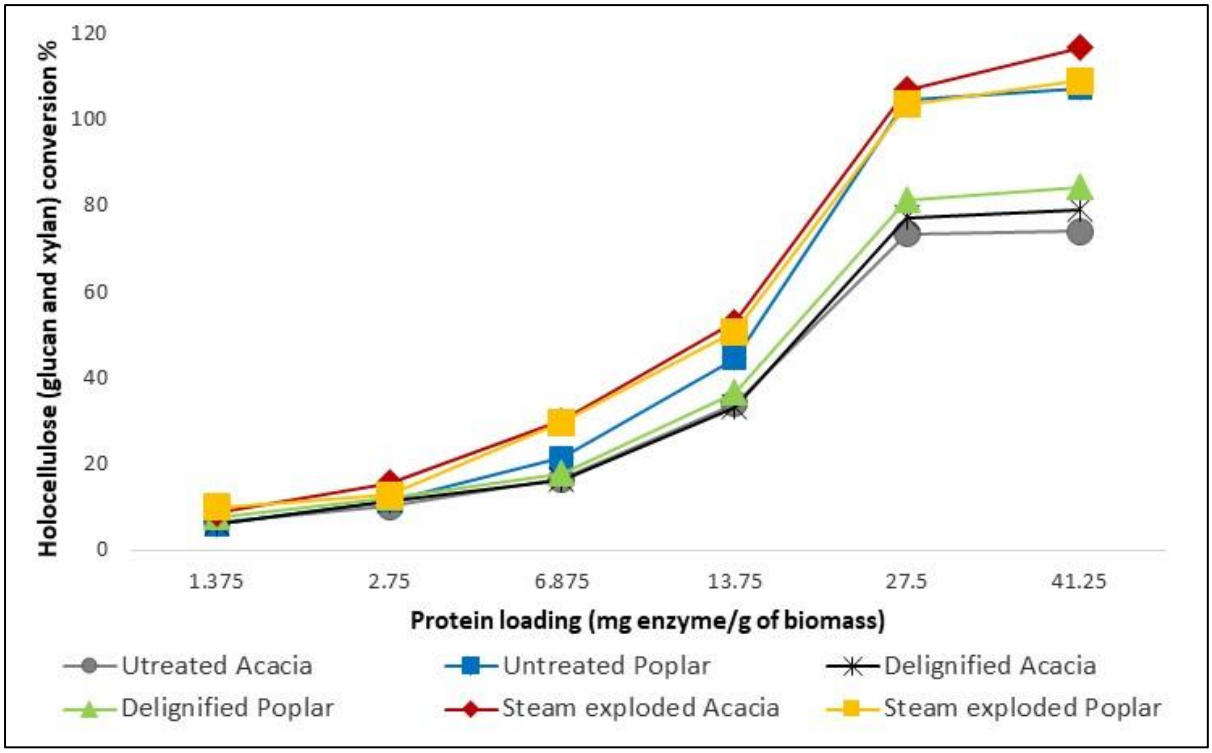

Figure 7.4. The extent of holocellulose hydrolysis after 24 h by HoloMix core sets and the total protein dosage (mg/g biomass) used in the hydrolysis of untreated (A) *Acacia* and (B) Poplar, delignified (C) *Acacia* and (D) Poplar, and steam exploded (E) *Acacia* and (F) Poplar. The optimized HoloMix core sets were CelMix: XynMix at 75: 25% for delignified hardwoods and at 93.75: 6.25% for untreated and steam exploded hardwoods. Values are represented as mean values ±SD (n=3).

Yields of up to 100% saccharification were achieved by 27.5 mg/g biomass during the degradation of steam exploded hardwoods, both Acacia and Poplar, and untreated Poplar. On the other hand, saccharification of delignified hardwoods and untreated Acacia plateaued at around 70% saccharification even when protein dosage was increased to levels as high as 41.25 mg/g biomass.

7.4.4. Enhancement of HoloMix activity on hardwoods by AbLac addition

Sequential application of the laccase, AbLac, and HoloMix on the hardwoods was conducted, Figure 7.5 below illustrates the effect of the laccase on hardwood hydrolysis. The data showed that the addition of AbLac improved xylan digestibility of hardwood biomass, except for steam exploded hardwood biomass where pre-treatment with AbLac led to a decrease in xylan saccharification of the biomass by the HoloMix.

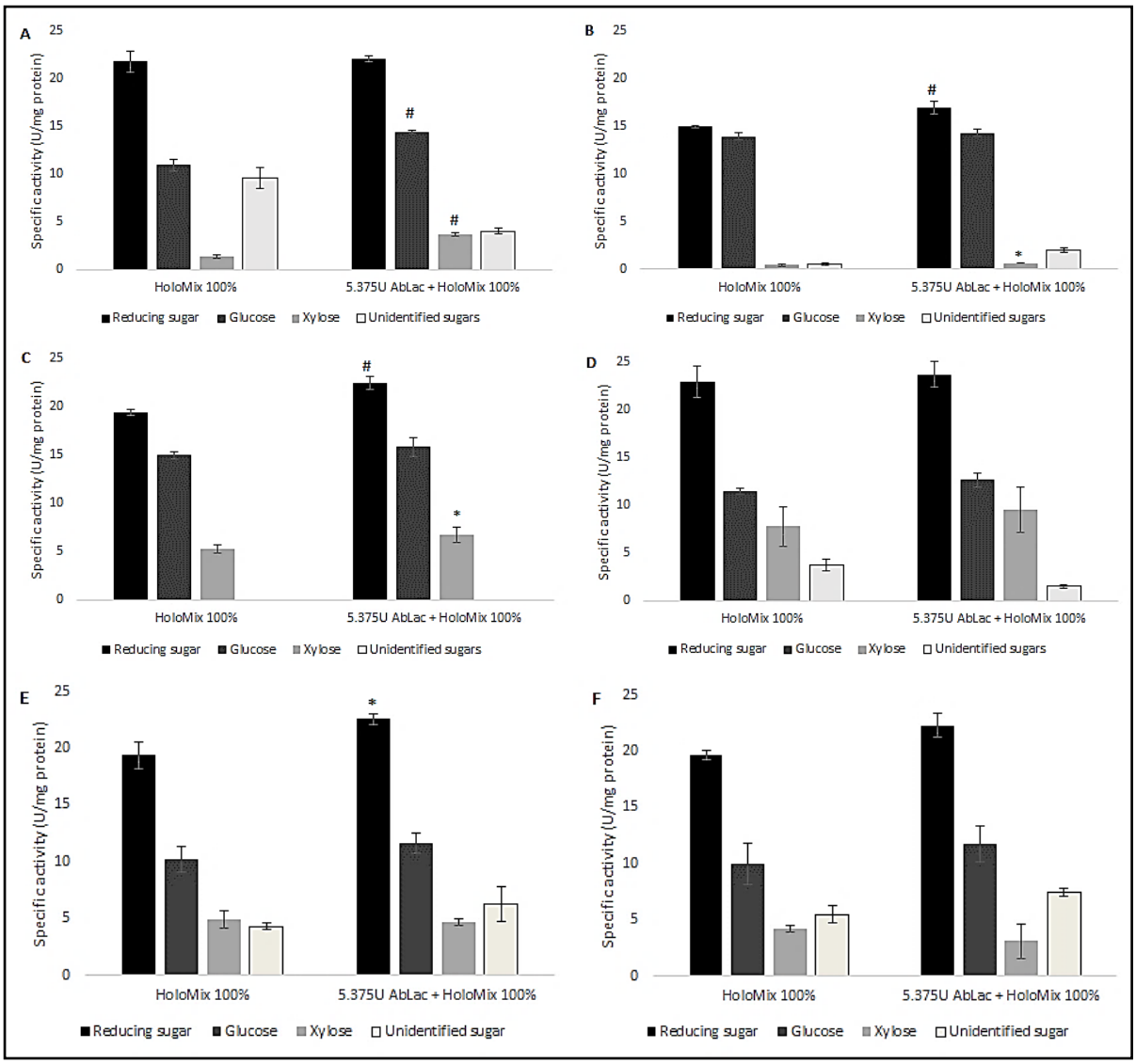

Figure 7.5. The effect of AbLac pre-treatment of untreated (A) *Acacia* and (B) Poplar, delignified (C) *Acacia* and (D) Poplar, and steam exploded (E) *Acacia* and (F) Poplar on the hydrolytic efficiency of HoloMix core sets. ANOVA analysis for improvement of hydrolysis with respect to reducing sugar, xylose and glucose release by HoloMix on AbLac treated wood compared to non AbLac treated wood, keys: * (p value<0.05) and # (p value<0.01). Values are represented as mean values ±SD (n=3).

7.4.5. Effect of the accessory enzymes addition on HoloMix efficiency

Improvement of HoloMix efficiency by various accessory enzymes during the hydrolysis of untreated *Acacia*, delignified *Acacia* and delignified Poplar hardwoods was evaluated as 100% saccharification yields could not be obtained with the optimised HoloMix – even at loadings as high as 41 mg/g biomass (see Fig 7.4). The data showed that replacing HoloMix with XynMix or AbLac exhibited no improvement in saccharification yield, while replacement with AccMix led to 100% saccharification for all the biomasses assessed (see Fig 7.6).

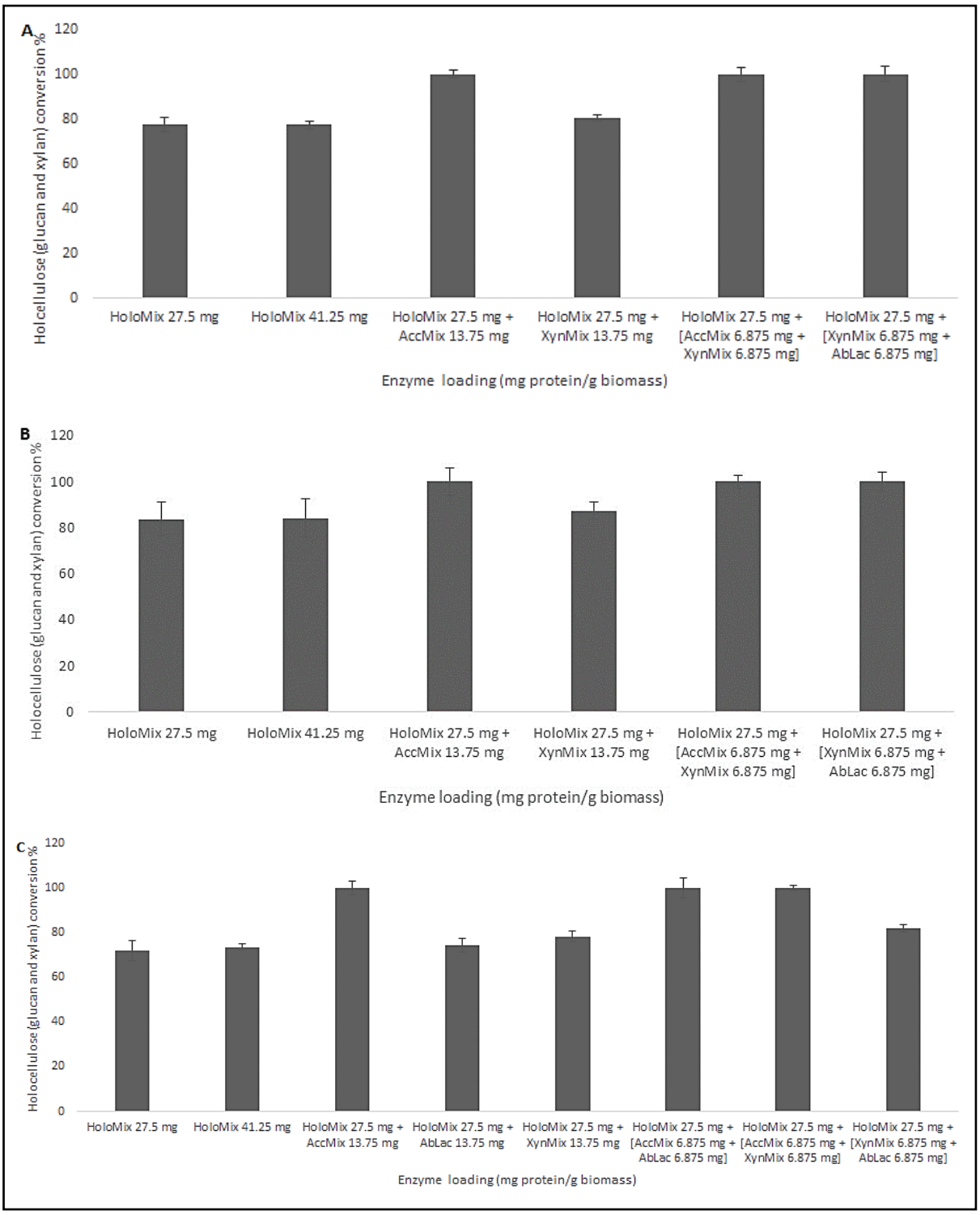

Figure 7.6. HoloMix replacement by accessory enzymes: AbLac, AccMix, XynMix, [AccMix + XynMix], [AbLac + XynMix] and [AccMix + AbLac] for improving the degradation of delignified (A) *Acacia* and (B) Poplar, and (C) untreated *Acacia*, respectively.

7.5. Discussion

7.5.1. HoloMix optimization during hardwood degradation

During lignocellulose degradation, xylan removal is said to enhance cellulose accessibility and thus its digestibility by cellulolytic enzymes. In this study, it was shown that the addition of a hemicellulolytic core set consisting of glucuronoxylan acting enzymes (Xyn2A 60: XT6 20%: AguA 11%: SXA 9%) could enhance the saccharification of hardwoods and as a result increased the overall yield of sugars obtained compared to when cellulolytic enzymes were used alone. Xylan removal by the glucuronoxylan acting enzyme mixture, XynMix, was improved during the degradation of delignified hardwoods compared to both untreated and steam exploded hardwoods. Delignified hardwoods contain a higher proportion of xylan compared to untreated and steam exploded hardwoods (see Table 5.1), it is therefore expected that the presence of a higher proportion of XynMix (25% protein loading) would be required for efficient degradation of delignified hardwoods (see Figs 7.1B and 7.1E), while lower proportions of XynMix (6.25% protein loading) would be required for untreated (see Figs 7.1A and 7.1D) and steam exploded hardwoods (see Figs 7.1C and 7.1F). Similarly, Hu and co-workers reported that only 5% xylanase in a cellulase: xylanase: AA9 combination was required for efficient degradation of steam pre-treated Poplar (Hu et al. 2015). The delignified biomass was also the substrate that showed strong synergistic interactions between CelMix and XynMix. We propose that the lowered synergistic interactions between these enzyme core sets on the untreated and steam exploded hardwoods could also be due to inaccessibility of their xylan to the XynMix enzymes. Also, and interestingly, similar specific activities by the optimized HoloMix were observed on both untreated and pre-treated hardwood substrates (15-20 U/mg protein); the implications of this fact being that: 1) the formulation can be used on untreated biomass, thus eliminating the costs associated with pre-treatment processes and 2), the formulation can be used on various hardwoods without the need to further optimize it before use.

With respect to comparing sequential versus simultaneous applications of the HoloMix components, CelMix and XynMix, during hardwood degradation, generally, no apparent differences between the two approaches were observed with regards to yields. However, delignified and steam exploded Poplar showed better saccharification yields when the biomasses were hydrolysed sequentially by the two HoloMix components. In general, it has been reported that the mode of synergy between enzymes that target different polysaccharides, such as that between xylanases and cellulases, favours the simultaneous synergy route over the

sequential route during lignocellulose hydrolysis (Hu et al. 2011; Murashima et al. 2003). Pauly and co-workers attributed this to be due to the xylan in dicot plants being entrapped within the cellulose micro-fibrils, thus requiring the action of both cellulases and xylanases for its removal (Pauly et al. 1999). In our study, we showed that steam explosion leads to high solubilisation of xylan leaving only about 6% per dry mass (see Table 5.1). SEM analysis showed smooth cellulose fibrils remaining after treatment (see Fig 5.2F); leaving only the inter-fibril spaces to be potentially occupied by the remaining xylan. In this regard, CelMix pre-incubation is therefore required to unravel the buried xylan, thus sequential synergism gave higher saccharification yields. Similar to our findings for delignified and steam exploded Poplar, Shao and co-workers are among a few that have found that sequential enzymatic hydrolysis of AFEX-treated corn stover and AFEX-treated whole corn plant by the cellulase produced 15–20% higher yields compared to simultaneous addition of the hydrolytic enzymes (Shao et al. 2010).

7.5.2. **Comparison of HoloMix hydrolytic efficiency to commercial enzyme preparations during the degradation of hardwoods**

In order to compare HoloMix to commercial enzymes (Celluclast 1.5L, Cellic CTec2, Primafast 200 and Viscozyme L) on hardwood biomass under identical conditions, all enzymes were evaluated with various pre-treated *Acacia* and Polar substrates using the same protein loading (1.375 mg protein per g biomass) (Figs. 7.3A-F). The optimised HoloMix cocktail out-performed Primafast 200, Viscozyme L, Celluclast 1.5L (except during delignified Poplar degradation) and Cellic CTec2 (except during delignified hardwoods degradation and steam exploded *Acacia*) during hardwood biomass degradation. Similar to our findings, Agrawal and colleagues showed that an optimised enzyme cocktail could out-perform the commercial preparations Celluclast, Bioconvert L1 and P10, and Accellerase during wheat straw degradation (Agrawal et al. 2015).

7.5.3. **Effect of enzyme loading on the hydrolysis yields of HoloMix on hardwoods**

In this study, as enzyme loading increased, sugar yields tended to plateau at 27.5 mg protein per g biomass with a maximum yield of ~73% and 100% for *Acacia* and Poplar pre-treated substrates, respectively (Fig 7.4). In addition, at 27.5 mg protein per g biomass, the enzyme mixture exhibited a relatively high saccharification performance (20-24 mg sugars/mg enzymes) on the various pre-treated hardwoods after 24 h. This saccharification performance is similar (27.3 mg sugars/mg enzymes) to that reported by Hu and colleagues during the hydrolysis of steam pre-treated corn stover (SPCS) after 72 h (Hu et al. 2011).

Our optimized enzyme preparation performed better than that reported by others (Agrawal et al. 2015) which showed that maximal hydrolysis yield (>95%) after 24 h on dilute acid wheat straw was achieved with OptEMix and CL enzyme at a protein loading of 54.75 mg protein per g biomass. Also, considering the hydrolysis of the steam pre-treated hardwoods and untreated Poplar, which are predominantly cellulosic with less than 7% xylan, our combination performed better than that reported by Banerjee et al. (2010), as our combination reached over 85% yield with 27.5 mg protein per g of biomass after 24 h, while the reported cocktail achieved only 60% yields on alkaline peroxide pre-treated corn stover after 48 h with half the protein loading (Banerjee et al. 2010). Interestingly, 100% saccharification could not be achieved for delignified *Acacia* and Poplar, and untreated *Acacia* – even at protein loadings as high as 41.25 mg/g biomass (see Fig 7.4). These hardwood substrates were the highest xylan containing substrates (see Table 5.1). Similarly, Hu and co-workers reported that three times higher protein loadings were required for efficient degradation of SPCS compared to those required for steam pre-treated Poplar. This is most likely due to the higher amount of xylan associated with the milder pre-treated SPCS substrate (Hu et al. 2011; Hu et al. 2015).

In summary, we were able to formulate a preparation that reached comparable saccharification efficiencies to those reported in literature at the same protein loadings, but with shorter saccharification times (24 h) to those reported for other preparations (~72 h). With Spezyme CP cellulase, other studies have reported high sugar yields for a mass loading of 120 mg of cellulase plus beta-glucosidase/g glucan, or cellulase to xylanase mixture (cellulase loading at 14.5 mg/g glucan and a xylanase supplementation at 84.2 mg/g glucan) on untreated and pre-treated corn stover and Poplar, respectively (Kumar & Wyman 2009b; Kumar & Wyman 2009c). These loadings were double that of HoloMix (55 mg/g glucan or 27.5 mg/g biomass) required to reach such high yields on untreated and pre-treated hardwoods in our study, while Shen and co-workers on steam pre-treated substrates reached similar sugar yields with a protein loading of 145.1 mg/g glucan (~20 mg/g glucan Spezyme CP, ~5.1 mg/g glucan Novozyme 188 and 120 mg/g glucan Multifect Xylanase) (Shen et al. 2011). It is noteworthy to mention that Shen and co-workers reached similar sugar yields with nearly three times the loading used in our study (55 mg protein/g glucan).

7.5.4. **Enhancement of HoloMix efficiency by AbLac pre-treatment of hardwoods**

The optimized HoloMix core sets for the various pre-treated hardwood substrates were evaluated for the effect of supplementation with AbLac (Figs. 7.5A-F). It was evident that AbLac improved xylan digestibility of hardwood biomass, except for steam exploded

hardwood biomass where pre-incubation with AbLac decreased xylan degradation of the biomass by the HoloMix. Tabka and co-workers also reported a negative effect of laccase addition on steam exploded wheat straw hydrolysis, possibly due to the release of phenolic inhibitory compounds in the medium that decreases the activities of the hydrolases (Tabka et al. 2006). Chen and co-workers have also evaluated the effect of laccase on the saccharification of biomass where they demonstrated that cellulose conversion of ensiled corn stover increased from 24.3% to 31.7% as the laccase loading rate increased from 0 to 4400 U/g (Chen et al. 2012). In hardwoods, lignin is linked to hemicellulose via ester linkages between lignin alcohols and the carboxyl groups of 4-O-methyl-D-glucuronic acid side residues (Jeffries 1990; Takahashi & Koshijima 1988). Due to this intimate interaction between the two polymers, the presence of lignin can impede xylanolytic enzymes during xylan degradation. Thus, laccase treatment likely leads to disruption of the hemicellulose to lignin interaction, leading to improved HoloMix activity towards xylan digestion. It is also possible that AbLac may be coupling the mono-aromatic phenolics generated into the liquor from the biomass during hydrolysis into oligomers − thus lowering the amounts of total phenolics that can (see Fig 6.5 in Chapter 6), and this resulting in lowered inhibition of XynMix.

Several studies have attempted to explain the inhibitory mechanism of the oxidative polymerization of phenols mediated by laccases on the enzymatic hydrolysis of agricultural residues (Wang et al. 2013), but unfortunately the inhibitory mechanism remains unclear. Oliva-Taravilla et al. (2016) tried to elucidate this mechanism by studying the effects of the oligomeric phenols and radicals formed via laccase oxidation on the enzymatic hydrolysis of model cellulose (Sigmacell)(Oliva-Taravilla et al. 2016). It was shown that the oligomeric phenols and the phenoxyl radicals play an important role in the enzymatic inhibition. Laccases also have the ability to oxidize the phenolic components of lignin (Oliva et al. 2003; Oliva-Taravilla et al. 2016). Laccase splits the aromatic rings of phenols, resulting in the formation of new quinonoid structures (Witayakran and Ragauskas, 2009; Shleev et al., 2006). The formation of these new substructures induces changes in the tridimensional structure of lignin, which could interact with the cellulosic polymer, increasing the number of lignin–cellulose interactions, and therefore, hampering the effective binding of cellulases onto cellulosic fiber.

7.5.5. **Enhancement of HoloMix efficiency by the addition of an accessory enzymes mixture during hardwood substrate degradation**

Untreated *Acacia*, delignified *Acacia* and delignified Poplar could not be degraded to completion by the optimized HoloMix - even at loadings as high as 41.25 mg protein per gram

biomass, yields only as high as 73% could be achieved (see Fig 7.4). These three hardwoods specifically displayed the highest xylan content (see Table 5.1, Chapter 5). Due to this biomass composition, it is possible that HoloMix generated xylo-oligosaccharides at too high a rate for the low quantities of SXA and AguA in the cocktail to process. This, in turn, led to the inhibition of the core enzymes (endoglucanases, cellobiohydrolases and xylanases) in the HoloMix by these oligosaccharides and, as a result, led to a plateau in yields. Kumar and co-workers have previously reported on the inhibition of enzyme cocktails by oligosaccharides (Kumar & Wyman 2014; Baumann et al. 2011; Qing et al. 2010).

Replacing a portion of the HoloMix with XynMix led to only a ~4% improvement with regards to saccharification of the hardwoods, while replacing HoloMix with a mixture of XynMix and AbLac led to an ~8% increase in untreated *Acacia* degradation. This demonstrated that HoloMix contained adequate quantities of endo-xylanase activities to unravel the hemicellulose that is intertwined with cellulose. Also, lignin linkages were not impeding this process as adding more AbLac led to no significant improvement in HoloMix activity. However, replacing HoloMix with AccMix, or a mixture of accessory enzymes containing AccMix and XynMix or AbLac, led to 100% saccharification of all the hardwoods. The data indicated that addition of the AccMix accessory enzymes to the HoloMix enhanced the action of the HoloMix. Other studies have also shown that, in order to reduce inhibition by xylo-oligomers and achieve a high xylose monomer recovery, in addition to xylanase supplementation, cellulases should also be supplemented with a β-xylosidase (Qing & Wyman 2011a; Qing & Wyman 2011b).

7.6. **Conclusions**

This study demonstrated that an optimized enzymatic cocktail comparable to/if not better than commercial preparations for hardwood saccharification, with a short hydrolysis time (24 h), could be formulated. Pre-treatment with a laccase, especially on wood biomass with a higher proportion of lignin, improved saccharification by the cocktail. The proportions of CelMix and XynMix in the cocktail appeared to be pre-treatment specific, with biomass with higher xylan content requiring a higher proportion XynMix in the cocktail for efficient hydrolysis. High xylan containing biomasses specifically required further dosage with xylanolytic accessory enzymes (i.e. AccMix containing β-glucosidase (Bgl1), β-xylosidase (SXA), α-arabinofuranosidase (Araf43), α-glucuronidase (AguA) and acetyl xylanesterase (AxeA) at a ratio of 1:1:1:1:1) to achieve yields of 100% saccharification, as further addition of cellulolytic (i.e. CelMix) or ligninolytic (i.e. AbLac) or backbone cleaving xylanolytic enzymes (i.e. XynMix) had no effect of saccharification yielded when the yields plateaued at 70%. The data presented in Figure 7.4 showed no correlation with biomass water holding/swelling capacity (see Table 5.2), crystallinity index (see Table 5.3) and lignin content (see Table 5.1) values with their recalcitrance. However, a high degree of correlation was observed between biomass recalcitrance and their xylan content – with those biomasses containing over 20% xylan being highly recalcitrant. This study suggests xylan degradation can assist in the realization of high hydrolysis yields as it strongly contributes towards recalcitrance.

Chapter 8: General discussion, Conclusions and Future perspectives

8.1. General Discussion and Conclusions

Utilisation of lignocellulosic biomass via a bio-refinery process for producing bioethanol and other value added products has emerged as the most prominent technology to replacing depleting petroleum reserves which are associated with climate change (Bhattacharya et al. 2015). However, the recalcitrant structure of biomass is associated with low enzyme saccharification yields and high costs in enzyme production processes pose major economic challenges for the production of cellulosic fuels and other value added products from lignocellulosic biomass (Himmel et al. 2007; Zhao et al. 2012). In this study we proposed combating these challenges by using biomasses (hardwoods) with high polysaccharide content as feedstocks in the bio-refinery process, which are easier to pre-treat as this will be accompanied with high enzyme saccharification yields. Secondly, we formulated a universal enzyme preparation specific for the saccharification of hardwoods, as currently available commercial preparations are tailored for agricultural grass residues. In order to meet the aims of this study the following questions had to be addressed;

a) What are the required enzyme activities for complete degradation of hardwoods based on their biomass composition?

b) What are the optimal combinations and ratios of enzymes and additives required to degrade hardwood biomass effectively?

c) How does the hydrolytic efficiency of the optimised enzyme mixture, HoloMix, compare to that achieved by commercial enzyme preparations during hardwood biomass saccharification?

First and foremost, it was important to select the necessary enzymes required for complete depolymerisation of hardwoods, and this was based on the major polysaccharides contained within the biomass, cellulose and glucuronoxylan, followed by understanding the structure, glycosidic linkages and the chemistry of these polysaccharides. Upon selection of the enzymes, these were then subjected to characterisation in order to understand the factors that influence their activities. A consortium of cellulolytic (Bgl1, Cel6A, Cel7A and EglA) (all fungal in origin), and xylanolytic (AguA, SXA, Xyn2A and XT6) (mostly bacterial) enzymes required for the efficient degradation of cellulose and glucuronoxylan, respectively, were successfully characterised with respect to both their physico-chemical characteristics and substrate

specificities. Based on their physico-chemical properties, all the assessed enzymes appeared to be active under the operating conditions of pH 5.0 and 50^{o}C. The characterisation of each holocellulolytic enzyme also led to an improved understanding of the optimal conditions for its activity and substrate specificities. Numerous enzymes appeared not to exhibit cross activity with others on most of the tested defined substrates, except for Xyn2A and XT6 – this was preferable so as to avoid substrate competition during biomass degradation by the consortium of hydrolytic enzymes. The obtained data facilitated a better understanding and interpretation of the synergy and interactions between these enzymes on model (Avicel and beechwood xylan) and complex (*Acacia* and Poplar) substrates in the following chapters.

Monosaccharide (glucose and xylose) product inhibition of the enzymes was also evaluated. Our results showed that the HoloMix mono-component to be extremely tolerant to inhibition by these products, except for Bgl1 and SXA which were inhibited by their products, glucose and xylose, respectively. Inhibition of HoloMix mono-component enzymes by sugar products may be not necessarily critical since these are consumed by fermentative microorganisms in a simultaneous saccharification and fermentation (SSF) or consolidated bioprocessing (CBP) system. Because many common fermentative organisms cannot utilize oligomers, evaluation of hardwood derived oligomers, such as cello-oligomers and xylo-oligomers (Qing & Wyman 2011a), on HoloMix mono-component enzymes would have been interesting. Numerous studies conducted by the Center for Environmental Research and Technology at University of California, Riverside, have shown that hemicellulose-derived oligomers such as manno-oligomers (Kumar & Wyman 2014) and xylo-oligomers (Qing & Wyman 2011a, 2011b) significantly hamper the activity of hydrolytic enzymes.

In the quest for lignocellulosic biomass conversion into biofuels and other commodity chemicals, efficient saccharification of the biomass is the major bottleneck (Zhao et al. 2012). Therefore, elucidation and improvement of synergistic interactions between lignocellulolytic enzymes to improve lignocellulose saccharification and also to lower protein loading, thus lowering biomass conversion costs, has been of interest over the past few decades (Ganner et al., 2012). In this study, the synergistic effects between the various enzyme combinations were investigated for the degradation of cellulosic and hemicellulosic fractions of lignocellulosic biomass. The cellulolytic and xylanolytic cocktails consisting of (Egl 75%: Cel7A 18.75%: Cel6A 6.25%) and (Xyn2A 56.25: XT6 18.75%: AguA 25%), respectively, were optimised for cellulose and beechwood glucuronoxylan degradation, and then selected for an investigation that studied synergy between these two binary enzyme preparations during the hydrolysis of

native/untreated, steam exploded and sodium chlorite/acetic acid delignified *Acacia* and Poplar wood, respectively (Chapter 7).

Following optimisation of the cellulolytic and xylanolytic cocktails, the pre-treatment of hardwoods, *Acacia* and Poplar, by steam explosion and SC/AA delignification, were described and discussed. During the enzymatic saccharification of lignocellulosic biomass, pre-treatment of the recalcitrant biomass is generally required to allow better digestibility of the biomass by enzymes. The effects of the pre-treatments on the hardwoods, such as the effects on the composition and structure of the biomass, were evaluated. The above mentioned biomass parameters were assessed as the effectiveness of enzymatic hydrolysis of the biomass material is critically dependent upon them. Similar to what Sun et al. (2014) reported on dilute acid pre-treatment of Poplar, our results suggested the increased cellulose accessibility on steam exploded hardwoods was mainly due to hemicellulose removal, lignin-hemicellulose phase separation and/or lignin redistribution (Sun et al. 2014).

Following biomass pre-treatment, we determined the individual inhibitory properties of each of the selected pre-treatment by-products and the effects of the wash streams from these hardwoods pre-treated by various technologies on the two optimised enzyme cocktails. From this study it was elucidated that the inhibition of the enzyme cocktails, CelMix and XynMix, relies on the particular relationship which occurs between the specific cocktail and pre-treatment by-product(s). Interestingly, the study revealed that CelMix was significantly activated by some of the individual by-products and a majority of the pre-treated biomass wash liquors from the pre-treated hardwoods. On the contrary, XynMix was inhibited gallic acid and a majority of the pre-treated biomass wash liquors from the pre-treated hardwoods, except those derived from delignified hardwoods. It is noteworthy to mention that three out of the four enzyme components in CelMix were of fungal origin, while three of the four enzyme components in XynMix were of bacterial origin. It still remains unclear whether these microbial source differences could be the reason for the reported catalytic properties of these cocktails in the presence of pre-treatment inhibitors. Recently, in a review, we discussed the fact that enzymes may differ with regards to their tolerance to chemicals (particularly to biomass pre-treatment by-products), and that this may occur as a result of the microorganisms from which a particular enzyme is derived (Malgas et al. 2017). For instance, Ximenes and co-workers have demonstrated that phenolics inhibit β-glucosidase from *T. reesei* about twice as much as that from *A. niger* (Ximenes et al. 2010; Ximenes et al. 2011). In our research group, we have also demonstrated that mannanolytic enzymes (mannanases, mannosidases and

galactosidases) from different sources interact differently with pre-treatment by-products (Malgas et al. 2016). From our studies, it also appeared that the inhibitory effects exerted by the individual pre-treatment by-products were concentration dependent. This implies that the inhibitory effect of these pre-treatment by-products can be limited by modifying their concentrations. Furthermore, this study provided insight into the impact of individual inhibitors on hydrolytic enzymes, which, in future, will inform the appropriate pre-treatment strategies for different substrates. We also demonstrated that lignin-blocking agents such as surfactants and BSA have no effect on the inhibitory effects of soluble by-products such as phenolics, indicating that their inhibition mechanism (on enzymes) is different from that of insoluble lignin. A better understanding of the interactions between pre-treatment by-products such as phenolics or lignin and individual enzymes will contribute to the rational design of an improved enzyme consortium (which would be composed of enzymes resistant to pre-treatment by-products) to break down the lignocellulose more effectively. These enzymes can be either prospected or rationally engineered, followed by screening for their tolerance to these pre-treatment inhibitors.

The present study demonstrated that an universal enzymatic cocktail, comparable to/if not better than commercial enzyme preparations for hardwood saccharification, with a short hydrolysis time (24 h), could be formulated. The proportions of the cellulolytic and xylanolytic components of the cocktail appeared to be pre-treatment specific, with pre-treatments that render a higher xylan proportion in the hardwoods requiring a higher proportion of xylanolytic protein in the enzyme cocktail for efficient hydrolysis. Our studies also showed that the pre-treatment and the type of biomass used may also have an effect on the enzyme loadings. For example, we found that loadings of 27.5 mg/g biomass could achieve 100% saccharification yields for steam exploded hardwoods and not for delignified samples, while 100% saccharification was achieved on untreated Poplar and not on *Acacia*. Similarly, Wyman and co-workers found that higher protein loadings were required for alkaline pre-treated substrates, whereas dilute acid, SO_2 and liquid hot water pre-treated substrates required lower enzyme loadings for the same levels of hydrolysis (Wyman et al. 2011). Both our laboratory and Wyman and co-workers related this phenomenon specifically to the hemicellulose content, which required additional enzymes such as xylanases, and to a lesser extent – lignin content. The mode of cooperation between the components of the enzyme cocktail was elucidated with the aid of sequential saccharification studies. Pre-treatment of the hardwoods with a laccase,

especially on wood biomass with a higher proportion of lignin, improved saccharification by the formulated enzyme cocktail.

In conclusion, results from this study demonstrated that a rationally designed enzyme cocktail, based on the enzymes required to degrade major components in wood biomass (cellulose and glucuronoxylan), can facilitate the efficient hydrolysis of hardwoods and can be used to improve conversion of a variety of hardwoods – even without the requirement of a pre-treatment step.

8.2. **Future perspectives**

In this study, improved biomass conversion using enzyme synergy was achieved with combinations of individual enzymes and led to a greater understanding of synergy and cooperation between enzymes for biomass degradation. However, challenges still remain and recommendations for future research to address these challenges are suggested below.

Enzyme costs are associated with low competitiveness of biofuels to petroleum fuels, as enzymes usually make up a huge portion of the total industrial costs for the lignocellulosic ethanol process. We therefore recommend the development of an onsite production strategy of the enzymes constituting the holocellulolytic cocktail, HoloMix, in a suitable microorganism such as *Aspergillus sp.* that can be grown on cheap carbon sources (i.e. agricultural residues) (rather than purchasing them from Megazyme and Sigma) to enable economic viability of the lignocellulose saccharification process. However, fungal strains generally secrete high levels of endoglucanase and cellobiohydrolase activities, while glucosidase and non-cellulolytic enzyme activities such as hemicellulases and pectinases are expressed in smaller quantities (Qing & Wyman 2011a). Therefore, co-cultivation of the cellulase-producing fungal strain with a high hemicellulase-producing microorganism may be a viable way for cheap enzyme production.

Secondly, pre-treatment of the plant biomass by various technologies resulted in the production of various pre-treatment products, which may have affected CelMix and XynMix activities differently. This possibly held implications for the synergistic interactions between these two cocktails during the degradation of pre-treated hardwoods. We suggest that protein engineering techniques be conducted on these enzymes, such as those constituting XynMix by methods such as negatively supercharging cellulases (Whitehead et al. 2017) and enzyme glycosylation (Vermaas et al. 2015), as these could reduce lignin inhibition.

Thirdly, protein loadings of 27.5 mg/g biomass for achieving 70-100% saccharification of lignocellulosic biomass are still relatively high for alleviating the industrial costs for the lignocellulosic ethanol process. We therefore suggest more studies on improving the synergistic associations between the enzymes in order to lower enzyme loadings. Reports have shown that, from a techno-economic analysis, the cost savings associated with decreasing the enzyme loading by 2-fold would be between $0.26-0.57 per gallon ethanol equivalent (Whitehead et al. 2017). There is still potential in establishing synergism between hydrolytic enzymes and auxillary activity enzymes such as LPMOs, expansins and swollenins in this regard.

Time course (24-96 h) evaluation of the hydrolytic efficiency of HoloMix on the hardwoods should be conducted as lower protein loadings could be required if the saccharification is run over longer than 24 h. Also, the evaluation of the enzyme cocktail's performance at higher than 2% (w/v) substrate loadings is required as industry standards of up to 20% biomass loadings yield ethanol level enough to make biofuel production from lignocellulosic biomass more economically viable.

Finally, another potential approach for lowering enzyme costs is through re-usability by the use of immobilization techniques. Advantages associated with enzyme immobilization include: improved activity, enzyme stability, process control and enzyme recyclability (Mubarak et al. 2014; Kim et al. 2006; Tsai & Meyer 2014). As Bhattacharya and co-workers have shown that glycoside hydrolases can be immobilized successfully using magnetic cross-linked enzyme aggregates (CLEAs**)** (Bhattacharya et al. 2015), we propose that immobilization of these glycoside hydrolases could be attempted. However, CLEAs may not be easy to scale up for large-scale production due to the cost of the process and technical challenges. Adsorption of enzymes on cheap supports such as lignin and recycling these may be a better option.

This study provided insights into the enzymatic hydrolysis of various pre-treated hardwood substrates (*Acacia* and Poplar) and showed that the same lignocellulolytic cocktail comparable to (if not better than commercial enzyme preparations) could be used to efficiently hydrolyse different hardwood species. The proportions of CelMix and XynMix in the cocktail appeared to be pre-treatment specific, with biomass with higher xylan content requiring a higher proportion of XynMix in the cocktail for efficient hydrolysis. Saccharification of the various pre-treated hardwoods by the optimised lignocellulolytic cocktail, HoloMix, at enzyme loadings of 27.5 mg protein/g biomass for 24 h achieved 70–100% sugar yields.

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Appendices

Appendix A: List of chemicals

Acetic acid	Merck (Cat. No. 1.00063.2500)
Acetic acid assay kit	Megazyme (Cat. No. K-ACETRM)
Acrylamide	Sigma (Cat. No. A8887)
Agarose	Sigma (Cat. No. A9539)
Aldouronic acids mixture	Megazyme (Cat. No. O-AMX)
Ammonium persulphate	Sigma Aldrich (Cat. No. A3678)
Bovine serum albumin (BSA)	Sigma (Cat. No. A7906)
Bradford reagent	Sigma (Cat. No. B6916)
Bromophenol blue	Sigma (Cat. No. B8026)
Cellotetraitol	Megazyme (Cat. No. O-CTERD)
Coomassie Brilliant Blue R250	Merck (Cat. No. 1.12553)
3,5-Dinitrosalicylic acid	Sigma (Cat No. D0550)
Di-potassium hydrogen phoshate	Merck (Cat. No. 1.05104.1000)
Di-sodium hydrogen orthophosphate	Saarchem (Cat. No. 5822860)
Ethanol	Merck (Cat. No. 8.18700)
Folin-Ciocalteu reagent	Merck (Cat. No. 1.09001.0500)
Formic acid	Merck (Cat. No. UN1779)
Formic acid assay kit	Megazyme (Cat. No. K-FORM)
Furfural	Sigma (Cat. No. W248908)
Glacial acetic acid	Merck (Cat. No. 1.00063)
D-Glucose	Saarchem (Cat. No. 2676020)
Glucose assay kit	Megazyme (Cat. No. K-GLUC)
Glucuronic acid assay kit	Megazyme (Cat. No. K-URONIC)
Glycerol	Saarchem (Cat. No. 2676520)
Glycine	Merck (Cat. No. 1.04169)

Hydroxymethylfurfural	Sigma (Cat. No. W501808)
Levulinic acid	Sigma (Cat. No. L2009)
Lignin, low sulfonate	Sigma (Cat. No. 471003)
Locust bean gum	Fluka (Cat. No. 62631)
N,N-methylenebisacrylamide	Sigma (Cat. No.M7279)
2-mercaptoethanol	Fluka (Cat. No. 63700)
Methanol	Merck (Cat. No. 8.22283)
p-Nitrophenol	Sigma (Cat. No. 42,575-3)
p-Nitrophenyl-cellobioside	Sigma-Aldrich (Cat. No. N5759)
p-Nitrophenyl-glucopyranoside	Sigma-Aldrich (Cat. No. N7006)
p-Nitrophenyl-xylopyranoside	Sigma-Aldrich (Cat. No. N2132)
Phenol	Sigma (Cat.No. P3653)
Potassium permanganate	Sigma (Cat. No. 223468)
Protein standards	Bio-Rad (Cat. No. 1610363)
Sodium azide	Merck (Cat. No. 8.22335)
Sodium carbonate	Merck (Cat. No. 1.06392.0500)
Sodium chloride	Saarchem (Cat. No. 5822320)
Sodium chlorite	Sigma (Cat. No. 244155)
Sodium dodecyl sulphate (SDS)	BDH biochemicals (Cat. No. 301754)
Sodium hydroxide	Saarchem (Cat. No. 5823200)
Sodium metabisulfite	Sigma-Aldrich (Cat. No.255556)
Sodium Potassium tartrate	Merck (Cat. No. 1.08087)
Tris (hydroxymethyl) aminomethane	Merck (Cat. No. 1.08382)
Tri-sodium citrate dehydrate	Merck (Cat. No. 1.06448)
Tween 20	Merck (Cat. No. 8.22184.0500)
Tween 80	Sigma-Aldrich (Cat. No. P4780)
Triton X-100	Sigma-Aldrich (Cat. No. T-8787)

D-Xylose	Sigma-Aldrich (Cat. No. X3877)
Xylose assay kit	Megazyme (Cat. No. K-XYLOSE)

Appendix B: Standard curves for protein and activity determination

B. 1. Protein standard curve

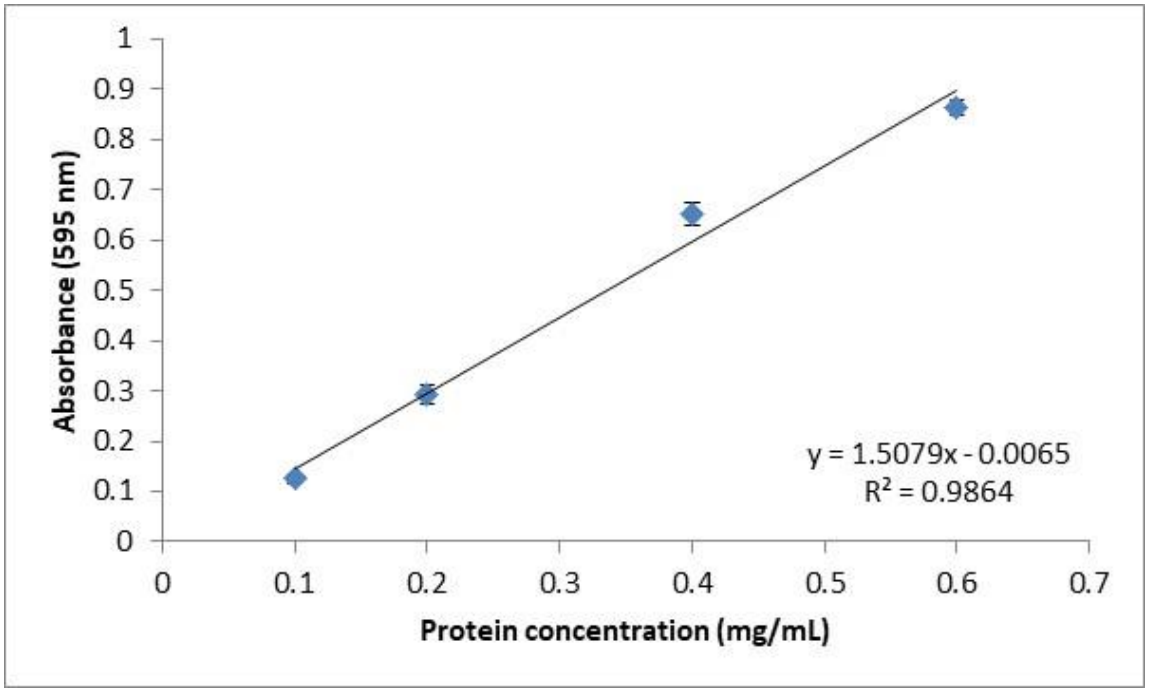

Figure B. 1. Bradford standard curve to determine protein concentration, BSA used as the protein standard. Values are represented as means ± SD, n=3.

B. 2. Enzyme activity curves

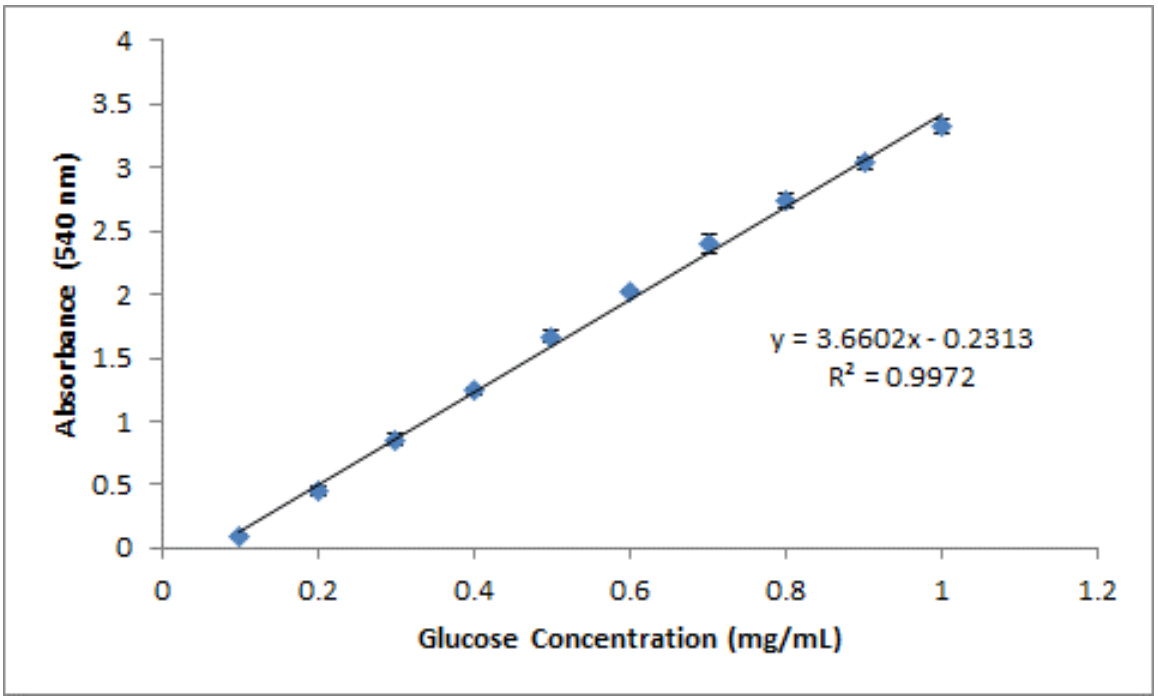

Figure B. 2. 1. Glucose standard curve using DNS assay. Values are represented as means ± SD, n=3.

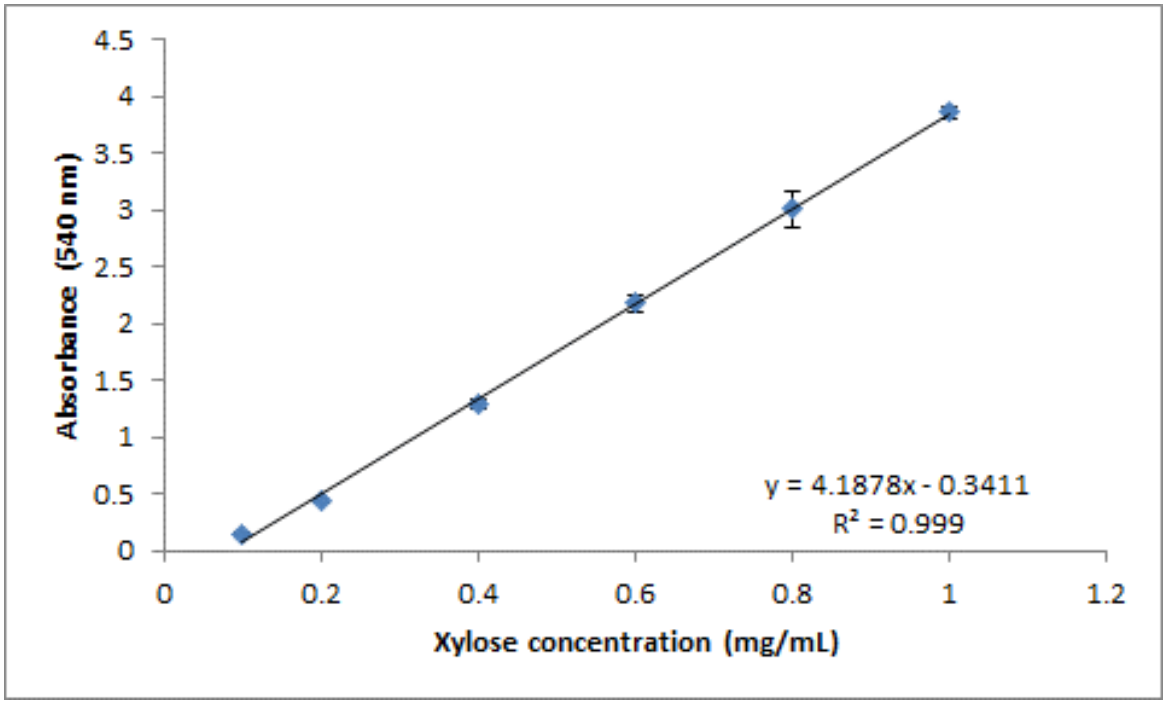

Figure B.2.2. Xylose standard curve using DNS assay. Values are represented as means ± SD, n=3.

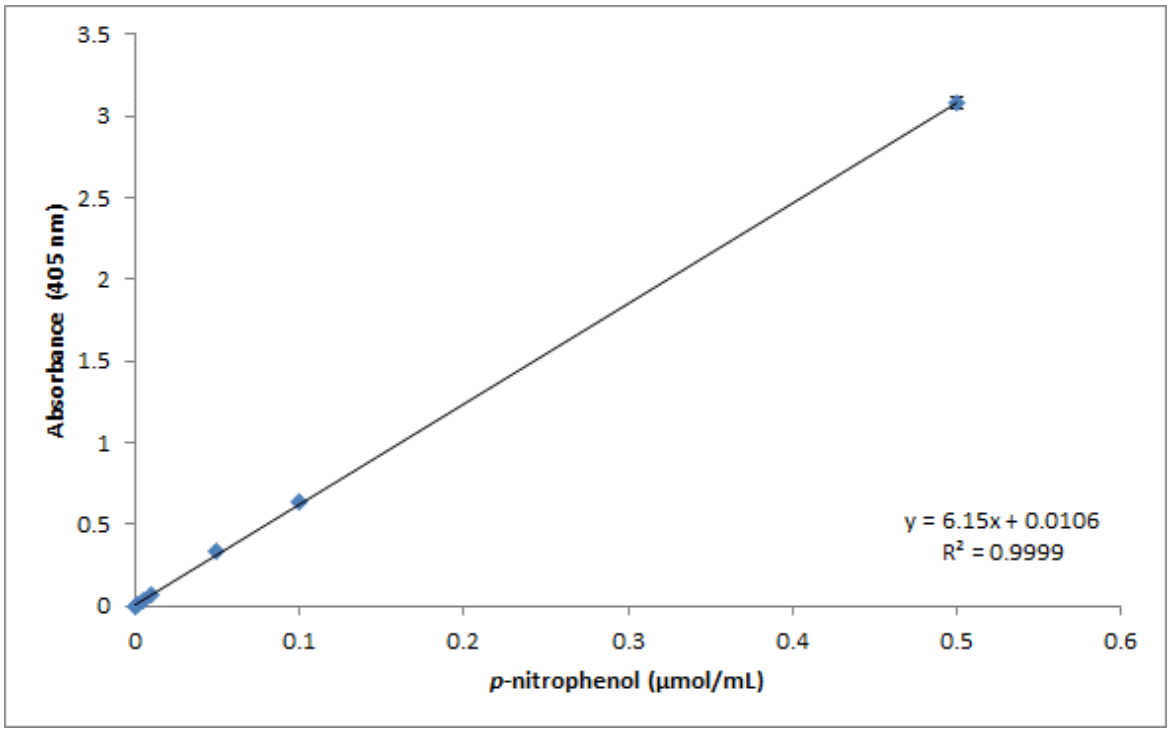

Figure B. 2. 3. *p*-nitrophenol standard curve. Values are represented as means ± SD, n=3.

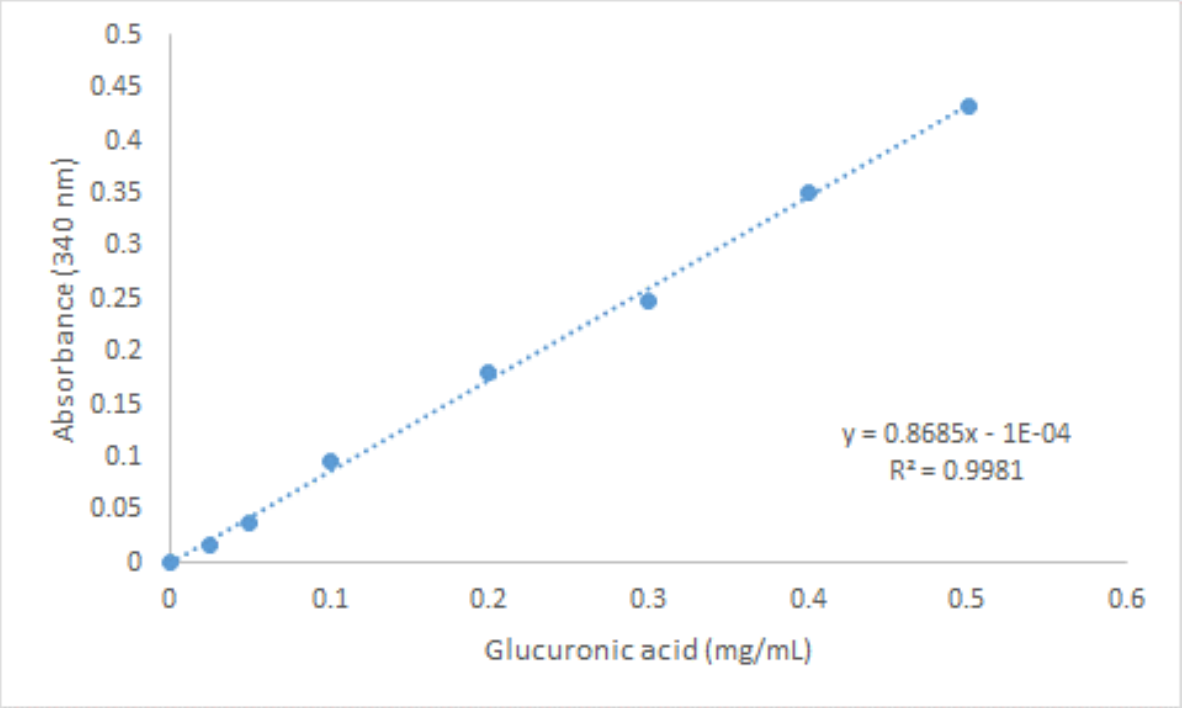

Figure B. 2. 4. Glucuronic acid standard curve using Megazyme™ kit for glucuronic and galacturonic acid determination (K-URONIC). Values are represented as means ± SD, n=3.

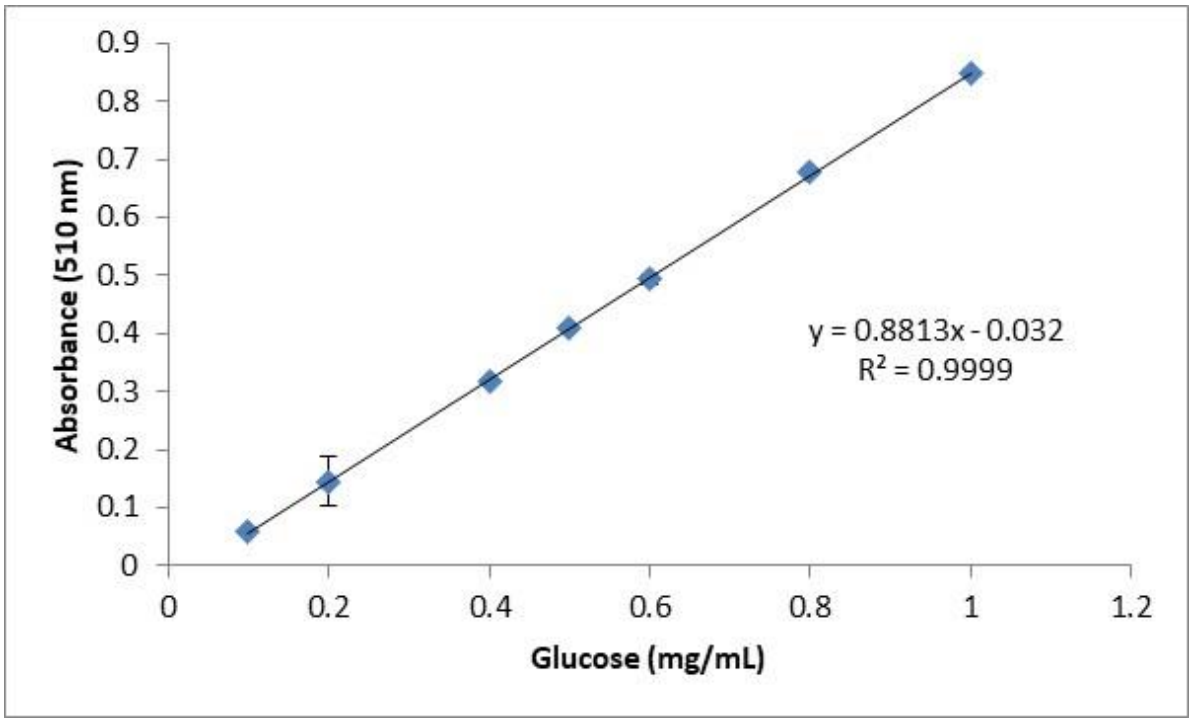

Figure B. 2. 5. Glucose standard curve using Megazyme™ kit for glucose determination (K-GLUC). Values are represented as means ± SD, n=3.

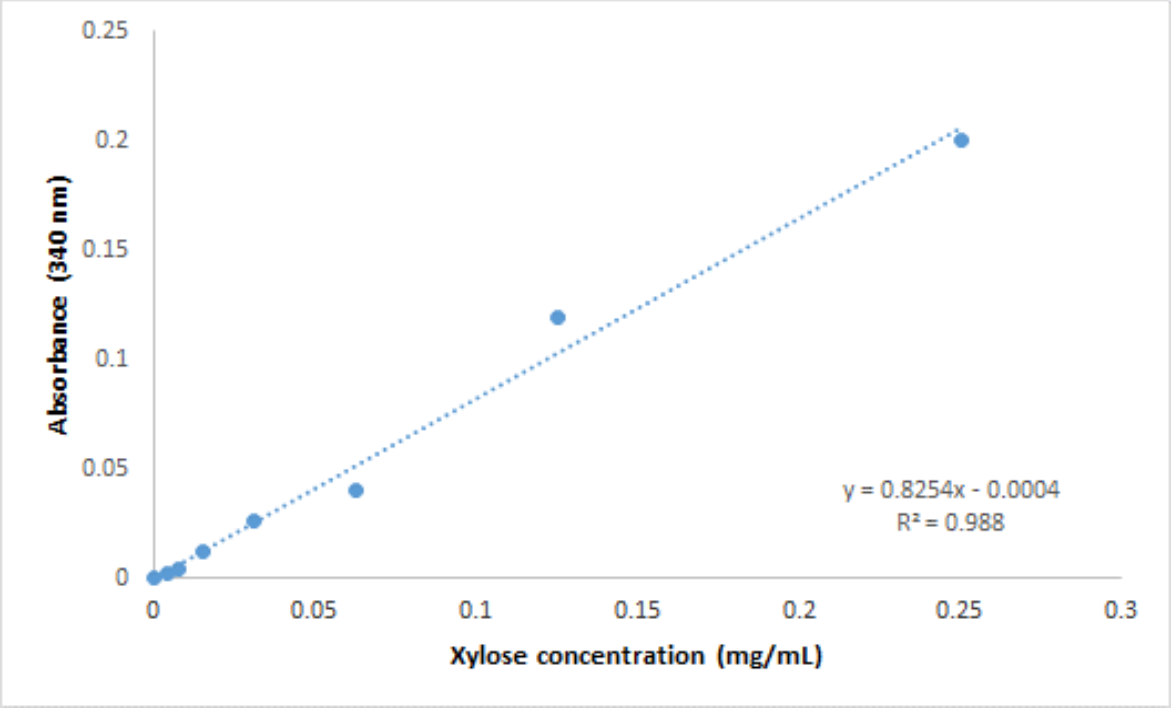

Figure B. 2. 6. Xylose standard curve using Megazyme™ kit for xylose determination (K-XYLOSE). Values are represented as means ± SD, n=3.

B.3. Phenolic content curve

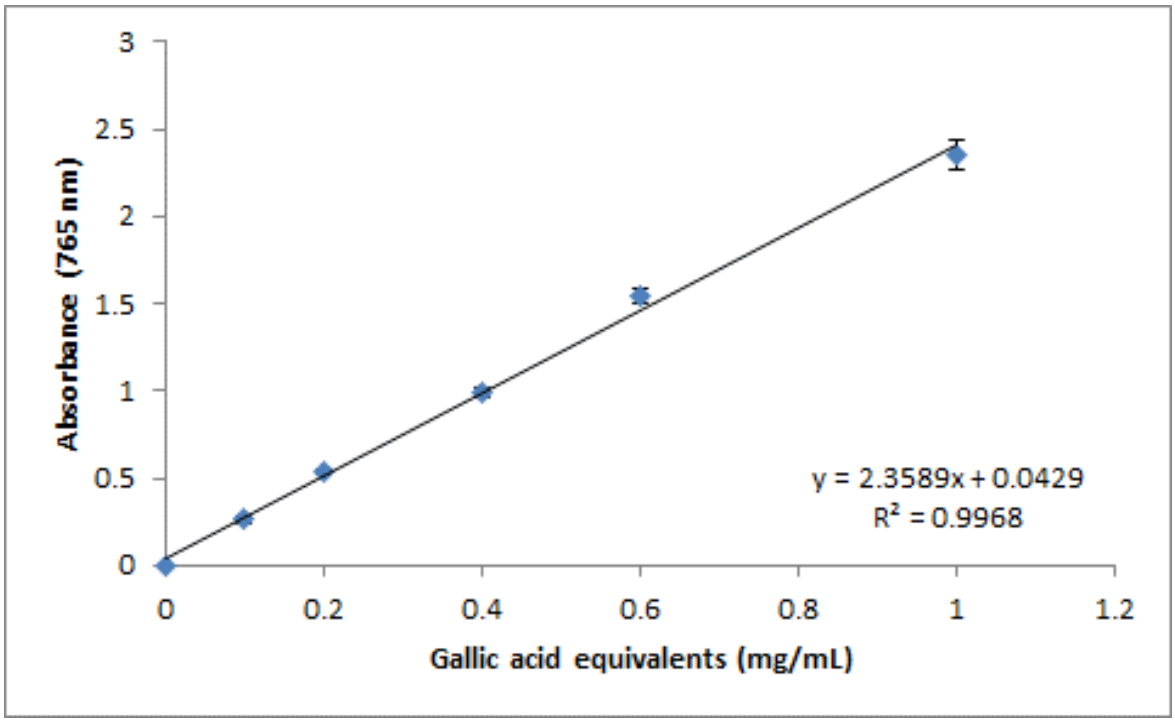

Figure B.3. Phenolics (Gallic acid equivalents) standard curve using the Folin-Ciocalteu method. Values are represented as means ± SD, n=3.

B.4. SDS-PAGE calibration curve

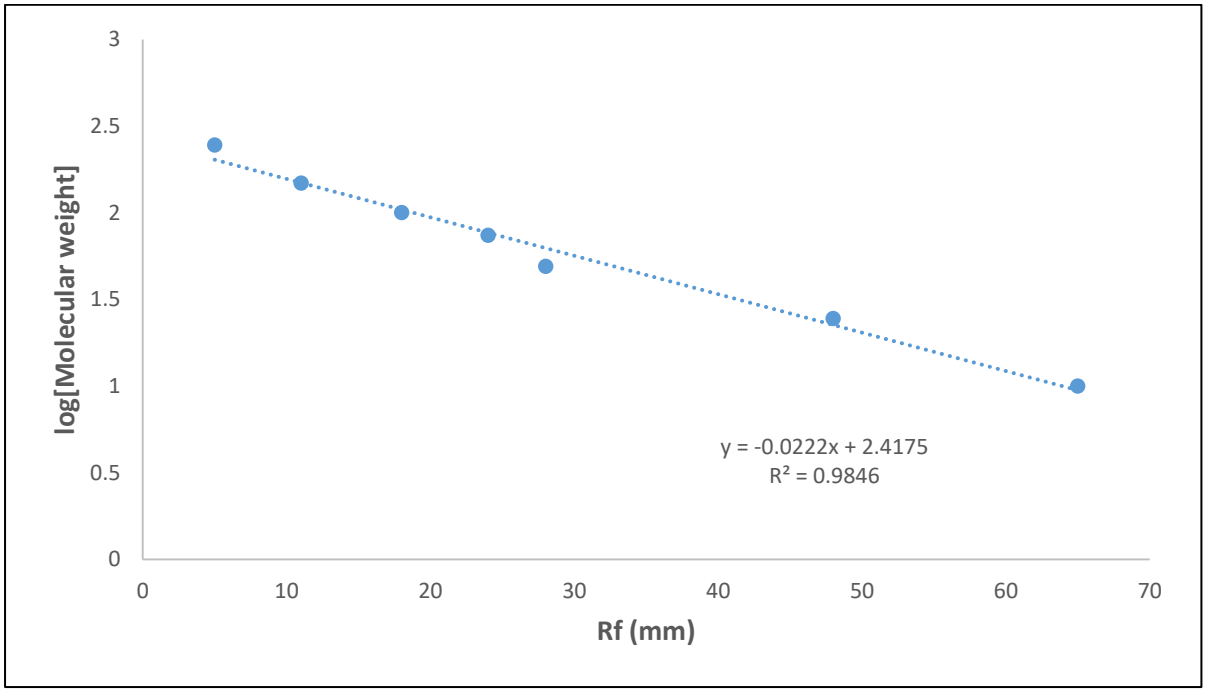

Figure B. 4. Standard curve of the BIO-RAD Precision Plus Protein Standards 1610363 protein marker run on 10% SDS-PAGE.