The effect of GH family affiliations of mannanolytic enzymes on their synergistic associations during the hydrolysis of mannan-containing substrates

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Abstract

Ethanol derived from lignocellulosic biomass is a sustainable route to the production of biofuels to replace fossil-based transportation fuels. However, enzyme hydrolysis of the biomass polysaccharides into fermentable sugars remains the rate limiting step in the process. This is attributable to the recalcitrance of biomass and low enzyme activities which are observed on untreated biomass substrates, leading to lengthy hydrolysis times and low sugar yields. There is limited data reported in literature on the enzymatic degradation of mannans and mannan-containing lignocellulosic biomass using synergistic mannanolytic enzyme mixtures and mannanolytic enzyme mixtures in synergy with cellulases, respectively.

This study first investigated the behavior of mannanolytic enzymes, specifically focusing on differences with respect to their substrate specificities and their synergistic associations with enzymes from different glycoside hydrolase (GH) families. Galactosidases from *C. tetragonolobus* seeds (Aga27A, GH27) and *A. niger* (AglC, GH36), mannosidases from *B. thetaiotaomicron* (Man2A, GH2) and *C. mixtus* (Man5A, GH5) were evaluated for their abilities to synergistically interact with mannanases from *C. cellulovorans* (ManA, GH5) and *A. niger* (Man26A, GH26) in hydrolysis of guar gum and locust bean gum. Among the mannanases, Man26A was more efficient at hydrolyzing both galactomannan substrates; while among the galactosidases, Aga27A was the most effective at removing galactose substituents on both galactomannan substrates and galactose-containing oligosaccharides; and among the mannosidases, Man5A was more efficient at releasing mannose from galactomannan substrates and galactose-containing oligosaccharides.

An optimal protein mass ratio of glycoside hydrolases required to maximize the release of both reducing sugar and galactose or mannose residues was determined. Clear synergistic enhancement of locust bean gum hydrolysis with respect to reducing sugar release was observed when both mannanases at 75% enzyme dosage were supplemented with 25% enzyme protein dosage of Aga27A or AglC and or Man5A. At a protein ratio of 75% Man26A to 25% Aga27A, the presence of Man26A significantly enhanced galactose release by 25% Aga27A (2.36 fold) with locust bean gum, compared to when Aga27A was used alone at 100% enzyme protein dosage. During locust bean gum hydrolysis, all the binary combinations between ManA and Man5A liberated mannose that was higher than that released by Man5A at 100% enzyme protein dosage. During guar gum hydrolysis, ManA to Man2A or Man5A at 75% to 25% protein content liberated the highest reducing sugar content, respectively. All the binary

combinations between ManA and Man5A liberated mannose that was higher than that released by Man5A at 100% enzyme protein dosage. With respect to reducing sugar release from guar gum, no significant synergistic associations were observed between Man26A and galactosidases or mannosidases. A dosage of Man26A and Aga27A at 75% to 25% protein content, liberated reducing sugar release equivalent to that when Man26A was used alone at 100% protein content. A dosage of Aga27A at 75% and ManA at 25% protein content liberated the highest reducing sugar during guar gum hydrolysis. Higher reducing sugar and monomeric sugar release was observed for the heterosynergistic binary combinations compared to the homeosynergistic binary combinations. From the findings obtained in this study, it was observed that the GH family classification of an enzyme affects its substrate specificity and synergistic interactions with other glycoside hydrolases from different families (more so than its EC classification). The GH26 Man26A and GH27 Aga27A enzymes appeared to be more promising for applications that involve the hydrolysis of galactomannan containing biomass.

It is generally well known that pre-treatment of lignocellulosic biomass to increase the accessibility of hydrolytic enzymes to the hydrolysable carbohydrates may result in the generation of compounds that hamper enzymatic hydrolysis and microbial fermentation. We therefore elucidated the individual inhibitory properties of each of the pre-treatment by-products and the synergistic effects that the inhibitor cocktails (obtained from washes of substrates pre-treated by various technologies) had on the mannanolytic enzymes used in this study. Lignin derivatives appeared to be the most inhibitory to the mannanolytic enzymes, followed by organic acids and furan derivatives. Lignin derivative inhibition appeared to be as a result of protein-phenolic complexation, leading to protein precipitating out of solution. The functional groups on the phenolic lignin derivatives appeared to be directly related to the enzyme-interfering capacity of the phenolic, with the phenolic containing the highest hydroxyl group content exhibiting the greatest inhibition. From this study it was also demonstrated that various pre-treatment methods render different pre-treatment soluble by-products which interact in various ways with the mannanolytic enzymes. It was also demonstrated that differences in the nature of the substrate (different plant species) would release different by-products that interact with the mannanolytic enzymes in a diverse manner even if the substrates are pre-treated with the same technology.

In this study, we also investigated how the binary combination of mannanolytic enzymes, Man26A and Aga27A, together with a cellulase preparation, Cellic® CTec2, could synergistically improve both the release of fermentable sugars and the rate of cellulose hydrolysis of delignified sugarcane bagasse. The use of CTec2 alone showed rapid cellulose hydrolysis within the first 24 hours of the reaction, followed by a significant decrease in the reaction rate. The synergistic combination of 75% CTec2 to 25% [75% Man26A: 25% Aga27A] (in terms of protein loading) exhibited a higher rate of biomass hydrolysis up to 48 hours, followed by a significant decrease in the reaction rate. The use of CTec2 alone (at 100% protein loading) liberated a total of approximately 4 mg/mL (68% conversion of cellulose and galactoglucomannan) of reducing sugars after 96 hours, while the synergistic combination of CTec2 at 75% to 25% [75% Man26A 75%: 25% Aga27A] (in terms of protein loading) liberated up to 5.5 mg/mL (80% conversion of cellulose and galactoglucomannan) of reducing sugars from delignified bagasse (an approximate 40% improvement in reducing sugar release). We postulate that the removal of the small amount of mannan, intertwined with cellulose, by the mannanolytic enzymes allowed cellulase (CTec2) accessibility to new hydrolysis sites, thus leading to an improvement in both the rate of biomass hydrolysis and fermentable sugar yield. High yields of fermentable sugars will allow the production of high ethanol yields which will lead to an improvement in cost-effectiveness.

In conclusion, this study demonstrated that a rationally designed mannanolytic enzyme cocktail (based on GH family affiliations) can facilitate the efficient hydrolysis of mannans and can be used in the optimisation of commercial or purified enzyme mixtures to improve the economic viability of the conversion of high mannan-containing biomass such as softwoods and sugarcane bagasse to bioethanol.

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List of abbreviations

Aga27A	Galactosidase 27A
AglC	Galactosidase C
°C	Degree(s) Celsius
CD	Circular dichroism
µg	Microgram
µL	Microlitre
µM	Micromolar
µmol	Micromole
DNS	Dinitrosalicylic
DS	Degrees of synergy
EC	Enzyme commission number
g	Gram
g	Gravity
GH	Glycoside hydrolase
h	Hour
His	Histidine
HPAEC	High-performance anion-exchange chromatography
HPLC-MS	High-performance liquid chromatography- Mass spectrometry
HPLC-RID	High-performance liquid chromatography-Refractive index detector
IMAC	Immobilised metal affinity chromatography
IPTG	Isopropyl β-D-1-thiogalactopyranoside
kDa	Kilo Daltons
L	Litre
Man2A	Mannosidase 2A
Man5A	Mannosidase 5A
Man26A	Mannanase 26A

ManA	Mannanase A
mg	Milligram
min	Minute
mL	Millilitre
mM	Millimolar
nm	Nanometer
NREL	National Renewable Energy Laboratory
OD	Optical density
PAD	Pulsed electrochemical detection
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	Scanning electron microscopy
TEMED	N, N, N’, N’-tetramethylethylenediamine
U	Units of enzyme activity
w	Weight

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Dedication and Acknowledgements

I dedicate my work to Jesus Christ whom I can do all things through. By His will I have been able finish this work.

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List of research outputs

a. National conference proceedings:

Malgas, S., van Dyk, J. S. and Pletschke, B. I. Synergistic actions of various GH family mannan-degrading enzymes on galactomannan substrates (Poster). 2nd ANNUAL ENERGY POST GRADUATE CONFERENCE (EPC), iTHEMBA LABS, CAPETOWN, 11-14 AUGUST 2013.

Malgas, S., van Dyk, J. S. and Pletschke, B. I. Synergistic actions of various GH family mannan-degrading enzymes on galactomannan substrates (Poster). 18th ANNUAL MEETING OF THE SASM, BELA BELA, LIMPOMPO, 24-27 NOVEMBER 2013.

Malgas, S., van Dyk, J. S. and Pletschke, B. I. Synergism between mannanolytic enzymes and CTec2 for the enhanced hydrolysis of delignified sugarcane bagasse (Oral). 25th ANNUAL CATSA CONFERENCE, SAINT GEORGES HOTEL AND CONFERENCE CENTRE, PRETORIA, 9-12 NOVEMBER 2014.

b. Publications in peer reviewed journals:

Malgas, S., van Dyk, J. S. and Pletschke, B. I. (2015). β-mannanase (Man26A) and α-galactosidase (Aga27A) synergism–a key factor for the hydrolysis of galactomannan substrates. Enzyme and Microbial Technology. 70, 1-8.

Malgas, S., van Dyk, J. S. and Pletschke, B. I. (2015). A review of the enzymatic hydrolysis of mannans and synergistic interactions between β-mannanase, β-mannosidase and α-galactosidase. World Journal of Microbiology and Biotechnology. "Under review".

c. Anticipated publications:

Malgas, S., van Dyk, J. S. and Pletschke, B. I. (2015). Determination of the inhibitory effects of various substrate pre-treatment by-products and wash liquors from substrates pre-treated by leading pre-treatment technologies on mannanolytic enzymes. Article in preparation for submission to "Enzyme and Microbial Technology".

Malgas, S., Thoresen, M., van Dyk, J. S. and Pletschke, B. I. (2015). The effect of time on the synergistic associations between various polysaccharide active enzymes on different lignocellulosic feedstocks. Article in preparation for submission.

Chapter 1: General Introduction and Literature Review

1.1. Introduction

The search for alternative fuels to replace the depleting petroleum-based fuels has been an ongoing quest for years. The major factors identified to be the causes of petroleum-based fuel depletion are the continual increase in the global population and growth in industrialization (Beukes and Pletschke, 2011). One of the most acknowledged and promising fields in terms of scientific and economic feasibility is the production of ethanol from lignocellulosic biomass (Van Dyk and Pletschke, 2012). The fact that lignocellulosic biomass is a renewable resource, abundant, and environmentally friendly makes it an appropriate candidate for replacing petroleum-based fuels (Beukes and Pletschke, 2011; Gao et al., 2011).

A number of reports have shown that lignocellulosic biomass has great potential in being used as a feedstock that can be refined to offer many value added products such as organic acids, biofuels, monosaccharides and sugar alcohols (Van Zyl et al., 2010). Other biorefinery concepts such as using hemicellulose in pulping and paper making have also received a great deal of attention in the past few years (Willför et al., 2003). In most of these biorefinery processes, the conversion of hemicellulose to value added products is aided by glycosyl hydrolases (enzymes) (Saha, 2003; Van Zyl et al., 2010). As a result of such extensive applications of enzymes in industries, their market worth was $2 billion by 2004 with a predicted annual growth of about 5% (Moreira and Filho, 2008).

Shi et al. (2011) provided a list of lignocellulosic feedstocks considered for biofuel and other value added products production, including 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.

1.1.1. Plant biomass

Lignocellulose is a material consisting of lignin, which forms a protective barrier around the holocellulose component, which is a combination of cellulose and hemicellulose in the plant biomass (Beukes and Pletschke, 2011). According to Van Zyl et al. (2010), cellulose, hemicellulose and lignin are present in an approximate ratio of 2:1:1 for each of the three components. Lignin is a polymer consisting of phenylpropane units and has been found to be covalently connected to hemicellulose residues (Várnai et al., 2011). In cell walls, lignin acts as an amorphous aromatic-rich barrier to microbial degradation (Van Zyl et al., 2010).

1.1.1.1. Cellulose

Cellulose is the most abundant polysaccharide globally and represents about 40-50% of lignocellulosic biomass. Cellulose consists of linear polysaccharides of β-1,4-linked D-glucose residues (Schwarz, 2001). Cellulose is arranged in the form of long and tightly packed polysaccharide chains that form insoluble, partly crystalline bundles, which provide strength and rigidity to cell walls (Van Zyl et al., 2010).

1.1.1.2. Lignin

Lignins are polymers consisting of phenylpropane units. Varnai et al. (2011) have indicated that lignin has also been found to be covalently connected to hemicellulose residues, forming lignin-carbohydrate residues. The main precursor of lignin in softwoods is trans coniferyl alcohol (Schwarz, 2001).

1.1.1.3. Hemicellulose

Hemicellulose is the second most abundant polysaccharide globally and represents about 20-35% of lignocellulosic biomass. According to Dhawan and Kaur (2007), within biomass, hemicellulose is situated between the lignin and the collection of cellulose fibres underneath. The fact that it is a hetero-polysaccharide made up of different combinations of numerous monosaccharides makes it quite diverse (Saha, 2003). Hemicellulose contained in wood can be categorized according to the source of wood i.e. soft wood (e.g. Douglas fir) and hard wood (e.g. *Eucalyptus*). Table 1.1 shows the contents of the main components of hardwoods and softwoods, including differences in composition. Hardwoods have a low mannan but high xylan content, while softwoods have a high mannan, but also a significant xylan content.

Table 1.1. Contents of the main components of dry wood (as a percentage of total dry mass):

Type of wood	Cellulose	Galactomannan	Xylan	Other polysaccharides	Lignin
Hardwood	38-51	1-4	14-30	2-4	21-31
Softwood	33-42	14-20	5-11	3-9	27-32

Xylans are found in hardwoods as acetylglucuronoxylans in association with variable percentages of galactose, arabinose, rhamnose, and methylglucuronic acid units and acetyl groups (Moreira and Filho, 2008; Saha, 2003).

Mannans, on the other hand, are found in the hemicelluloses of softwoods, and also in specialized structures; such as plant seeds of the Leguminoseae family and fruits (Saha, 2003; Van Zyl et al., 2010). The mannans contain a β-1,4-linked backbone which can contain only D-mannose residues (mannans) or a combination of mannose and D-glucose residues. Based on the variable mannan backbone, mannans can be classified into four subfamilies: linear mannan, glucomannan, galactomannan and galactoglucomannan (Moreira and Filho, 2008; Van Zyl et al., 2010).

1.1.1.3.1. Mannans

In plants, mannans play a structural role, acting as hemicelluloses that bind cellulose. They also display a storage function as a non-starch carbohydrate reserve in endosperm walls and vacuoles of seeds and vacuoles in vegetative tissues, as well as function as signaling molecules in plant growth and development (Moreira and Filho, 2008; Van Zyl et al., 2010).

The simplest type of mannans are linear mannans which are homopolysaccharides composed of linear main chains of 1,4-linked β-D-mannopyranosyl residues and contain no more than 5% of galactose, see Figure 1. 1 (Moreira and Filho, 2008; Van Zyl et al., 2010).

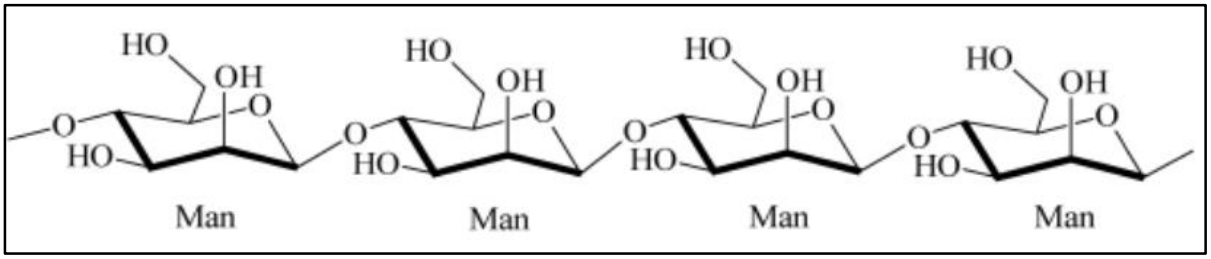

Figure 1.1. An illustrative structure of linear mannan (obtained from Dhawan and Kaur, 2007)

Glucomannans constitute the major hemicellulose fraction in softwoods and can represent up to 50% of the hemicellulose in coniferous woods. They typically contain linear chains of β-1,4-linked D-mannose and D-glucose residues in a 3:1 ratio (Van Zyl et al., 2010). Willför et al. (2008) reported the presence of 2–5% (w/w) of glucomannans with no galactose side groups attached in hardwoods (Angiospermae). Glucomannans in hardwoods have a glucose: mannose ratio of 1: 1.5-2. Glucomannans may also be present in an acetylated form with acetylation up to 18% (Van Zyl et al., 2010). Figure 1. 2 shows a structure of glucomannan.

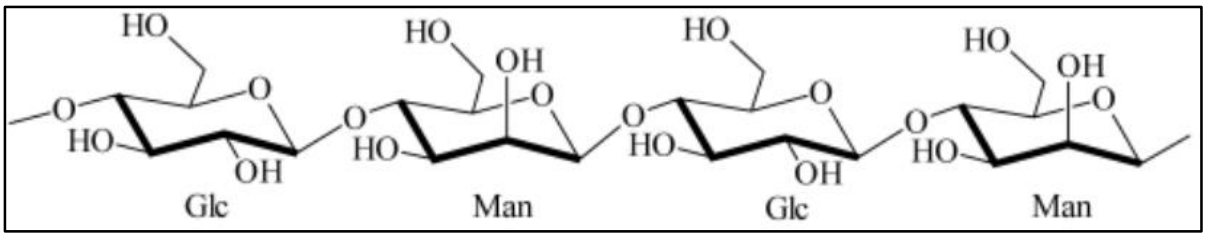

Figure 1.2. An illustrative structure of glucomannan (obtained from Dhawan and Kaur, 2007)

The most complex type of mannans in the glucomannan major group is galactoglucomannan. Galactoglucomannans have a main chain which consists of randomly distributed β-1,4-linked D-mannose and D-glucose residues, and D-galactose residues that are present as single substituents on the mannose units in the main chain (Van Zyl et al., 2010; Willför et al., 2008). According to Moreira and Filho (2008), the mannose, glucose, and galactose residues are reported to be in a molar ratio of 3:1:1 (Figure 1. 3). Van Zyl et al. (2010) and Willför et al. (2008) stated that galactoglucomannans may also contain *O*-acetyl groups and that these acetylated galactoglucomannans form the major hemicellulose of softwoods. The amounts in stemwood usually range from 10–25% (w/w), thus fulfilling structural functions similar to that of xylans in hardwoods.

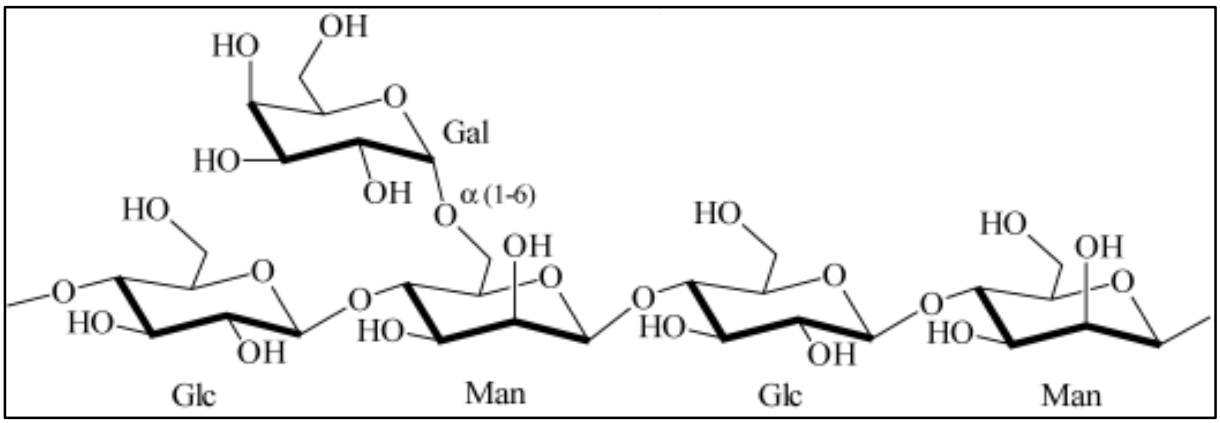

Figure 1.3. An illustrative structure of galactoglucomannan (obtained from Dhawan and Kaur, 2007)

Galactomannans are composed of linear chains of β-1,4-linked D-mannose residues with α-1,6-linked D-galactose side groups (Figure 1.4), these side chain groups prevent close associations between adjacent polymers, resulting in a more amorphous structure that retains water and contributes to its water-solubility (Van Zyl et al., 2010). Moreira and Filho (2008) stated that the D-galactosyl side branches of the polymer are the hydrophilic parts of the molecule, and that the solubility in water increases when the galactose yield increases. Furthermore, the mannose residues can be acetylated to varying degrees at the C-2 and C-3 positions on the

average of one group per three to four hexose residues (Dhawan and Kaur, 2007; Klyosov et al., 2012). Galactomannans are mainly found in the seeds of the family of Leguminoseae and are located in the endospermic part of the seeds (Moreira and Filho, 2008). This project will focus on the degradation and enzymes involved in the degradation of this type of hemicellulose.

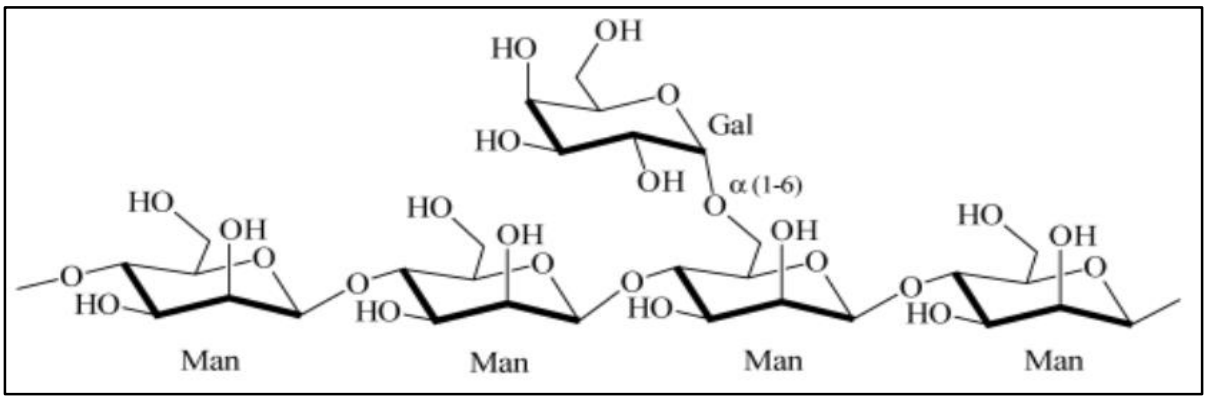

Figure 1.4. An illustrative structure of galactomannan (obtained from Dhawan and Kaur, 2007)

According to Klyosov et al. (2012), there are four primary plant sources of galactomannans, i.e. locust bean or carob tree (*Ceretonia siliqua* L.), guar (*Cyamopsis tetragonolobus* L. Tabu.), tara plant (*Caesalpinia spinosa*) and fenugreek (*Trigonella*). Galactomannans from these four plant sources have distinctive structural characteristics, for example, the mannose to galactose ratio (M/G) varies from 4:1, 3:1, 2:1 and 1:1 for locust been gum, tara gum, guar gum and fenugreek gum, respectively (Moreira and Filho, 2008; Klyosov et al., 2012).

Dea et al. (1986) proposed that a regular (ordered) or block and or random distribution of D-galactosyl groups occur along the main chains of galactomannans. Daas et al. (2000) reported that a more random distribution of D-galactosyl residues occurs in tara gum. Non-regular distributions of D-galactosyl residues with a higher proportion of unsubstituted blocks of intermediate length are said to be found in locust bean gum (Daas et al., 2000; Dea et al., 1986; McCleary, 1985). Guar is said to be found to have few, if any, non-substituted regions (Daas et al., 2000). (See Figure 1. 5 for an illustration of the different degrees of distribution of galactose on galactomannans).

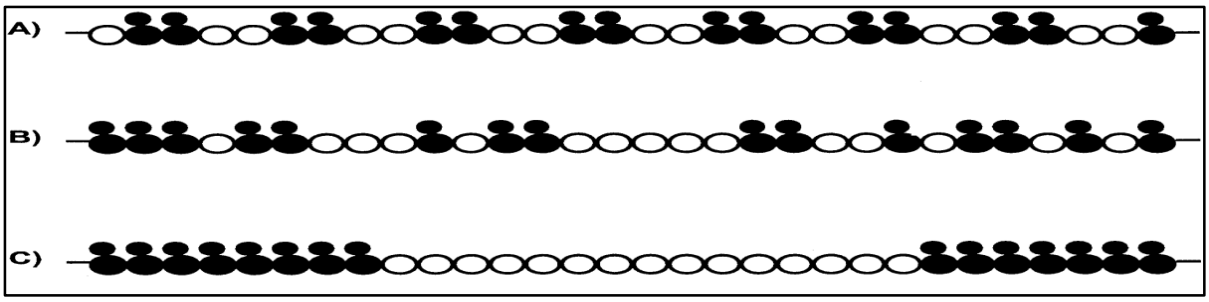

Figure 1.5. The three typical distributions of galactose substituents on galactomannans are shown: A, ordered; B, random; C, block-wise. Galactose substituted mannose are shown as black circles and non-substituted ones shown as open circles (obtained from Daas et al., 2000)

According to Daas et al. (2000), the variations in distribution of galactose in different gums results in different interactions between hydrolytic enzymes and the substrate and in the formation of dissimilar amounts and different types of degradation products after enzymatic hydrolysis.

1.1.2. Enzymatic hydrolysis of biomass

Enzymatic hydrolysis seems to be most promising among the lignocellulosic biomass-to-ethanol conversion processes used in industries. According to Al-Zuhair et al. (2013), this is because the utility cost of enzymatic hydrolysis is much lower since it is carried out at mild conditions and does not require any expensive treatment steps prior to fermentation. A number of different enzymes with different specificities are required to efficiently degrade all the components of lignocellulose. The variety of enzymes required in the hydrolysis of lignocellulosic biomass includes cellulases and hemicellulases (Saha, 2003; Van Dyk et al., 2010). These enzymes are produced by microorganisms in two types of enzyme systems for lignocellulose degradation, namely free and complexed systems, which are also called cellulosomes (Van Dyk et al., 2010; Zhang and Lynd, 2004).

1.1.2.1. Glycoside Hydrolases

Mannan is a very complex and a diverse substrate and thus requires an array of enzymes to degrade it into its monomeric sugar residues. Mannan-degrading enzymes are enzymes that form part of a large arsenal of glycoside hydrolases produced by wood-degrading fungi and bacteria (Van Zyl et al., 2010). Glycoside hydrolases (GHs) include cellulose, xylan, mannan and starch degrading enzymes. These enzymes are classified into glycoside hydrolase families based on amino acid sequence, structural and mechanistic similarity rather than catalytic activity according to the EC nomenclature (Van Zyl et al., 2010). A list of the glycoside

hydrolase families is updated continuously and can be found on the Carbohydrate-Active Enzyme database (CAZy) (http://www.cazy.org).

According to Moreira and Filho (2008), the mannan-degrading glycoside hydrolase (GH) enzymes show a double displacement mechanism with retention of an anomeric configuration. This mechanism involves the attack of a nucleophile at the anomeric center with general acid-catalyzed displacement of the leaving group. This forms a covalent glycosyl-enzyme acylal intermediate. A general base catalyzed process takes place which involves water attacking the anomeric center of the intermediate, yielding the product and releasing the enzyme to its original state (Van Zyl et al., 2010). The mechanism described above is shown in Figure1. 6.

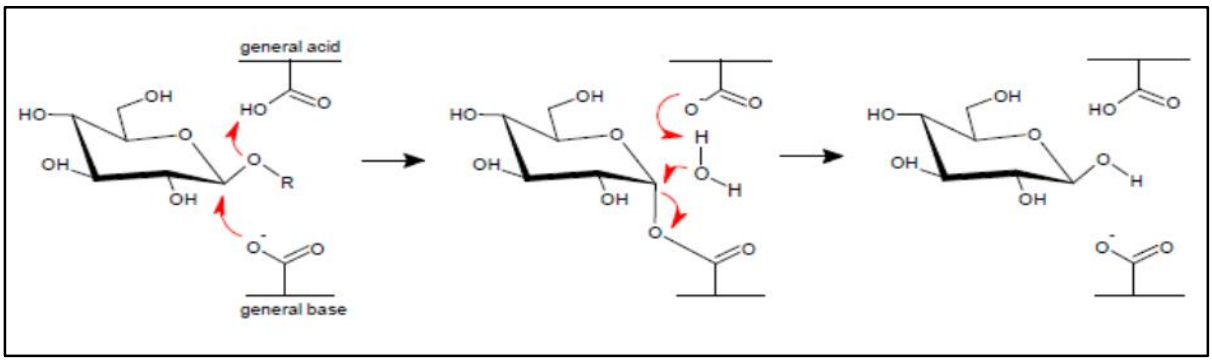

Figure 1.6. A net retention with a double displacement with water mechanism (obtained from McCarter & Withers, 1994)

McCarter & Withers (1994) and Moreira & Filho (2008) further stated that some glycoside hydrolases operate via single-displacement reactions with inversion of anomeric configuration. In this case, the reactions require the participation of a general acid and a general base with nucleophilic attack by a molecule of water. The mechanism described above is shown in Figure 1. 7.

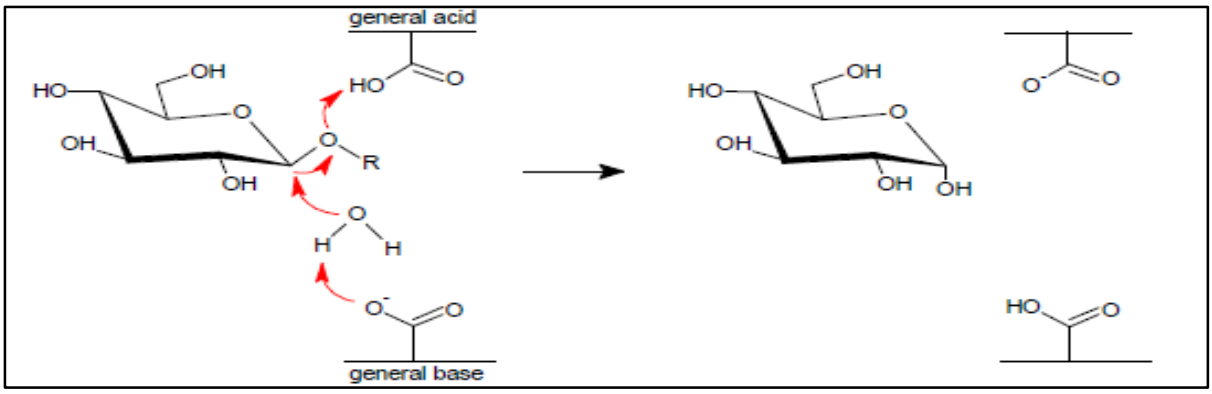

Figure 1.7. A net inversion with a direct displacement with water mechanism (adapted from McCarter and Withers, 1994)

According to Moreira and Filho (2008), these enzymes are often modular, and in addition to catalytic domains, have modules for carbohydrate binding (CBM) and cellulose surface modification and disruption. The role of CBMs is to localize the soluble enzyme to its target substrate, and in some cases it is also suggested that they are able to disrupt the structural integrity of the polysaccharide matrix, making it more accessible to hydrolysis (Shallom and Shoham, 2003; Shosevoy et al., 2006).

According to Boraston et al. (2004), there are three types of CBMs; namely Type A, B, and C modules. Type A CBM modules are those that bind to the surfaces of crystalline polysaccharides and show little or no affinity for soluble carbohydrates (Boraston et al., 2004). Type B CBM modules interact with single polysaccharide chains and bind to polysaccharides that are the substrates for the cognate catalytic module of the enzyme. Type C CBM modules bind optimally to mono-, di- or tri-saccharides (Boraston et al., 2004).

According to Dhawan and Kaur (2007), some GH family 26 β-mannanases which have family 23, 27 and 35 CBMs have been shown to bind to mannan. Also, it has been stated that β-mannanases from *Aspergillus aculeatus* and *Aspergillus niger* appear to lack CBMs (Dhawan and Kaur, 2007).

1.1.2.2. Mannan-degrading enzymes

The convention used in literature to describe the types of enzymes required to degrade mannans involves grouping these mannan degrading enzymes as: exohydrolases which act on the terminal glycosidic linkages and release terminal monosaccharide units from the non-reducing end (example: β-mannosidase), and endohydrolases which cleave internal glycosidic bonds at random or at specific positions (example: β-mannanase) (Moreira and Filho, 2008). It is said

that endo-acting enzymes frequently possess a cleft-shaped active site, while exo-active enzymes frequently have pocket shaped active sites (Juers et al., 1999). All the enzymes required to completely degrade mannans are shown in Figure 1. 8.

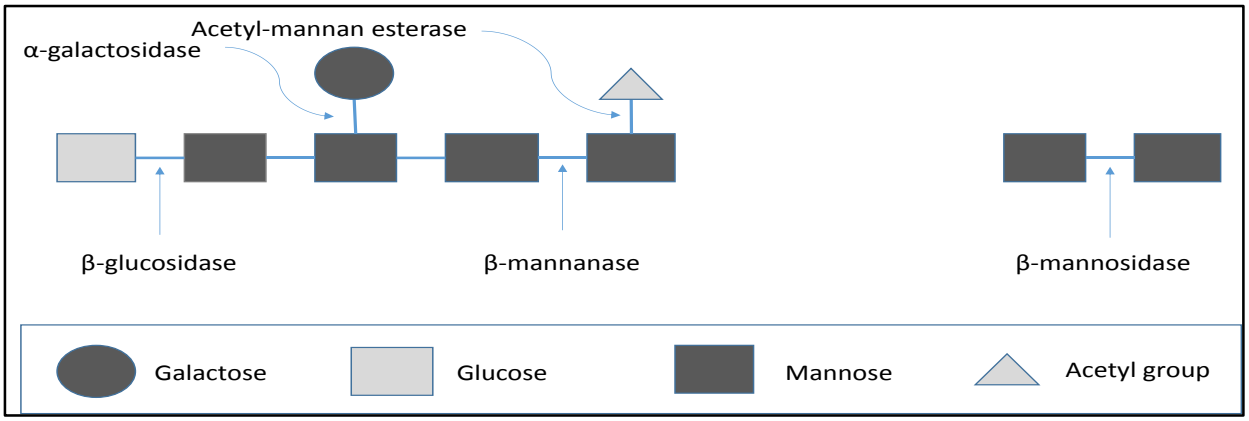

Figure 1.8. An illustration of the enzyme sites of the enzymes required to completely degrade mannans (adapted from Shallom and Shoham, 2003).

1.1.2.2.1. Endo-β-1,4-mannanase

The 1,4-β-D-mannan mannohydrolase (called β-mannanase, EC 3.2.1.78) is an endo-enzyme which is responsible for the random cleavage of β-1,4-linked internal linkages of the mannan backbone to produce new chain ends (Moreira and Filho, 2008; Wang et al., 2010). The β-mannanases fall into the GH family 5 and GH family 26 (Dhawan and Kaur, 2007). Both bacterial and eukaryotic mannanases are found in GH family 5 and those in GH family 26 are of bacterial origin, with the exception of a few anaerobic fungi (Dhawan and Kaur, 2007). It has been reported that the main products obtained during the hydrolysis of mannan by β-mannanases are mannobiose and mannotriose. Additional traces of higher oligosaccharides can also be produced by β-mannanases from *Aspergillus tamarii*, *Trichoderma reesei* and *Aspergillus niger* (Dhawan and Kaur, 2007; Gübitz et al., 1996).

The β-mannanases require a minimum chain length of four sugar residues for binding to ensure hydrolysis (Dhawan and Kaur, 2007). The substrate binding surface is split into different subsites where the subsites are numbered from –3; -2; -1; +1 and +2, which are equivalent to: alpha; beta; gamma; delta and epsilon (Dhawan and Kaur, 2007). Between subsite +1 and -1 of the mannan backbone is where cleavage of the glycosidic bond occurs. According to Van Zyl et al. (2010), complete mannan hydrolysis is affected by the extent and pattern of substitution of the backbone. Moreira and Filho (2008) also reported this observation in their review. When the D-galactosyl and D-glucosyl side chains are bound to residue B and D or

when a D-glucosyl replaces C or E then hydrolysis by the mannanase is blocked (Dhawan and Kaur, 2007). See Figure 1. 9 for an illustration of an enzyme-substrate interaction of mannanase on mannans.

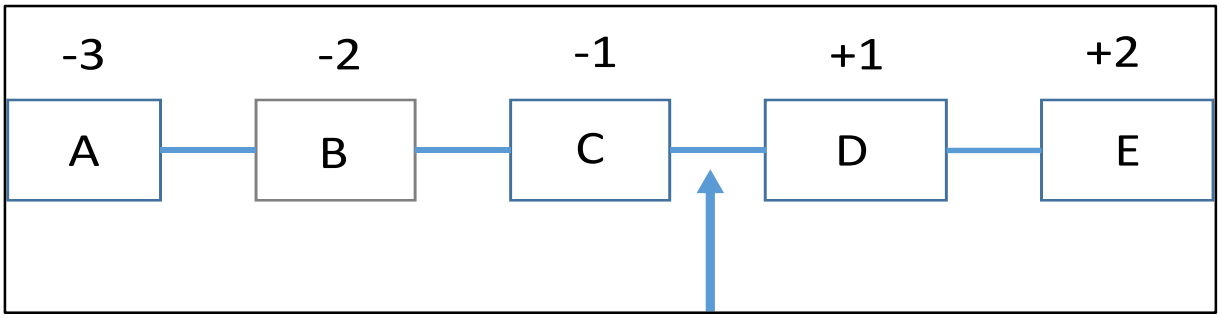

Figure 1.9. An illustration of an enzyme-substrate binding specificity of mannanase on mannan. -3 to +2 represents the subsites, the arrow represents the point of cleavage and A to E represents the mannan backbone (Adapted from Dhawan and Kaur, 2007).

It has been reported, that apart from hydrolysis, it is common for mannanases, especially those from GH family 5, to have transglycosylation activity (Klyosov et al., 2012). Klyosov et al. (2012) stated that this transglycosylation induces the decrease in reducing sugar content. This can lead to an observed decrease in the degree of mannan conversion in comparison to that which really occurred in the reaction.

1.1.2.2.2. β-mannosidase

A second enzyme, 1,4-β-D-mannopyranosidase (also called β-mannosidase, EC 3.2.1.25), is an exo-enzyme which cleaves β-1,4-linked mannosides, releasing mannose from the non-reducing end of mannans and mannan oligosaccharides (Moreira and Filho, 2008; Van Zyl et al., 2010; Wang et al., 2010). The β-mannosidases fall into GH family 1, GH family 2 and GH family 5 (Shallom and Shoham, 2003). Families 1 and 2 (http://www.cazy.org) form part of the GH-A clan. Some fungi such as *A. niger*, *A. awamori*, and *T. reesei* secrete β-mannosidases that act preferentially on shorter manno-oligosaccharides (Stoll et al., 1999).

Pre-incubation of mannan substrates with β-mannanase leads to the hydrolysis products mannobiose, mannotriose, and additional traces of higher oligosaccharides. Subsequent hydrolysis of the β-mannanase products with β-mannosidase is expected to lead to a synergistic effect that improves mannan hydrolysis. On the other hand, pre-incubation of mannan with β-mannosidase and subsequent hydrolysis with a β-mannanase leads to a negative synergistic effect (Gübitz et al., 1996).

1.1.2.2.3. β-glucosidase

A third enzyme, 1,4-β-D-glucoside glucosidase (also called β-glucosidase, EC 3.2.1.21), is an exo-enzyme which hydrolyzes 1,4-β-D-glucopyranose at the non-reducing end of the oligosaccharides released from glucomannan and galactoglucomannan by β-mannanase (Moreira and Filho, 2008; Van Zyl et al., 2010). It has been reported that most β-glucosidases are inhibited by glucose and are not able to hydrolyze long β-1,4-chains (Chauve et al., 2010; Xiao et al., 2004). The β-glucosidases are grouped into glycosyl hydrolase families 1 and 3, where GH family 1 forms part of the GH-A clan (www.cazy.org).

This enzyme is required in the synergistic hydrolysis of glucomannans and galactoglucomannans which have backbones of randomly distributed mannose and glucose residues. The β-mannanases hydrolyse substrates into oligosaccharides. The β-glucosidase then hydrolyzes 1,4-β-D-glucopyranose at the non-reducing ends of the oligosaccharides produced by β-mannanase, leading to the liberation of monosaccharides.

1.1.2.2.4. α-galactosidase

A fourth enzyme is 1,6-α-D-galactoside galactohydrolase (called α-galactosidase, EC 3.2.1.22), a de-branching enzyme which catalyzes the hydrolysis of α-1,6-linked D-galactopyranosyl side chains of galactomannan and galactoglucomannan. The α-galactosidases fall into the GH families 4, 27 and 36 (Van Zyl et al., 2010). According to Wang et al. (2010), α-galactosidases can be divided into two groups based on substrate specificity: one group has specificity for α-1,6-linked galactose units linked to inner mannose residues of galactomannans and galactoglucomannans, and the other has specificity for substrates that have a galactose residue linked to the non-reducing end. The GH family 27 α-galactosidases release galactose from intact galactomannan polymers, while GH family 36 α-galactosidases have their substrate specificity restricted to small oligosaccharides (Wang et al., 2010).

Pre-incubation of galactomannans or galactoglucomannans with a GH family 27 α-galactosidase and subsequent hydrolysis with β-mannanase should lead to a synergistic effect that improves mannan hydrolysis, but pre-incubation with a GH family 36 α-galactosidase would not lead to a synergistic effect since the enzyme lacks specificity for polymeric substrates. On the other hand, pre-incubation of mannan substrates with β-mannanase, will lead to the formation of small oligosaccharides. Subsequent hydrolysis with a GH family 36 α-galactosidase, which has substrate specificity for oligomers, should lead to a synergistic effect that improves mannan hydrolysis.

1.1.2.2.5. Acetyl mannan esterase

An accessory enzyme, acetyl mannan esterase (EC 3.1.1.6), may be required in some mannan types. This enzyme is a de-branching enzyme which releases acetyl groups from galactoglucomannan and galactomannan (Moreira and Filho, 2008; Van Zyl et al., 2010). Acetyl groups are expected to create steric hindrances for β-mannanase, which will induce a decrease in the degree of mannan conversion (Klyosov et al., 2012). The addition of this accessory enzyme in a substrate hydrolysis procedure is expected to drastically improve mannan degradation, especially if it is added in a sequential or pretreatment step before the addition of β-mannanase, as it will remove the acetyl groups which would have hindered β-mannanase from effectively degrading mannans.

1.1.3. Enzyme synergy

For the mannan structure of lignocellulosic material to be efficiently degraded, synergistic actions by the main-chain and side-chain-cleaving enzymes are required (Moreira and Filho, 2008). Beukes and Pletschke (2011) defined synergy as the interaction between two or more hydrolytic components, producing a total effect greater than the theoretical sum of the effects of the individual components.

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).

Zhang and Lynd (2004) indicated that, with respect to synergy on cellulose, endo-exo synergy increased with increased enzyme loadings below the point of saturation. It was also noted that synergy decreased when enzyme loadings went above saturation level. The other important observation made was that synergy becomes higher if inhibition by soluble products is minimised, even though this was not observed under all circumstances (Zhang and Lynd, 2004).

Studies performed by Doi (2008) showed that, when the enzymes are used simultaneously or sequentially in the reaction mixture to hydrolyse a substrate, the effects of synergy between the enzymes may vary. Determining whether synergistic effects occur simultaneously or

sequentially may be important in understanding the characteristics of enzymes and the mechanism of cooperation between them. In the case of sequential synergy, Vardakou et al. (2004) argued that a main-chain cleaving enzyme will have enhanced activity if substituents are first removed through de-branching enzymes; the rationale behind being that the substituent poses a steric hindrance to the main-chain cleaving enzyme. However, this is not always the case as it depends on the specificity of the de-branching enzyme.

Beukes and Pletschke (2011) indicated that a synergistic association is present when the degree of synergy is greater than 1. 0. If the synergy is less than 1. 0, then no synergy has taken place between the enzymes, or there could be anti-synergy between the enzymes due to competition for the same substrate.

1.1.3.1. Synergy studies on defined mannan substrates

The following is a list of some of the synergy studies that have been reported in literature and the synergistic interactions that have been identified between mannanolytic enzymes on model mannan substrates:

- The addition of Man5A-TM2 (mannanase with a truncational mutation that lacks the SL, CBM, and SLH repeats) to the reaction mixture did not influence the activity of Man5A-TM1 (mannanase with a truncational mutation that lacks the SP and SLH repeats) on locust bean gum and glucomannan. This result could have occurred since both enzymes display substrate specificity for polymeric mannans and not oligosaccharides. In guar gum the above mentioned combination was additive and the combination stimulated reducing sugar release on linear mannan as a substrate. This occurred as guar gum hydrolysis released oligosaccharides with a DP of 5 to 6 which these two enzymes could act on. The reducing sugar release was additive from guar gum and locust bean gum when Man5B (a mannanase that lacks SP, SLH, and CBM repeats) was used in the presence of Man5A-TM1. This is possible since Man5B showed β-mannosidase activity and would therefore hydrolyse the oligosaccharide hydrolysis products released by Man5A-TM1 (Han et al., 2010).

- The mixture of the mannanase Man5A and α-galactosidase r-AgalB showed a synergistic increase (3-fold) on guar gum hydrolysis, this occurred since r-AgalB de-branched the galactose side chains in guar leading to an increased interaction between guar and Man5A, which is usually hindered by the presence of galactose side chains in substrates (Wang et al., 2010).

- The ability of β-mannosidase to release mannose and galactose from galactomannan was enhanced in the presence of either one of the mannanases from *S. rolfsii*. The observation occurred since β-mannosidase acts on the hydrolysis products (oligomers) of β-mannanases. Pre-incubation of galactomannan with this β-mannosidase and subsequent hydrolysis with one of the mannanases resulted in an anti-synergistic effect. This anti-synergy occurred since the β-mannanase could not act on the hydrolysis products of the β-mannosidase. Anti-synergy was also encountered when both β-mannanases were added in the reaction mixture; this occurred due to the fact that both these enzymes had the same substrate specificity and competed for the same substrate sites (Gübitz et al., 1996).

It is clear that the synergy studies performed on the hydrolysis of mannans were conducted to define enzyme combinations and ratios that provided the greatest synergy and yield of reducing sugar. Such findings are of interest to the design of cost-effective enzyme cocktails for the biofuels industry.

1.1.3.2. Synergy studies on mannan containing lignocellulosic biomass

The three dimensional structure of plant cell walls and the complex interactions that exist between different components pose a barrier to their enzymatic degradation. Access to cellulose is hindered by hemicellulose as well as lignin. This complexity in lignocellulose therefore requires numerous enzymes, working in synergy, for its hydrolysis (Van Dyk and Pletschke, 2012). The variety of enzymes required in the hydrolysis of lignocellulosic biomass includes cellulases (cellobiohydrolase, endoglucanase and β-glucosidase) and hemicellulases (endo-xylanase, acetyl xylan esterase, β-xylosidase, endo-mannanase, β-mannosidase, α-L-arabinofuranosidase, α-glucuronidase, ferulic acid esterase, α-galactosidase and *p*-coumaric acid esterase) (Saha, 2003; Van Dyk et al., 2010). These enzymes are produced by microorganisms in two types of enzyme systems for lignocellulose degradation, namely free and complexed systems, which are also called cellulosomes (Van Dyk et al., 2010; Zhang and Lynd, 2004).

Listed below are some of the synergy studies that have been reported in literature and the synergistic interactions that have been identified between mannanolytic enzymes and other glycoside hydrolases on mannan-containing lignocellulosic substrates:

- Clarke et al. (2000) demonstrated that the combination of a *P. fluorescens* ManA and a *C. thermocellum* XylA was significantly more effective than using a *C. thermocellum*

ManA on its own during the hydrolysis of softwood paper pulp. In addition, incubation of the softwood pulp with both enzymes plus a galactosidase, AgaA, resulted in the largest enhancement of bleach-ability, and was accompanied by an increase in the total reducing sugar release (Clarke et al., 2000). These data suggested that the activity of the α-galactosidase enhanced the mannanase activity, and that a combination of these two enzymes is able to enhance the activity of XylA.

- Jørgensen et al. (2010) also reported synergism between the mannanolytic enzymes: mannanase and mannosidase with a cellulase on the hydrolysis of Palm Kernel Press Cake. The presence of the mannanolytic enzymes in the enzyme cocktail did not only improve mannose release to yields as high as 88%, but also greatly improved glucose release by the cellulase (Jørgensen et al., 2010).

Both these studies demonstrated that intramolecular synergism between mannanolytic enzymes on the mannan portion of a lignocellulosic biomass greatly enhances the hydrolysis of other polysaccharides within the biomass since these various polysaccharides are said to cover each other and are intertwined within each other (Banerjee et al., 2010; Várnai et al., 2011).

Synergy studies on lignocellulosic biomass have been performed to determine the minimum number of enzymes required for maximum hydrolysis of a particular substrate, as variations exist between substrates from different sources. These studies were conducted to elucidate the structures of complex lignocellulosic substrates and the production of cost effective enzyme cocktails for each substrate (Van Dyk and Pletschke, 2012). Synergy studies in the degradation of lignocellulosic biomass that have been performed in the literature can be grouped into studies of cellulase interaction, hemicellulase interaction and studies with combined enzymes on complex substrates. This thesis examines synergy studies relating to the degradation of model mannan substrates (galactomannan) and the degradation of a mannan-containing lignocellulosic biomass (delignified sugarcane bagasse) in greater detail.

1.1.4. 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 (Shi et al., 2011). 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. The significant percentage of lignin contained in biomass has been demonstrated to be the most important factor in plant recalcitrance, limiting hydrolysis of the biomass by cellulolytic and hemicellulolytic enzymes (Varnai et al., 2010). A correlation between the percentage lignin and release of sugars from lignocellulose substrates has been established; providing evidence that lignin has an effect on hydrolysis (Van Dyk and Pletschke, 2012).

Zhang and Lynd (2004) have indicated that, since enzymatic hydrolysis of native lignocellulose usually results in solubilization 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 lignocellulose. An in-depth discussion on plant recalcitrance and biomass pretreatment for removal of lignin is not further addressed in this thesis.

There are numerous pre-treatment technologies such as steam explosion, H_2SO_4 (Li et al., 2013) and alkaline (NH_4OH, lime, NaOH) (Beukes and Pletschke, 2011; Jabasingh and Nachiyar, 2011; Parvón-Orozco et al., 2012) which have been performed on lignocellulosic biomass. Most of the pre-treatment methods result in the removal or modification of lignin and or hemicellulose, and in the disruption of cellulose crystallinity and increased porosity of the biomass (Alvira et al., 2010; Hendriks and Zeeman, 2009). Although most of the various pre-treatment technologies have been extensively studied and are well understood, limited studies have focused on using sodium chlorite/acetic acid to oxidatively delignify lignocellulosic biomass. As a result, oxidative delignification is the method that was chosen for biomass pre-treatment in this study.

Chapter 2: Research Motivation and Hypothesis

2.1. Problem statement

According to Penttilä et al. (2013), the current use of enzymes to degrade biomass is costly due to the high loadings of enzymes that are used and the low activities they exhibit, and the use of enzymes contributes about 10% of the overall costs in biofuel production. The obstacles in this process step are mainly attributable to plant recalcitrance against enzymatic degradation (Shi et al., 2011). Improvement in the efficiency of enzyme mixtures would greatly improve the economic viability of lignocellulosic ethanol (Banerjee et al., 2010). Woody species such as softwoods (Douglas fir and red pine) and some agricultural residues specifically have a lower pentose content. They contain more hexoses in their backbones (instead of xylan) than non-woody biomass, and this favours their easy conversion to ethanol, since fermentation of pentoses to ethanol is relatively more difficult (Ayeni et al., 2013). This therefore implies that focusing on high hexose sugar feedstocks as a source of biofuels is more economically feasible than the currently used xylan containing feedstocks.

Hemicellulose (mannans) is found in close association with the cellulose fibrils and do, to some extent, cover the cellulose fiber surface (Banerjee et al., 2010; Várnai et al., 2011). This, in turn, limits cellulase activity on the mannan coated/intertwined cellulose fibrils. Improvement of and addition of mannanolytic activities in cellulase enzyme cocktails used in the lignocellulosic conversion process can improve cellulose and mannan hydrolysis in mannan rich biomass through the synergistic interaction of these various glycoside hydrolase (GH) enzymes. This, in turn, would lead to the improvement of the conversion of polysaccharides to fermentable sugars for biofuel production.

2.2. Hypothesis

Supplementation of enzyme cocktails with various mannanolytic activities can improve the overall degradation of mannan rich lignocellulosic feedstocks. Mannanolytic enzymes from different GH families show different synergistic behaviour with each other in the hydrolysis of mannans and mannan containing lignocellulosic biomass.

2.3. Aims and Objectives

1. To over-express and purify *C. cellulovorans* mannanase, ManA.

2. To fully characterize various GH family mannanolytic enzymes (expressed and purchased from Megazyme™ and Prozomix™).
3. To establish the effect of synergistic associations between various GH family mannanolytic enzymes on the degradation of galactomannan.
4. To determine the inhibitory effects of pre-treatment by-products and sugar products on mannanolytic enzymes.
5. To delignify sugarcane bagasse using a sodium chlorite/acetic acid pre-treatment method.
6. To determine the synergistic effect of supplementing a cellulase preparation with an effective binary mannanolytic combination on delignified sugarcane bagasse degradation.

2.4. Overview of Thesis

The over-expression of the *manA* gene and its enzyme purification was successful and is described in Chapter 3. In Chapter 4, the various GH family mannanolytic enzymes used in this work were characterized for their physicochemical properties, substrate specificities and kinetic parameters. The hypothesis that not only enzyme classifications (EC affiliations) of mannanolytic enzymes affect their synergy, but also their GH families and substrate specificities was explored and developed in Chapter 5. Chapter 6 of this thesis dealt with determining which potential products (released from leading pre-treatment technologies and reactor products) would pose inhibitory effects on the activities of the mannanolytic enzymes. Chapter 7 of the thesis was the last experimental chapter and dealt with assessing how effective the synergistic mannanolytic binary cocktail (optimized in Chapter 5) would perform in synergistically hydrolysing sodium chlorite/acetic acid delignified bagasse with CTec2. Chapter 8 of the thesis provides a general discussion, and highlights to the conclusions and future perspectives regarding the work entailed in this thesis.

Chapter 3: Expression and Purification of ManA

3.1. Introduction

Recombinant protein expression has become a common method for the production of proteins used in the biological and biomedical sciences. These recombinant proteins can be expressed in a number of organisms such as in bacteria, yeasts, insect cells and in human cells. The *Escherichia coli* (*E. coli*) expression system is suitable for expressing stable and globular proteins such as catalytic domains or protein interaction domains, protein complexes and some integral membrane proteins (Peti and Page, 2007). According to Braun and LaBaer (2003), up to 50% of proteins from Eubacteria and Archaea and 10% of proteins from Eukarya can be expressed in *E. coli* in soluble form. These qualities make *E. coli* the first choice as an expression host for producing any protein, whatever the source. For achieving high protein quantities, the choice of *E. coli* strain to use as an expression system is BL21 (DE3). The advantage of this strain (as a high protein expression system) is attributable to it being deficient in both *lon* and *ompT* proteases, its compatibility with the T7 *lac*O promoter and the fact that it also contains a plasmid that encodes for three tRNA genes that recognize rare codons (Peti and Page, 2007; Studier et al., 1990).

To successfully purify a recombinant protein of interest, proteins should be fusions to affinity tags when expressed by recombinant means, because the use of affinity methods might make it possible to purify proteins that are difficult or even impossible to obtain by traditional techniques (Waugh et al., 2005). Examples of fusion tags include, but are not only limited to, hexahistidine, maltose-binding protein, biotin acceptor peptide, N-utilization substance A and glutathione *S*-transferase (Peti and Page, 2007; Waugh et al., 2005). One of the most widely used affinity tags for purification of high-throughput recombinant proteins is the hexahistidine tag (Cheung et al., 2012; Waugh et al., 2005). According to Porath (1992), immobilized metal affinity chromatography (IMAC) for the purpose of purifying recombinant proteins has been in use since 1975. IMAC relies on hexahistidine (His-tag) interactions with transition metal ions that are bound to the chelating groups of a chromatographic resin resulting in retention of the recombinant protein of interest on the column. Elution of the protein of interest from metal chelate gels can be accomplished by two principal elution protocols: (1) a decreasing pH protocol whereby the protonation of His_6 residues occurs and (2) an imidazole elution protocol whereby competitive complexation of free imidazole with metal occurs (Sulkowski, 1996). Advantages of the His-tag include its small size, inexpensive matrices that are able to withstand

multiple regeneration cycles, high binding capacity and the option of conducting purification under both native and denaturing conditions (Cheung et al., 2012; Waugh et al., 2005).

Clostridium cellulovorans (ATCC 35296) is an anaerobic, spore forming and mesophilic bacterium that can utilize a variety of carbon sources such as cellulose, xylan, pectin and various others for its growth (Tamaru and Doi, 2000). The bacterium is reported to degrade these various carbon sources via the use of a cellulolytic multi-enzyme complex called the cellulosome (Tamaru and Doi, 2001). A gene for a GH5 mannanase lying in gene clusters of the *C. cellulovorans* cellulosome has been reported previously (Tamaru and Doi, 2001). The mannanase is cellulosomal and contains two highly conserved, identical dockerin domains in its sequence, but these domains are not present in the heterologously expressed version of the enzyme (Tamaru and Doi, 2001). In this study, the mannanase encoding gene of *Clostridium cellulovorans* was expressed in *E. coli* BL21 (DE3) using a pET-29a vector. The protein was finally purified to a high purification fold using immobilized metal affinity chromatography.

3.2. Aims and objectives

3.2.1. Aim

To express and purify *C. cellulovorans* mannanase ManA in *E. coli* BL21 in large quantities at a sufficiently high fold purification for research purposes.

3.2.2. Objectives

- To express ManA with pET-29a expression system;
- To purify ManA with IMAC;
- To use SDS-PAGE to visualize ManA protein fractions from IMAC;
- To conduct ManA protein determination with the Bradford assay;
- To quantify ManA enzyme specific activity using the DNS method;
- To construct a protein purification table for ManA.

3.3. Methods and Materials

3.3.1. Expression of recombinant ManA

Competent *E. coli* BL21 (DE3) (Novagen) cells were transformed with ManA containing plasmid construct, pET-29a-ManA (Beukes et al., 2008). The pre-inocula culture was inoculated (1% (v/v)) and incubated at 37^{o}C in 250 mL of Luria Broth (1% (w/v) tryptone; 1% (w/v) NaCl and 0.5 % (w/v) yeast extract powder) with 50 µg/mL of kanamycin, shaking at 150 rpm. After 18 to 24 hours of incubation, once the optical density measured at 600 OD, had reached 0.8 to 1 units, translation of the recombinant ManA was induced with isopropyl β-D-1-thiogalactopyranoside (IPTG) addition at 0.1 mM. After this, incubation of the culture at 18^{o}C for 24 hours with shaking at 150 rpm was conducted. Sufficient levels of the recombinant protein for subsequent purification were reached after 24 hours of expression.

3.3.2. Purification of recombinant ManA

The culture was transferred to Beckman centrifuge tubes (250 mL) and centrifuged at 15, 316 x *g* (Beckman Avanti centrifuge) for 10 minutes at 4°C. After centrifugation, the supernatant was removed and the cell pellet re-suspended in 10 mL lysis buffer (50 mM NaH_2PO_4; 300 mM NaCl; pH 8.0) at 4°C. The solution was then sonicated at 40 Hz (Vibra Cell Sonics Materials) five times for 10 seconds each, with 30 second rest intervals between each sonication. A second centrifugation step was performed at 15, 300 x *g* under identical conditions to those previously used. The protein containing supernatant was removed and the pellet, containing cellular debris, disposed. The supernatant was then filtered through a 0.20 µm syringe filter to remove any residual cellular debris. The supernatant of the cell lysate was then treated as the crude ManA sample for the remaining steps of the protein purification protocol.

Two bed volumes of the supernatant (1 mL) was added to an eppendorf tube containing the Ni^{2+} charged resin. The resin was incubated at 4^{o}C for 4 hours with rocking using a rotor. For subsequent elution steps, the eppendorf tube was placed in an upright position. The resin was then sedimented by centrifugation using a bench-top centrifuge (Biofuge *pico* Heraeus instruments) at 9, 503 x *g* for 3 minutes, the supernatant carefully decanted and kept as the flow through fraction (unbound protein sample). Two bed volumes (1 mL) of ice cold wash buffer (50 mM NaH_2PO_4; 300 mM NaCl; 20 mM imidazole; pH 8.0) were added to the resin and mixed for 5 minutes using a rotor. The resin was then sedimented by centrifugation at 9, 503 x *g* for 3 minutes, the supernatant carefully decanted and kept as the wash. This step was

conducted to remove all non-specifically bound protein. This step was repeated another three times. To elute the His-tagged ManA protein, two bed volumes of ice cold elution buffer (50 mM NaH_2PO_4; 300 mM NaCl; 250 mM imidazole; pH 8.0) were added to the resin and mixed for 5 minutes using a rotor. The resin was then sedimented by centrifugation at 9, 503 x *g* for 3 minutes; the supernatant was carefully decanted and kept as the eluate (E1). This elution step was repeated once to elute any remaining protein (E2). A final wash step was conducted to remove the imidazole using the washing buffer. The two elution fractions (E1 and E2) were combined and constitute the ManA fraction that will subsequently be used in the remainder of the study. A volume of glycerol giving a final concentration of 20% (v/v) was added to the pool of the two elutions to act as a protein stabilizer. The resin was then stored in a 20% (w/v) ethanol solution. SDS-PAGE analysis was conducted to determine which fraction contained the majority of the polyhistidine-tagged ManA and the Bradford's method was conducted to determine the protein concentration of each fraction.

3.3.3. SDS-PAGE

All the fractions were 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 were made up to 25 µL and placed at 100°C for 3 min (Labnet AccuBlock™ digital dry bath). Approximately 12 µL of protein sample was added to each well and separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970). A discontinuous gel was used. The gel was composed of 10% resolving and 4% stacking gels. 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; and 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. 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.

Gels were then 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.

3.3.4. Protein determination

The Bradford method was used to determine the protein concentration of the IMAC fractions (Bradford, 1976). A standard curve for protein concentrations from 0.1 to 0.6 mg/mL of protein was constructed using bovine serum albumin (BSA) as the standard. For protein sample analysis, appropriate dilutions of solutions were made to produce protein content readings within the standard curve reading region and 25 µL of sample was added to 230 µL of Bradford's reagent. A lysis/wash buffer control was used as a negative control and a blank was constructed for each set of samples. After 10 minutes at room temperature, the absorbance values of the samples were measured at 595 nm with a Power Wave$_x$ Spectroquant using KC Junior software.

3.3.5. Enzyme activity determination

3.3.5.1. Enzyme activity assay

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 0.66% (w/v) substrate volume. Sodium citrate buffer (50 mM) at pH 5 was used to buffer all the solutions. A buffer control was served as a negative control and acted as a blank for each sample set. Substrate and enzyme controls were used whereby either the enzyme or the substrate of the mixture volume was replaced by buffer to make up the respective volume.

To establish ManA activity, the defined substrate, locust bean gum (LBG), was used (0.5% final concentration). All reaction mixtures were kept at 37°C until the addition of any enzyme and were assayed for 15, 30 and 60 minutes at 37°C with tumbling at 20 rpm. The assays were then centrifuged at 16, 060 x *g* (desktop centrifuge-biofuge pico Heraeus) for 5 min to pellet out insoluble substrates. The resulting reducing sugars produced from the inclusion of ManA was determined using the DNS method. One unit of activity was defined as 1 µmol/min of product (reducing sugar) per mg enzyme under the specified assay conditions.

3.3.5.2. Reducing sugar determination

Reducing sugars released were analyzed to determine enzyme activity using a dinitrosalicylic (DNS) assay (Miller, 1959). The standard curve was constructed from the absorbance values produced from the DNS assay using D-mannose as a suitable standard.

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 measured (Power $Wave_x$ Spectroquant on KC Junior software). An appropriate regression equation from the established reducing sugar standard curve was used to convert absorbance values into reducing sugar concentrations.

3.4. Results

3.4.1. SDS-PAGE of *C. cellulovorans* ManA

Through the induction of IPTG, the *manA* gene was successfully expressed. There was no need for assessing the efficacy of the expression method through an induction study on the pET-29a-ManA competent cultures since such data was previously reported by Beukes et al. (2008) and Dredge et al. (2011).

The IMAC purification fractions generated from the purification of ManA were visualized in order to assess the efficacy of the IMAC purification method. Visualization of relative protein contents and molecular masses of the proteins in each purification step usig SDS-PAGE was conducted. A SDS-PAGE of the ManA purification fractions is represented in Figure 3.1 below.

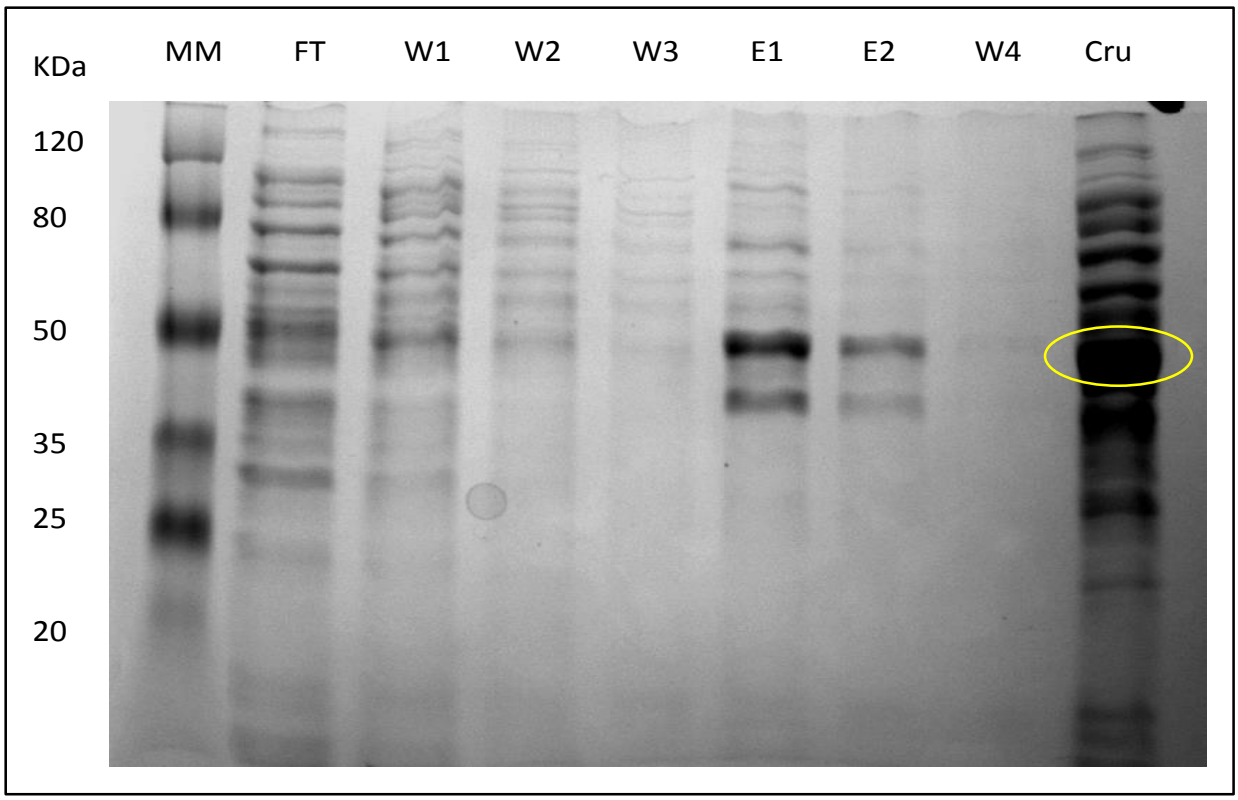

Figure 3.1. SDS-PAGE analysis of ManA purification with Nickel Affinity chromatography. Lane MM: Molecular weight Thermo scientific® protein molecular marker 26612 ladder; Lane FT: Sample of the flow through elution from ManA crude showing intracellular protein from *E. coli*; Lane W1, W3 and W2: Samples of the wash elution fractions of ManA; Lane E1: Samples of the elution fractions of ManA, a protein matching ManA size was detected at 47.2 kDa; Lane E2: Sample of the elution fraction after additional elution wash step, a minor quantity of protein equivalent in weight to ManA was observed; Lane W4: Samples of the wash elution fractions of ManA, no protein was detected; Lane Cru: Sample of crude for ManA.

According to Tamaru and Doi (2000), ManA has a molecular weight of approximately 45 kDa. There appeared to be ManA eluted in the flow-through (lane 2) and wash 1 (lane 3) with an approximate molecular weight of 47.2 kDa. The reasons for this elution could be because ManA was in excess to the number of available Ni^{2+} ions binding sites or because there may have been competition between the His-tagged ManA and endogenous *E. coli* histidine containing proteins for the available Ni^{2+} ions binding sites. These fractions could be re-applied to the column to generate a larger fraction of the purified ManA. Successful elution of ManA from the nickel resins required two steps, as a large amount of ManA was observed in lanes E1 and E2. No further ManA was observed in the wash step (W4) after the elution steps.

3.4.2. Protein concentration of ManA elution fractions

The protein concentration of the purification fractions was used to act as a supporting method to assess the effectiveness of the IMAC purification method. Using the Bradford method, protein concentrations of each of the protein elution samples for ManA purification was measured (Figure 3.2). The protein concentration of the purified ManA, in the E1 and E2 elution steps, was used after dilutions of the enzyme component in subsequent activity assays.

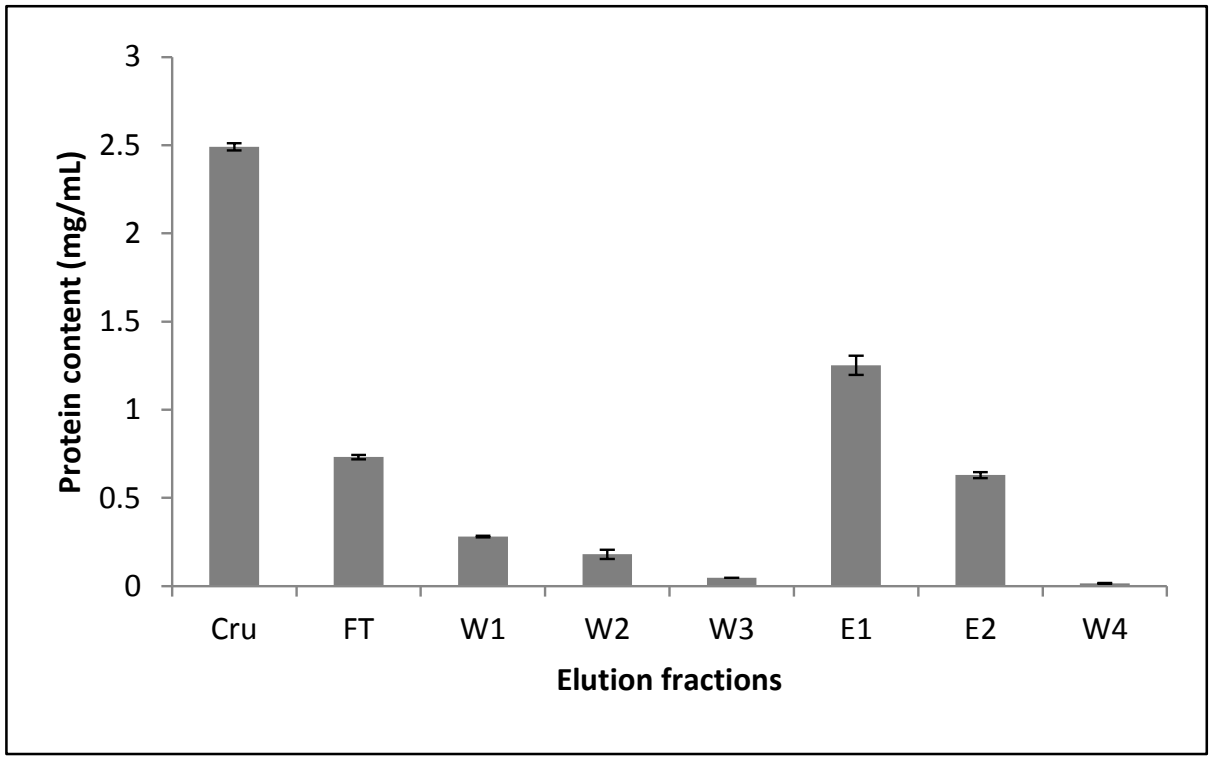

Figure 3.2. Protein content estimation for ManA elution fractions from the Nickel affinity chromatography purification step, each fraction = 1 mL. Values represent means ± SD (n = 3).

To the eppendorf tube, 1 mL of cell lysate containing 2.49 mg/mL of total protein was loaded. The initial wash fraction (W1) contained 0.28 mg/mL of protein with the second wash fraction (W2) having 0.18 mg/mL protein and the third wash fraction (W3) with no significant quantity of protein present. The selective retention and elution of ManA was confirmed with the elution of ManA containing protein at 1.25 mg/mL for the E1 fraction and 0.63 mg/mL for the E2 fraction, with the flow through fraction containing 0.73 mg/mL of protein consisting of the *E. coli* cellular proteins. No further detectable amounts of protein were measured in the fourth wash fraction (W4).

3.4.3. Purification table of ManA

A purification table of the purified ManA was constructed using the information obtained from the protein estimation and activity assay results (Table 3.1). The defined substrate, 0.5% (w/v) locust bean gum, was used under standard enzyme assay conditions.

Table 3.1. Protein purification table for ManA purification fractions. Values represent means ±SD (n = 3).

ManA Fractions	Volume (mL)	Total protein (mg)	Activity (U/mL)	Total activity (U)	Specific activity (U/mg)	Fold purification	Yield (%)
Crude	10	24.4	3.79	37.90	1.55	1	100
Ni^{2+} affinity	20	18.8	1.812	36.24	9.25	5.97	95.62

U = µmol mannose released per min

As seen in Table 3.1, a single step of Ni^{2+} affinity chromatography was used to purify crude ManA. The ManA was purified with a 95.62% yield after Ni^{2+} affinity chromatography with a fold purification of about 5.97. The success of the purification procedure of used for ManA purification was confirmed using the above mentioned findings. The elution fractions, collected after IMAC purification, constituted the enzyme solutions used in all subsequent enzyme assays.

3.5. Discussion

A protein band corresponding to ManA (47.2 kDa), in Lanes E1 and E2, and the minimal presence of a corresponding band in the flow through lane, was observed in Figure 3.1. This demonstrated that a protein matching ManA's mass had been selectively retained and eluted through the manipulation of pH and addition of imidazole. This protein was present in a relatively large quantity in the crude lane, yet absent in the flow-through lane, as per Figure 3.2. Some faint banding was observed in both elution fractions E1 and E2, Figure 3.1. These were most likely due to some endogenous *E. coli* histidine containing proteins that also displayed affinity for Ni^{2+} ions binding sites. Another reason could be attributed to the fact that there were proteins that nonspecifically bound to the recombinant proteins. Such examples could include chaperones that naturally occur in *E. coli* (Hennessy et al., 2005). The presence

of endogenous proteins did not significantly affect the specific activity of the ManA purified in this study (9.25 U/mg protein on locust bean gum), as its specific activity value is similar to that reported by Tamaru and Doi (2000) of 12.90 U/mg protein on locust bean gum.

According to Morrison (2013), a purification step involving IMAC could be considered a success when a benchmark of above 90% yield and a 3 fold purification was achieved. In the current study, a high yield of 95.62% and a fold purification of 5.97 represent a successful purification of the recombinant β-mannanase, ManA. Thus, a relatively pure solution of enzyme was produced for subsequent enzyme activity assays.

3.6. Conclusions

The *manA* gene was successfully expressed and purified in *E. coli* BL21 (DE3) cells. The long induction period of 24 hours produced large quantities of ManA that would be sufficient for this study. The methods for purification proved sufficient and the analytical techniques were conclusive and supported the validity of the purification procedure.

Chapter 4: Characterization of mannanolytic enzymes

4.1. Introduction

Since mannan is a major hemicellulosic component of plant material, the presence of mannan-degrading (also called mannanolytic) enzymes in the lignocellulase consortium should contribute to the optimal degradation of plant cell walls. Organisms that express these plant cell wall hydrolases (mannanases) can host large numbers of closely related enzymes (same EC classification/enzyme activity, but different GH families). This likely reflects 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). The major enzymes required to completely degrade mannans into fermentable sugars are mentioned below.

β-mannanases (also called 1,4-β-D-mannan mannohydrolases, EC 3.2.1.78) catalyze the random hydrolysis of β-1,4-mannosidic linkages in the main chain of mannans (Moreira and Filho, 2008; Wang et al., 2010). Mannanases have been shown to belong to glycoside hydrolase (GH) family 5 or family 26 and or family 113 (www.cazy.com). It has been reported that GH family 5 mannanases often possess carbohydrate binding modules (CBMs), while most GH family 26 mannanases lack these CBMs (Tailford et al., 2009). It has been also reported that the main products obtained during the hydrolysis of mannan by β-mannanases are mannobiose and mannotriose (Dhawan and Kaur, 2007; Gübitz et al., 1996).

The second enzyme, 1,4-β-D-mannopyranosidase (also called β-mannosidase, EC 3.2.1.25), is an exo-enzyme which cleaves β-1,4-linked mannosides, releasing mannose from the non-reducing end of mannans and mannan oligosaccharides (Moreira and Filho, 2008; Van Zyl et al., 2010; Wang et al., 2010). The β-mannosidases fall into the GH family 1, GH family 2 and GH family 5 (Shallom and Shoham, 2003). Studies have shown that β-mannosidases from both GH families possess two aglycone sugar binding subsites (+1 and +2) and a glycone sugar binding subsite (-1) (Dias et al., 2004; Tailford et al., 2007).

α-galactosidases (also called 1,6-α-galactose galactohydrolases, EC 3.2.1.22) are enzymes that hydrolyze α-galactoside substituents from simple oligo- (e.g. raffinose and stachyose) to polymeric substrates (e.g. galactomannans) (Blöchl et al., 2008; Jindou et al., 2001; Van Zyl et al., 2010). It has been observed that most eukaryotic α-galactosidases belong to the GH family 27 cluster, while those of prokaryotic origin mostly belong to GH family 36 (Wang et al.,

2010). Wang et al. (2010) have also proposed that the GH family 27 galactosidases have higher substrate specificity for galactose substituents on galactomannan polymers and oligosaccharides, while GH family 36 galactosidases have restricted substrate specificity to small galactose-containing oligosaccharides.

In the present study an *A. niger* α-galactosidase (AglC, GH36) and guar seed (*C. tetragonoloba*) α-galactosidase (Aga27A, GH27), an *A. niger* β-mannanase (Man26A, GH27) and a *C. cellulovorans* β-mannanase (ManA, GH5), and a *C. mixtus* β-mannosidase (Man5A, GH5) and a *B. thetaiotaomicron* β-mannosidase (Man2A, GH2) were subjected to detailed comparative biochemical characterization and analyzed for substrate specificities.

4.2. Aims and objectives

4.2.1. Aims

To conduct comparative biochemical characterization and substrate specificity determination of mannan-degrading enzymes from different GH families

4.2.2. Objectives

- To conduct *in silico* characterization of the mannanolytic enzymes (ProtParam);
- To determine substrate specificities of the mannanolytic enzymes;
- To determine temperature optima of the mannanolytic enzymes;
- To determine temperature stability of the mannanolytic enzymes;
- To determine pH optima of the mannanolytic enzymes;
- To determine kinetic parameters of the mannanolytic enzymes.

4.3. Materials and Methods

4.3.1. Materials

Two α-galactosidases, one from guar (*C. tetragonolobus*) seeds (Aga27A, GH27), and the other from *A. niger* (AglC, GH36) were purchased from Megazyme™. A β-mannanase from *A. niger* (Man26A, GH26) was also purchased from Megazyme™, another mannanase (ManA, GH5) from *C. cellulovorans* was expressed and purified as described in Chapter 3. The two β-mannosidases were purchased from Prozomix™; *C. mixtus* mannosidase (Man5A, GH5) and *B. thetaiotaomicron* mannosidase (Man2A, GH2). All the substrates (guar gum, *p*-nitrophenyl-β-

D-mannopyranoside and *p*-nitrophenyl-α-D-galactopyranoside) were purchased from Megazyme™, except for the locust bean gum which was purchased from Sigma Aldrich.

4.3.2. SDS-PAGE and *in silico* characterization of mannanolytic enzymes

The molecular weights of the six mannanolytic enzymes assessed in this study were estimated using a 12% reducing SDS-PAGE gel following the protocol described in Section 3.3.3.

From the Carbohydrate-Active Enzyme (CAZy) database (http://www.cazy.org), sequences of the mannan-degrading enzymes were retrieved (uniprot ID): Aga27A (P14749), AglC (Q9UUZ), Man2A (Q8AAK6), Man5A (Q6QT42), Man26A (A2R6F5) and ManA (C5RQ36). Computation of various physical and chemical parameters for the given proteins was conducted using the ProtParam tool in the ExPasy Bioinformatics Resource Portal (Gasteiger et al., 2005).

4.3.3. Specific activity determination

4.3.3.1. Mannanase activity

The enzyme assays and reducing sugar release for Man26A and ManA were assessed using the protocols described in Section 3.3.5 of Chapter 3. Enzyme activity was measured in units (U), where 1 unit is defined as the amount of enzyme releasing 1 µmol of reducing sugar per minute. Mannose was used as the sugar standard. The substrates for Man26A and ManA using the DNS method were guar gum and locust bean gum. The mannanases were also assessed for mannosidase and galactosidase cross activity using the protocols described in sections 4.3.3.2 and 4.3.3.3.

4.3.3.2. Mannosidase activity

The specific activities of Man2A and Man5A were measured by their ability to hydrolyze *p*-nitrophenyl-β-D-mannopyranoside (*p*NPM). The assay reaction mixture was in the ratio 1:9 with 50 µL of appropriately diluted enzyme: 450 µL of 2.25 mM *p*NPM in 50 mM sodium citrate buffer (pH 5.0). The reaction was conducted at 37°C for 15 minutes and terminated by the addition of 500 µL of 2 M sodium carbonate. The released *p*-nitrophenyl product was measured at 405 nm with Power Wave$_x$ Spectroquant on Kc Junior software. *p*-Nitrophenol was used as a suitable standard. Standard mannanase and galactosidase activities of the mannosidases, Man2A and Man5A were performed using the protocols mentioned in Sections 4.3.3.1 and 4.3.3.3.

4.3.3.3. Galactosidase activity

The α-galactosidase activity for Aga27A and AglC was performed using the same protocol for determining β-mannosidase activity, but with 2.25 mM *p*-nitrophenyl-α-D-galactopyranoside (*p*NPG) in 50 mM citrate buffer (pH 5.0) as substrate. The reaction was conducted at 37ºC for 15 minutes and terminated with the addition of sodium carbonate. The activity of the α-galactosidases on the polymeric galactomannan substrates (guar and locust bean gum) and galacto-oligomers (galactosyl-mannotriose and di-galactosyl-mannopentaose) was determined using the protocol mentioned in Section 4.3.3.1. Galactose released by the enzymes on the galactomannan substrates was monitored using the modified AOAC Official Method 984.15 for lactose detection (Beutler, 1988). The assay was monitored using a 96 well plate at 340 nm for 20 minutes with readings taken at 1 minute intervals (25ºC being the operating temperature).

4.3.4. Temperature optimum and temperature stability determination

A range of temperatures (30-90ºC) was used to determine the temperature optima of the mannanolytic enzymes (Labnet AccuBlock™ digital dry bath) at pH 5.0 (50 mM sodium citrate buffer). The activities of Man26A and ManA were measured with the DNS method as described in section 4.3.3.1, the activities of Man2A and Man5A with the mannosidase assay as described in Section 4.3.3.2, and the activity of Aga27A and AglC with the galactosidase assay as described in Section 4.3.3.3.

The stabilities of the individual mannan-degrading enzymes were investigated in triplicate over time at 37 and 50ºC (Labnet AccuBlock™ digital dry bath) at pH 5.0 (50 mM sodium citrate). Each enzyme was incubated at these different temperatures for selected different time periods, up to 24 hours and the residual activity determined. The DNS method (see Section 4.3.3.1) for Man26A and ManA, mannosidase method (see Section 4.3.3.2) for Man2A and Man5A, and galactosidase method (see Section 4.3.3.3) for Aga27A and AglC were performed.

4.3.5. pH optimum determination

A range of pH values (pH 4-8) was used to determine the optimal pH (PHS-3BW BANTE instruments pH meter) for the individual Aga27A, AglC, Man2A, Man5A, Man26A and ManA mannanase enzymes. The buffers were: 50 mM sodium citrate (pH 4.0 – 6.0) and 50 mM potassium phosphate (pH 6.0 - 8.0). The enzyme assays were performed in triplicate at 37°C (Labnet AccuBlock™ digital dry bath) using the protocols described in Section 4.3.3.

4.3.6. Kinetic parameters determination

The kinetic parameters K_M and V_{max} were determined from Lineweaver-Burk plots using seven concentrations of *p*-nitrophenyl based substrates (1-5 mM) for galactosidases and mannosidases. Up to six concentrations of galactomannan substrates (0.5-10 mg/mL) were used for mannanase kinetic parameter determination.

4.4. Results

4.4.1. SDS-PAGE and *in silico* characterization of mannanolytic enzymes

Purity and molecular weight analysis of the individual commercial mannanolytic enzymes used were conducted using SDS-PAGE. The commercial proteins were observed and found to be mostly single bands, confirming their purity (see Figure 4.1).

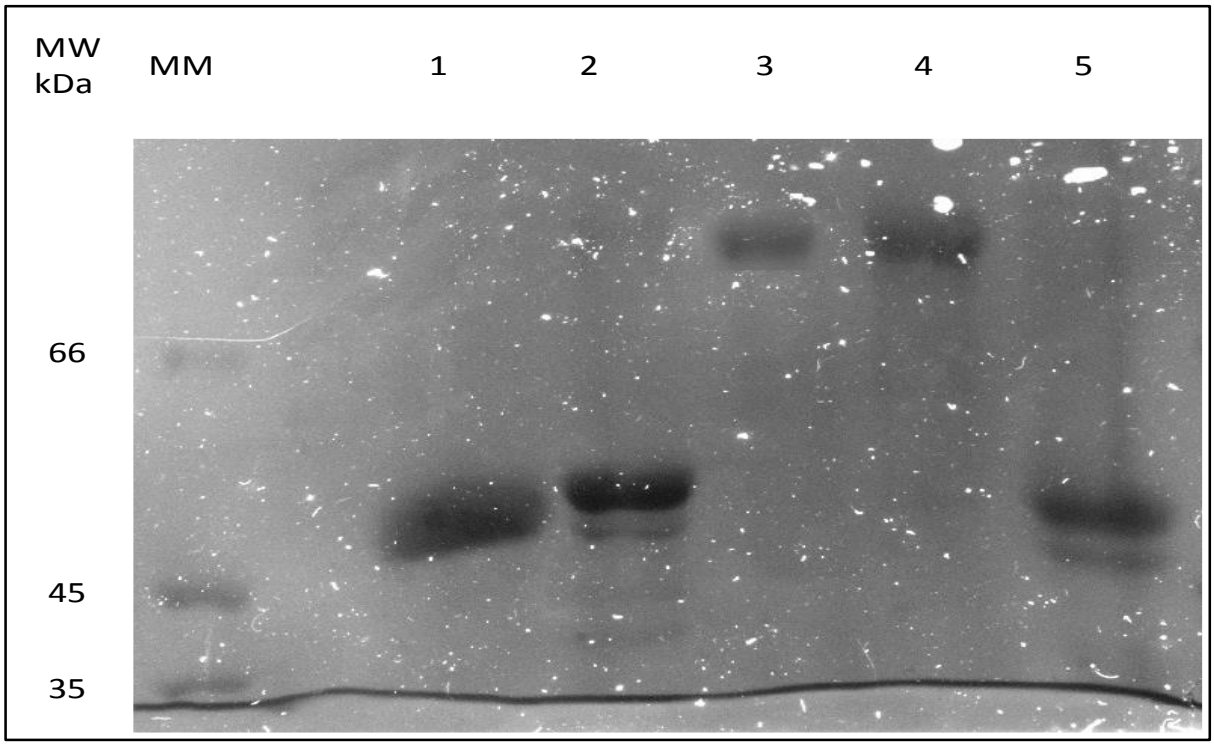

Figure 4.1. SDS-PAGE analysis of commercial mannanolytic enzymes. Lane MM: Molecular weight Thermo scientific® protein molecular marker 26612 ladder; Lane 1: Man26A; Lan2 2: Man5A; Lane 3: Man2A; Lane 4: AglC and Lane 5: Aga27A.

The molecular masses of the commercial mannanolytic enzymes estimated from SDS-PAGE were: 50.1 kDa for *C. tetragonolobus* seeds Aga27A, 85.1 kDa for *A. niger* AglC, 81.3 kDa for *B. thetaiotaomicron* Man2A, 55 kDa for *C. mixtus* Man5A, 47 kDa for C. cellulovorans ManA (see Section 3.4.1 in Chapter 3) and 46.8 kDa for *A. niger* Man26A.

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 4.1 below displays results obtained from sequence analysis of each mannanolytic enzyme using ProtParam.

Table 4.1. Data obtained from Uniprot and ProtParam for mannanolytic enzymes.

Name (Uniprot id)	Sequence length (aa)	Mass (kDa)	pI	Active site residues	Binding site
Aga27A (P14749)	411	45	3.7-4.1	177, 232	-
AglC (Q9UUZ4)	747	81.8	4.9	508, 534	Mg, NAD
Man2A(Q8AAK6)	864	99.5	5.90	462, 555	Cl
Man5A(Q6QT42)	456	51.6	4.91	-	SO_4
Man26A(A2R6F5)	335	37.1	5.81	-	-
ManA (D9SS67)	425	47.1	5.62	-	-

Any annotated post-translational modifications were not taken into account

4.4.2. Specific activity 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 (Table 4.2).

Table 4.2. Specific activities of the mannan-degrading enzymes on defined substrates. Values are represented as means ±S.D. (n=3). "nd" = not detected.

Substrate	α-galactosidases (U/mg protein)		β-mannosidases (U/mg protein)		β-mannanases (U/mg protein)	
	Aga27A	**AglC**	**Man2A**	**Man5A**	**Man26A**	**ManA**
Di-galactosyl-mannopentaose	13.7	0.49	0.082	0.041	nd	nd
Galactosyl-mannotriose	4.50	1.61	0.015	0.32	nd	nd
Guar gum	11	0.97	nd	0.70	217.18	4.63
Locust bean gum	3.68	1.01	0.41	0.71	436.41	9.61
***p*NPG**	80.3	1168	nd	nd	nd	nd
***p*NPM**	nd	nd	0.44	0.048	nd	nd

1 U is the amount of enzyme releasing 1 µmol of product per minute, unless otherwise stated

4.4.3. Temperature optima and temperature stability determination

The temperature optimum and temperature stability for each of the mannan-degrading enzymes was determined using 50 mM sodium citrate buffer (pH 5.0). The figures below illustrate the optima and stability temperatures of each enzyme. Figure 4.2 illustrates thermo-properties of Aga27A.

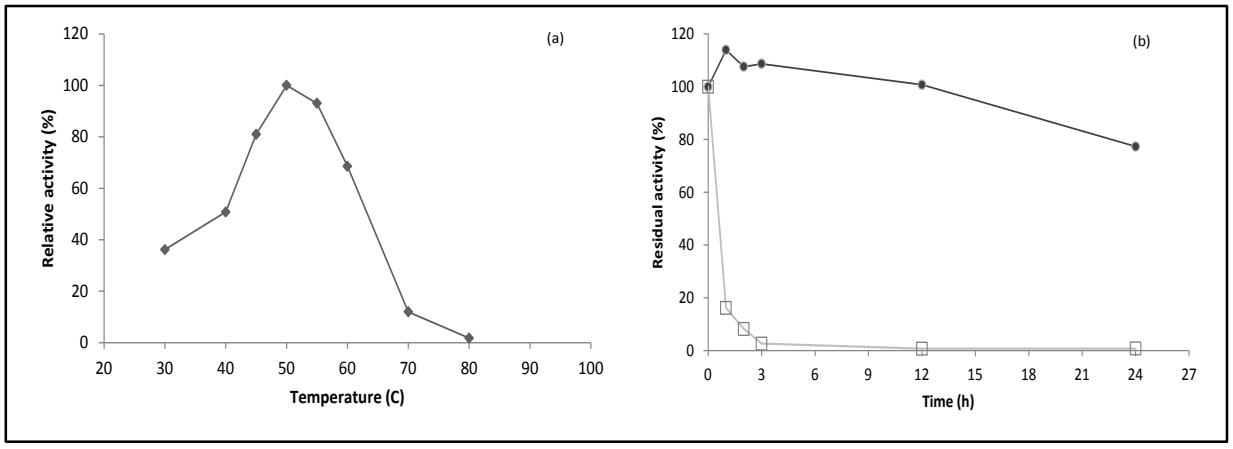

Figure 4.2. Temperature profile of the α-galactosidase Aga27A, (a) temperature optimum and (b) temperature stability at 37 (●) and 50°C (□) over a 24 hour period.

The temperature profile for Aga27A showed a temperature optimum at 50°C and then a decline of activity afterwards with complete loss of activity beyond 80°C (Figure 4.2.a). Aga27A was most stable at 37°C with also an activation of residual activity compared to the initial activity during the first 12 hours of incubation (see Figure 4.2.b).

The optimum temperature of AglC was at 60ºC (Figure 4.3.a). The stability of AglC was lower than that of Aga27A at 37ºC, with more than 50% reduction in residual activity within an hour. But, the stability of AglC at 50ºC was greater than that of Aga27A (Figure 4.2.b and 4.3.b).

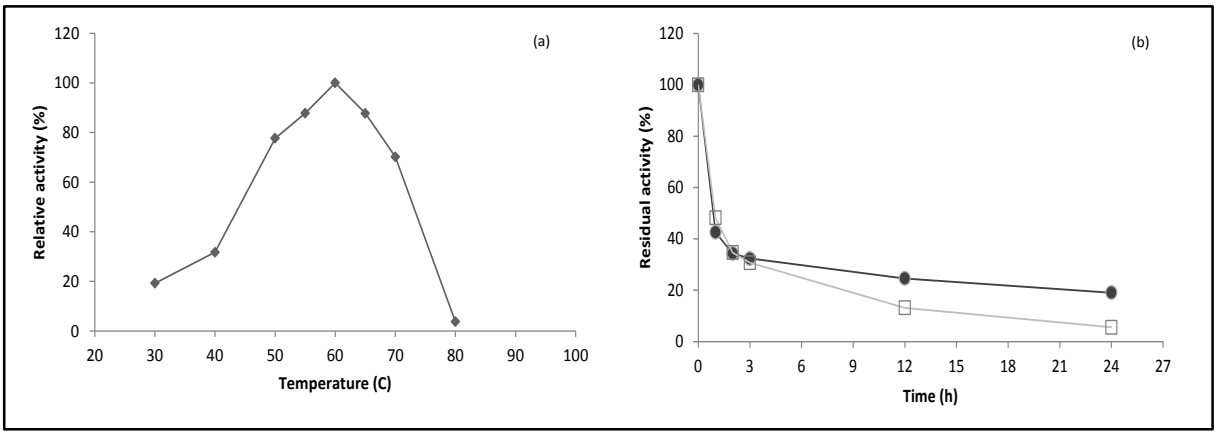

Figure 4.3. Temperature profile of the α-galactosidase AglC, (a) temperature optimum and (b) temperature stability at 37 (●) and 50ºC (□) over a 24 hour period.

The optimum temperature of Man2A was at 40ºC (Figure 4.4.a). The stability of Man2A was less at 50ºC compared to at 37ºC, with more than 90% reduction in residual activity within an hour (Figure 4.4.b).

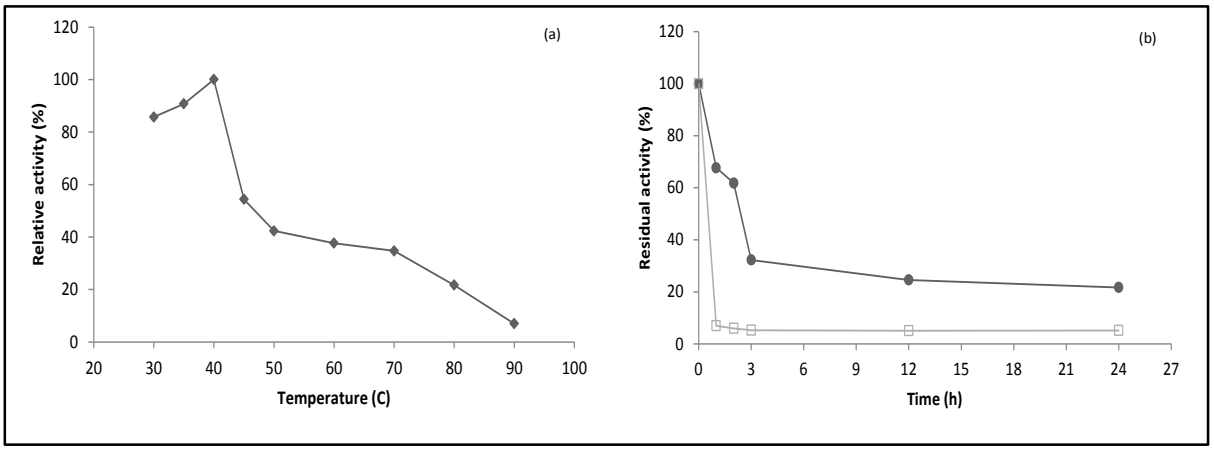

Figure 4.4. Temperature profile of the β-mannosidase Man2A, (a) temperature optima and (b) temperature stability at 37 (●) and 50ºC (□) over a 24 hour period.

The optimum temperature for Man5A was at 70ºC (Figure 4.5.a). The enzyme was also fairly stable at both 37 and 50ºC, with retention of about half its activity after 24 hours at both incubation temperatures (Figure 4.5.b).

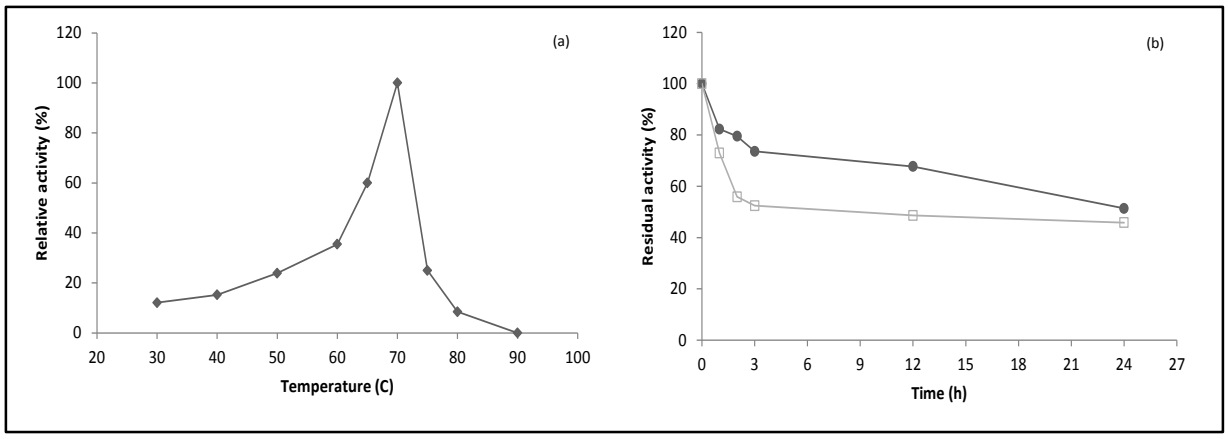

Figure 4.5. Temperature profile of the β-mannosidase Man5A, (a) temperature optima and (b) temperature stability at 37 (●) and 50°C (□) over a 24 hour period.

The Man26A temperature profile in Figure 4.6 showed an increase in activity as the temperature increased from 30 to 80°C. There was a sudden decline of activity after the temperature optimum (80°C). Man26A retained more than 50% of its residual activity at all the temperatures (37 and 50°C) assessed for stability up to 24 hours (Figure 4.6.b).

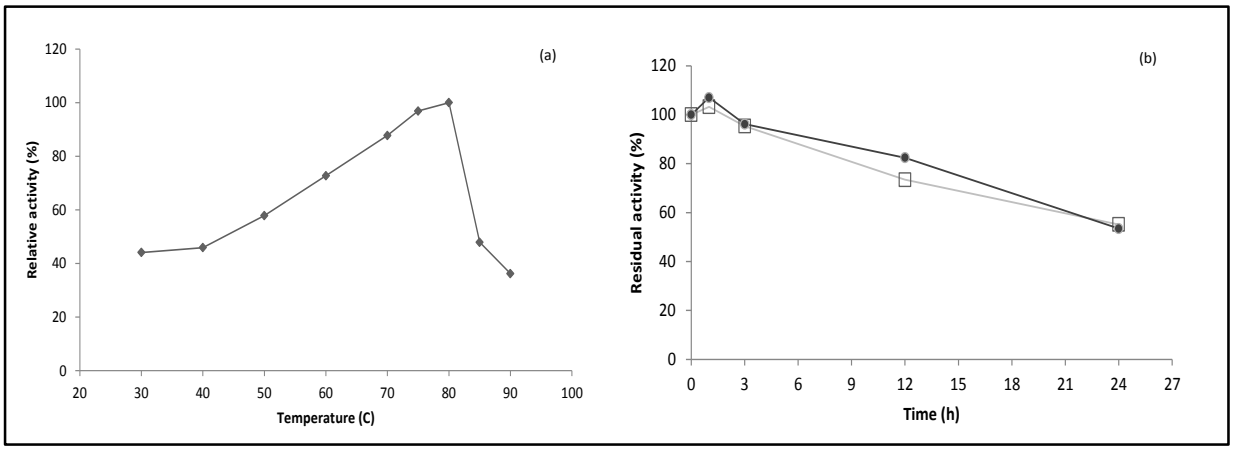

Figure 4.6. Temperature profile of the β-mannanase Man26A, (a) temperature optima and (b) temperature stability at 37 (●) and 50°C (□) over a 24 hour period.

The mannanase, ManA displayed a temperature optimum at 40°C (Figure 4.7.a). The enzyme was very unstable at 50°C (nearly 90% loss of activity within an hour) compared to incubation conducted at 37°C. After 12 hours of incubation, the enzyme had lost all its activity at both temperatures used for the thermal study.

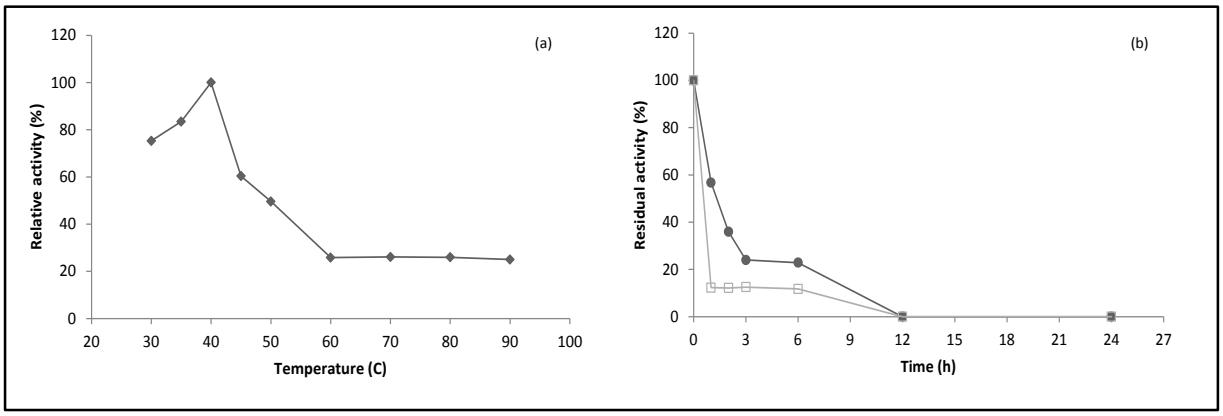

Figure 4.7. Temperature profile of the β-mannanase ManA, (a) temperature optima and (b) temperature stability at 37 (●) and 50°C (□) over a 24 hour period.

4.4.4. pH optimum determination

The pH optimum profiles of the mannan-degrading (mannanolytic) enzymes were determined using buffers in a pH range from 4 to 8. The range selected was based on the pH optima and ranges used in studies reported in literature for all three mannan-degrading enzyme classes. The buffers that were used to assess the pH optima of the mannanolytic enzymes were: 50 mM sodium citrate (pH 4.0 – 6.0) and 50 mM potassium phosphate (pH 6.0 - 8.0). The reactions were conducted at 37°C using the standard activity assays described in Section 4.3.3 (see Figure 4.8).

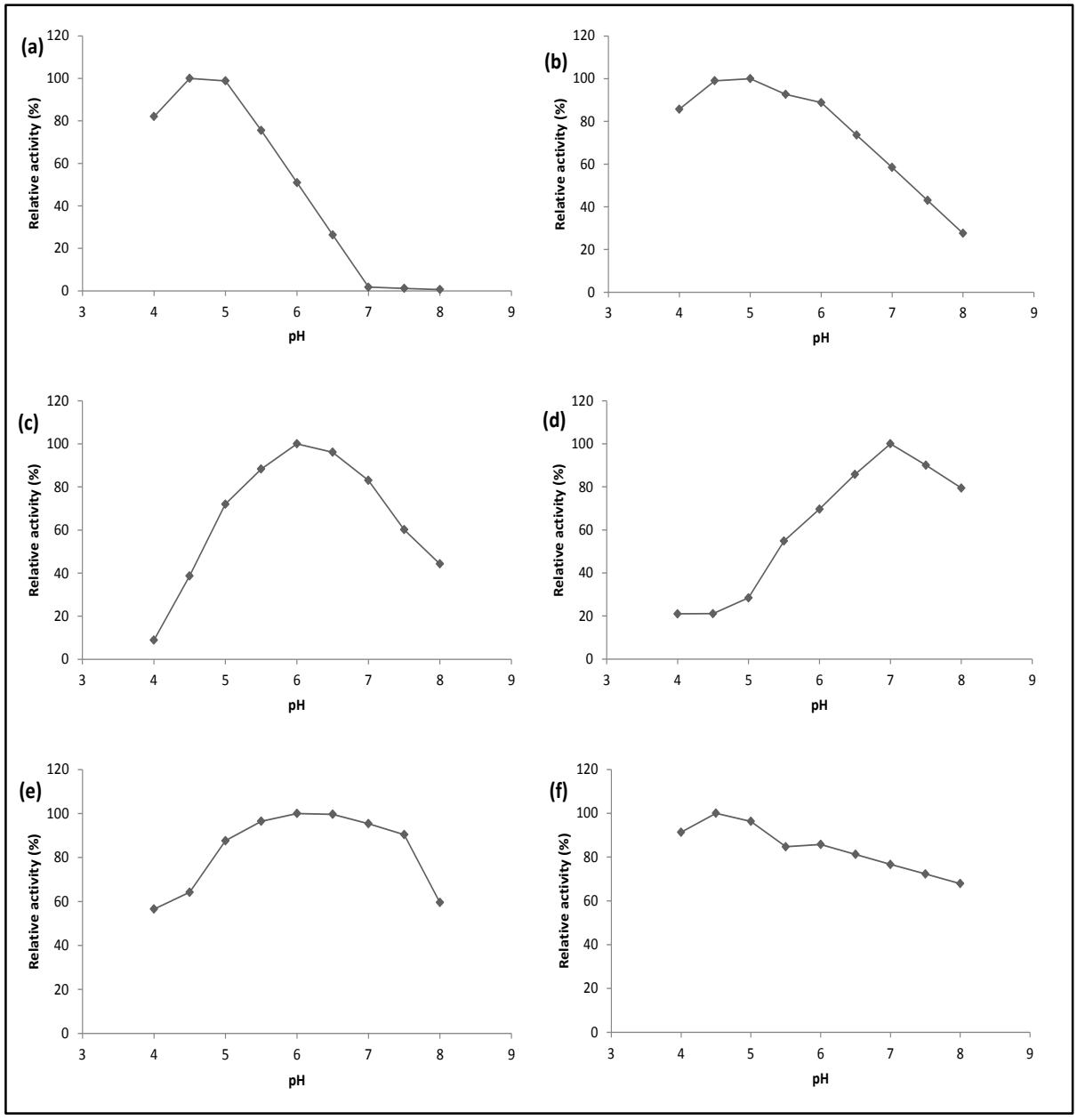

Figure 4.8. The pH optima profiles of the mannanolytic enzymes; (a) Aga27A, (b) AglC, (c) Man2A, (d) Man5A, (e) ManA and (f) Man26A on their respective defined substrates from pH 4.0 to 8.0 using 50 mM sodium citrate (pH 4.0-6.0) and 50 mM sodium phosphate (pH 6.0-8.0) buffers.

Aga27A, AglC and Man26A exhibited their pH optima around pH 4.5 to 5.0. This pH range is similar to the pH range reported in literature for most glycoside hydrolases. The two β-mannosidases, Man2A (pH 6.0) and Man5A (pH 7.0) exhibited higher pH optima compared to the other mannanolytic enzymes. The β-mannanase, ManA, had a very broad pH optimum ranging from 5.5 to 7.

4.4.5. Kinetic parameters of the mannanolytic enzymes

From Lineweaver-Burk plots, the K_M and V_{max} values of the mannanolytic enzymes were calculated (Table 4.3). From these two parameters and molecular weight of the enzymes, k_{cat} and k_{cat}/K_M were calculated (Table 4.3). For Aga27A and AglC, the K_M and V_{max} values were estimated towards *p*NPG. For Man2A and Man5A, the kinetic parameters estimated towards *p*NPM. For ManA and Man26A, the K_M (expressed in mg/mL because these are polymeric substrates) and V_{max} values were estimated using guar gum and locust bean gum, respectively.

Table 4.3. Kinetic parameters of the different mannan-degrading enzymes.

Enzyme	Substrate	V_{max} (µmol/min)	K_M (mM)	k_{cat} (min^{-1})	k_{cat}/K_M (min^{-1} mM^{-1})
Aga27A	*p*NPG	131.5	1.472	6588	4476
AglC	*p*NPG	2132	1.558	1.814×10^5	1.164×10^5
Man2A	*p*NPM	0.1017	0.520	8.268	15.90
Man5A	*p*NPM	0.1087	6.893	5.979	0.867

Enzyme	Substrate	V_{max} (µmol/min)	K_M (mg/mL)	k_{cat} (min^{-1})	k_{cat}/K_M ($min^{-1}mg^{-1}$ mL)
Man26A	Guar gum	577.1	6.19	3.278×10^4	5.295×10^3
	Locust bean gum	835.4	3.08	3.910×10^4	1.269×10^4
ManA	Guar gum	12.24	1	575.3	575.3
	Locust bean gum	20.79	0.526	977.1	1858

4.5. Discussion

4.5.1. SDS-PAGE and *in silico* characterization of mannanolytic enzymes

The α-galactosidase Aga27A (GH 27) has a 411-amino-acid polypeptide with a deduced molecular weight of 45 kDa and an isoelectric point of 3.7-4.5, based on its amino acid sequence, while on SDS-PAGE the molecular mass is 50.1 kDa. AglC (GH 36) has a 747-amino-acid polypeptide with a deduced molecular weight of 81.8 kDa and an isoelectric point of 4.9, based on its amino acid sequence (Table 3.1). Based on SDS-PAGE analysis, AglC has a molecular mass of 85.1 kDa. The molecular mass of the guar seed α-galactosidase (Aga27A)

was similar to that of other family 27 α-galactosidases, such as rice α-Gal (40-41 kDa) (Kim et al., 2002), *M. thermophilia* aGal1 (48.6 kDa and pI 4.6) (Dotsenko et al., 2012) and the *P. simplicissium* AGLI (417 amino acids sequence length, 46 kDa) (Luonteri et al., 1998). For AglC, these results were similar to those of other family 36 α-galactosidases, MelA (738-amino-acid polypeptide with a deduced molecular weight of 84 kDa and an isoelectric point of 5.12) and Aga-F75 (with a deduced molecular weight of 82 kDa) (Cao et al., 2008; Silvestroni et al., 2002). The molecular weights of the family 27 Aga27A and family 36 AglC were in accordance with a report by Ademark et al. (2001b) which stated that GH family 36 α-galactosidases are usually large and possess a tri-or tetra-meric structure, while GH family 27 α-galactosidases are smaller and some of them monomeric.

The deduced sequence length and molecular mass of the glycoside hydrolase family 2 β-mannosidase, Man2A were 864 amino acids and 99.5 kDa, respectively. The actual molecular mass of the enzyme as determined by SDS-PAGE was 81.3 kDa. The molecular mass of Man2A was similar to that of other family 2 β-mannosidases; *T. maritima* Man2 which was about 90-95 kDa (Parker et al., 2001), *M. thermophile* bMann9 (97 kDa) (Dotsenko et al., 2012) and an *A. niger* β-mannosidase (130 kDa) (Fliedrová et al., 2012). The family 5 β-mannosidase, Man5A, had a sequence length of 456 amino acids and a deduced molecular weight of 51.6 kDa. On SDS-PAGE, Man5A, exhibited a molecular weight of 55 kDa. No reports were available in literature to compare the physical characteristics of the family 5 β-mannosidase, Man5A to.

According to Tamaru and Doi (2000), the *C. cellulovorans* mannanase, ManA, has a molecular mass of approximately 45 kDa. Using the sequence data obtained from Uniprot, ManA has a sequence length of approximately 425 amino acids and a molecular mass of 47.1 kDa with a pI around 5.6. The estimated molecular mass of the enzyme was in agreement with that estimated by SDS-PAGE (47 kDa). A sequence of a putative *A. niger* β-mannanase, Man26A (Uniprot ID A2R6F5) was found in the CAZy database, this sequence possessed 335 amino acids with a deduced mass of 37.7 kDa, but this mass did not coincide with the protein mass estimated by SDS-PAGE (46.8 kDa) and that reported by a previous study (45 kDa) (Zhao et al., 2011). This difference between the polypeptide and the mature expressed protein is probably due to glycosylation at multiple sites. Amino acid sequence analysis of the polypeptide was conducted using the protein sequence analysis tool NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/) and four putative N-glycosylation sites were

detected at the following positions: 21Asn-Gln-Thr-Lys24, 142Asn-Val-Ser-Glu145, 152Asn-Gly-Thr-Gln155 and 323Asn-Asp-Thr-Arg326. According to Zhang et al. (2011), glycosylation in β-mannanases has been identified before, such as in an *A. sulphureus* β-mannanase expressed in *P. pastoris*.

4.5.2. Substrate specificities of the mannanolytic enzymes

The α-galactosidase, β-mannanase and β-mannosidase activities of the mannanolytic enzymes were determined using appropriate mannan-based substrates. The specific activities of each of the mannanolytic enzymes on their respective substrates are shown in Table 3.2.

The substrate specificities of *A. niger* and guar seed α-galactosidases were investigated by using galactose-containing oligosaccharides (6^3, 6^4-α-galactosyl-mannopentaose and 6^1-α-galactosyl-mannotriose), polysaccharides (guar gum and locust bean gum) and *p*NPG. AglC (1168 U/mg) exhibited higher activity towards *p*NPG compared to Aga27A (80.29 U/mg). As previously described, α-galactosidases can be classified into two GH families depending on their specificity to galactose-containing substrates. The family 27 guar seed enzyme was more active on both polymeric galactomannan substrates (11 U/mg for guar gum and 3.68 U/mg for locust bean gum) compared to the family 36 *A. niger* α-galactosidase (see Table 3.2). Other family 27 α-galactosidases; rice αGal (Kim et al., 2002) and *C. josui* Aga27A (Jindou et al., 2001) have been reported to exhibit high activity on galactomannans relative to other enzymes.

The *A. niger* AglC exhibited comparable activity on locust bean gum (1.01 U/mg) and guar gum (0.97 U/mg), and this same effect has been reported for other microbial α-galactosidases (Mi et al., 2002; Puchart et al., 2000). On α-6^1-galactosyl-mannotriose and α-6^4,6^3-di-galactosyl-mannopentaose, Aga27A exhibited activity of 4.50 U/mg and 13.7 U/mg, respectively. AglC exhibited 1.61 U/mg for α-6^1-galactosyl-mannotriose and 0.49 U/mg for α-6^4,6^3-di-galactosyl-mannopentaose. It has been previously reported that family 36 α-galactosidases prefer oligomers with terminal galactose substituents, while family 27 α-galactosidases exhibit high activity on deconstructing internally linked galactose substituents on oligomers (Ademark et al., 2001a; Ademark et al., 2001b). Kaneko et al. (1990) have reported an *A. niger 4-15* that was able to cleave off galactose substituents that are internally attached to manno-oligosaccharides, but did not cleave off the terminal galactoses attached to the non-reducing-end.

Ademark et al. (2001b) have reported GH36 galactosidases to be usually large and possess tri- or tetra-meric structures, while GH27 ones are usually smaller and monomeric. We believe the smaller size of the GH27 galactosidases allows the enzyme to be able to deconstruct block-wise galactose substituents even from polymers, while the large multimeric structures of the GH36 galactosidases may be sterically hindered when required to deconstruct inner and block-wise galactose residues from polymers. Due to its significant ability to hydrolyze both polymeric mannans and oligosaccharides, the guar seed family 27 Aga27A appears more promising for biomass degradation applications compared to the *A. niger* family 36 AglC.

The *Bacteroides* Man2A exhibited minimal activity towards the doubly substituted substrate 6^3, 6^4-α-galactosyl-mannopentaose (0.082 U/mg) and 0.015 U/mg towards 6^1-α-galactosyl-mannotriose; these findings are similar to those reported by Tailford et al. (2009). An earlier report by Ademark et al. (1999) also demonstrated that an *A. niger* mannosidase was active to a lesser extent towards substituted manno-oligosaccharides compared to non-substituted ones. Interestingly, the *Bacteroides* Man2A was active on locust bean gum galactomannan (0.41 U/mg) and released about 1% mannose after an hour of incubation. The ability of Man2A to hydrolyze galactomannan substrates is interesting, but a more detailed kinetic study is needed to fully understand the mode of action of this enzyme.

Analysis of substrate specificity of Man5A (Table 4.2) indicates that among the considered *p*NP, oligomeric and polymeric substrates (guar gum and locust bean gum (0.71 U/mg)), this enzyme prefers *p*NPM as its substrate. Man5A prefers 6^1-α-galactosyl-mannotriose (0.32 U/mg) over 6^3, 6^4-α-galactosyl-mannopentaose (0.041 U/mg). A similar study by Dias et al. (2004) showed that hydrolysis of 6^1-α-galactosyl-mannotriose by Man5A released only a single mannose per substrate molecule, and the enzyme displayed no activity against 6^3,6^4-α-digalactosyl- mannopentaose. This is a clear indication that Man5A can accommodate a galactose side chain in the +2 subsite (Dias et al., 2004). The enzyme did not exhibit any significant activity towards polymeric galactose containing substrates. These findings are similar to those reported by Dotsenko et al. (2012) with their *M. thermophile* bMann9. In this study, the *C. mixtus* Man5A was more active on *p*NPM compared to the *B. thetaiotaomicron* Man2A. These findings were contrary to those reported by Tailford et al. (2009).

The family 5 mannanase, ManA (9.61 U/mg for locust bean gum and 4.63 U/mg for guar gum) had significantly lower mannanase activity compared to its family 26 homolog, Man26A (217.18 U/mg for guar gum and 436.41 U/mg for locust bean gum) for the hydrolysis of both

galactomannan substrates. The recorded specific activity data of ManA on these two galactomannan substrates correlated well with the 12.9 U/mg on locust bean gum and 3 U/mg with guar gum published by Tamaru and Doi (2000). The specific activity of ManA on both locust bean gum and guar gum hydrolysis was comparable to that of another documented *C. cellulovorans* mannanase (12.2 U/mg for locust bean gum and 2.2 U/mg for guar gum), ManB (Jeon et al., 2011). The specific activity of Man26A was higher than that reported for another *A. niger* (231.14 U/mg with locust bean gum) β-mannanase (Ademark et al., 1998), but lower than that reported for *B. stearothermophilus* β-mannanase of 455.60 U/mg (Dhawan and Kaur, 2007).

Both β-mannanases, ManA from *C. cellulovorans* and Man26A from *A. niger* exhibited high activity on locust bean gum (100% relative activity), but considerably lower activity on guar gum (approximately 50% relative activity). These findings support the hypothesis that the enzyme activity of a mannanase is limited by the extent and pattern of D-galactosyl substituents on the mannan backbone. These findings were similar to those reported for other mannanases, such as that of the WL-mannanase (Yoon et al., 2008) and Man26A (Zhao et al., 2011) which both displayed high activity towards the low galactose decorated locust bean gum and reduced activity on the higher galactose substituted guar gum. Both mannanases exhibited no activity towards *p*NPG and *p*NPM; the lack of activity in this case suggests that the enzymes possess no galactosidase activity and are also unable to cleave terminal mannosides, thus the enzymes are classical endo-β-1,4-mannanases. Both ManA and Man26A exhibited no activity towards the decorated oligosaccharides: α-6^1-galactosyl-mannotriose and α-6^4,6^3-di-galactosyl-mannopentaose. The lack of activity towards α-6^1-galactosyl-mannotriose could be due to the fact that this substrate is too short to allow the mannanase to bind, since mannanases have been reported to require a minimum of four subsites on the substrate for backbone cleavage to occur (Couturier et al., 2013; Tailford et al., 2009). The lack of activity towards α-6^4,6^3-di-galactosyl-mannopentaose may be due to D-galactosyl substituents binding to substites -2 and or +1, such action by galactose residues has been documented to block mannanase activity (Dhawan and Kaur, 2007).

4.5.3. Temperature profiles of the mannanolytic enzymes

The temperature optimum of the guar seed Aga27A was lower than that of another family 27 galactosidase, aGal1 (60^{o}C) which was reported by Dotsenko et al. (2012). The enzyme was found to display maximum catalytic activity at 50^{o}C and this optimum was similar to that of another GH27 α-galactosidase, *Cm*Aga27A reported by Centeno et al. (2006). The family 36

galactosidase from *A. niger* (AglC) displayed a temperature optimum of 60^{o}C. This optimum temperature was similar to that of other fungal galactosidases from *A. corymbifera* expressed in *E. coli* (Baik et al., 2000) and from *Gibberella* sp. F75 (Cao et al., 2009).

A temperature optimum of 40^{o}C was exhibited by the *Bacteroides* Man2A on *p*NPM. The *C. mixtus* mannosidase displayed a temperature optimum of 70^{o}C and this optimum was comparable to that of an *A. niger* mannosidase (65^{o}C) (Fliedrová et al., 2012) and that of another mannosidase purified from *A. niger* (70^{o}C) by Ademark et al. (1999). Man5A was more thermally stable at elevated temperatures (50^{o}C in this study) compared to recombinant and wild type versions of the *A. niger* mannosidase which lost nearly 100% and 85% of their activities after incubation for 80 minutes at 65^{o}C, respectively (Fliedrová et al., 2012).

The mannanase temperature activity and stability of the two mannanases was tested using the DNS method and the substrate locust bean gum (0.5% w/v). The *A. niger* β-mannanase, Man26A displayed maximal activity at 80^{o}C, similar temperature optima have been reported in literature for other thermostable β-mannanases; from *T. neapolitana* with maximal activity at 90^{o}C and *C. saccharolyticum* with a temperature optimum of 80^{o}C (Duffaud et al., 1997). The *A. niger* Man26A was also very thermally stable, with retention of approximately 50% residual activity after incubation at either 37 or 50^{o}C for 24 hours. The GH5, *C. cellulovorans* mannanase, ManA exhibited maximal activity at 40^{o}C and exhibited thermal stability for up to 12 hours at 37^{o}C. The optimum temperature of 40°C correlated well with the 45°C published by Tamaru and Doi (2000), while the temperature stability of less than 12 hours reported in this study also correlated well with the 11 hours reported by Dredge (2011).

The most thermally stable mannanolytic enzymes appeared to exhibit higher temperature optima values compared to the least thermally stable mannanolytic enzymes, with only Aga27A being the exception to this trend. For ManA and Man26A, the thermal stability of each enzyme appeared to display an association with the specific activity of the individual mannanases, with the highly stable Man26A being the most active among the mannanases. For the galactosidases, Aga27A and AglC, and the mannosidases, Man2A and Man5A, thermal stability did not show any association with optimal activity. Future studies where the enzymes' thermal stability is assessed in the presence of substrates are needed to further understand the underlying differential activities of mannanolytic enzymes from the same EC affiliation, but different GH families. We propose that thermal stability and temperature optima properties of

these enzymes are not associated with the respective GH family affiliation, but are ascribed to the thermophilic origin of the organism which each enzyme is isolated from.

4.5.4. pH profiles of the mannanolytic enzymes

The pH optimum of Aga27A (pH 4.5-5) fell within the range of aGal1 (pH 3.5-6.5), another family 27 galactosidase studied by Dotsenko et al. (2012). The family 36 galactosidase, AglC exhibited a pH optimum of 5.0, this pH optimum was comparable to those reported on *A. corymbifera* galactosidase and *Gibberella* sp. Aga-F75, other GH36 galactosidases (Baik et al., 2000; Cao et al., 2008).

The *Bacteroides* Man2A possessed a pH optimum of 6.0 and was found to be similar to a previous report by Tailford et al. (2009) of 5.6 and also similar to that of a recombinant *A. niger* mannosidase (pH 5.5) reported by Fliedrová et al. (2012). The *C. mixtus* mannosidase, Man5A possessed a pH optimum (7.0) similar to that of *Streptomyces sp. S27* ManAS27 (Shi et al., 2011).

The *A. niger* mannanase (Man26A) exhibited a pH at 4.5. Data on the physico-chemical properties of Man26A has not been published previously. The family 5 *C. cellulovorans* mannanase (ManA) exhibited a broad pH optimum from pH 5 to 7, with maximal activity recorded at pH 6.0. Tamaru and Doi (2000) however, reported a higher pH optimum of 7.0, while Dredge (2011) recorded a pH optimum of 5.5.

4.5.5. Kinetic parameters of the mannanolytic enzymes

The K_M and V_{max} for the family 27, guar seed galactosidase, Aga27A on *p*NPG were estimated to be 1.47 mM and 131.5 μmol/min, respectively. Similar findings to these were reported for another GH27 galactosidase (0.81 mM and 92.9 μmol/min) from *C. josui* on *p*NPG (Jindou et al., 2001). From the Lineweaver-Burk plot, the K_M and V_{max} values of the GH36 galactosidase, AglC were estimated to be 1.558 mM and 2132 μmol/min, respectively. This reported K_M value was lower than that reported for another GH36 galactosidase from *C. stercorarium*, Aga36A (4.3 mM) (Suryani et al., 2003), but higher than that of a *Penicillium janczewskii zaleski* (1 mM) agl1 (Zhang et al., 2011) for the same substrate. The V_{max} value of AglC was also higher than the 430 μmol/min reported for Aga36A by Suryani et al. (2003) and 227.3 μmol/min reported for agl1 by Zhang et al. (2011). The V_{max} of AglC was lower than that documented for a *L. fermentum* (2838 μmol/min) galactosidase (Garro et al., 1995). The kinetic studies indicated that the two different GH family galactosidases, Aga27A and AglC, have similar

affinities for *p*NPG (comparable K_M values), but that AglC had a higher rate of hydrolysis (higher V_{max}) for *p*NPG compared to Aga27A.

The estimated values for V_{max} and K_M for the *Bacteroids* Man2A were 0.1017 μmol/min and 0.520 mM, respectively. An earlier investigation on *Bacteroids* mannosidase reported a K_M value of 0.19 mM and a k_{cat} of 7689 min^{-1} (Tailford et al., 2009). The Bacteroids enzyme characterized in this study was found to have a lower catalytic efficiency compared to that characterized by Tailford et al. (2009). The differences in the kinetic parameters could be due to differences in the pH, buffer system, enzyme and substrate loadings used in the two studies. The K_M value for this study's Man2A was similar to K_M values of other mannosidases characterized in literature, such as the recombinant *A. niger* β-mannosidase with a K_M of 0.48 mM (Fliedrová et al., 2012). The K_M and V_{max} values for the *C. mixtus* Man5A were 0.1087 mol/min and 6.893 mM, respectively. An earlier report on the *C. mixtus* mannosidase mentioned a different K_M value of 1.6 mM (Dias et al., 2004). The catalytic efficiency (k_{cat}/K_M) value reported in our current study (0.867 min^{-1} mM^{-1}) was comparable to that of 1.25 min^{-1} mM^{-1} reported by Dias et al. (2004) on *p*NPM. Other significantly higher K_M values for mannosidases have been reported before i.e. 6.5 mM was determined a β-mannosidase from *A. fulica* (Zahura et al., 2012).

The estimated values for V_{max} and K_M for *C. cellulovorans* ManA were 20.79 μmol/min and 0.526 mg/mL for locust bean gum, and 12.24 μmol/min and 1 mg/mL for guar gum, respectively. The K_M of ManA for locust bean gum was similar to that reported for a *T. neopolitana* 5068 β-mannanase (0.55 mg/mL) (Duffaud et al., 1999). The K_M and V_{max} parameters for Man26A with guar gum and locust bean gum were 6.19 mg/mL and 3.08 mg/mL, and 577.1 μmol/min and 835.4 μmol/min, respectively. The K_M and V_{max} parameters for Man26A on locust bean gum were comparable to those documented for an *A. fumigatus* Man I (3.07 mg/ml and 1935 μmol/min) (Dhawan and Kaur, 2007). The k_{cat}/K_M of the *A. niger* Man26A (3.910 x10^4 $min^{-1}mg^{-1}mL$) was considerably higher than that reported for a *C. fimi* Man26A (1150 $min^{-1}mg^{-1}mL$) for locust bean gum hydrolysis (Stoll et al., 1999).

The kinetic studies indicated that ManA had a higher affinity for galactomannan substrates (low K_M) compared to Man26A, while Man26A had a higher rate of hydrolysis (high V_{max}) for galactomannan substrates compared to ManA. The higher V_{max} observed on locust bean gum hydrolysis compared to guar gum is presumed to be related to the steric hindrance present in the more highly and block-wise galactose substituted mannan backbone associated with guar.

These findings for the two mannanases were comparable to those of a *C. saccharolyticum* β-mannanase, which displayed a V_{max} of 63.2 U/mg with locust bean gum but only 2.5 U/mg with guar gum (Bicho et al., 1991). Based on Table 4.3, Man26A appears to have a higher k_{cat} value compared to ManA for galactomannan substrates. This finding is similar to that reported for two mannanases, *Cj*Man26A (2.8 $x10^5$ min^{-1}) and *Cj*Man5A (1.4 $x10^5$ min^{-1}) (Tailford et al., 2009).

In summary, the results obtained from the biochemical characterisation of the mannanolytic enzymes indicated the following and are illustrated in Table 4.4:

Table 4.4. Comparison of the properties of different GH family mannanolytic enzymes used in this study

Origin	Enzyme name	pH_{opt}[a]	T_{opt}[b] (ºC)	Thermal stability$_\tau$ [c]	Family (GH)
α-galactosidases					
C. tetragonolobus	Aga27A	4.5-5.0	50	>24 h, 37ºC 30 min, 50ºC	27
A. niger	AglC	4.5-5.0	60	60 min, 37ºC 45 min, 50ºC	36
β-mannanases					
C. cellulovorans	ManA	5.5-7.0	40	90 min, 37ºC 30 min, 50ºC	5
A. niger	Man26A	4.0-5.0	80	>24 h, 37ºC and 50ºC	26
β-mannosidases					
B. thetaiotaomicron	Man2A	5.5	40	150 min, 37ºC <30 min, 50ºC	2
C. mixtus	Man5A	7	70	>24 h, 37ºC and 50ºC	5

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

4.6. Conclusions

The different family glycoside hydrolases were successfully characterized with respect to substrate specificities and kinetic parameters. The galactosidases, Aga27A and AglC, showed different substrate specificities. The GH27 galactosidase, Aga27A, preferred inner block-wise substituted galactose residues, while the GH36 AglC exhibited affinity for terminal galactoses attached to the non-reducing ends of substrates. Aga27A was the most active between the

galactosidases on polymeric galactomannans. Man2A displayed high preference for *p*NPM compared to the galactose-containing oligosaccharides (galactosyl-mannotriose and di-galactosyl-mannopentaose). On the other hand, Man5A exhibited substrate preference for polymeric galactose containing substrates > galactose-containing oligomers > *p*NPM. These findings are similar to those previously reported for Man5A which indicate low activity against decorated soluble polysaccharides or insoluble mannans (Dias et al., 2004). The mannanases, Man26A and ManA showed similar substrate specificities. Both mannanases displayed high affinity for the low galactose substituted locust bean gum and reduced preference for the highly galactose decorated guar gum. Man26A was the most active between the two mannanases on polymeric galactomannans. Both mannanases did not exhibit activity on the galactose decorated oligosaccharides and *p*-nitrophenyl based substrates used in this study.

In most instances, similar conclusions were drawn from our physicochemical data compared to that previously published. Aga27A was maximal at pH 4.5-5.0 and 50°C. AglC exhibited a pH optimum at 5.0 and a temperature optimum at 60^{o}C. These optimums were similar to those observed on *A. corymbifera* and Aga-F75 α-galactosidases, other family 36 galactosidase (Baik et al., 2000; Cao et al., 2008). The optimal physicochemical properties of the ManA from our study were pH 6.0 and 40°C; however, these were slightly different to those reported by Tamaru and Doi (2000) of pH 7.0 and 45°C and those reported by Dredge (2011) of pH 5.5. Data on the mannanase, Man26A confirmed the optimal activity at pH 4.5 and 80°C. The temperature stability studies indicated a complete loss of activity in ManA after 12 hours. However, Man26A retained about 50% activity and was still active after 24 hours. It is worth noting that these studies investigated enzyme stability in the absence of substrate and thus the stability of these enzymes could be significantly higher in the presence of substrate.

The characterization of each mannanolytic enzyme led to an improved understanding of the optimal conditions of its activity, kinetic parameters and substrate specificities. This data facilitated a better understanding and interpretation of the synergy and interactions between these enzymes on model (defined) galactomannan substrates in the next chapter.

Chapter 5: Synergy between mannanolytic enzymes from various GH families on galactomannan hydrolysis

5.1. Introduction

Mannan is a complex and diverse substrate and requires an array of enzymes to degrade it into its monomeric sugar residues. Mannan-degrading (mannanolytic) enzymes are enzymes that form part of a large arsenal of glycoside hydrolases produced by wood-degrading fungi and bacteria (Van Zyl et al., 2010). Glycoside hydrolases (GHs) include cellulose, xylan, mannan, pectin, chitin, chitosan and starch degrading enzymes. These enzymes are classified into glycoside hydrolase families based on amino acid sequence, structural and mechanistic similarity (Van Zyl et al., 2010). A list of the glycoside hydrolase families is updated continuously and can be found on the Carbohydrate-Active Enzyme database (CAZy) (http://www.cazy.org).

The 1,4-β-D-mannan mannohydrolase (called β-mannanase, EC 3.2.1.78) is an endo-enzyme which is responsible for the random cleavage of β-1,4-linked internal linkages of the mannan backbone to produce new chain ends (Moreira and Filho, 2008; Wang et al., 2010). The β-mannanases fall into GH families 5, 26 and 113 (www.cazy.org). Both bacterial and eukaryotic mannanases are found in GH family 5 and those in GH family 26 are of bacterial origin, with the exception of a few anaerobic fungi (Dhawan and Kaur, 2007). It has been reported that the main products obtained during the hydrolysis of mannan by β-mannanases are mannobiose and mannotriose. Additional traces of higher oligosaccharides are also be produced by β-mannanases from *Aspergillus tamarii*, *Trichoderma reesei* and *Aspergillus niger* (Dhawan and Kaur, 2007; Gübitz et al., 1996).

The second enzyme, 1,4-β-D-mannopyranosidase (also called β-mannosidase, EC 3.2.1.25), is an exo-enzyme which cleaves β-1,4-linked mannosides, releasing mannose from the non-reducing end of mannans and mannan oligosaccharides (Moreira and Filho, 2008; Van Zyl et al., 2010; Wang et al., 2010). The β-mannosidases fall into GH family 1, GH family 2 and GH family 5 (Shallom and Shoham, 2003). Studies have shown that β-mannosidases from GH families 2 and 5 possess two aglycone sugar binding subsites (+1 and +2) and a glycone sugar binding subsite (-1) (Dias et al., 2004; Tailford et al., 2007). Comparison of the properties of the various reported GH family mannanases is difficult due to the lack of genetic data and data on the activities of the enzymes towards different mannan substrates.

The third enzyme is 1,6-α-D-galactoside galactohydrolase (called α-galactosidase, EC 3.2.1.22), a de-branching enzyme which catalyzes the hydrolysis of α-1,6-linked D-galactopyranosyl side chains of galactomannan and galactoglucomannan. The α-galactosidases fall into the GH family 27 and GH family 36 (Van Zyl et al., 2010). According to Wang et al. (2010), α-galactosidases can be divided into two groups based on substrate specificity: one group has specificity for α-1,6-linked galactose units linked to inner mannose residues of galactomannans and galactoglucomannans, and the other has specificity for substrates that have a galactose residue linked to the non-reducing end. The GH family 27 α-galactosidases release galactose from intact galactomannan polymers, while GH family 36 α-galactosidases have their substrate specificity restricted to small oligosaccharides (Wang et al., 2010).

In the present study the various GH family mannanolytic enzymes characterized in Chapter 4 were assessed for their synergistic interactions and associations on galactomannan (guar gum and locust bean gum) hydrolysis.

5.2. Aims and Objectives

5.2.1. Aims

To conduct synergy studies on galactomannan substrates using Aga27A, AglC, Man2A and Man5A with Man26A and ManA.

To determine which GH family β-mannosidase and GH family α-galactosidase synergistically cooperates the best with the GH5 and 26 β-mannanases in galactomannan hydrolysis.

5.2.2. Objectives

- To conduct binary simultaneous synergy studies between mannanases and galactosidases, and between mannanases and mannosidases with guar gum and locust bean gum;
- To quantify the reducing sugar (DNS method) , galactose and mannose (using monosaccharide detection kits) release from the synergy studies;
- To establish synergistic associations between the glycoside hydrolases using the hydrolysis data from synergy studies.

5.3. Methods

5.3.1. Synergy studies

In the enzyme synergy studies, enzyme mixtures were used in various binary combinations: a β-mannanase and anα-galactosidase, and a β-mannanase and a β-mannosidase. The enzyme mixtures contained mannanolytic enzymes loaded at a total protein loading of 0.05 mg/g of galactomannan for the binary combinations with Man26A and 2.35 mg/g of galactomannan for the ManA containing binary combinations. The mannanase loadings used in the experiments were based on loadings that release at least 0.5 mg/mL (quantifiable concentration using the DNS assay, reached about 10% substrate hydrolysis) of reducing sugar per hour on locust bean gum hydrolysis. The experiments were carried out in triplicate with a 0.5% (w/v) galactomannan content in 50 mM sodium citrate buffer (pH 5.0) in a 400 µL total volume at 37^{o}C, mixing at 25 rpm for up to 1 h. The hydrolysis was then terminated by boiling for 5 minutes at 100^{o}C to inactivate the enzymes. Hydrolysis controls included substrate (without the enzyme) and enzyme controls (without substrate). The samples were stored at 4^{o}C for further analysis. Analysis of reducing sugar, galactose and mannose release was conducted according to the methods described in Section 4.3.3 of Chapter 4.

5.3.2. Data analysis

One way analysis of variance (ANOVA) was used to analyze the activity of the enzymes. Evaluation was conducted to elucidate significant increases exhibited by the enzyme combinations with respect to galactose or mannose and or reducing sugar release compared to that released by 100% protein enzyme loading. All pairwise comparison procedures were conducted using the Data analysis feature in Microsoft Excel.

5.4. Results

The synergistic associations between the enzymes were determined through the quantification of the reducing sugars released and galactose or mannose released during the degradation of galactomannan substrates (guar gum and locust bean gum).

5.4.1. Synergy on locust bean gum hydrolysis

Figure 5.1 demonstrates that homeosynergy was found between GH5 and GH26 mannanases (ManA and Man26A) with GH2 and GH5 mannosidases (Man2A and Man5A) on locust bean gum hydrolysis.

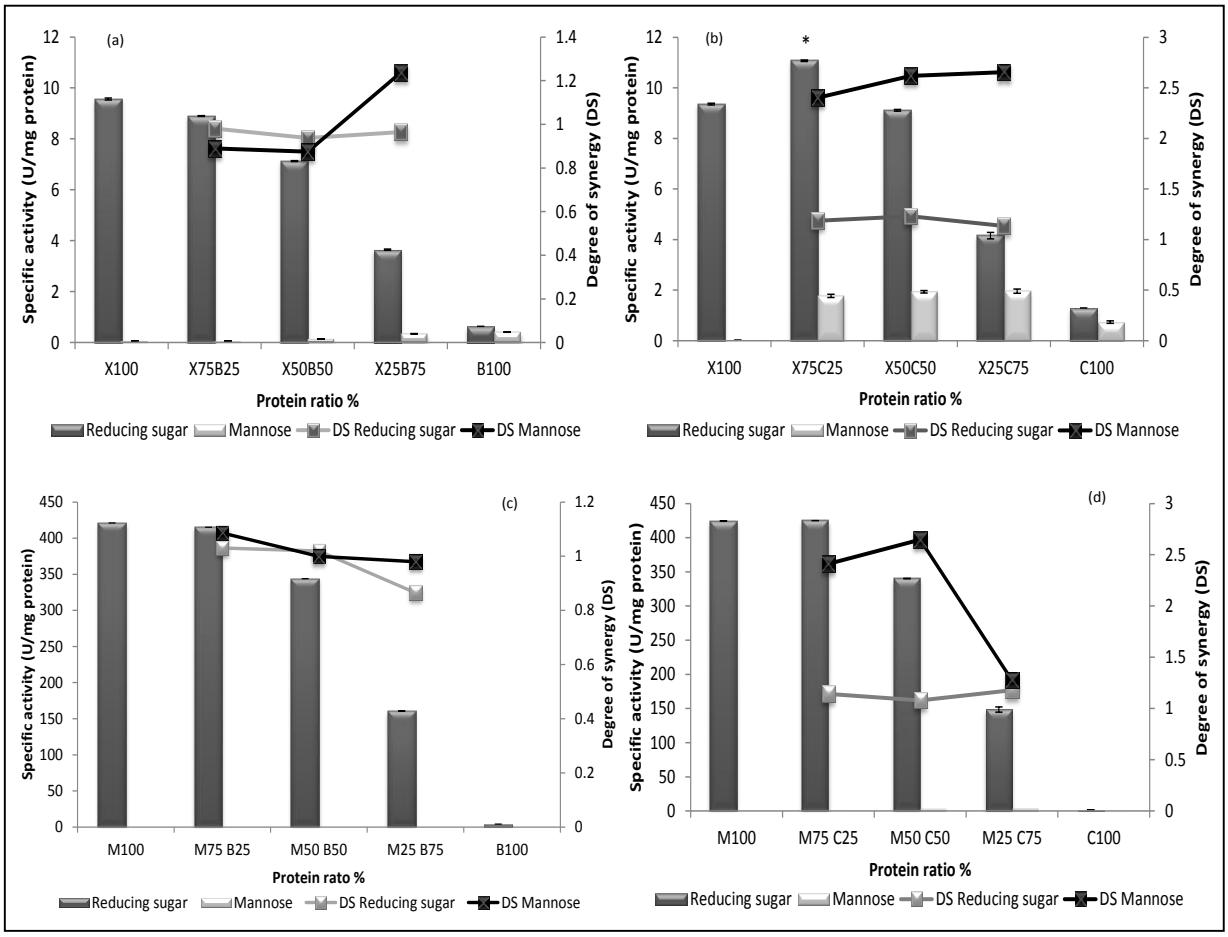

Figure 5.1. Mannose released, reducing sugar released and synergistic associations obtained on locust bean gum by the various combinations of enzymes, (5.1a) ManA (X) and Man2A (B), (5.1b) ManA (X) and Man5A (C), (5.1c) Man26A (M) and Man2A (B), and (5.1d) Man26A (M) and Man5A (C). ANOVA analysis for improvement of hydrolysis with respect to reducing sugar and galactose 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).

The maximum specific activity (9.57 U/mg protein) produced by the synergistic association of *C. cellulovorans* ManA (X) and *Bacteroides* Man2A (B) was observed when ManA was at 100% protein dosage (Fig. 5.1a). Among the combinations considered, the degree of synergy with respect to reducing sugar release appeared to be around 1; this implies that there was no cooperation between ManA and Man2A in reducing sugar liberation. With respect to mannose release, Man2A at 100% protein loading displayed the highest specific activity of 0.41 U/mg

protein. The highest degree of synergy (1.24) with respect to mannose release was observed at ManA 25% to Man2A 75% (0.35 U/mg protein) (Fig. 5.1a).

Fig. 5.1b illustrates that the protein ratio X75:C25 produced significantly ($p<0.05$) higher reducing sugar of 11.07 U/mg protein compared to that liberated by ManA alone (9.36 U/mg protein). Reducing sugar release appeared to increase with an increase in the ratio of ManA to Man5A (Fig. 5.1b). All the considered ManA to Man5A combinations liberated mannose that was higher than that liberated by ManA alone (0.74 U/mg protein). Mannose release appeared to increase with an increase in the ratio of Man5A to ManA (Fig. 5.1b).

With respect to both reducing sugar and mannose release, there was an apparent degree of synergy around 1 among most of the combinations between mannanase Man26A (M) and mannosidase Man2A (B) that were considered. In addition, all the combinations considered had lower reducing sugar release than that observed when Man26A (420.99 U/mg protein) was used alone, as well as lower mannose release than by ManA alone at 100% protein loading. The Man26A 75% to Man2A 25% combination liberated reducing sugar content (415.12 U/mg protein) similar to that liberated by Man26A alone at 100% protein dosage ($p>0.05$) (Fig. 5.1c).

All the combinations used between the mannanase Man26A (M) and *C. mixtus* mannosidase Man5A (C) had a degree of synergy greater than one, but none of them had a reducing sugar release significantly ($p>0.05$) greater than that observed when Man26A was used alone at the same protein dosage (424.41 U/mg protein). The Man26A and Man5A combination at the ratio 75 to 25% exhibited the greatest reducing sugar release (424.90 U/mg protein), equivalent to that released when Man26A was used alone (424.41 U/mg protein). The highest degree of synergy (1.18) with respect to reducing sugar release was observed with the combination of Man26A to Man5A at 25:75%.

Figure 5.2 demonstrates heterosynergy between GH5 and 26 mannanases (ManA and Man5A) with GH27 and 36 galactosidases (Aga27A and AglC) during locust bean gum hydrolysis.

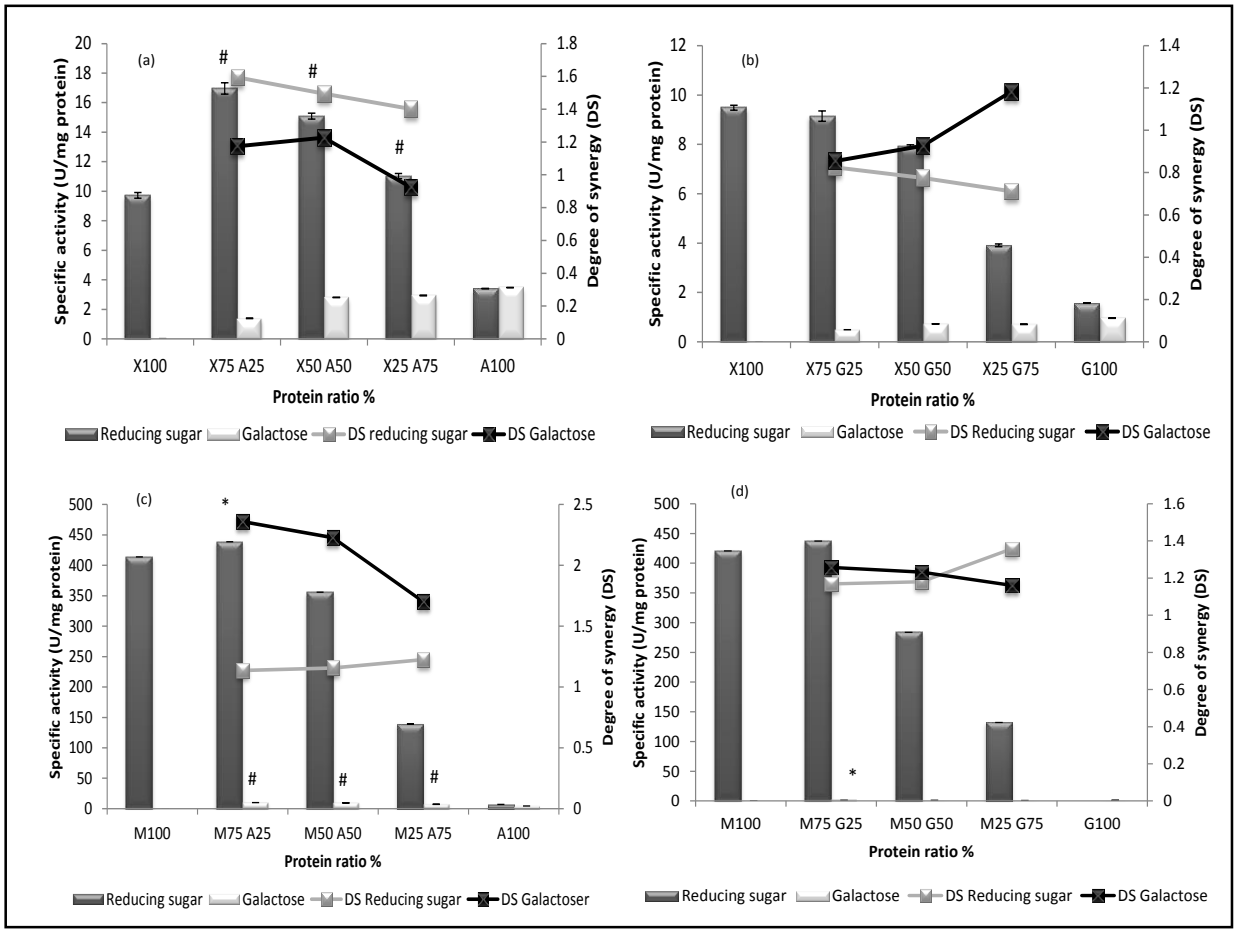

Figure 5.2. Galactose released, reducing sugar released and synergistic associations obtained on locust bean gum by the various combinations of enzymes, (5.2a) ManA (X) and Aga27A (A), (5.2b) ManA (X) and AglC (G), (5.2c) Man26A (M) and Aga27A (A) and (5.2d) Man26A (M) and AglC (G). ANOVA analysis for improvement of hydrolysis with respect to reducing sugar and galactose 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).

All *C. cellulovorans* ManA (X) and guar seed Aga27A (A) combinations showed a significant ($p<0.01$) increase with respect to reducing sugar release compared to when ManA or Aga27A were used alone at 100% protein mass dosage (Fig. 5.2a). The ManA and Aga27A combination, in a protein ratio of 75% ManA to 25% Aga27A produced the highest quantity of reducing sugar (16.96 U/mg protein) with the highest degree of synergy of 1.59 (Fig. 5.2a). None of the combinations led to an increase with respect to galactose release compared to that released by Aga27A (3.48 U/mg protein) alone at 100% protein loading (Fig. 5.2a). From this data, it is evident that the ManA did not assist Aga27A in galactose release, while Aga27A greatly assisted ManA activity by removing galactose decorations on the polymer that would have hindered ManA. A similar relationship between a mannanase, Man5A and a galactosidase, AgalB on guar gum hydrolysis has been reported by Wang et al. (2010).

The ManA (X) to *A. niger* AglC (G) combination produced the highest degree of synergy (0.82) at a protein ratio of 75%:25% and the reducing sugar released (9.15 U/mg protein) was not statistically different ($p>0.05$) from that released by ManA alone at 100% protein ratio (9.49 U/mg protein) (Fig. 2b). The highest degree of synergy (1.18) with respect to galactose release was observed when the ManA to AglC combination was at 25%:75% and the galactose released (0.71 U/mg protein) was not statistically different ($p>0.05$) from that released by AglC alone at a 100% protein ratio (0.96 U/mg protein) (Fig. 5.2b). It appears that, with respect to reducing sugar release (mostly attributable to ManA activity), the two enzymes exhibited an antisynergistic relationship with each other (i.e. a degree of synergy of less than 1). The antisynergistic relationship observed is proposed to be due to competition that occurred between the two enzymes for subsites on the substrate.

A degree of synergy greater than 1 was observed in all the Man26A (M) and guar seed galactosidase Aga27A (A) combinations assessed. Reducing sugar release was significantly ($p<0.01$) greater than when Man26A (M 100%) (413.8 U/mg protein) was used alone at a protein ratio of Man26A to Aga27A of 75:25% (438.74 U/mg protein). This combination also exhibited the highest degree of synergy (2.36) with respect to galactose release and the lowest (1.14) with respect to reducing sugar release The Man26A to Aga27A protein ratio of 25:75% showed the greatest degree of synergy (1.23).

All the *A. niger* mannanase Man26A (M) and *A.niger* galactosidase AglC (G) combinations had a degree of synergy greater than 1, demonstrating that the two enzymes interacted synergistically to degrade the substrate. A significant increase with respect to galactose (1.30 U/mg protein) and an insignificant ($p>0.05$) increase in reducing sugar (437.19 U/mg protein) release compared to when Man26A or AglC were used alone at 100% was observed when Man26A was dosed at 75% and AglC at 25% of total protein content. This combination also exhibited the highest degree of synergy (1.26) with respect to galactose release and the lowest (1.17) with respect to reducing sugar release. The greatest degree of synergy with respect to reducing sugar release (1.36) was observed with the Man26A (M 25%) to AglC (G 75%) protein ratio.

5.4.2. Synergy on guar gum hydrolysis

The main difference between the two substrates (guar and locust bean gum) is the extent and pattern of galactose substitution on the mannan backbone. As expected, the specific activity of most of the mannanolytic enzymes on guar gum was lower than that obtained on locust bean gum, due to the higher extent of galactose substitution in guar gum (see Section 4.4.2). Homeosynergistic associations between the four mannanolytic enzymes (Man2A, Man5A, Man26A and ManA) were determined on guar gum hydrolysis (Fig. 5.3).

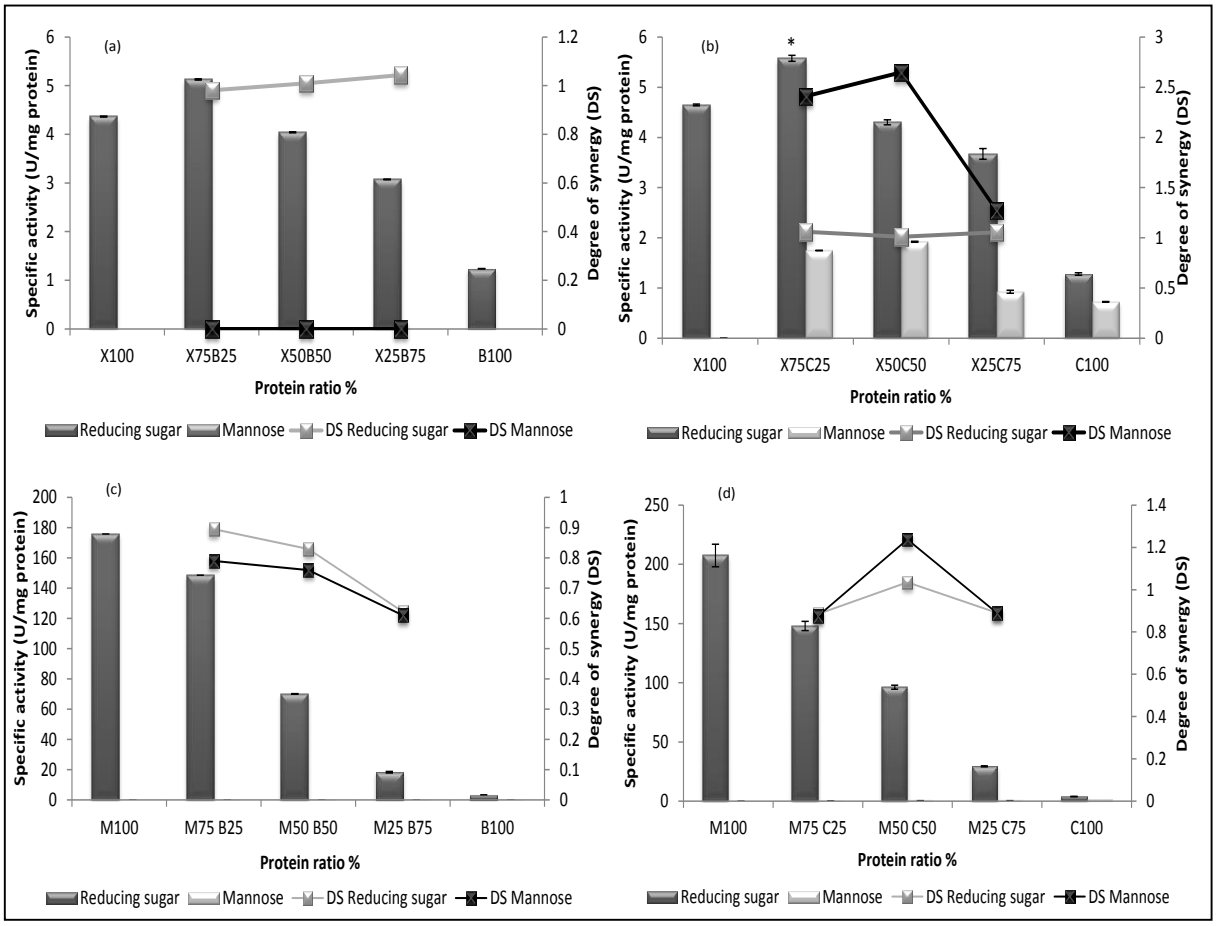

Figure 5.3. Mannose released, reducing sugar released and synergistic associations obtained on guar gum by the various combinations of enzymes, (5.3a) ManA (X) and Man2A (B), (5.3b) ManA (X) and Man5A (C), (5.3c) Man26A (M) and Man2A (B), and (5.3d) Man26A (M) and Man5A (C). ANOVA analysis for improvement of hydrolysis with respect to reducing sugar and galactose 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).

The homeosynergistic association between ManA and Man2A at all the combinations considered could not release greater levels of reducing sugar than that released by ManA alone at a 100% protein dosage (U/mg protein) (Fig. 5.3a). With respect to mannose release, none of

the binary combinations between these enzymes could liberate detectable levels of mannose (Fig. 5.3a). The homeosynergistic combination of ManA to Man5A at 75%:25% protein ratio liberated significantly ($p<0.05$) higher reducing sugars (5.58 U/mg protein) than those liberated by Man alone at 100% protein loading (4.65 U/mg protein) (Fig. 5.3b). The binary combination of ManA to Man5A at the protein ratios of 75:25% (1.75 U/mg protein) and 50:50% (1.92 U/mg protein), respectively, liberated higher levels of mannose than that liberated by Man5A at 100% protein ratio (0.72 U/mg protein) (Fig. 5.3b). All the considered synergistic combinations between Man26A (M) and *B. thetaiotaomicron* mannosidase Man2A (B) on guar gum exhibited a degree of synergy less than one. None of the considered combinations liberated reducing sugar content greater than that displayed by Man26A alone (175.83 U/mg protein) (Fig. 5.3c). These two enzymes exhibited antisynergy with respect to reducing sugar release. With respect to mannose release, all the combinations considered could not liberate any mannose from guar gum (Fig. 5.3c).

None of the considered combinations between the *A. niger* mannanase Man26A and *C. mixtus* β-mannosidase Man5A liberated reducing sugars greater than when Man26A was used on its own (207.46 U/mg protein). The Man26A to Man5A combination at the ratio 50 to 50% exhibited the highest degree of synergy (1.04) on guar gum (see Fig. 5.3d). With respect to mannose release, all the considered combinations liberated lower mannose than that released by Man5A at 100% protein content (0.92 U/mg protein).

Heterosynergy studies between the four mannanolytic enzymes (Aga27A, AglC, Man26A and ManA) were also conducted for guar gum hydrolysis (Fig. 5.4).

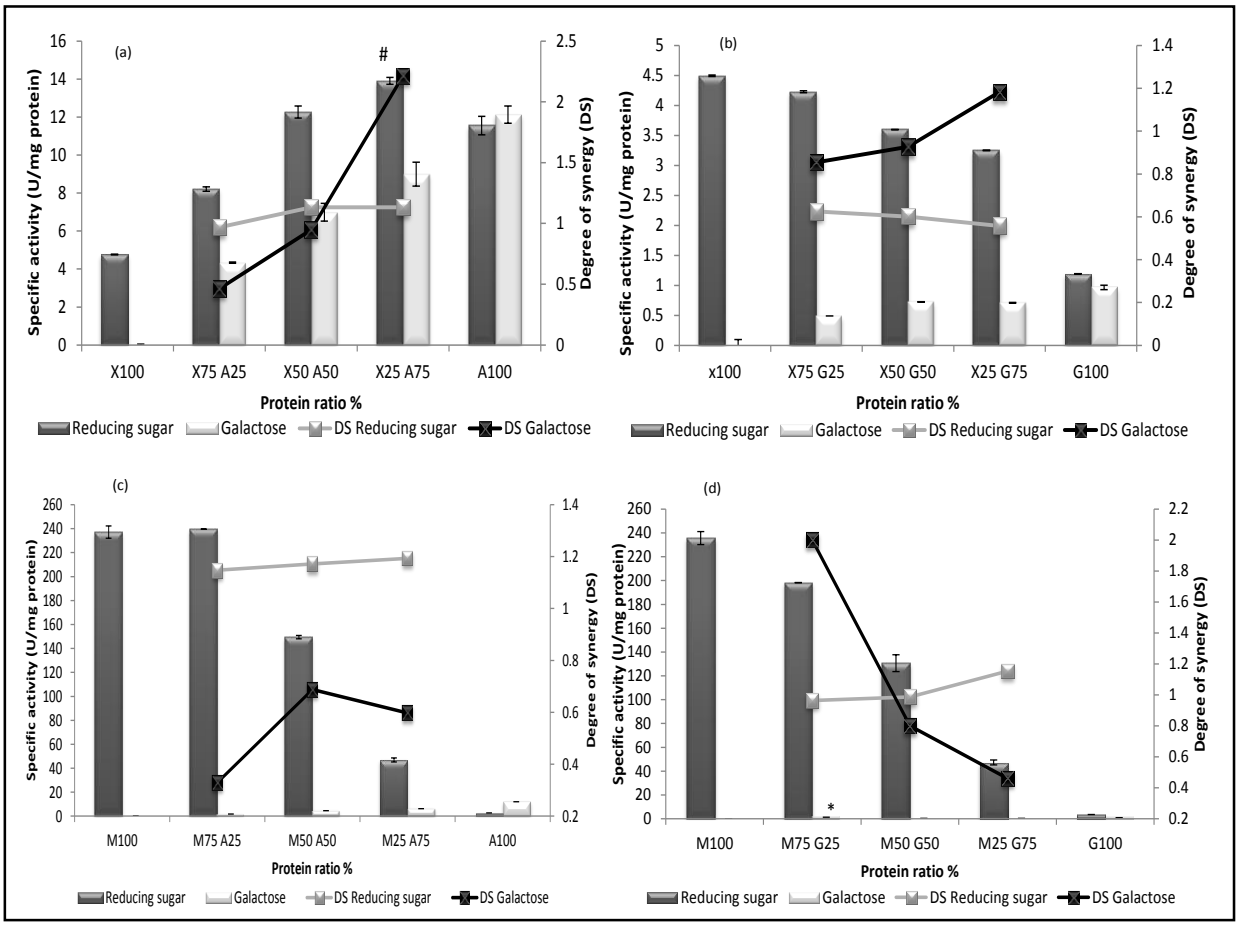

Figure 5.4. Galactose released, reducing sugar released and synergistic associations obtained on guar gum by the various combinations of enzymes, (5.4a) ManA (X) and Aga27A (A), (5.4b) ManA (X) and AglC (G), (5.4c) Man26A (M) and Aga27A (A), and (5.4d) Man26A (M) and AglC (G). ANOVA analysis for improvement of hydrolysis with respect to reducing sugar and galactose 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).

The protein ratio of ManA to Aga27A of 25%:75% produced the highest synergistic association of 1.13 and displayed the highest significant ($p<0.01$) reducing sugar release of 13.91 U/mg protein on guar gum hydrolysis compared to when Aga27A (11.56 U/mg protein) or ManA (4.76 U/mg protein) were used alone at 100% protein loading (Fig. 5.4a). None of the ManA to Aga27A combinations liberated higher galactose content than that liberated by Aga27A (12.13 U/mg protein) at 100% protein loading (Fig. 5.4a).

ManA 75% to AglC 25% produced reducing sugar (4.23 U/mg protein) comparable to ManA alone at 100% dosage (4.49 U/mg protein) (Fig. 5.4b). All the ManA to AglC combinations considered liberated less galactose than that released by AglC when used alone at 100%. All the ManA to AglC combinations, with respect to reducing sugar release, exhibited competition

(i.e. a degree of synergy of < 1). This implies that the two enzymes were not cooperating with each other and were actually hindering each other during guar gum hydrolysis.

All the Man26A to Aga27A combinations assessed exhibited a degree of synergy greater than 1, but Man26A at 75% to Aga27A at 25% displayed the highest reducing sugar release (239.54 U/mg protein) and was comparable ($p>0.05$) to that released by Man26A alone at 100% protein enzyme loading (237.14 U/mg protein) (Fig. 5.4c). All the Man26A to Aga27A combinations exhibited competition and could not liberate galactose comparable to that released by Aga27A at 100% protein loading (Fig. 5.4c).

Using Man26A alone at 100% total protein loading exhibited the highest reducing sugar release (235.66 U/mg protein) compared to all the assessed Man26A to AglC combinations (Fig. 5.4d). The enzyme combination of Man26A at 75% to AglC at 25% displayed the highest synergy (2.0) and galactose release (1.25 U/mg protein) compared to when AglC was loaded at 100% total protein loading (Fig. 5.4d).

5.5. Discussion

Synergy is defined as the interaction that occurs between two or more hydrolytic enzymes, producing a total effect greater than the theoretical sum of the effects of the individual enzymes (Beukes et al., 2008). 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). Homeosynergistic associations between mannanases and mannosidases (Gübitz et al., 1996; Shi et al., 2011; Zahura et al., 2012), and heterosynergistic associations between mannanases and galactosidases with respect to galactomannan degradation have been documented previously (Margolles-Clark et al., 1996; Wang et al., 2010). These studies concluded that there is general cooperation between these classes of mannanolytic enzymes on galactomannan degradation. However, an assessment of synergistic relationships between mannanases of

different GH families with galactosidases and mannosidases of different GH families has not been carried out previously.

5.5.1. Synergy on locust bean gum

In the homeosynergy studies between the β-mannanases (GH5 ManA and GH26 Man26A) and the β-mannosidases (GH2 Man2A and GH5 Man5A) on locust bean gum, the highest reducing sugar content was found with a mixture including 75% of mannanases and 25% of mannosidases. Charrier and Rouland (2001) reported a different result from this study, namely that the best synergistic effect was found with a mixture including 25% of mannanase and 75% of mannosidase (Fig. 5.4a). We propose that the high content of small fragment-products released by the high protein ratio of β-mannanases, such as manno-oligosaccharides with non-reducing ends, was preferred substrates of the β-mannosidases and is suggested to be the reason for this trend in synergy amongst these two main-chain cleaving enzyme classes.

With respect to mannose release by the homeosynergistic associations between mannanases and mannosidases, the binary combination between ManA and Man5A was the only combination that released higher mannose levels than those released by Man5A alone at 100% protein loading (Fig. 5.4a). As suggested earlier, synergy between these two enzyme classes is postulated to be due to the production of short manno-oligosaccharides by ManA that are the preferred substrates of Man5A. It is still unclear why the binary combination between Man26A and Man5A couldn't liberate high levels of mannose compared to that released by Man5A alone at 100% content. However, the absence of synergy between β-mannanases and β-mannosidases has been reported before between Man2S27 and Man5S27 in locust bean gum hydrolysis (Yao et al., 2011).

Among the heterosynergy binary studies between the β-mannanases (ManA and Man26A) and α-galactosidases (GH27 Aga27A and GH36 AglC) on locust bean gum, the highest reducing sugar content was found with a mixture including 75% of mannanases and 25% of α-galactosidases. Higher reducing sugar content was observed in the binary combinations between GH5 or GH26 mannanases and Aga27A (Fig. 5.4b). With the significant increase in reducing sugar release by the combinations, we propose that Aga27A removes galactose decorations from locust bean gum that would have hindered the mannanases.

With respect to galactose release, all the binary combinations between Man26A and Aga27A, and Man26A to AglC at 75:25% liberated higher levels of galactose than that liberated by the

galactosidases alone at 100% on the polysaccharide (Fig. 5.4b). With regards to the significant galactose release by the combinations, we postulate that it is attributable to Man26A releasing oligomeric fragments from locust bean gum that are a preferable substrate for Aga27A, thus resulting in the pronounced activity of Aga27A in the presence of Man26A.

5.5.2. Synergy on guar gum

The data obtained from the binary combination of ManA and Aga27A suggests that increasing the protein mass ratio of Aga27A in the combination leads to a reducing sugar release greater than that exhibited by Aga27A at 100% protein loading (Fig. 5.4a). But none of the combinations between these two enzymes led to a galactose release greater than that released by Aga27A when used on its own. These two findings suggest that the presence of Aga27A in the combination aids/improves the activity of ManA by removing galactose substituents that would have hindered ManA activity. On the other hand, the presence of ManA in the combination did not improve Aga27A activity in releasing galactose. This could be due to the low rate of small oligosaccharide forming activity of ManA, thus the low quantity of these oligosaccharides could not significantly improve Aga27A activity.

With regards to the binary combination of ManA and AglC, no synergistic associations led to reducing sugar release greater than that observed when ManA was used alone and galactose release greater than that when AglC was used alone at 100% dosage (Fig. 5.4b). These two findings suggest that the presence of AglC in the combination has no effect on the activity of ManA since AglC has insignificant activity on block-wise galactose-containing polymers (see Table 4.2 of Chapter 4). In addition, the presence of ManA in the combination did not improve AglC activity, which could be due to the low rate of small oligosaccharide forming activity of ManA, thus the low quantity of these oligosaccharides could not significantly improve AglC activity.

From the data obtained from Man26A and Aga27A combinations (Fig. 5.4c), it can be proposed that the increase in reducing sugar release by the combinations is due to Aga27A activity which removes galactose substituents from the guar gum thus increasing Man26A activity on the polymer. The antisynergy observed with respect to galactose release may be due to unproductive binding of Man26A at highly decorated regions of guar gum leading to a blockage or shielding of Aga27A access to these preferred galactose rich regions.

The data illustrated on Figure 5.4d suggests that AglC has negligible activity on guar gum and does not influence Man26A activity on the polymer. On the other hand, the high galactose release by Man26A 75% to AglC 25% is hypothesized to be attributable to Man26A releasing small galactose-containing oligosaccharides that are a preferable substrate for AglC, thus the pronounced activity of AglC on guar gum in the presence of Man26A.

5.6. Conclusions

The results showed that the α-galactosidase from *A. niger*, belonging to GH family 27, exhibits better hydrolytic activity on galactose-containing polymers and can act hetero-synergistically with β-mannanases from either GH5 or GH26 in the hydrolysis of galactomannan substrates. The data also showed that the β-mannosidase from *C. mixtus* (GH5) has higher hydrolytic activity on galactomannans and can act homeosynergistically with β-mannanases in the hydrolysis of galactomannan substrates.

From the findings obtained in this study, it was elucidated that the GH family classification of an enzyme affects its substrate specificity and synergistic interactions with other glycoside hydrolases from different GH families to a larger degree than its EC classification. This method of screening for maximal compatibility between various GH families should ultimately lead to a more rational development of tailored enzyme cocktails for lignocellulose degradation.

The mannanolytic cocktail consisting of Man26A and Aga27A was selected for an investigation that studied synergy between a commercial cellulase preparation (Cellic® CTec2) and mannanolytic enzymes on the hydrolysis of sodium chlorite/acetic acid delignified sugarcane bagasse (Chapter 7).

Chapter 6: Determination of the inhibitory effects of various substrate pre-treatment by-products on mannanolytic enzymes

6.1. Introduction

Increased interest in renewable energy and biochemical value-added products has led to the use of lignocellulosic biomass as a low-cost feedstock for the production of these commodity chemicals. Lignocellulosic biomass is composed of polysaccharides such as cellulose and hemicellulose, estimated to be approximately 70% of the dry mass of the biomass, and lignin. Thus it has been suggested as a model feedstock for cellulosic ethanol production (Morales et al., 2014; Zeng et al., 2014). The hydrolysate of cellulose is mainly used for bioethanol production, while the hydrolysate of hemicellulose is used for production of artificial sweeteners such as xylitol and mannitol, prebiotic oligomers and biofuels. However, lignocellulose has a very complex structure and is therefore very recalcitrant to enzymatic hydrolysis (Lou et al., 2013; van Dyk and Pletschke, 2012). This necessitates the use of a pre-treatment method to increase the accessibility of the enzymes to the hydrolysable carbohydrates (Morales et al, 2014; van Dyk and Pletschke, 2012).

A number of pre-treatment approaches have been developed empirically with a combined effort of reducing particle size and to change the physical and chemical characteristics of lignocellulosic biomass so that enzymatic hydrolyzability can be improved. However, most of these processes result in the generation of compounds that hamper enzymatic hydrolysis and microbial fermentation (Kim et al., 2011; Oliva et al., 2003; Palmqvist and Hahn-Hägerdal, 2000). In addition, plants are reported to naturally release phenolic compounds that inhibit enzyme hydrolysis as a defense mechanism against pathogens that use hydrolases to gain entry into the plant cells (Ximenes et al., 2010). The soluble 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 *p*-coumaric acid, vanillin, vanillic acid and gallic acid) (Kim et al., 2011; Morrison et al., 2011).

Methods to mitigate the inhibitory effects of phenolic compounds on the hydrolytic enzymes and fermentative microorganisms have been developed; these include extraction and detoxification methods using anhydrous ethanol, deionized water and ethyl acetate (Li et al, 2013; Li et al., 2014). However, it is still unclear how the effects of different solvents used in

the extraction impact the subsequent enzymatic hydrolyzability of the solid fractions after solvent extraction (Li et al., 2013). As a result, research has focused more on procuring tolerant hydrolases to be used in the biomass-to-ethanol process.

Although several studies on pre-treatment liquor effects have been reported, the effect of by-products, liberated through various pre-treatment methods on enzymatic hydrolysis is not completely understood. The mechanism of inactivation caused by these compounds is also still unclear. The aim of this study was to elucidate the individual inhibitory properties of each of the pre-treatment by-products and the synergistic effect that inhibitor cocktails obtained from washes of substrates pre-treated by various technologies would have on mannanolytic enzymes.

6.2. Aims and Objectives

6.2.1. Aim

To determine the individual inhibitory effects of each of the pre-treatment by-products and the synergistic inhibitory effect of an inhibitor cocktail has on mannanolytic enzymes.

6.2.2. Objectives

- To conduct mannanolytic enzyme inhibition studies using pre-treatment by-products;
- To determine the mode of mannanolytic enzyme inhibition;
- To conduct sugar feedback inhibition studies on rate limiting mannanolytic enzymes;
- To conduct mannanolytic enzyme inhibition studies using washes from substrates pre-treated by different pre-treatment technologies.

6.3. Methods and Materials

6.3.1. Product inhibition profiles of the rate limiting mannanolytic enzymes

Inhibition of α-galactosidases (Aga27A and AglC) by D-galactose (2-100 mM) was determined using 2 mM *p*NPG as the substrate (see method in Section 4.3.3.3). The inhibition of β-mannosidases (Man2A and Man5A) by D-mannose (2-100 mM) was also determined using 2 mM *p*NPM as the substrate (see Section 4.3.3.2).

6.3.2. Pretreatment by-products inhibition assays

Enzyme activity assays were carried out with each individual inhibitor (1-2 g/L) and activity assays for the various mannanolytic enzymes were set up as described in Section 4.3.3. The inhibitors assessed were: acetic acid, formic acid, 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.

6.3.3. Precipitation of BSA by phenolics

To directly measure the extent of protein precipitation by the phenolic compounds, the phenolics at 0.05-0.1 mg bovine serum albumin (BSA)/g total phenolic were incubated for 60 minutes at 37^{o}C in 50 mM sodium citrate buffer (pH 5.0). At the end of the incubation period, the mixture was centrifuged to collect the supernatant and the Bradford method was used to measure non-precipitated protein. A control reaction consisted of BSA only in pH 5.0 sodium citrate buffer. The Bradford method was then used to determine the quantity of protein present in solution after incubation with the phenolics (Bradford, 1976). Bovine serum albumin was used as a suitable protein standard.

The total amount of gallic acid participating in protein precipitation was determined using a modified Folin-Ciocalteu method. The aqueous solutions from the protein and gallic acid incubations (10 µL) were pipetted in 96 well plates with 180 µL of water, 20 µL of Folin reagent, and left to stand for a minute before the addition 50 µL of 2 M sodium carbonate. The mixture was then covered, incubated at 40^{o}C for 30 minutes before reading at 765 nm using a Powerwave$_{x}$ microtiter plate reader.

6.3.4. Preparation of inhibitor cocktail washes from pre-treated substrates and inhibition studies

6.3.4.1. Preparation of inhibitor cocktail washes from substrates pre-treated by various pre-treatment technologies

Sugarcane bagasse that had been pre-treated the following ways was used: (1) delignified by sodium chlorite/acetic acid, (2) steam exploded and (3) lime pre-treated; while (4) steam exploded Douglas-fir was also used to assess inter-substrate (plant species) variations effects on pre-treatment by-products liberation. About 100 mg of each substrate was weighed out, added to a 50 mL Schott bottle and mixed with 15 mL of 50 mM sodium citrate buffer (pH

5.0). This gave a substrate concentration of 0.66 % (w/v) pretreated substrate. The substrates were mixed using a magnetic stirrer for 24 hours. After this, the insoluble components of the substrate were left to settle down for 30 minutes. The aqueous layer was then transferred to eppendorf tubes and centrifuged at 13,000 x *g* for 5 minutes. 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).

6.3.4.2. Enzyme inhibition studies using washes from biomass pre-treated by various pre-treatment technologies

Galactosidase, mannanase and mannosidase activity assays were set up as previously described in Section 4.3.3. 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 citrate buffer and another prepared using washes 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 washes contained a final concentration of pre-treatment soluble compounds corresponding to those that would be liberated if the pre-treated biomass were loaded at 0.5% (w/v).

6.3.5. Data analysis

One way analysis of variance (ANOVA) was used to analyze 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.

6.4. Results

6.4.1. Product inhibition profiles of the rate limiting mannanolytic enzymes

Inhibition of the two α-galactosidases by D-galactose and that of the two β-mannosidases by D-mannose was conducted over a concentration range of 0-100 mM of each of the sugar products. The figure below (Fig. 6.1) shows the tolerance levels of each α-galactosidase to galactose and each β-mannosidase to mannose.

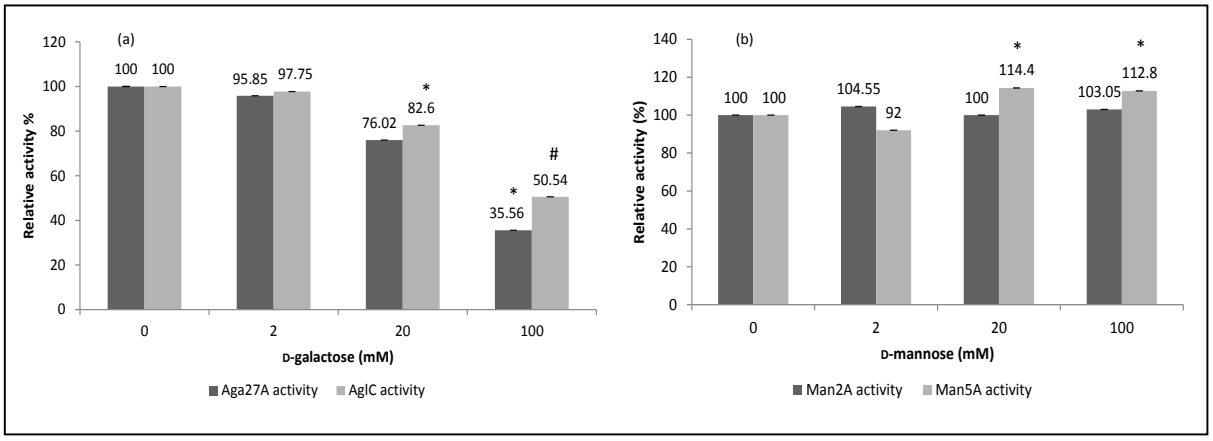

Figure 6.1. Tolerance of: (a) the two α-galactosidases (Aga27A and AglC) to the presence of galactose at different concentrations and that of (b) the β-mannosidases (Man2A and Man5A) in the presence of mannose at different concentrations. Values are represented as mean values ±SD. (n=3). ANOVA analysis 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).

The family 36 α-galactosidase, AglC was more tolerant of the presence of D-galactose compared to the family 27 α-galactosidase, Aga27A (Figure 6.1.a). Among the β-mannosidases, Man5A seemed to be significantly ($p<0.05$) activated by the presence of mannose at high concentrations while Man2A activity was not affected by the presence of mannose at all the given concentrations (see Figure 6.1.b).

6.4.2. Pre-treatment by-products inhibition assays

Inhibition of the mannanolytic enzymes by lignocellulose pretreatment based byproducts was conducted over a concentration range of 1 to 2 g/L of each compound. Figures 6.2 and 6.3 below show the tolerance levels of the α-galactosidase (Aga27A and AglC), β-mannosidase (Man2A and Man5A) and β-mannanase (Man26A and ManA) to the various pre-treatment compounds. The figure below displays the inhibitory effects of the pretreatment sugar degradation products (acetic acid, formic acid, furfural, hydroxymethylfurfural and levulinic acid) on the activity of mannanolytic enzymes (Fig. 6.2). It is noteworthy to mention that the concentrations of the inhibitors used did not significantly affect the pH of the reaction medium (±0.5 pH unit variation) (data not shown).

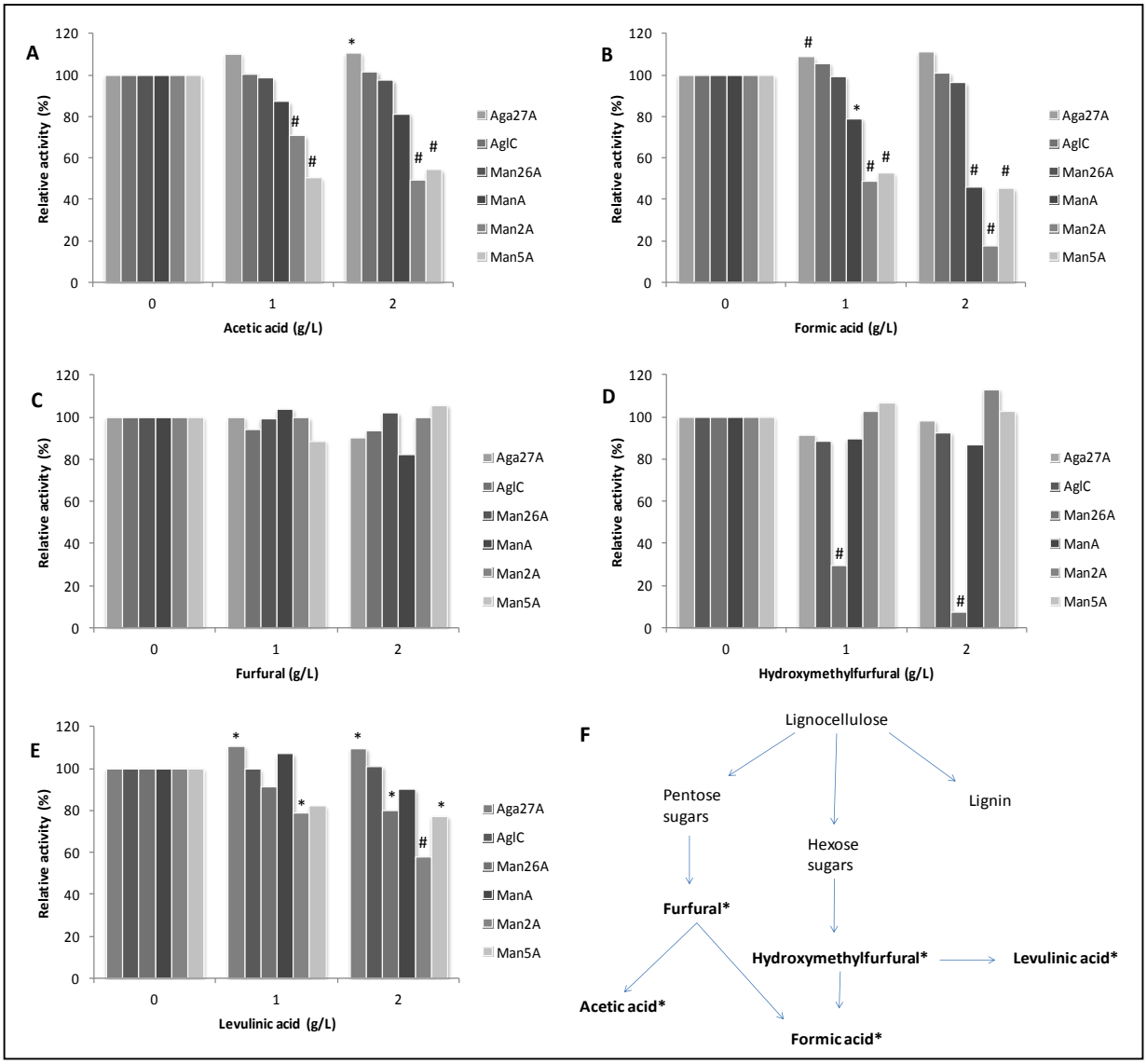

Figure 6.2. Inhibition of mannanolytic enzymes by (A) acetic acid, (B) formic acid, (C) furfural, (D) hydroxymethylfurfural and (E) levulinic acid at 1 and 2 g/L concentrations. Values are represented as mean values ±SD. (n=3). ANOVA analysis of inhibition or activation of enzymes by the different sugar 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 pre-treatment of biomass.

The figure below displays the inhibitory effects of the pre-treatment lignin degradation products (gallic acid, low sulfonated lignin, *p*-coumaric acid, vanillic acid and vanillin) on the activity of mannanolytic enzymes (Fig. 6.3).

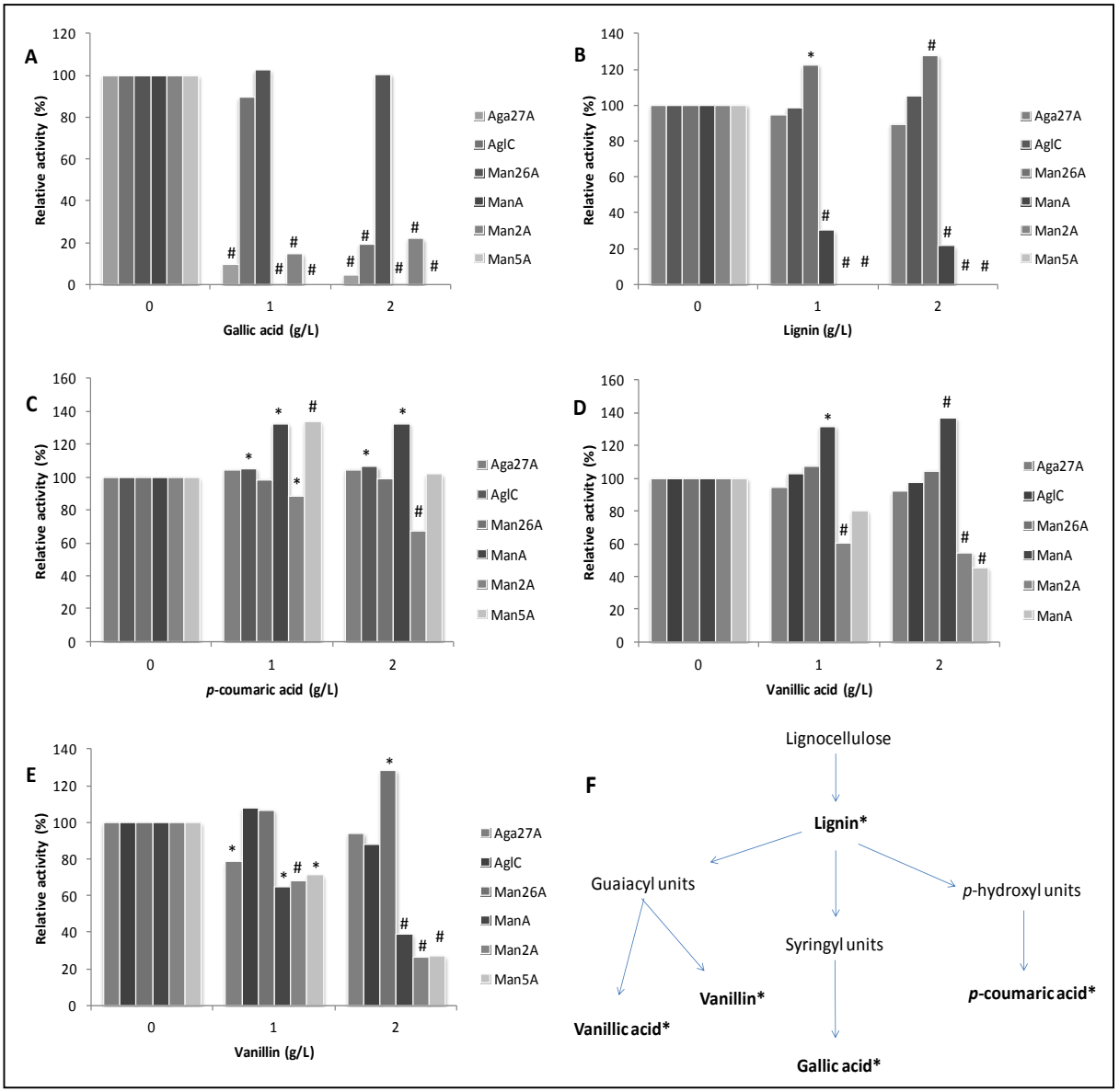

Figure 6.3. Inhibition of mannanolytic enzymes by (A) gallic acid, (B) lignin, (C) *p*-coumaric acid, (D) vanillic acid and (E) vanillin at 1 and 2 g/L concentrations. Values are represented as mean values ±SD. (n=3). ANOVA analysis of inhibition or activation of enzymes by the different lignin degradation 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 pre-treatment of biomass.

Most of the compounds had no significant effect on the activity of the galactosidases (Aga27A and AglC), while some of the compounds seemed to activate enzymatic activity and some drastically inhibiting the activity of the enzymes. Acetic acid and *p*-coumaric acid were found to be the least inactivating compounds at both concentrations considered towards the galactosidase AglC. These findings are similar to those reported for an endo-glucanase by Canatarella et al. (2014). The family 27 galactosidase, Aga27A was significantly activated by the presence of acetic acid ($p<0.05$) at a concentration of 2 g/L, *p*-coumaric acid ($p<0.05$) at a

concentration of 1-2 g/L, levulinic acid ($p<0.05$) at a concentration of 1-2 g/L and formic acid ($p<0.01$) at a concentration of 1 g/L (Fig. 6.2). A report by Kim et al. (2011) also showed that concentrations of acetic acid as high as 13 g/L was not inhibitory to enzymes. The GH36 galactosidase, was not significantly affected by the presence of sugar degradation products (acetic acid, formic acid, furfural, hydroxymethylfurfural and levulinic acid) at a concentration range of 1-2 g/L (Fig. 6.2).

Inhibition of the two mannanases by the lignocellulose pretreatment degradation by-products was also assessed. Among the sugar degradation products, the GH26 mannanase, Man26A was significantly ($p<0.01$) inhibited by hydroxymethylfurfural at the concentrations of 1-2 g/L, and by levulinic acid at the concentration of 2 g/L ($p<0.05$) (Fig. 6.2). Among the lignin degradation by-products, Man26A was significantly activated by the presence of lignin at the concentrations 1g/L ($p<0.05$) and 2 g/L ($p<0.01$), and by vanillin at 2 g/L ($p<0.05$) (Fig. 6.3). The GH5 mannanase, ManA was significantly inhibited by formic acid at the concentrations 1 g/L ($p<0.05$) and 2 g/L ($p<0.01$). The other sugar degradation products exhibited no significant ($p>0.05$) inhibitory effects on ManA activity (Fig. 6.2). All the lignin degradation products significantly inhibited ManA at the concentration range considered (Fig. 6.3).

Among the sugar degradation products, the GH2 mannosidase, Man2A was significantly inhibited by acetic acid, formic acid and levulinic acid at a concentration range of 1-2 g/L (Fig. 6.2). All the considered lignin degradation products exhibited significant inhibitory effects on Man2A activity (Fig. 6.3).

6.4.3. Precipitation of BSA by phenolics

Haslam (1974) and Kim et al. (2011) have reported the existence of interactions between phenolics and enzymes that leads to complexes, resulting in enzyme precipitation. In this study the interaction between gallic acid and BSA was used to validate this theory as the reason for mannanolytic enzyme(s) inhibition by phenolics (Fig. 6.4).

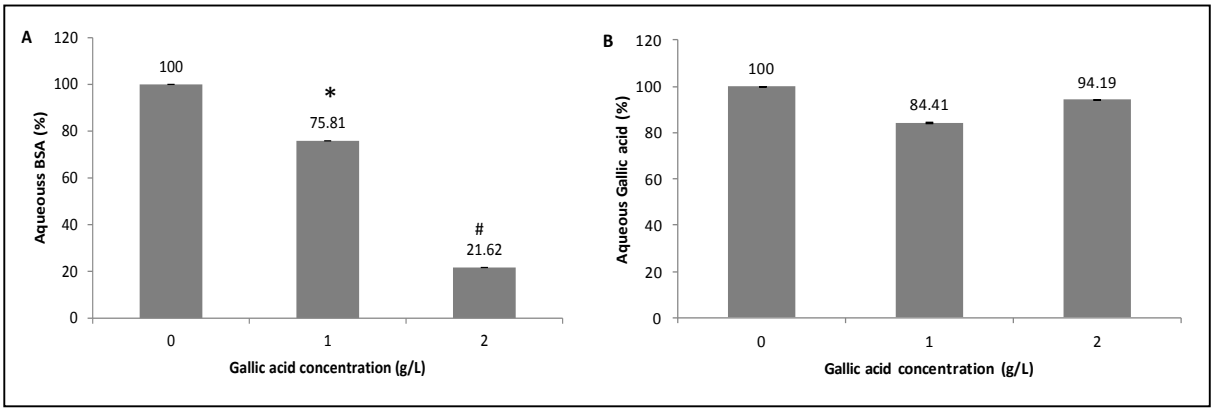

Figure 6.4. (A) Precipitation of BSA into phenolics-protein complexes at 1-2 g/L gallic acid per 0.010 mg protein (BSA). (B) Precipitation of gallic acid into phenolics-protein complexes at 1-2 g/L of gallic acid per 0.010 mg protein. Values are represented as mean values ±SD. ANOVA analysis of protein-phenolic complexation was conducted and compared to when only protein or phenolic was on its own, test keys; * (p value<0.05) and # (p value<0.01).

Gallic acid appeared to significantly precipitate protein out of solution, this effect appeared to be more pronounced with an increase in gallic acid in the reaction mixture (Fig. 6.4A). Irrespective of the gallic acid concentration in the reaction mixture only about 100 mg/L of gallic acid appeared to be responsible for phenolics-protein complexation, with 0.84 g/L of 1 g/L concentration of gallic acid remaining in solution after incubation with BSA and with 1.90 g/L of 2 g/L concentration of gallic acid in solution remaining in solution after incubation with BSA (Fig. 6.4B).

6.4.4. Enzyme inhibition studies using washes from biomass pre-treated by various pre-treatment technologies

Lignocellulosic biomass pre-treatment can result in the release of several lignocellulose degradation products, such as furans and organic acids from polysaccharides and mono-lignols from lignin. We assessed the effects of soluble pre-treatment by-products from biomass pre-treated by various pretreatment technologies on the mannanolytic enzymes (see Fig. 6.5 below). Sugarcane bagasse was used as the model substrate to assess the impact of various pre-treatment technologies on the liberation of by-products and their concentrations. Steam exploded Douglas-fir was used as a control to assess the impact of different biomass on the by-products produced from a particular pre-treatment technology. As mentioned in Section 6.3.4.1, the pH values of the different substrate washes were determined and were found to be not significantly different from that of citrate buffer at pH 5.0 (data not shown).

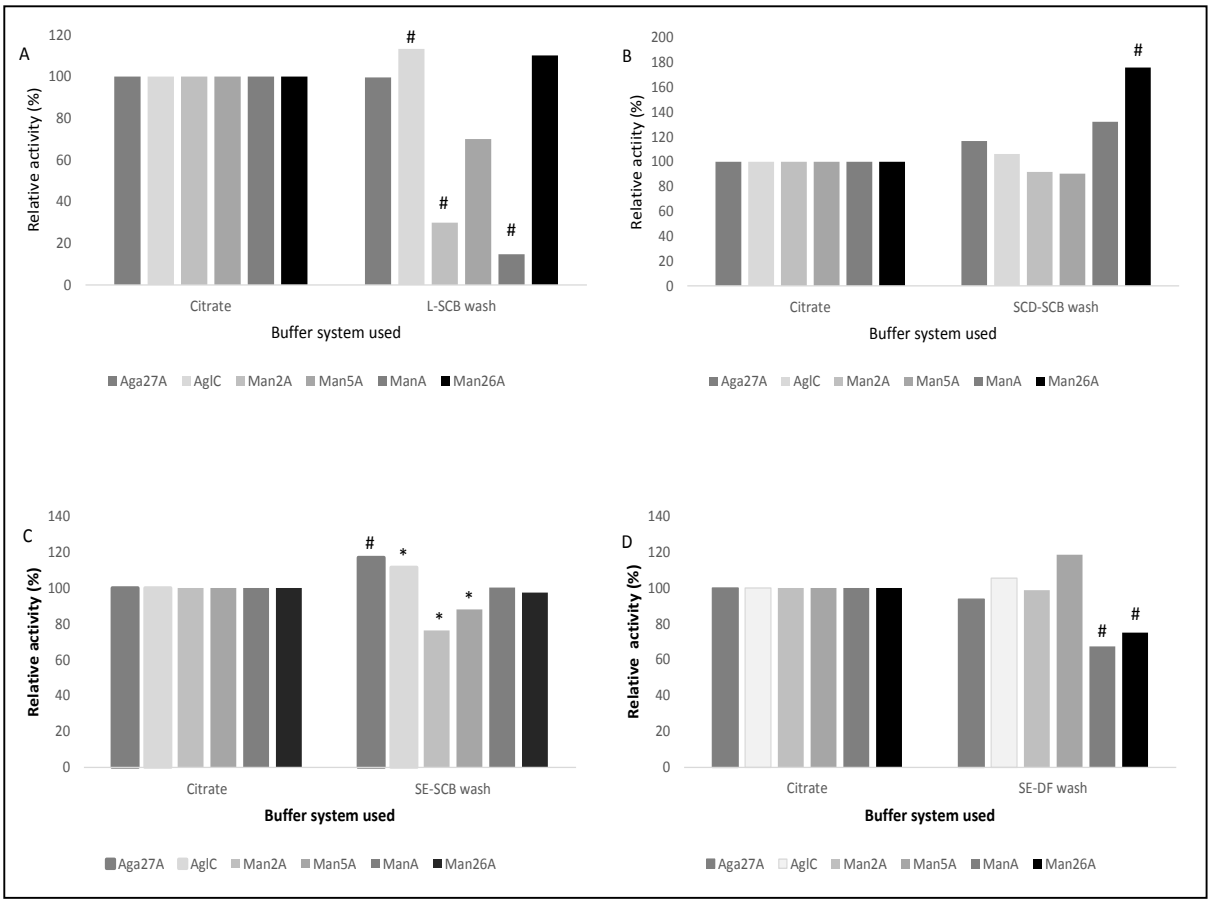

Figure 6.5. Determination of the inhibitory effects of soluble pre-treatment byproducts in washes of substrates pre-treated by various pre-treatment technologies. The washes used were from: (A) lime treated bagasse (L-SCB), (B) sodium chlorite/acetic acid treated bagasse (SCD-SCB), (C) steam exploded bagasse (SE-SCB) and (D) steam exploded Douglas-fir (SE-DF). ANOVA analysis for inhibition or activation of mannanolytic enzyme in a buffer system of pre-treated substrate wash compared to mannanolytic enzyme 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=3).

The various pre-treatment technologies seemed to affect the mannanolytic enzymes differently and the various mannanolytic enzymes also appeared to respond to each substrate pre-treatment wash differently. Lime pre-treated sugarcane bagasse wash appeared to contain the most inhibitory pre-treatment soluble compounds (particularly inhibiting Man2A, Man5A and ManA to the highest extent) (Fig. 6.5A), while sodium chlorite/acetic acid delignified sugarcane bagasse wash appeared to contain the least inhibitory pre-treatment soluble compounds, with none of the mannanolytic enzymes exhibiting significant inhibition by this substrate wash (Fig. 6.5B).

6.5. Discussion

6.5.1. Product inhibition profiles of the rate limiting mannanolytic enzymes

Inhibition of the α-galactosidases by galactose was studied using *p*NPG as a substrate (Fig. 6.1a). Aga27A lost 65% of its activity when 100 mM (18 g/L) galactose was added (with a K_i of 30.73 mM), whereas AglC retained about half of its activity at this galactose concentration (with an estimated K_i of 66.92). Inhibition of α-galactosidase *p*NPG activity by high concentrations of galactose has also been observed in a study by Luonteri et al. (1998) who proposed this inhibition to be due to the formation of a complex of the enzyme with the sugar and substrate molecules, and also powerful competition by D-galactose for the enzymes active site. Galactose has also been reported to competitively inhibit an α-galactosidase from *C. melo* (K_i of 13 mM) and another galactosidase from *Tachigali multijuga* seeds (K_i of 2.74 mM) for *p*NPG hydrolysis (da Silva Fialho et al., 2008).

The two β-mannosidases, Man2A and Man5A were not inhibited by the hydrolysis product, mannose at the considered range of 0 to 100 mM. According to Dias et al. (2004), GH1 and GH2 mannosidases often exhibit product inhibition at substrate concentrations as low as 400 µM, whereas very high concentrations of mannose (K_i of about 400 mM) inhibit the GH5 Man5A. The inhibition profile for Man2A in this study displays findings contrary to those expected of GH2 mannosidases (low K_i value for mannose). A β-mannosidase from *Trichoderma reesei* was reported to be competitively inhibited by mannose (with a K_i of 0.2 mM) on *p*NPM hydrolysis (Kulminskaya et al., 1999).

Product inhibition effects, while important, are less critical than the impact of phenolics and sugar degradation products (furans and organic acids) since these are not consumed (at least not at the same level as sugars) by fermentative microorganisms.

6.5.2. Pre-treatment by-products inhibition assays

The pre-treatment by-products concentration explored in this study falls in the range estimated in samples from aqueous pretreatment of lignocelluloses, where a release of about 2–16% (2–5 g/L) of the mass of lignin, as phenols, is expected in the aqueous solution (Canatarella et al., 2014; Ximenes et al., 2011). Figure 6.6 illustrates the various chemical structures of the pre-treatment by-products used in the inhibition studies conducted using the mannanolytic enzymes.

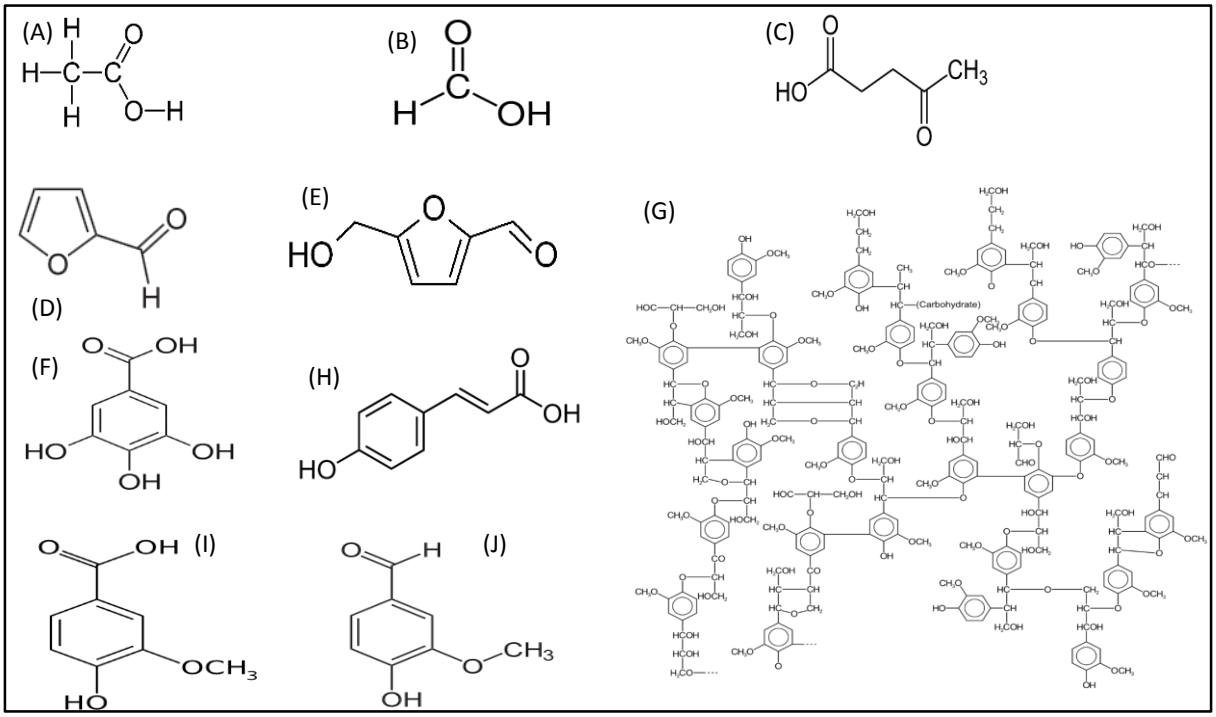

Figure 6.6. Chemical structures of the various substrate pre-treatment by-products used in this study. Where sugar degradation products are: (A) Acetic acid, (B) Formic acid, (C) Levulinic acid, (D) Furfural and (E) Hydroxymethylfurfural, and lignin degradation products are: (F) Gallic acid, (G) Lignin, (H) p-coumaric acid, (I) Vanillic acid and (J) Vanillin.

Based on Figure 6.6, the most common functional group amongst the considered substrate pre-treatment by-products was the carbonyl group followed by hydroxyl, methoxyl and ethoxy groups. The sugar degradation products listed in A-E were either simple hydrocarbons or furans, while the lignin degradation products listed from F-J were phenol derivatives.

The side chain cleaving α-galactosidases were the least inhibited enzymes among the mannanolytic enzyme classes examined, while the main chain cleaving β-mannosidases were prone to inhibition by a large array of the lignocellulose pre-treatment degradation products considered in this study. Among the mannanases, ManA exhibited inhibition profiles similar to those observed on the mannosidases in the presence of numerous inhibitors, such as acetic acid, formic acid, gallic acid, lignin and vanillin. With respect to acetic acid and formic acid inhibition, the two galactosidases were not inhibited, but in the presence of these acids, Aga27A activity appeared to be activated. A previous study by Lima et al. (2004) demonstrated that acetic acid could activate the action of a xylose reductase (XR) (EC 1.1.1.21) and that of a xylitol dehydrogenase (XDH) (EC 1.1.1.9). The two mannosidases, Man2A and ManA, and the mannanase, ManA were greatly inhibited by increasing concentrations of formic acid. A similar trend on cellulase and xylanase activity was observed by Panagiotou and Olsson (2007)

where the addition of 4 g/L formic acid exhibited a strongly negative effect on filter paper hydrolysis and led to at least a 20% reduction in the glucose and xylose concentrations, respectively.

The effect of furfural and hydroxymethylfurfural on the mannanolytic enzymes at 1 g/L and 2 g/L concentrations was also assessed. From these studies it was observed that furfural did not significantly inhibit the activity of the various mannanolytic enzymes, while hydroxymethylfurfural significantly inhibited Man26A activity in a concentration dependant manner (Figs. 6.2C and D). A study by Modig et al. (2002) assessing the effects of furfural and hydroxymethylfurfural on dehydrogenases (ADH, AIDH and PDH) also demonstrated that various enzymes are not affected to the same extent by the same sugar degradation products and also, various sugar degradation products interacted differently with each enzyme.

Gallic acid, lignin and vanillin or vanillic acid exhibited the greatest inhibitory effect on the mannanolytic enzymes compared to the other compounds that were assessed for their inhibitory properties. Kim et al. (2011) have also reported an inhibitory effect of gallic acid and tannic acid on a *T. reesei* β-glucosidase. The mode of action behind phenolic compound inhibition is suggested to be via phenol-to-protein complexation that leads to protein precipitation (Haslam, 1974; Kim et al., 2011). Our data using BSA as a protein and gallic acid as a representative mono-phenolic showed that proteins could be precipitated out of solution by phenolics, and that this phenomenon increased with an increase in phenolics concentration (Fig 6.4A). However, the concentration of gallic acid (phenolic) participating in the protein-to-phenolic precipitation was similar (about 100 mg/L) at both 1 g/L and 2g/L phenolics concentration. This suggested that protein-to-phenolic complex precipitation might not be the only mode of enzyme inactivation.

The data for inhibitor effects on mannanolytic enzymes activity show that changing the inhibitor concentration from 1 to 2 g/L leads to increased inhibition. The lower inhibition obtained with 1 g/L, however, indicated the feasibility of preventing enzyme inhibition by reducing the concentration of phenolic compounds, with an appropriate detoxification procedure. Such an approach to combat enzyme inactivation has been previously proposed in literature by Cantarella et al. (2004) and Kim et al. (2011), but they also suggested that increasing the amount of enzyme can prevent enzyme inactivation, even though the specific reaction rate would always be lower.

Vanillic acid and vanillin were found to be inhibitory to the rate-limiting mannanolytic enzymes such as galactosidases and mannosidases, but they were found to activate the mannan backbone cleaving Man26A and ManA in a concentration dependent fashion, respectively. A similar observation was also made by Kaya et al. (2000) where-by xylan hydrolysis rate by a xylanase was enhanced in the presence of increasing vanillic acid up to concentrations of 0.05% (w/v). Kaya et al. (2000) made use of CD spectroscopy and 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 effect of lignin on the mannanolytic enzymes was assessed by physically mixing lignin with the various mannan-based substrates for each mannanolytic enzyme. Due to this approach of substrate preparation, the inhibitory effect of lignin as a result of it forming a barrier around the mannan substrates that shields mannanolytic enzymes from acting was excluded (lignin did not coat or cover the substrates). But inhibition could be due to non-specific adsorption and other chemical interactions with the actual enzymes (Nakagame et al., 2011). The addition of lignin clearly inhibited the action of mannanolytic enzymes and the inhibition appeared to increase with an increase in lignin content in the reaction. Man26A was the only exception to this phenomenon; the enzyme appeared to be greatly activated by an increase in lignin concentration in the reaction (Fig. 6.3B). Studies on the impact of lignin on glycoside hydrolases (cellulases and xylanases) have been conducted and they showed that inhibition of these enzyme activities increase with increasing lignin concentrations (Berlin et al., 2006; Sewalt et al., 1997). Lou et al. (2013) proposed that inhibition is due to glycoside hydrolases binding to lignin through hydrophobic, electrostatic and hydrogen bonding interactions.

With respect to mannanolytic enzyme inhibition by the phenolics, the mono-methoxylated mono-phenolics (vanillin and vanillic acid) (Figs. 6.3D and E) appeared to be more inhibitory than the non-methoxylated mono-phenolics (*p*-coumaric acid) (Fig. 6.3C), except for gallic acid. Pan (2008) also reported that mono-methoxylated compounds seem to have slightly higher inhibitory effect than non-methoxylated compounds. The position of the methoxyl substituent on the phenolic ring appears not to affect the phenolic's inhibitory effect on the mannanolytic enzymes, this can be seen by the similar inhibitory profiles exhibited by vanillin (Fig. 6.3D) and vanillic acid (Fig. 6.3E). The tri-hydroxylated gallic acid was the most inhibitory compound on the mannanolytic enzymes among the mono-phenolics used in the study (Fig. 6.3A). Pan (2008) proposed that the inhibition caused by phenolic hydroxyl group

is via the quinone methide intermediate and that this interaction may be more inhibitory than non-specific adsorption.

6.5.3. Inhibition by washes from substrates pre-treated by various technologies

From this study it was apparent that the use of different pre-treatment technologies leads to the release of different pre-treatment soluble products that interact with the mannanolytic enzymes in different ways. It is possible that the different profiles exhibited by these different pre-treatment washes on the activity of the mannanolytic enzymes could be due to synergistic effects of these various by-products. We hypothesize that the different inhibitory profiles exhibited by steam exploded Douglas-fir compared to those exhibited by steam exploded sugarcane bagasse demonstrate that different types of biomass interact differently with each type of pre-treatment technology leading to possibly different types of by-products to be liberated. But characterization and analysis techniques such as LC-MS and HPLC will be required to analyze the different compounds and their amounts in these two types of biomass (feedstocks) pre-treated by the same technology to confirm the validity of this hypothesis.

6.6. Conclusions

The order of the inhibition strength of the lignocellulose degradation products to mannanolytic enzymes is lignin derivatives > organic acids > furan derivatives. This study could not provide insights on the half-lives of these enzymes in the presence of the inhibitory compounds, first order inhibition kinetics and also estimation of the irreversibility/reversibility of the inhibiting effect of the compounds. Synergism and cumulative concentration effects of these compounds were also not assessed in this study. But potential major biomass pretreatment by-products such as gallic acid were identified and the above mentioned studies could be pursued further on such inhibitors.

With respect to inhibition of the mannanolytic enzymes by lignin derived mono-phenolics, there appeared to be a relationship between the functional groups on the phenolic and enzyme-interfering capacity of the phenolic, with high hydroxyl group containing phenolics exhibiting the highest inhibition.

From this study it was also demonstrated that various pretreatment methods render different pre-treatment soluble by-products which interact in various ways with the mannanolytic

enzymes. It was also demonstrated that differences in the nature of the substrate (different plant species) would release different by-products that interact with the mannanolytic enzymes in a diverse manner even if the substrates are pre-treated with the same technology.

To achieve high levels of fermentable sugars, we propose that the removal of poly- and mono-phenols using methods such as extraction and detoxification using anhydrous ethanol, deionized water, activated charcoal and ethyl acetate, which will, in turn, preserve enzyme activity and enhance overall yields of these sugars.

Chapter 7: Synergism between mannanolytic enzymes and CTec2 for the hydrolysis of delignified sugarcane bagasse

7.1. Introduction

Sugarcane (*Saccharum officinarum*) is a perennial grass that is cultivated and harvested primarily for its sucrose content (Beukes and Pletschke, 2011). During sugarcane processing, the sugarcane stalk is crushed to extract the sucrose sap and sugarcane bagasse is the chief residue that remains afterwards (Beukes and Pletschke, 2011; Jabasingh and Nachiyar, 2011). Sugarcane bagasse is composed of cellulose fibres associated with lignin and a matrix of hemicellulose, thus it is lignocellulosic in its nature (Beukes and Pletschke, 2011; Chang, 2007; Siqueira et al., 2013).

It is estimated that approximately 100 million tons of dry sugarcane bagasse are produced globally every year (Li et al., 2014). Since the inception of the sugar industry, which is mainly built around sugarcane, sugarcane bagasse has been considered a great environmental hazard and a disposal problem with few beneficial uses in many parts of the world (Jabasingh and Nachiyar, 2011). However, due to its high polysaccharide content, sugarcane bagasse is a potential feedstock for the biotechnological production of biofuels and other value added commodity products (Pavón-Orozco et al., 2012).

The presence of lignin in lignocellulosic biomass such as bagasse is known to contribute to its recalcitrance and hinders the accessibility of cellulases to the substrate for hydrolysis (Siqueira et al., 2013). A pre-treatment method is therefore required to disrupt or remove the lignin content while keeping the cellulosic and hemicellulosic portions unchanged. Various pre-treatment technologies such as steam explosion, H_2SO_4 (Li et al., 2013) and alkaline (NH_4OH, lime, NaOH) (Beukes and Pletschke, 2011; Jabasingh and Nachiyar, 2011; Parvón-Orozco et al., 2012) have been applied and their effects studied, but few studies have focused on using sodium chlorite/acetic acid to oxidatively delignify bagasse.

Since sugarcane bagasse is lignocellulosic in its nature, its structural complexity necessitates a consortium of various lignocellulolytic enzymes to degrade it efficiently. Therefore, it is important to determine the enzymes and their suitable ratios that are required to efficiently and synergistically degrade sugarcane bagasse. Although it is a well-established phenomenon that cellulase action benefits from accessory enzymes during the hydrolysis of lignocellulose, the

synergy between cellulases and hemicellulases (specifically mannanase) has not been well studied (Pavón-Orozco et al., 2012).

The current investigation studied the potential synergy between a commercial cellulase preparation (Cellic® CTec2) and mannanolytic enzymes (α-galactosidase, Aga27A and β-mannanase, Man26A) during the hydrolysis of sodium chlorite/acetic acid delignified sugarcane bagasse.

7.2. Aims and Objectives

7.2.1. Aim

To establish synergistic associations between mannanolytic enzymes and CTec2 on delignified sugarcane bagasse hydrolysis.

7.2.2. Objectives

- To delignify sugarcane bagasse by sodium chlorite/acetic acid;
- To conduct microscopic analysis of natural and delignified bagasse;
- To characterize the components in natural bagasse and in delignified bagasse;
- To conduct enzyme substrate specificity studies using natural and delignified sugarcane bagasse;
- To conduct synergy studies between Man26A and CTec2 on delignified sugarcane bagasse;
- To conduct synergy studies between a mannanase cocktail (Man26A 75% to Aga27A 25%) and CTec2 on delignified bagasse;
- To compare synergism efficiency between Man26A and a mannanase cocktail with CTec2 on delignified sugarcane bagasse;
- To compare simultaneous versus sequential synergy efficiency on delignified sugarcane bagasse hydrolysis.

7.3. Materials and Methods

7.3.1. Delignification of sugarcane bagasse

Sugarcane bagasse obtained from Ushukela Milling (Pty) Ltd., Durban, South Africa was used in this study. A total of five grams of bagasse was treated using sodium chlorite/acetic acid for

up to 4 hours according to a modified protocol by Siqueira et al. (2013). For each gram of sugarcane bagasse, 0.3 g of sodium chlorite, 0.1 mL of anhydrous acetic acid and 32 mL of tap 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 further for 2 hours. Finally, the samples were removed from the bath, allowed to cool down to room temperature, and filtered using a cheese cloth and washed with a litre of deionized water.

7.3.2. Microscopic analysis of sugarcane bagasse

Various microscopic techniques were used to assess the impact of the sodium chlorite/acetic acid oxidative pre-treatment on sugarcane bagasse morphology and lignin content.

7.3.2.1. Scanning electron microscopy (SEM)

For scanning electron microscopy (SEM), the natural and delignified sugarcane bagasse were mounted on a metal stub, dried using critical point-drying process and coated with a thin layer of gold prior to SEM analysis (Cross, 2001).

7.3.2.2. Light microscopy (Histochemical assays for lignin)

7.3.2.2.1. Wiesner test

Prior to use, two parts of a 5% (w/v) phloroglucinol prepared in 95% (v/v) ethanol were mixed with one part of concentrated HCl and then applied to the natural and the delignified bagasse (Dashek et al., 1997; Tao et al., 2009). 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.

7.3.2.2.2. Mäule test

The plant tissues from natural and delignified sugarcane bagasse were immersed in 1% (w/v) potassium permanganate solution for 5 minutes. The samples were then rinsed thoroughly with water up until no potassium permanganate could be observable in the washing solution (Dashek et al., 1997). The dark red-brown colour produced by the presence of syringyl lignin from the staining procedure was visualized using an Olympus BX40 light microscope and photographed using an Olympus DP72 digital camera.

7.3.3. Substrate composition analysis

The sugarcane bagasse substrates were characterized using a modified sulphuric acid method by Sluiter et al. (2010) (National Renewable Energy Laboratory-NREL). About 300 mg of the samples (untreated and delignified bagasse) in glass test tubes were hydrolysed with 3 mL of 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 74 mL of 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^{o}C for 48 hours or until no change in the mass of the fraction was apparent. The acid soluble lignin present in the sugar solutions was detected by the Folin-Ciocalteu method for phenolics estimation. The presence of glucose, galactose, xylose and mannose was estimated through the use of Megazyme sugar kits. Cellobiose and arabinose were detected using a Shimadzu HPLC equipped with a Reflective Index detector and Shodex column (8.0 mm ID x 300 mm L, SP-0810, Japan) according to Abboo (2014). Reducing sugars were estimated using the DNS method as described before in Section 3.3.5.2.

7.3.4. Substrate specificity determination

Both untreated and delignified sugarcane bagasse were used to assess the specificity of each enzyme to be used for the synergy studies. The enzymes assessed were the commercial cellulase reparation, Cellic® CTec2, an *A. niger* mannanase, Man26A, and a mannanolytic binary enzyme cocktail [Man26A 75%: Aga27A 25%] (optimal ratio in galactomannan hydrolysis identified in Chapter 5). The enzymes were loaded at a protein loading of 2.25 mg per gram of substrate. The experiments were conducted according to the protocol described in Section 3.3.5.1. The reactions were finally terminated after 24 hours of incubation and reducing sugar release quantified using the DNS assay.

The enzyme assays were performed in triplicate, and the activities were expressed in units (U), where 1 unit was defined as the quantity of enzyme required to release 1 µmol of reducing sugar per hour, with glucose and mannose as the reducing sugar equivalents.

7.3.5. Synergism between CTec2 and mannanolytic enzymes on delignified bagasse

The binary combination studies between two enzymes was at a protein ratio of CTec2 to Man26A or [Man26A 75%: Aga27A 25%] at 75:25% total protein loading. This contained a

total protein loading of 2.25 mg per gram of delignified bagasse. The commercial β-glucosidase preparation, Novozyme 188 was also loaded at 10% total protein loading (0.225 mg protein per gram of substrate) in all the reaction mixtures. Samples were taken after 24, 48 and 96 hours. The hydrolysed samples were assayed for reducing sugar release using the DNS assay as described in Section 3.3.5.2. The efficient combination was then assessed for sequential application via pre-treatment of the substrate for 24 hours by CTec2 and then adding the mannanolytic enzyme(s) during hydrolysis for a total period of 48 hours. This was conducted in parallel with a simultaneous addition of the enzyme cocktail and compared.

The degree of synergy that was obtained in the synergy assays was calculated by dividing the sum of the actual activities of the enzymes obtained with the delignified bagasse samples, by the theoretical sum of the individual enzyme activities.

7.4. Results

7.4.1. Delignification of sugarcane bagasse

This study made use of sodium chlorite/acetic acid delignification to remove the majority of the lignin present in sugarcane bagasse. Untreated bagasse normally displays a dark brown colour, but after the delignification the bagasse was converted to an off-white colour. This was visual evidence that a majority of the lignin was removed from the substrate. Similar results with respect to visual changes on substrates subsequent to bleaching were obtained on the delignification of lignin coated cellulose by sodium chlorite/acetic acid delignification in a study conducted by Hubbell et al. (2010). Figure 7.1 displays untreated bagasse and sodium chlorite/acetic acid delignified bagasse.

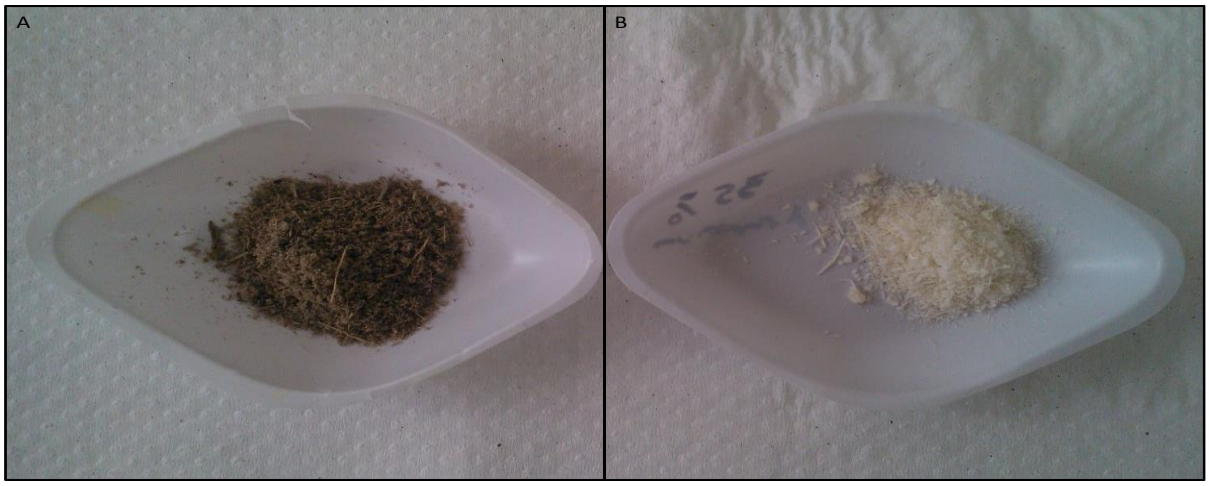

Figure 7.1. An illustration of the observable differences between (A) untreated sugarcane bagasse and (B) sodium chlorite/acetic acid pre-treated sugarcane bagasse.

From Figure 7.1, it can be observed that the natural untreated sugarcane bagasse has a dark brown hue attributable to the presence of lignin, while the pre-treated sugarcane bagasse exhibits a white colour attributable to a high polysaccharide content and the removal of lignin.

7.4.1.1. Microscopy

7.4.1.1.1. Scanning electron microscopy (SEM)

The natural untreated bagasse and delignified bagasse samples were mounted onto metal stubs and coated with a thin layer of gold under vacuum. The samples were then analysed under various magnifications using SEM (Fig. 7.2).

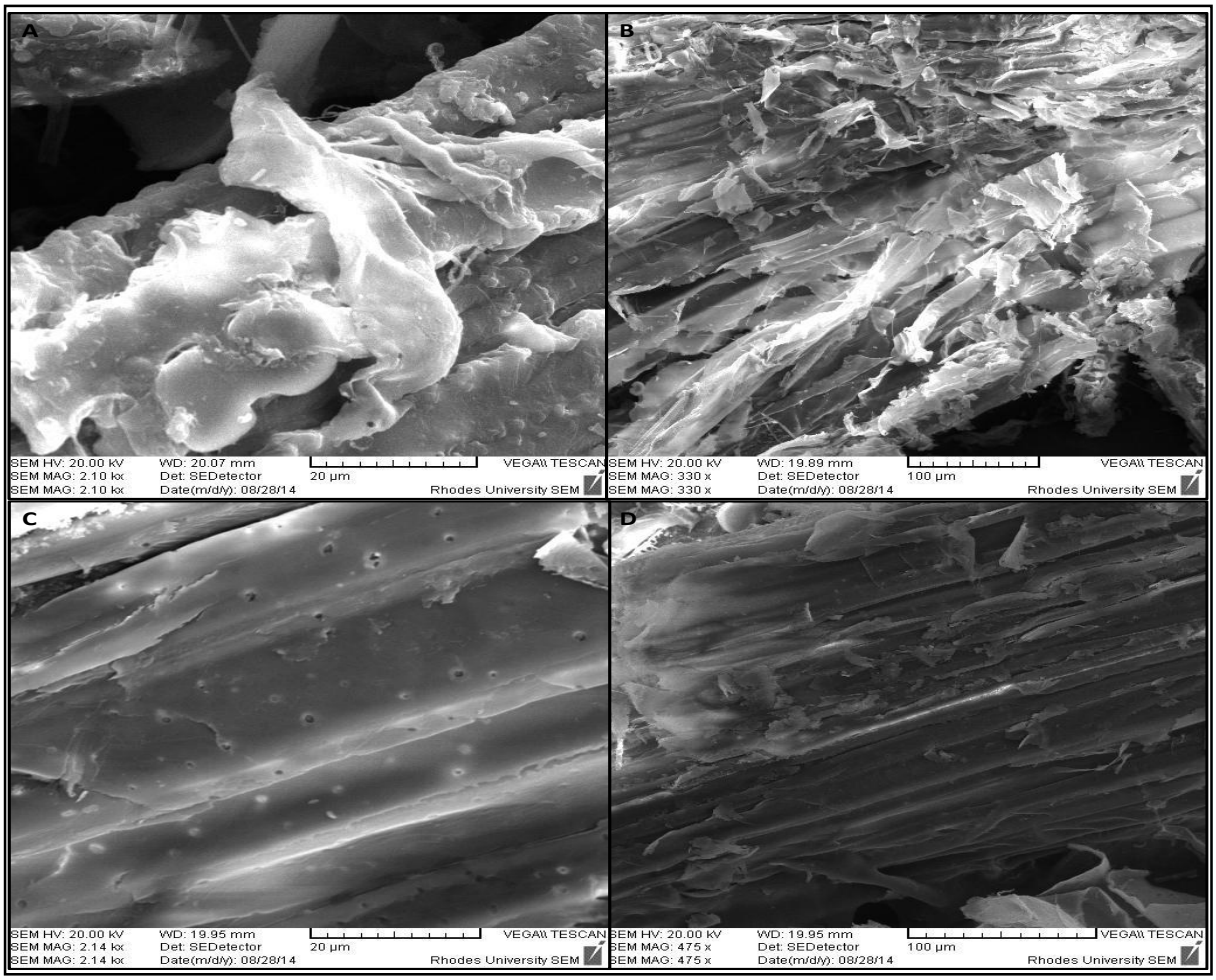

Figure 7.2. SEM analysis of untreated sugarcane bagasse at (A) 20 µm and (B) 100 µm, and sodium chlorite/acetic acid delignified sugarcane bagasse at (C) 20 µm and (D) 100 µm. Bars: (A and C is 20 µm, 2.1 kx mag) and (B and D is 100 µm, 400 x mag).

Based on the SEM images shown in Fig 7.2, it can be observed that the untreated sugarcane bagasse possesses matrices which appears to be coating the cellulose fibrils (Figs. 7.2A and B), while the sodium chlorite/acetic acid delignified sugarcane bagasse exhibits smooth and bare fibrils that are without any matrices coating them. The delignified fibrils exhibited a presence of pores on their surfaces when viewed at high magnification (Fig. 7.2C). In summary, there was a prominent morphological change in the bagasse structure due to the delignification.

7.4.1.1.2. Light microscopy

After the sugarcane bagasse was pre-treated, the untreated and delignified samples were stained for detecting the presence of lignin. The light microscope images taken on the sodium chlorite/acetic acid pre-treated sugarcane bagasse showed that the method effectively bleached lignin off the substrate, which is reflected in the decrease in magenta stain observed on the

Wiesner stained samples and a decrease in dark red-brown stain observed on Mäule stained samples (Fig. 7.3).

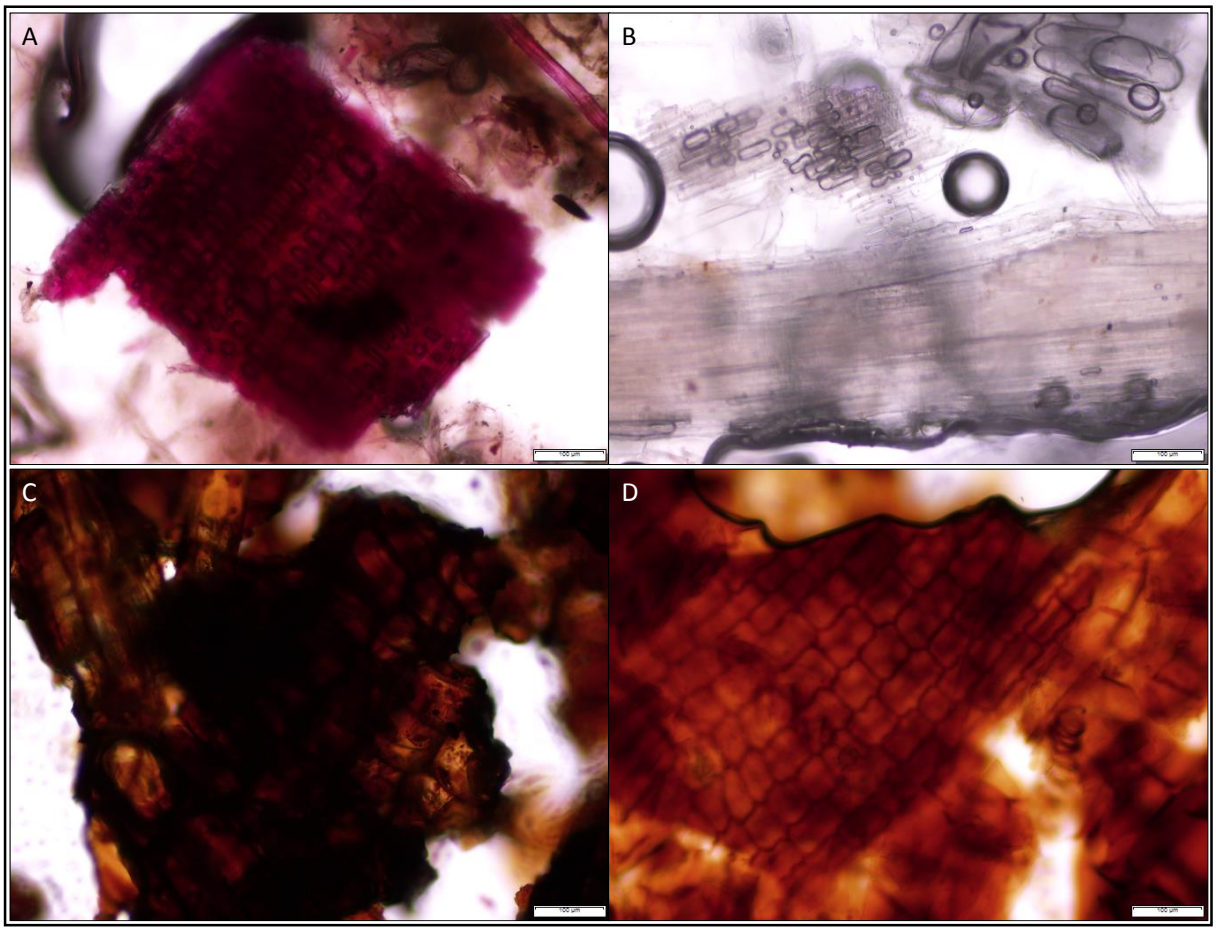

Figure 7.3. Histochemical localization of cinnamyl lignin moieties in (A) untreated and in (B) delignified sugarcane bagasse by Weisner method (Phloroglucinol-HCl). Histochemical localization of syringyl lignin moieties in (C) untreated and in (D) delignified sugarcane bagasse by Mäule method (Potassium permanganate). Bars: 100 µm, 10 x mag.

Both the Weisner and Mäule lignin staining procedures demonstrated that sodium chlorite/acetic acid oxidative pre-treatment was successful in delignifying sugarcane bagasse.

7.4.2. Substrate composition analysis

In order to determine the efficiency and the effect of the sodium chlorite/acetic acid delignification pre-treatment method on sugarcane bagasse, the determination of the chemical composition of the different samples was essential (Tables 7.1 and 7.2).

Table 7.1. Chemical composition of the sugarcane samples.

Content (mg)	Delignified SCB (mg/ 300 mg biomass)	Untreated SCB (mg/ 300 mg biomass)
Total reducing sugars[a]	**197.78**	**162.67**
Glucose[b]	91.58	80.53
Cellobiose[c]	-	-
Xylose[b]	69.46	41.79
Mannose + Fructose[b]	61.05	49.15
Galactose[b]	24.29	17.46
Arabinose[c]	17.66	16.10
Soluble phenolics[d]	2.82	2.39
Insolubles (Lignin + Ash)[e]	50.29	143
Total mass/ 300 mg biomass	314.33	348.03

[a]-DNS method, [b]-Megazyme sugar kits, [c]-HPLC, [d]-Folin-Ciocalteu method, [e]-Weighing balance

The composition for total sugars and insolubles (Acid insoluble lignin + Ash) for untreated sugarcane bagasse were 59% and 41%, while for sodium chlorite/acetic acid delignified bagasse they were 84% and 16%, respectively. The acid soluble lignin content in both sugarcane bagasse substrates (untreated and delignified) was comparable.

7.4.3. Substrate specificity determination

The specific activities of the enzymes used in this study were determined on untreated and on delignified sugarcane bagasse. If the sodium chlorite/acetic acid pre-treatment performed in this study was effective, then an enhancement of enzyme activity should be observed on the pre-treated substrate in relation to the untreated substrate (Table 7.2).

Table 7.2. Substrate specificity determination of CTec2 and Man26A on bagasse and sodium chlorite pre-treated bagasse.

Enzyme	Specific activity (U/mg protein)	
	Untreated bagasse	**$NaCl_3$ pre-treated bagasse**
Cellic® CTec2	1.56	18.64
Man26A	0.00	0.52
[Man26A75%:Aga27A25%]	0.00	0.51

The delignified bagasse was more easily hydrolysed by the enzymes compared to the lignin rich untreated bagasse. This finding agrees with previous reports that classify lignin as one of the most important components in preventing the efficient hydrolysis of polysaccharides in the cell walls of lignocellulosic materials (Siqueira et al., 2013).

7.4.4. Synergism between CTec2 and mannanolytic enzymes on delignified bagasse

Synergy between Man26A or [Man26A 75%: Aga27A 25%] and CTec2 was determined by measuring the reducing sugars released during hydrolysis of delignified sugarcane bagasse. The β-glucosidase, Novozyme 188 was added in all reactions to avoid potential CTec2 inhibition by cellobiose. In the enzyme reaction controls, Novozyme 188 on its own did not liberate any reducing sugars from the delignified bagasse.

Considerable conversion of cellulose to glucose equivalents was reached within 24 hours of hydrolysis when the cellulase preparation was used on its own (Fig. 7.4). This kinetic behaviour showed rapid cellulose hydrolysis within the first hours of the reaction, followed by a significant decrease in the reaction rate. Easily hydrolysable fractions are digested by cellulase first, whereas the remaining cellulose is less accessible to enzymes due to hemicellulose intertwining the cellulose (Hu et al., 2011; Pavón-Orozco et al., 2012).

However, in the reaction that contained a binary mixture of cellulase and mannanase (Man26A), the rate of hydrolysis was still high after 24 hours and started decreasing only after 48 hours of hydrolysis (Fig. 7.4). We propose that the removal of the little amount of mannan intertwined with cellulose allowed cellulase accessibility to new hydrolysis sites.

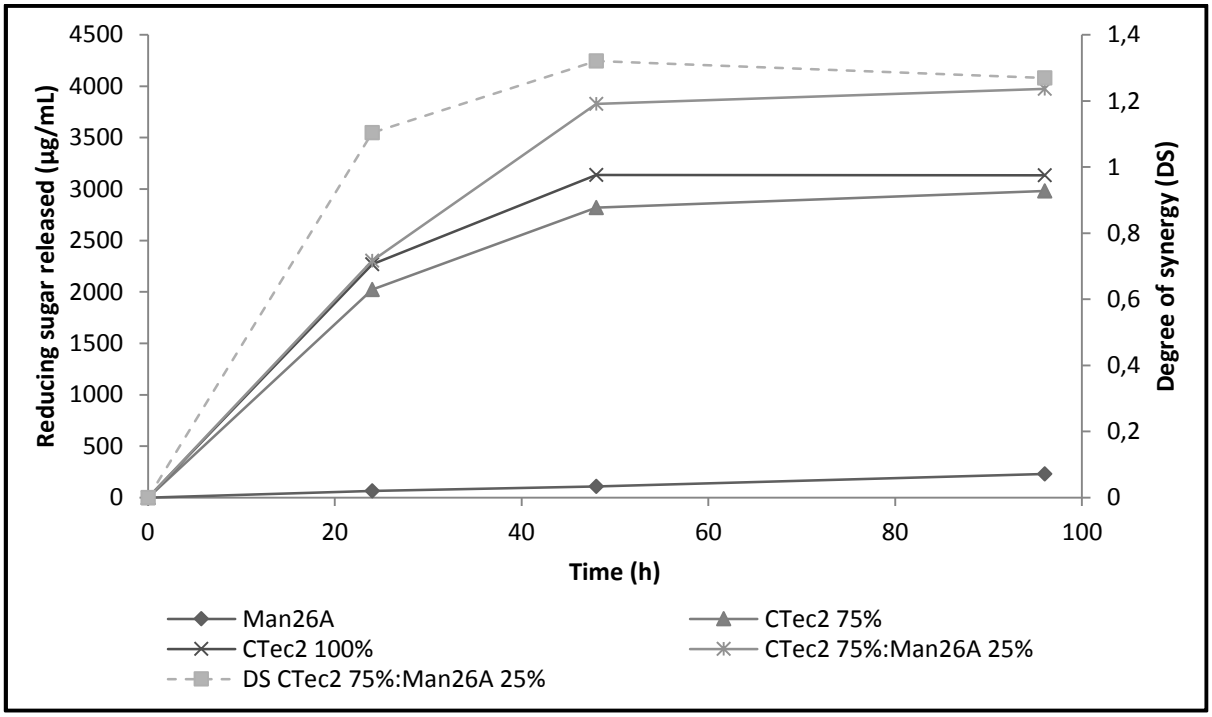

Figure 7.4. Synergism between Man26A and CTec2 on delignified sugarcane bagasse. Synergy quantified by reducing sugar release.

Comparison of synergism efficiency between Man26A and the binary mannanolytic enzyme cocktail with CTec2 on delignified sugarcane bagasse was conducted and is illustrated in Figure 7.5. The enzyme cocktail, [Man26A75%:Aga27A25%], exhibited higher reducing sugar release and synergism with CTec2 compared to that displayed by combining only Man26A and CTec2 on delignified sugarcane bagasse hydrolysis.

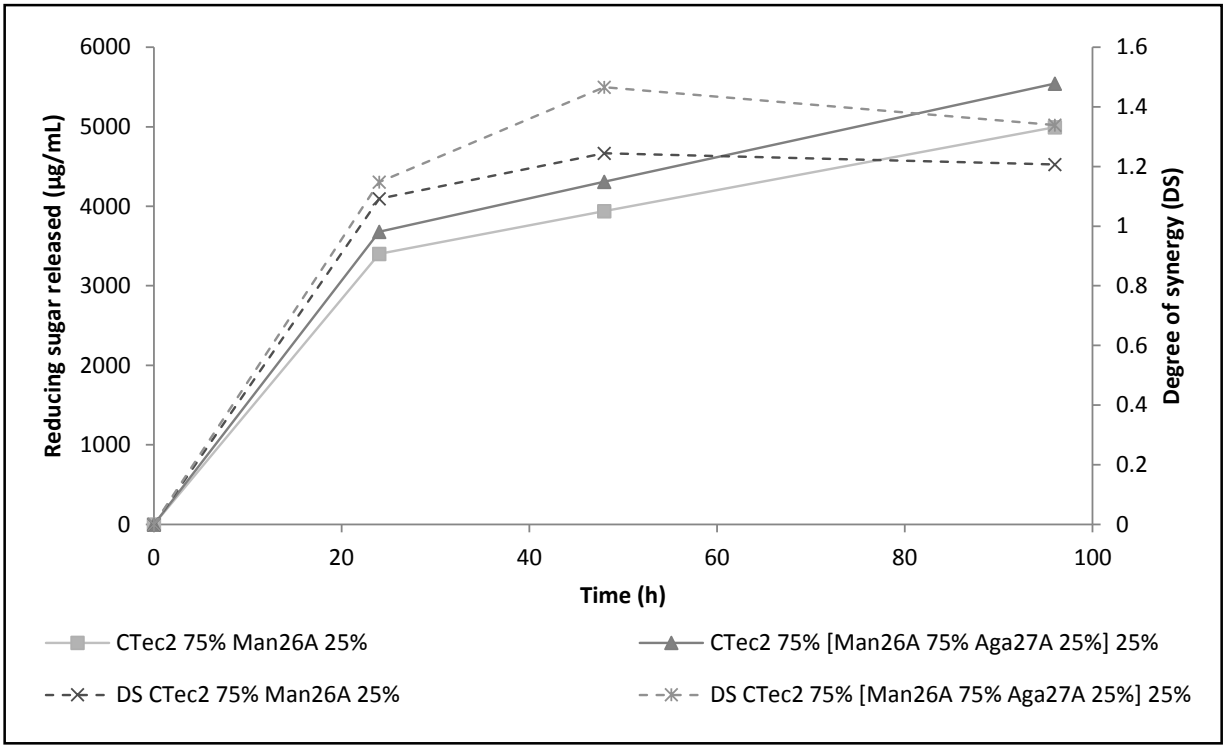

Figure 7.5. Comparison of synergism efficiency between Man26A and a mannanolytic enzyme cocktail, [Man26A75%:Aga27A25%] with CTec2 on delignified sugarcane bagasse.

The degree of synergy exhibited by the two combinations assessed, CTec2 75% to Man26A 25% and CTec2 75% to [Man26A75%:Aga27A25%] 25%, was evaluated after 24, 48 and 96 hours. In both cases, the degree of synergy was the lowest at the initial stages of hydrolysis i.e. 24 hours, but gradually increased over time. The degree of synergy appeared to be at its highest at 48 hours and then gradually decreases when the hydrolysis reached 96 hours. The CTec2 75% to [Man26A75%:Aga27A25%] 25% combination exhibited higher degrees of synergy compared to the CTec2 75% to Man26A 25% binary combination through-out the entire course of hydrolysis. Figure 7.6 and Figure 7.7 below demonstrate the various extents of delignified bagasse solubilisation by the various enzyme applications using SEM micrographs and photography of the actual hydrolysates after 96 hours of incubation, respectively.

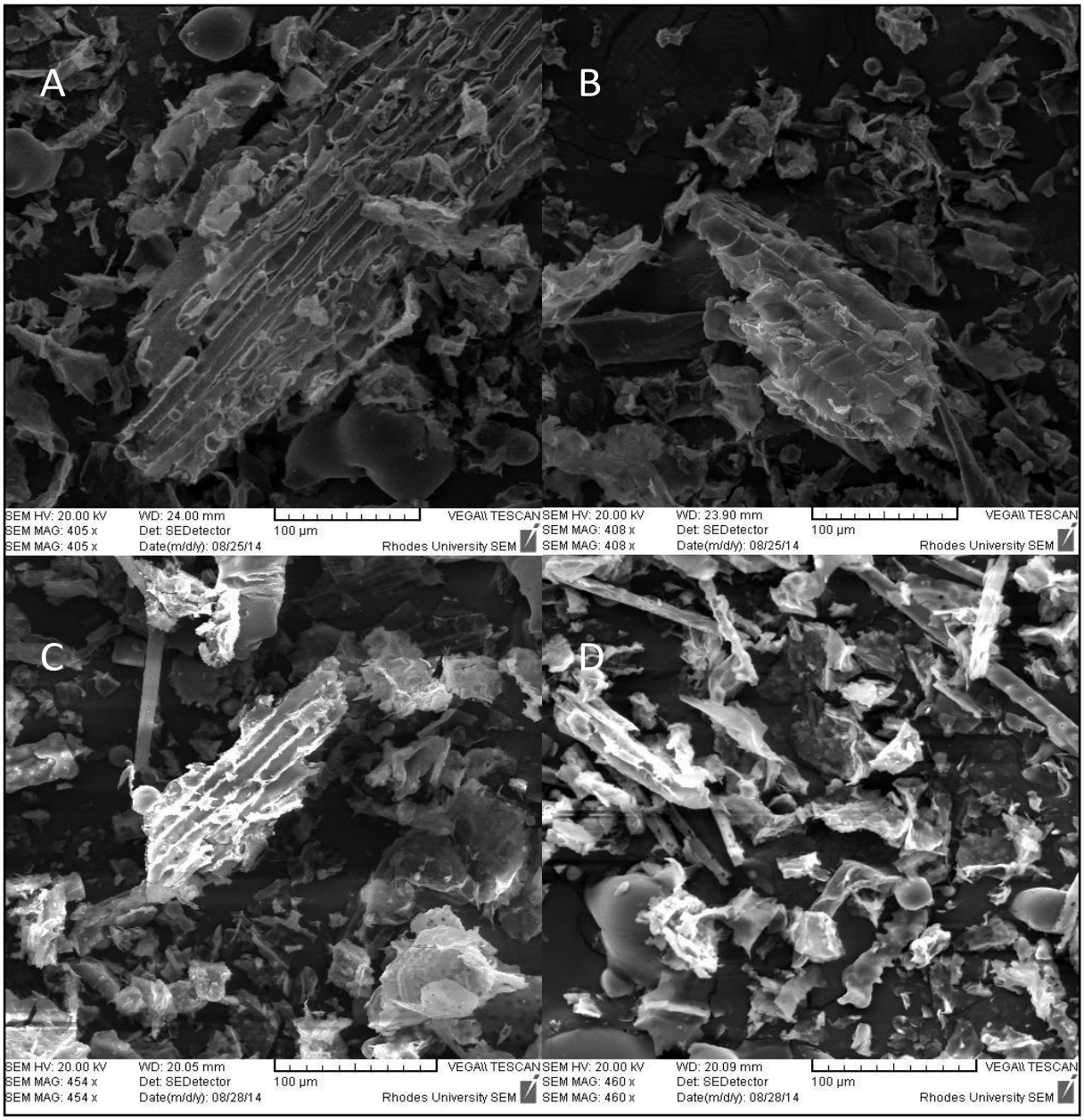

Figure 7.6. SEM analysis of (A) delignified sugarcane bagasse at 100 µm, (B) [Man26A 75%: Aga27A 25%] hydrolysed delignified sugarcane bagasse 100 µm, (C) CTec2 hydrolysed delignified sugarcane bagasse 100 µm and (D) CTec2 and [Man26A 75%: Aga27A 25%] hydrolysed sugarcane bagasse 100 µm, 400 x magnification.

Results from SEM analysis showed that treatment of the delignified bagasse with only the mannanolytic enzyme cocktail had no effect on the size of the fibres in the substrate (Figs. 7.6A and B), while treatment with CTec2 greatly reduced the biomass fibres size (Fig. 7.6C). Synergistic application of the mannanolytic enzyme cocktail with CTec2 had an even greater effect in decreasing the size of the biomass fibres (Fig. 7.6D). Figure 7.7 below illustrates the physical extent of solubilisation of delignified sugarcane bagasse by the various enzyme cocktails used.

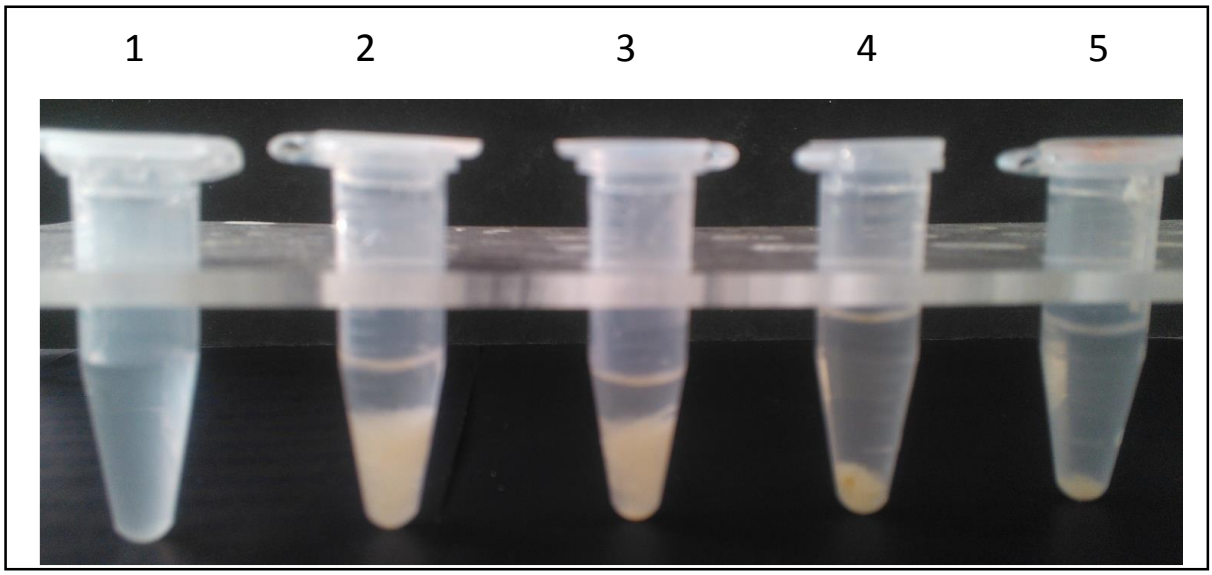

Figure 7.7. Visual inspection of (1) citrate buffer (control), (2) 1% (w/v) delignified sugarcane bagasse, (3) [Man26A 75%: Aga27A 25%] hydrolysed delignified sugarcane bagasse, (4) CTec2 hydrolysed delignified sugarcane bagasse and (5) CTec2 and [Man26A 75%: Aga27A 25%] hydrolysed sugarcane bagasse. Photographs taken after 96 hours of hydrolysis.

The greater the extent of delignified sugarcane bagasse hydrolysis the greater the solubilisation of the biomass in solution (Fig. 7.7). Based on SEM imaging, the size of the fibres decreased with a higher extent of hydrolysis, thus leading to a greater degree of solubilisation of the biomass in solution. Table 7.3 below illustrates the change in the degree of synergy between mannanolytic enzymes and CTec2 over time during the hydrolysis of delignified sugarcane bagasse.

Table 7.3. Degree of synergy between CTec2 and mannanolytic enzymes during delignified sugarcane bagasse hydrolysis.

Time (hours)	**Degree of synergy[a]**		
	C 100[b]	**C 75[b]:M 25[c]**	**C 75[b]:[M75: A25] 25[c]**
24	1	1.10	1.15
48	1	1.29	1.46
96	1	1.24	1.34

[a]- *Degree of synergy was calculated as the ratio of the activities of the enzymes in the hydrolysis when they are together divided by the sum of the individual activities used*

[b]- *Protein ratio of CTec2 used in the hydrolysis mixture*

[c]- *Protein ratio of mannanolytic enzyme(s) used in the hydrolysis mixture*

7.4.5. Sequential application of CTec2 and the binary mannanolytic cocktail on delignified sugarcane bagasse

Sequential synergism between CTec2 and [Man26A 75%:Aga27A 25%] at 75%:25% protein ratio on delignified bagasse hydrolysis over 48 hours was conducted and compared to simultaneous application of the enzymes (Fig. 7.5). Sequential application of CTec2 and the binary mannanolytic cocktail on delignified sugarcane bagasse was conducted as described in Section 7.3.3 and compared to simultaneous application of these enzymes (Fig 7.8).

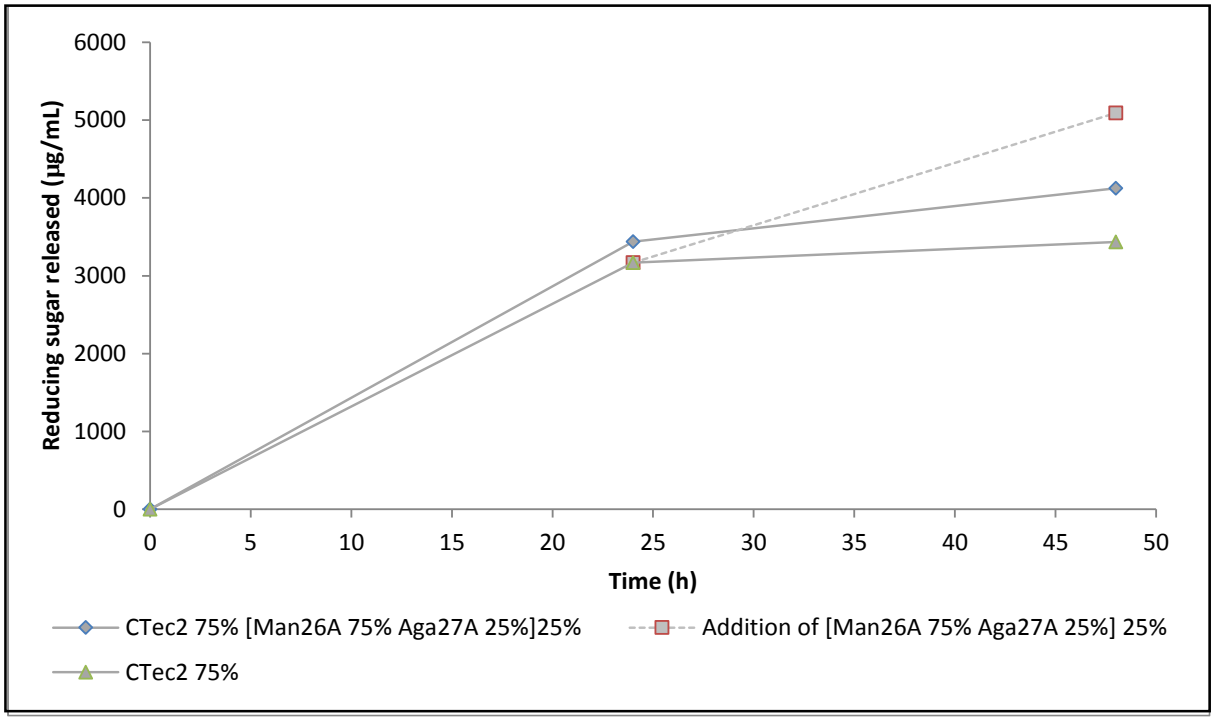

Figure 7.8. Comparison of synergism efficiency between sequential and simultaneous application of a mannanolytic enzyme cocktail, [Man26A75%:Aga27A25%], with CTec2 on delignified sugarcane bagasse.

Sequential application of the mannanolytic enzyme to CTec2 cocktail proved to be more synergistic compared to applying this cocktail in a simultaneous fashion. Reducing sugar content realized in 96 hours by the simultaneous cocktail (Fig. 7.5) were obtained within 48 hours by the sequentially applied mannanase to cellulase cocktail (Fig 7.8). The degree of synergy between CTec2 and [Man26A 75%: Aga27A 25%] was improved by approximately 0.3 units when the enzymes were applied sequentially compared to their simultaneous application.

7.5. Discussion

7.5.1. Substrate composition and substrate specificity determination

SEM micrographs exhibited a change in the surface morphology of the sugarcane bagasse after pre-treatment, with the fibre surfaces becoming smoother and porous after delignification (Fig. 7.2). The sodium chlorite/acetic acid pre-treatment was successful at delignifying sugarcane bagasse, thus decreasing substrate recalcitrance and improving enzyme activities on bagasse hydrolysis. The possible reason for the increase in enzyme activity on the delignified bagasse could be due to the removal of lignin (approximately 60-65% of acid insoluble lignin was removed) which hinders polysaccharide accessibility and also non-specifically adsorbs enzymes. The other reason for increased activity could be the increase in the relative cellulose and hemicellulose content (58% polysaccharide content in untreated bagasse and 83% polysaccharide content in delignified bagasse). This decreases the likelihood of the enzymes non-specifically and non-productively binding to other components of the biomass besides polysaccharides. The amount of lignin removed by this severity of delignification of sugarcane bagasse using sodium chlorite/acetic acid, and reported in this study, was similar to that reported by Siquera et al. (2013) at the same severity, which was 63.2% of lignin extraction during delignification of sugarcane bagasse.

7.5.2. Synergism between CTec2 and mannanolytic enzymes on delignified bagasse

There are two different types of synergism reported between hydrolytic enzymes, the synergism which occurs between enzymes that hydrolyse a single type of a polymer such as that between a galactosidase, mannosidase and mannanase on galactomannan hydrolysis, and this is termed intra-molecular synergy (van Dyk and Pletschke, 2012). There is also synergism that occurs between enzymes that hydrolyse different polymers that are associated with each other such as that between xylanases and cellulases on lignocellulose hydrolysis, and this is called inter-molecular synergy. In this study, inter-molecular synergism and a combination of both intra-molecular and inter-molecular synergistic hydrolysis of delignified sugarcane bagasse were evaluated.

Since mannan interacts with cellulose and is believed to be intertwined among the cellulose fibers in plant cell walls, it needs to be disrupted in order to expose the cellulose fibers to cellulases thus enhancing their activity (Banerjee et al., 2010; Várnai et al., 2011). The synergistic effect between mannanolytic enzymes and cellulase can be attributed to the removal

of the mannan polysaccharide, creating more binding sites for cellulases to act on. Other studies have also reported that the removal of polysaccharide coating and intertwined with cellulose by their respective accessory enzymes stimulated cellulose hydrolysis by cellulases (Berlin et al., 2007; Li et al., 2014a; Visser et al., 2013). In our study, the rate of reducing sugar release increased when the binary cellulase to mannanolytic enzyme(s) mixture was used compared to when a cellulase preparation (CTec2) was used on its own on delignified sugarcane bagasse hydrolysis (Figs. 7.4 and 7.5). This showed that cooperation between cellulolytic enzymes and mannanolytic enzymes is required to effectively unravel and degrade lignocellulosic biomass.

The [Man26A 75%: Aga27A 25%] enzyme cocktail liberated higher levels of reducing sugar and demonstrated a high degree of synergy compared to the reducing sugar and degree of synergy exhibited when only Man26A was used at the same protein loading with the commercial cellulase, CTec2, on delignified sugarcane bagasse hydrolysis. Our results show that the action of Aga27A facilitated the action of Man26A, presumably as a result of cleavage of galactose side-chain groups from galactoglucomannan that would have hindered the mannanase. This, in turn, led to a higher extent of galactoglucomannan hydrolysis in the presence of Aga27A, which in turn exposes more sites for the cellulase, CTec2, to act on compared to when only Man26A was used.

A study by Clarke et al. (2000) demonstrated that the combination of a *P. fluorescens* ManA and a *C. thermocellum* XylA was significantly more effective than using a *C. thermocellum* ManA on softwood paper pulp. Also, incubation of pulp samples with both enzymes plus a galactosidase, AgaA, resulted in the largest enhancement of bleach-ability, and was accompanied by an increase in the observed total reducing sugar release (Clarke et al., 2000). These data suggested that the activity of the α-galactosidase enhanced the mannanase activity, and that a combination of these two enzymes is able to enhance the activity of XylA.

At initial incubations (24 and 48 hours) synergy increased from 1.10 and 1.15 reaching up to 1.29 and 1.46 for CTec2 75% to Man26A 25% and CTec2 75% to [Man26A 75%: Aga27A25%] 25%, respectively (Table 7.2). This trend with respect to synergy between hemicellulases and cellulases on bagasse hydrolysis is contrary to observations reported by previous studies (synergy was initially high and then decreased over prolonged incubation) (Beukes and Pletschke, 2010; Li et al., 2014b; Pavón-Orozco et al., 2012). At prolonged incubations (48 and 96 hours) synergy slightly decreased to values reaching up to 1.24 and 1.34 for CTec2 75% to Man26A 25% and CTec2 75% to [Man26A 75%: Aga27A 25%] 25%,

respectively (Table 7.3). An increase in the degree of synergy towards the late stages of hydrolysis has been reported in literature in the hydrolysis of steam exploded sugarcane bagasse (Li et al., 2014a). This phenomenon has also been demonstrated in the hydrolysis of mildly steam exploded corn stover by Zhang et al. (2014). The enhanced and high synergy at mid-to-late stages of incubation evident in this study represents a new interesting finding. We propose that the low degree of synergy observable at the initial stages of hydrolysis is due to the substrate being compact and intertwined; this limits the hydrolysis to the enzymes that have cleavage sites available. But as hydrolysis proceeds over time, the substrate opens up and more binding sites are available for enzymes to act upon and this leads to an increase in synergy. Finally, a state where the substrate is fully accessible is reached towards the end of hydrolysis and at this stage the enzymes don't rely on each other for hydrolysing their respective subsites and synergy decreases. The results show that hydrolysis time is one of the key factors that affect the degree of synergy between enzymes (van Dyk and Pletschke, 2012).

Sequential application of CTec2 to the [Man26A 75%: aga27A 25%] combination was conducted to explain the likely mechanism behind the cooperative interaction between both types of enzymes on delignified bagasse hydrolysis (Fig. 7.8). It was apparent that sequential addition of the enzymes during the degradation of the cellulose and mannan fractions reached completion within 48 hours, while simultaneous application of the enzymes took 96 hours to reach the same level during sugarcane bagasse hydrolysis. After 48 hours, no further reducing sugar release was observed when the enzymes were applied sequentially (data not shown). Hydrolysis of the delignified bagasse by Man26A alone or with the mannanolytic enzyme cocktail liberated less than 0.2 mg reducing sugars per 10 mg of biomass (Fig 7.4). It is likely that the readily accessible mannan on the fibre surfaces of cellulose is minimal, while a substantial amount of mannan is that which is buried and intertwined in-between the fibres. To reach the mannan that is more closely associated with the cellulose and intertwined within the cellulose fiber structure, we postulate that the synergistic interaction between mannanolytic enzymes with CTec2 is required for more extensive mannan degradation thus the observed improvement in reducing sugar release (Figs. 7.4, 7.5 and 7.8). The lower synergistic association observed when the enzymes are applied simultaneously compared to sequential application of the enzymes could be due to enzyme deactivation of the mannanolytic enzymes during the lengthy incubation periods. In Section 4.4.3, it is reported that Aga27A and Man26A have half-lives of about >24 hours and 24 hours, respectively. Simultaneous application of the enzymes is likely to lead to thermal deactivation of these enzymes as they will not be highly

active during the first 24 hours of hydrolysis since only about 0.2 mg of substrate is available to them for hydrolysis and no substantial buried mannan would have been released from within the fibres by CTec2 by this time.

It is noteworthy that the holocellulose in the form of glucose, galactose, fructose and mannose constitutes about 55-59% of the overall delignified sugarcane bagasse biomass (5.5-5.9 mg/10 mg of biomass). Therefore, synergistic application of the mannanolytic enzyme cocktail with CTec2 was successful at degrading delignified sugarcane bagasse as yields above 80% were achieved (approximately 5.0 mg reducing sugars released per 10 mg of biomass).

7.6. Conclusions

This chapter described and discussed the delignification of sugarcane bagasse using sodium chlorite/acetic acid oxidative pre-treatment and its synergistic hydrolysis using CTec2 and a mannanolytic enzyme cocktail optimized on galactomannan hydrolysis in Chapter 5. The [Man26A 75%: Aga27A 25%] enzyme cocktail synergistically interacted better than the use of Man26A at the same protein dosage with the commercial cellulase, CTec2, on delignified sugarcane bagasse hydrolysis. It was also demonstrated that hydrolysis time is important for investigating the degree of synergy. Sequential application of these enzymes whereby CTec2 was used as a pre-treatment enzyme, followed by the addition of the mannanolytic enzyme cocktail, rendered higher synergism compared to simultaneous application of the same enzyme during the hydrolysis of delignified sugarcane bagasse. In this study, a rationally designed mannanolytic enzyme cocktail was found to facilitate the total hydrolysis of biomass and can be used in the optimisation of commercial or purified enzyme mixtures to improve the economic viability of the conversion of biomass to bioethanol.

Chapter 8: General discussion, conclusions and future recommendations

8.1. General discussion and Conclusions

A number of mannan-containing lignocellulosic substrates are available globally for use as feedstocks in the biomass-to-bioethanol process and for the production of other value added products (Yamabhai et al., 2014). These feedstocks include endosperm walls of seeds such as coconut, coffee bean, locust bean or the vacuoles in vegetative tissue of plants such as konjac, ivory nut and guar; softwoods such as pine, spruce, and douglas-fir; and agricultural residues such as sugarcane bagasse and sugar beet (Moreira and Filho, 2008; Yamabhai et al., 2014). This study first investigated the behavior of mannanolytic enzymes, specifically focusing on their synergistic associations with enzymes from different glycoside hydrolase (GH) families in order to design a mannanolytic enzyme cocktail. The use of a rationally designed synergistic binary mannanolytic enzyme cocktail consisting of an α-galactosidase, Aga27A and a β-mannanase, Man26A to synergistically interact with a commercial cellulase preparation (Cellic® CTec2) to hydrolyse a complex mannan-containing lignocellulosic substrate (delignified sugarcane bagasse) was also investigated in this study.

The presence of hemicellulose (mannan) in lignocellulosic biomass is thought to act as a barrier to cellulose fibrils limiting access of cellulases to their substrate and thus cellulose hydrolysis (Banerjee et al., 2010; van Dyk and Pletschke, 2012; Várnai et al., 2011). Hemicellulose is suspected to be intertwined around cellulose fibrils and hampers cellulose hydrolysis, but its polysaccharides and oligomers have also been reported to inhibit the action of cellulases on cellulose hydrolysis (Kumar and Wyman, 2011 and 2014). Therefore, the depolymerisation of hemicellulose is not only necessary for providing additional fermentable sugars, but also for improving cellulose hydrolysis by cellulases and alleviating cellulase inhibition by higher hemicellulose polymers and oligomers released during lignocellulose hydrolysis.

Most of the research performed on lignocellulosic biomass has focused on the utilization of both the cellulose and xylan portion of the lignocellulosic biomass for bioethanol production. As a result, the mannan component is usually disregarded as a source of fermentable sugars. Therefore, this study investigated the following aspects:

1. Elucidation of how the GH family classification of a mannanolytic enzyme affects its substrate specificity and synergistic interactions with other mannanolytic glycoside

hydrolases from different GH families-more so than its EC classification. Then using this method of screening for maximal compatibility and cooperation between various GH families to design a rational tailored mannanolytic cocktail for mannan-rich lignocellulose degradation.

2. Determining the individual inhibitory properties of each of the selected pre-treatment by-products and the effect that washes from substrates pre-treated by various technologies have on mannanolytic enzymes.
3. Studying the potential synergy between a commercial cellulase preparation (Cellic® CTec2) and the rationally designed binary mannanolytic enzyme cocktail during the hydrolysis of sodium chlorite/acetic acid delignified sugarcane bagasse.

The expression and purification of a *C. cellulovorans* GH5 β-mannanase, ManA, was successfully accomplished and was comparable to previous studies expressing this very same construct using *E. coli* as a host (Beukes et al., 2011; Morrison et al., 2013; Olver et al., 2011). In addition, the specific activity of the purified fraction of the enzyme (9.25 U/mg protein) was comparable to a previous study assessing the specific activity of the very same enzyme (12.90 U/mg protein) (Tamaru and Doi, 2000). This is the only enzyme that was expressed and purified in this study as there were no GH5 mannanases commercially available at the time.

Following the production of ManA, the enzyme was biochemically characterized with the other mannanolytic enzymes used in this study (which were purchased from commercial suppliers). The enzymes that were biochemically characterized for the purpose of this study included: an *A. niger* α-galactosidase (AglC, GH36) and a guar seed (*C. tetragonoloba*) α-galactosidase (Aga27A, GH27), an *A. niger* β-mannanase (Man26A, GH27) and a *C. cellulovorans* β-mannanase (ManA, GH5), and a *C. mixtus* β-mannosidase (Man5A, GH5) and a *B. thetaiotaomicron* β-mannosidase (Man2A, GH2). The enzymes used in this study were selected based on their GH family affiliations and their commercial availability.

The results obtained from the biochemical characterisation of the mannanolytic enzymes are summarized in Table 4.4 of Chapter 4. In summary, the enzymes were found to have pH optima in the range of 4.5-7.0, variable temperature optima and displayed high thermal stabilities at 37^{o}C compared to 50^{o}C. The various GH family enzymes were then tested for their respective specific activities on different mannan-based substrates at 37^{o}C using 50 mM sodium citrate buffer at pH 5.0 since these conditions assured fairly good and stable activity for all the enzymes assessed in this study (no denaturation).

From the substrate specificity studies it was found that the GH27 galactosidase, Aga27A, was more active on polymeric substrates (guar gum and locust bean gum) and galactose-containing oligomers compared to the GH36 galactosidase, AglC. It was also determined that the GH27 galactosidase preferred internally linked galactose substituents that were grouped next to each other, while the GH36 preferred lone and/-or terminal galactoses as substrates. The variation in substrate specificities among these two galactosidases was attributed to differences in enzyme structures and sizes (Ademark et al., 2001b; Kaneko et al., 1990). The GH2 mannosidase, Man2A, exhibited lower activity on both polymeric galactose-containing substrates and on galactose-containing oligomers compared to the GH5 mannosidase, Man2A. With regards to galactomannan hydrolysis, both the GH5 mannanase, ManA, and the GH26 mannanase, Man26A, exhibited higher activity on locust bean gum, a randomly and low galactose substituted substrate compared to guar gum, a block-wise and ordered highly galactose substituted substrate. These findings appeared to support the hypothesis that the enzyme activity of a mannanase is limited by the extent and pattern of D-galactosyl substituents on the mannan backbone (Yoon et al., 2008, Zhao et al., 2011). Both β-mannanases did not exhibit activity on the galactose decorated oligomers used in this study.

Clear synergistic interactions between β-mannanases, α-galactosidases and β-mannosidases were demonstrated. Among the homeosynergistic associations, the family 5 mannosidase, Man5A, at a 25% protein ratio, exhibited the highest synergy and reducing sugar release with either one of the β-mannanases (ManA or Man26A) at a 75% protein ratio. We propose that the observed synergy between these classes of enzymes (EC 3.2.1.78 and EC 3.2.1.25) is as a result of the high content of small fragment-products released by the high protein ratio of β-mannanases, such as manno-oligosaccharides with non-reducing ends, which were the preferred substrates of the β-mannosidases compared to the intact galactomannan polymers. Among the heterosynergistic associations, both of the mannanases and Aga27A exhibited a high degree of synergy and reducing sugar release when the enzyme that exhibited high reducing sugar release (higher specific activity) in the binary combination was at a higher protein ratio to the enzyme liberating minimal reducing sugar (lower specific activity) (i.e. 75%:25%). Synergy with respect to reducing sugar release was mainly attributed to the initial removal of galactose side chains by the polymer active Aga27A; this likely increased the action of the β-mannanases on the galactomannan polymers. With respect to synergistic galactose removal, no clear trends were observed among the ratios of mannanase to galactosidase, but

synergy was the result of the mannanases releasing oligomeric fragments from the galactomannan polymers that are preferred substrates for the galactosidases.

Higher reducing sugar release was observed for the heterosynergistic binary combinations compared to the homeosynergistic binary combinations. Man26A and Aga27A appeared to be more promising for biomass degradation due to their high activities on their respective substrates and a good synergistic relationship with each other on galactomannan. From the findings obtained in this study, it was concluded that the GH family classification of an enzyme affects its substrate specificity and synergistic interactions with glycoside hydrolases from other families-more so than its EC classification. The findings from this study also demonstrated that the patterns and extents of galactose substitution on mannans affect the synergistic associations between mannanolytic enzymes. Therefore, an empirical approach in enzyme cocktail formulation would be required to develop an enzyme mixture that would be effective in mannan degradation regardless of the galactose/mannose ratio in the complex substrates (softwoods or agricultural residues). This method of screening for maximal compatibility between various GH families should ultimately lead to a more rational development of tailored enzyme cocktails for efficient lignocellulose degradation.

In the production of biofuel from lignocellulosic biomass, pre-treatment of the recalcitrant biomass to allow better digestibility of the biomass by enzymes is required, but many soluble products may result from this treatment which could potentially affect enzymes within the bioreactor. Elucidation of the individual inhibitory properties of each of the pre-treatment by-products and the synergistic effects that inhibitor cocktails obtained from washes of substrates pre-treated by various technologies could have on mannanolytic enzymes was conducted. The order of the inhibition strength of the lignocellulose degradation products to mannanolytic enzymes was found to be lignin derivatives > organic acids > furan derivatives. With regards to lignin-derived mono-phenolic inhibition of the mannanolytic enzymes, a relationship between the functional groups of the phenolic compound and its enzyme-interfering capacity was established, with the high hydroxyl group containing phenolics exhibiting the highest inhibition followed by methoxyl group containing phenolics.

Sugarcane bagasse is a waste product from the sugarcane industry and is considered a suitable substrate for production of cellulosic ethanol in South Africa and the world. This high mannan-containing agricultural residue, was selected to assess the efficacy of the mannanolytic enzyme cocktail developed in Chapter 5. The binary mannanolytic enzyme cocktail [Man26A 75%:

Aga27A 25%] at 25% or just Man26A at a 25% protein loading was combined with Cellic® CTec2 at 75% protein loading for the synergistic hydrolysis of sodium chlorite/acetic acid delignified sugarcane bagasse. The use of the [Man26A 75%: Aga27A 25%] enzyme cocktail was shown to release higher levels of reducing sugar and demonstrated a more prominent degree of synergy compared to that exhibited when only Man26A was used at the same protein loading during the hydrolysis of delignified sugarcane bagasse with CTec2. Our results showed that the action of Aga27A facilitated the action of Man26A, presumably as a result of cleavage of galactose side-chain groups from galactoglucomannan that would have hindered the mannanase. This, in turn, led to a higher degree of galactoglucomannan hydrolysis in the presence of Aga27A, which in turn exposed more sites for the cellulase to act on, compared to when only Man26A was used. The degree of synergy between these enzymes over time during the hydrolysis of delignified sugarcane bagasse was assessed and found to increase over the incubation time followed by a slight decrease during the final stages of hydrolysis.

This type of synergism is similar to that reported by Clarke et al. (2000) who demonstrated that the combination of a *P. fluorescens* ManA and a *C. thermocellum* XylA was significantly more effective than using a *C. thermocellum* ManA on softwood paper pulp. Also, incubation of pulp samples with both enzymes plus a galactosidase, AgaA, resulted in the largest enhancement of bleach-ability, and was followed by an increase in the observed total reducing sugar release (Clarke et al., 2000). This data suggested that the activity of the α-galactosidase enhanced the mannanase activity, and that a combination of these two enzymes was able to enhance the activity of XylA. Jørgensen et al. (2010) also reported synergism between the mannanolytic enzymes mannanase and mannosidase, with cellulase on Palm Kernel Press Cake. Both these studies demonstrated that intramolecular synergism between mannanolytic enzymes on the mannan portion of a lignocellulosic biomass greatly enhances the hydrolysis of other polysaccharides within the biomass since these various polysaccharides cover each other and are intertwined within each other.

In conclusion, this study demonstrated that a rationally designed mannanolytic enzyme cocktail based on GH family affiliations can facilitate the efficient hydrolysis of mannans and can be used in the optimisation of commercial or purified enzyme mixtures to improve the economic viability of the conversion of high mannan-containing biomass such as softwoods and sugarcane bagasse to bioethanol.

8.2. Future recommendations

This research study indicated that differences in mannan substrates affects the synergistic associations between various GH family mannanolytic enzymes. One of the recommendations for future research is to establish the presence of synergistic associations between the enzymes used in this study in a wide variety of mannan-based substrates that were not assessed in this work, such as glucomannan and galactoglucomannan. This research will allow the empirical design of the optimal mannanolytic enzyme cocktail for the effective degradation of high mannan-containing complex substrates, such as sugarcane bagasse and softwoods.

We also recommend development of an onsite production strategy of Aga27A and Man26A in a suitable microorganism (rather than purchasing them from Megazyme) to reach economic viability of the lignocellulose saccharification process as enzymes usually makes up a huge portion of the total industrial costs for lignocellulosic ethanol process.

In addition to synergy studies on more mannan-based substrates, isolation ad characterization of the oligosaccharides produced by the cocktails, using methods such as acid hydrolysis and HPAEC-PAD (carbohydrate composition), HPLC-RI and HPAEC-MS (to determine the DP of the products), enzymatic hydrolysis (determination of non-reducing end carbohydrate) and NMR (both 1D and 2D, to verify structure and purity of purified compounds) can provide valuable information on how the enzymes in these mannanolytic cocktails interact with each other to synergistically degrade these substrates. This information can be very valuable when selecting mannanolytic enzymes for a feedstock that has a known mannan type.

Pre-treatment of plant biomass by various pre-treatment technologies resulted in the production of various pre-treatment products, which may affect mannanolytic enzyme activities in various ways. The use of techniques such as CD spectroscopy that permit the observation of changes in the secondary structure of the enzyme in the presence of inhibitors and bioinformatic modeling of enzyme-to-inhibitor interactions would also provide useful insights into the mode of inhibition of these mannanolytic enzymes by soluble pre-treatment by-products. The identification of these potential inhibitors derived from the pre-treated biomass washes by means of LC-MS and HPLC will allow the proper selection of mannanolytic enzymes tolerant to these inhibitors or proper selection of a pre-treatment technology when certain enzymes are going to be used, thus preventing significant denaturation of enzymes by these inhibitors in a bioreactor setting.

Synergistic associations between the rationally designed binary mannanolytic enzyme cocktail and the cellulase preparation, CTec2, were established during delignified sugarcane bagasse hydrolysis. From the chemical composition of the delignified sugarcane bagasse, it was evident that arabinoxylan constitutes a large portion of the total substrate polysaccharide content. Establishing synergistic associations between this mannanolytic and cellulolytic enzyme optimized cocktail with arabinofuranosidases and xylanases for the hydrolysis of delignified sugarcane bagasse would be very interesting.

References

Abboo, S. (2015). TILE-Bioreactor system for fruit waste water treatment: reactor kinetics and analysis of digestion products. PhD Thesis (In preparation). Rhodes University, South Africa.

Ademark, P., Lundqvist, J., Hägglund, P., Tenkanen, M., orto, N., Tjerneld, F. and Stälbrand, H. (1999). Hydrolytic properties of a β-mannosidase purified from *Aspergillus niger*. Journal of Biotechnology. 75, 281-289.

Ademark, P., Varga, A., Medve, J., Harjunpää, V., Brakenberg, T., Tjerneld F. and Stålbrand, H. (1998). Softwood hemicellulose-degrading enzymes from *Aspergillus niger*: Purification and properties of a β-mannanase. Journal of Biotechnology. 63, 199-210.

Ademark, P., de Vries R. P., Hägglund, P., Stålbrand, H. and Visser, J. (2001a). Cloning and characterization of *Aspergillus niger* genes encoding an α-galactosidase and a β-mannosidase involved in galactomannan degradation. European Journal of Biochemistry. 268, 2982-2990.

Ademark, P., Larsson, M., Tjerneld, F. and Stålbrand, H. (2001b). Multiple α-galactosidases from *Aspergillus niger*: purification, characterization and substrate specificities. Enzyme and Microbial Technology. 29, 441-448.

Alvira, P., Tomás-Pejó, E., Ballesteros, M. and Negro, M. J. (2010). Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: A review. Bioresource Technology. 101, 4851-4861.

Al-Zuhair, S., Ahmed, K., Abdulrazak, A. and El-Naas, M. H. (2013). Synergistic effect of pretreatment and hydrolysis enzymes on the production of fermentable sugars from date palm lignocellulosic waste. Journal of Industrial and Engineering Chemistry. 19, 413-415.

Ayeni, A. O., Banerjiee, S., Omoleye, J. A., Hymore, F. K., Giri, B. S., Deshmukh, S. C., Pandey, R. A. and Mudliar, S. N. (2013). Optimization of pretreatment conditions using factorial design and enzymatic convertibility of shea tree sawdust. Biomass and Bioenergy. 48, 130-138.

Baik, S. H., Saito, K., Yokota, A., Asano, K. and Tomita, F. (2000). Molecular Cloning and High-Level Expression in *Escherichia coli* of Fungal α-Galactosidase from *Absidia corymbifera* IFO 8084. Journal of Bioscience and Bioengineering. 90 (2), 168-173.

Banerjee, G., Scott-Craig, J. S. and Walton, J. D. (2010). Improving enzymes for biomass conversion: a basic research perspective. Bioenergy Research. 3, 82–92.

Berlin, A., Balakshin, M., Gilkes, N., Kadla, J., Maxienko, V., Kubo, S. and Saddler, J. (2006). Inhibition of cellulase, xylanase and β-glucosidase activities by softwood lignin preparations. Journal of Biotechnology. 125, 198-209.

Beukes, N, Chan, H, Doi, R. H. ans Pletschke, B. I. (2008). Synergistic associations between *Clostridium cellulovorans* enzymes XynA, ManA and EngE against sugarcane bagasse. Enzyme Microbial Technology. 42; 492-498.

Beukes, N. and Pletschke, B. I. (2011). Effect of alkaline pre-treatment on enzyme synergy for efficient hemicellulose hydrolysis in sugarcane bagasse. Bioresource Technology. 102, 5207-5213.

Beutler, H. O. Lactose and D-galactose. In: Bergmeyer HU, editor. Methods of Enzymatic Analysis, Cambridge, UK: VCH Publishers Ltd; 1988, 3(4), p. 104-112.

Blöchl, A., Peterbauer, T., Hofmann, J. and Richter, A. (2008). Enzymatic breakdown of raffinose oligosaccharides in pea seeds. Planta. 228, 99-110.

Boraston, A. B., Bolam, D. N., Gilbert, H. J. and Davies, G. J. (2004). Carbohydrate-binding modules: fine-tuning polysaccharide recognition. Biochemistry Journal. 382, 769-781.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Analytical Biochemistry. 72 (1-2), 248-254.

Braun, P. and LaBaer, J. (2003). High throughput protein production for functional proteomics. Trends in Biotechnology. 21, 283-388.

Cantarella, M., Mucciante, C. and Cantarella, L. (2014). Inactivating effects of lignin-derived compounds released during biomass pretreatment on the endo-glucanase catalyzed hydrolysis of carbomethylcellulose: A study in continuous stirred ultrafiltration-membrane reactor. Bioresource Technology. 156, 48-56.

Cao, Y., Wang, Y., Meng, K., Bai, Y., Shi, P., Luo, H., Yang, P., Zhou, Z., Zhang, Z. and Yao, B. (2009). A novel protease-resistant α-galactosidase with high hydrolytic activity from *Gibberella* sp. F75: gene cloning, expression, and enzymatic characterization. Applied Microbial Biotechnology. 83, 875-884.

Centeno, M. S. J., Guerreiro, C. I. P. D., Dias, F. M. V., Morland, C., Tailford, L. E., Goyal, A., Prates, J. A. M., Ferreira, L.M.A., Mongodin, E. F., Nelson, K. E., Gilbert, H J. and Fontes, C. M. G. A. (2006). Galactomannan hydrolysis and mannose metabolism in Cellvibrio mixtus. FEMS Micriobiology Letters. 261, 123-132.

Chang, M. C. Y. (2007). Harnessing energy from pant biomass. Current Opinion in Chemical Biology. 11, 677-684.

Charrier, M. and Rouland, C. (2001). Mannan-Degrading Enzymes Purified From the Crop of Brown Garden Snail *Helix aspersa* Muller (Gastropoda Pulmonata). Journal of Experimental Zoology. 290, 125-135.

Chauve, M., Mathis, H., Huc, D., Casanave, D., Monot, F. and Ferreira, N. L. (2010). Comparative kinetic analysis of two fungal β-glucosidases. Biotechnology for Biofuels. 3 (3).

Cheung, R. C. F., Wong, J. H. and Ng, T. B. (2012). Immobilized metal ion affinity chromatography: a review on its applications. Applied Microbiology and Biotechnology. 96, 1411-1420.

Clarke, J.H., Davidson, J. K., Rixon, J. E., Halstead, J. R., Fransen, M. P., Gilbert, H. J. and Hazlewood. (2000). A comparison of enzyme-aided bleaching of softwood paper pulp using combinations of xylanase, mannanase and α-galactosidase. Applied Microbial Biotechnology. 53, 661-667.

Couturier, M., Roussel, A., Rosengren, A., Leone, P., Stålnbrand, H. and Berrin, J. (2013). Structural and Biochemical Analyses of Glycoside Hydrolase Families 5 and 26 β-(1,4)-Mannanases from *Podospora anserine* Reveal Differences upon Manno-ologosaccharide Catalysis. Journal of Biological Chemistry. 288 (20), 14624-14635.

Cross, R. (2001). The preparation of biological material for electron microscopy part3: The preparation of material for scanning electron microscopy (SEM). Rhodes University Press. Grahamstown.

da Silva Fialho, L., Guimarães, V. M., Callegari, C. M., Reis, A. P., Barbosa, D. S., de Lima Borges, E. E., Moreira, M. A and de Rezende, S. T. (2008). Characterixzation and biotechnological application of an acid α-galactosidase from *Tachigali multijuga* Benth. Seeds. Phytochemistry. 69, 2579-2585.

Daas, P. J. H., Schols, H. A. and Jongh, H. H. J. (2000). On the galactosyl distribution of commercial galactomannans. Carbohydrate Research. 329, 609-919.

Dashek, W. V. (1997). Methods in plant biochemistry and molecular biology. Photosynthetica. 53 (4), 560.

Dea, I. C. M., Clark, A. H. and McCleary, B. V. (1986). Effect of galactose-substitution-patterns on the interaction properties of galactomannans. Carbohydrate Research. 147, 275-294.

Dhawan, S. and Kaur, J. (2007). Microbial mannanases: an overview of production and applications. Critical Review in Biotechnology. 27(4), 197-216.

Dias, F. M. V., Vincent, F., Pell, G., Prates, J. A. M., Centeno, M. S. J., Tailford, L. E., Ferreira, L. M. A., Fontes, C. M. G. A., Davies, G. J. and Gilbert, H. J. (2004). Insights into the Molecular Determinants of Substrate Specificity in Glycoside Hydrolase Family 5 Revealed by the Crystal Structure and Kinetics of *Cellvibrio mixtus* Mannosidase 5A. The Journal of Biological Chemistry. 279, 25517-25526.

Doi, R. H. (2008). Cellulases of mesophilic microorganisms. Cellulosome and noncellulosome producers. Ann New York Academy of Science. 267-279.

Dotsenko, G. S, Semenova, M. V., Sinitsyna, O. A., Hinz, S. W. A., Wery, J., Zorov, I. N., Kondratieva, E. G. and Sinitsyn, A. P. (2012). Cloning, Purification, and Characterization of Galactomannan-Degrading Enzymes from *Myceliophthora thermophila*. Biochemistry. 77 (11), 1303-1311.

Dredge, R. (2010). Enhancing the saccharolytic phase of sugar beet pulp via hemicellulase synergy. MSc Thesis. Rhodes University, South Africa.

Fliedrová, B., Gerstorferová, D., Křen, V., Weignerová, L. (2012). Production of *Aspergillus niger* β-mannosidase in *Pichia pastoris*. Protein Expression and Purification. 85, 159-164.

Gao, D., Uppugundla, N., Chundawat, S. P., Yu, X., Hermanson, S., Gowda, K., Brumm, P., Mead, D., Balan, V. and Dale, B. E. (2011). Hemicellulases and auxiliary enzymes for improved conversion of lignocellulosic biomass to monosaccharides. Biotechnology Biofuels. 4 (5).

Garro, M. S., de Valdez, G. F., Oliver, G. and de Giori, G. S. (1996). Purification of α-galactosidase from *Lactobacillus fermentum*. Journal of Biotechnology. 45, 103-109.

Gasteiger, E., Hoogland, C., Gattiker, A., Duvaud, S., Wilkins, M. R., Appel, R. D. and Bairoch. In: John M. Walker editors. The Proteomics Protocols Handbook, Humana Press, 2005, 571-607.

Gübitz, G. M., Hayn, M., Sommerauer, M. and Steiner, W. (1996). Mannan-Degrading enzymes from *Sclerotium Rolfsii*: Characterisation and synergism of two endo β-mannanases and β-mannosidase. Bioresource Technology. 58, 127-135.

Han, Y., Dodd, D., Hespen, C. W., Ohene-Adjei, S., Schroeder, C.M., Mackie, R. I. and Cann, I. K. O. (2010). Comparative analyses of two thermophilic enzymes exhibiting both β-1,4 Mannosidic and β-1,4 glucosidic cleavage activities from *Caldanaerobius polysaccharolyticus*. Journal of Bacteriology. 192(16), 4111-4121.

Haslam, E. (1974). Polyphenol-protein interactions. Biochemistry Journal. 1, 285-288.

Hendriks, A. T. W. M. and Zeeman, G. (2009). Pretreatments to enhance the digestibility of lignocellulosic biomass. Bioresource Technology. 100, 10-18.

Hennessy, F., Nicoll, W. S., Zimmermann, R., Cheetham, M. E. and Blatch, G. L. (2005). Not all J domains are created equal: Implications for the specificity of Hsp40-Hsp70 interactions. Protein Science. 14, 1697-1709.

Himmel, M. E., Ding, S. Y., Johnsons, D. K., Adney, W.S., Nimlos, M. R., Brady, J. W. and Foust, T. D. (2007). Biomass recalcitrance: Engineering plants and enzymes for biofuel production. Science 2007. 315, 804-807.

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

Hubbell, C. A. and Ragauskas, A. J. (2010) effect of acid-chlorite delignification on cellulose degree of polymerization. Bioresource Technology. 7410-7415.

Jabasingh, S. A. and Nachiyar, C. V. (2011). Utilization of pretreated bagasse for the sustainable bioproduction of cellulase by Aspergillus nidulans MTCC344 using response surface methodology. Industrial Crops and Products. 34, 1564-1571.

Jindou, S., Karita, S., Fujino, T, Hayashi, H., Kimura, T., Sakka, K. and Ohmiya, K. (2002). α-Galactosidase Aga27A, an Enzymatic Component of the *Clostridium josui* Cellulosome. Journal of Bacteriology. 184, 600-604.

Jørgensen, H., Sanadi, A. R., Felby, C., Lange, N. E. K., Fischer, M. and Ernst, S. (2010). Production of Ethanol and Feed by High Dry Matter Hydrolysis and Fermentation of Palm Kernel Press Cake. Applied Biochemistry Biotechnology. 161, 318-332.

Kaneko, R., Kusakabe, I., Ida, E. and Murakami, K. (1990). Substrate Specificity of α-Galactosidase from *Aspergillus niger* 5-16. Agricultural Biological Chemistry. 55 (1), 109-115.

Kaya, F., Heitmann, J. A., and Joyce, T. W. (2000). Influence of lignin and its degradation products on enzymatic hydrolysis of xylan. Journal of Biotechnology. 80 (3), 241-247.

Kim, W., Kobayashi, O., Kaneko, S., Sakakibara, Y., Park, G., Kusakabe, I., Tanaka, H. and Kobayashi, H. (2002). α-Galactosidase from cultured rice (*Oryza sativa* L. var. Nipponbare) cells. Phytochemistry. 61, 621-630.

Kim, Y., Ximenes, E., Mosier, N. S. and Ladisch, M. R. (2011). Soluble inhibitors/deactivators of cellulase enzymes from lignocellulosic biomass. Enzyme and Microbial Technology. 48, 408-415.

Klyosov, A. A., Dotsenko, G. S., Hinz, S. W. A. and Sinitsyn, A. P. (2012). Structural features of β-(1→4)-D-galactomannans of plant origin as a probe for β-(1→4)-mannanase polymeric substrate specificity. Carbohydrate Research. 352, 65-69.

Kulminskaya, A. A., Eneiskaya, E. V., Isaeva-Ivanova, L. S., Savel'ev, A. N., Sidorenko, I. A., Shabalin, K. A., Golubev, A. M. and Neustroev, K. N. (1999). Enzymatic activity and β-galactomannan binding property of β-mannosidase from *Trichoderm reesei*. Enzyme and Microbial Technology. 25, 372-377.

Kumar, R. and Wyman, C. E. (2009). Effect of xylanase supplementation of cellulase on digestion of corn stover solids prepared by leading pretreatment technologies. Bioresource Technology. 100, 4203-4213.

Kumar, R. and Wyman, C. E. (2014). Strong Cellulase Inhibition by Mannan Polysaccharides in Cellulose Conversion to Sugars. Biotechnology and Bioengineering. 111 (7).

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 227 (5259), 680-685.

Li, J., Lin, J., Xiao, W., Gong, Y., Wang, M., Zhou, P. and Liu, Z. (2013). Solvent extraction of antioxidants from steam exploded sugarcane bagasse and enzymatic convertibility of the solid fraction. Bioresource Technology. 130, 8-15.

Li, J, Wu, K., Xiao, W., Zhang, J., Lin, J., Gong, Y. and Liu, Z. (2014a). Effect of antioxidant extraction on the enzymatic hydrolysis and bioethanol production of the extracted steam-exploded sugarcane bagasse. Biochemical Engineering Journal.82, 91-96.

Li, J., Zhou, P., Liu, H., Xiong, C., Lin, W. Gong, Y. (2014b). Synergism of cellulase, xylanase and pectinase on hydrolysing sugarcane bagasse resulting from different pretreatment technologies. Bioresource Technology. 155, 258-265.

Li, J., Zhou, P., Liu, H., Lin, J., Gong, Y., Xiao, W. and Lui, Z. (2014c). Monosaccharides and Ethanol Production from Superfine Ground Sugarcane Bagasse Using Enzyme Cocktail. Bioresources. 9 (2), 2529-2540.

Lima, L. H. A., de Almeida Felipe, M. d., Vitolo, M. and Torres, F. A. G. (2004). Effect of acetic acid present in bagasse hydrolysate on the activities of xylose reductase and xylitol dehydrogenase in Candida guilliermondii. Applied Microbial Biotechnology. 65, 734-738.

Lou, H., Wang, M., Lai, H., Lin, X., Zhou, M., Yang, D. and Qui, X. (2013). Reducing non-productive adsorption of cellulase and enhancing enzymatic hydrolysis of lignocelluloses by noncovalent modification of lignin with lignosulfonate. Bioresource Technology. 146, 478-484.

Luonteri, E., Alatalo, E., Siika-aho, M., Pettilä., M. and Tenkanen, M. (1998). α-Galactosidases of *Penicillium simplicissium*: production, purification and characterization of the gene encoding AGLI. Biotechnology Applied Biochemistry. 28, 179-188.

Margolles-Clark, E., Tenkanen, M., Luonteri, E. and Penttila, M. (1996). Three α-galactosidase genes of *Trichoderma reesei* cloned by expression in yeast. European Journal of Biochemistry. 240, 104-111.

McCarter, J. D. and Withers, S. G. (1994). Mechanism of enzymatic glycoside hydrolysis. Current Opinion in Structural Biology. 4, 885-892.

McCleary, B. V. (1985). The fine structures of carob and guar galactomannans. Carbohydrate Research. 139, 237-260.

Mi, S., Meng, K., Wang, Y., Bai, Y., Yuan, T. and Luo, H. (2007). Molecular cloning and characterization of a novel α-galactosidase gene from *Penicillium sp*. F63 CGMCC 1669 and expression in Pichia pastoris. Enzyme Microbial Technology. 40, 1373-1380.

Miller, G. L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. Analytical Chemistry. 31(3), 426-428.

Modig, T., Lidén, G. and Taherzadeh, M. J. (2002). Inhibition effects on alcohol dehydrogenase, aldehyde dehydrogenase and pyruvate dehydrogenase. Biochemistry Journal. 363, 769-776.

Morales, L. O., lakovlek, M., Martin-Sampedro, R., Rahikainen, J. L., Laine, J., van Haininen, A. and Rojas, O. J. (2014). Effects of residual lignin and heteropolysaccharides on the

bioconversion of softwood lignocellulose nanofibrils obtained by SO_2–ethanol-water fractionation. Bioresource Technology. 161, 55-62.

Moreira, L. R. and Filho, E. X. (2008). An overview of mannan structure and mannan-degrading enzyme systems. Applied Microbiology and Biotechnology. 79,165-178.

Morrison, D. (2013). Lignocellulosic waste degradation using enzyme synergy with commercially available enzymes and *Clostridium cellulovorans* XylanaseA and MannanaseA. MSc Thesis. Rhodes University, South Africa.

Nakagame, S., Chandra, R. P. and Sagger, J. N. (2011). The Effect of Isolated Lignins, Obtained From a Range of Pretreated Lignocellulosic Substrates, on Enzymatic Hydrolysis. Biotechnology and Bioengineering. 105 (5).

Oliva, J. M., Sáez, F., Ballesteros, I., González, A., Negro, M. J., Manzaneres, P. and Ballesteros, M. (2003). Effect of Lignocellulose Degradation Compounds from Steam Explosion Pretreatment on Ethanol Fermentation by Thermotolerant Yeast *Kluyveromyces marxianus*. Applied Biochemistry and Biotechnology. 105-108.

Olver, B., van Dyk, J. S., Beukes, N. and Pletschke, B. I. (2011). Synergy between EngE, XynA and ManA from *Clostridium cellulovorans* on corn stalk, grass and pineapple pulp substrates. Biotechnology. 1, 187-192.

Palmqvist, E. and Hahn-Hägerdal, B. (2000). Fermentation of Lignocellulosic hydrolysates. II: inhibitors and mechanisms of inhibition. Bioresource Technology. 74, 25-33.

Pan, X. (2008). Role of Functional Groups in Lignin Inhibition of Enzymatic Hydrolysis of Cellulose to Glucose. Journal of Biobased Materials and Bioenergy. 2 (1), 25-32.

Panagiotou, G. and Olsson, L. (2007). Effect of compounds Released During Pretreatment of Wheat Straw on Microbial Growth and Enzymatic Hydrolysis. Biotechnology and Bioengineering. 96 (2), 250-258.

Pavón-Orozco, P., Santiago-Hernández, A., Rosengren, A., Hidalgo-Lara, E. H. and Stålbrand, H. (2012). The family II carbohydrate-binding module of xylanase CflXyn11A from *Cellulomonas flavigena* increases the synergy with cellulase TrCel7B from *Trichoderma reesei* during the hydrolysis of sugar cane bagasse. Bioresource Technology. 104, 622-630.

Peti, W. and Page, R. (2007). Strategies to maximize heterologous protein expression in *Escherichia coli* with minimal cost. Protein Expression and Purification. 51, 1-10.

Porath, J. (1992). Immobilized Metal ion Affinity Chromatography. Protein Expression and Purification. 3, 263-281.

Saha, B. C. (2003). Hemicellulose bioconversion. Journal of Industrial Microbiology and Biotechnology. 30 (5), 279-291.

Schwarz, W. H. (2001). The cellulosome and cellulose degradation by anaerobic bacteria. Appl Microbiol Biotechnol. 56, 634-649.

Sewalt, V. J. H., Glasser, W. G. and Beauchemim, K. A. (1997). Lignin impact on fiber degradation. 3. Reversal of inhibition of enzymatic hydrolysis by chemical modification of lignin and by additives. Journal of Agriculture Food Chemistry. 45 (5), 1823-1828.

Shallom, D. and Shoham, Y. (2003). Microbial hemicellulases. Current Opinion in Microbiology. 6, 219-228.

Shi, P., Yao, G., Cao, Y., Yang, P., Yuan, T., Huang, H., Bai, Y. and Yao, B. (2011). Cloning and characterization of a new β-mannosidase from *Streptomyces* sp. S27. Enzyme and Microbial Technology. 49, 277-283.

Shoseyov, O., Shani, Z. and Levy, I. (2006). Carbohydrate Binding Modules: Properties and Novel Applications. Microbiology and Molecular Biology Reviews. 283-295.

Silvestroni, A., Connes, C., Sesma, F., de Giori G. S. and Piard, J. (2002). Characterization of the melA Locus for α-Galactosidase in *Lactobacillus plantarum*. Applied Environmental Microbiology. 68 (11), 5464-5471.

Siqueira, G., Várnai, A., Ferraz, A. Milagres, A. M. F. (2013). Enhancement of cellulose hydrolysis in sugarcane bagasse by selective removal of lignin with sodium chlorite. Applied Energy. 10, 399-402.

Sluiter, J. B., Ruiz, R. O., Scarlata, C. J., Sluiter, A. D. and Templon, D. W. (2010). Compositional Analysis of Lignocellulosic Feedstocks 1: Review and Description of Methods. Journal of Agricultural and Food Chemistry. 58 (16), 9043-9053.

Stoll, D., Stålbrand, H. and Warren, A. J. (1999). Mannan-Degrading enzymes from *Cellulomonas fimi*. Applied and Environmental Microbiology. 2598-2605.

Studier, F. W., Rosenberg, A. H., Dunn, J. J. and Dubendorff, J. W. (1990). Use of T7 RNA polymerase to direct expression of cloned genes. Methods in Enzymology. 185, 60-89.

Sulkowski, E. (1996). Immobilized Metal-ion Affinity Chromatography: Imidazole Protein Pump and Chomatographic Sequelae. II. Chromatographic Sequelae. Journal of Molecular Recognition. 9, 494-498.

Suryani., Kimura, T., Sakka, K. and Ohmiya, K. (2003). Cloning, Sequencing and Expression of the Gene Encoding the *Clostridium stercorarium* α-Galactosidase Aga36A in *Escherichia coli*. Bioscience Biotechnology Biochemisrty. 67 (10), 2160-2166.

Tailford, L. E., Money, V. A., Smith, N. L., Dumon, C., Davies, G. J. and Gilbert, H. J. (2007). Mannose Foraging by *Bacteroides thetaiotaomicron*: structure and specificity of the β-mannosidase, *Bt*Man2A. The Journal of Biological Chemistry. 282 (15), 11291-11299.

Tailford, L. E, Ducros, V. M. A., Flint, J. E., Roberts, S. M., Morland, C. and Zechel, D. L. (2009). Understanding How Diverse β-Mannanases Recognize Heterogeneous Substrates. Biochemistry. 48, 7009-7018.

Tamaru, Y. and Doi, R. H. (2000). The engL Gene Cluster of *Clostridium cellulovorans* Contains a Gene for Cellulosomal ManA. Journal of Bacteriology. 182 (1), 244-247.

Tamaru, Y. and Doi, R. H. (2001).pectate lyase A, an enzymatic subunit of the *Clostridium cellulovorans* cellulosome. Proceeding of the National Academy of Science. 98 (7), 4125-4129.

Tao, S.,Khanizadeh, S., Zhang, H. and Zhang, S. (2010). Anatomy, ultrastructure and lignin distribution of stone cells in two Pyrus species. Plant Science. 176, 413-419.

Van Dyk, J. S. and Pletschke, B. I. (2012). A review on lignocellulose bioconversion using enzymatic hydrolysis and synergistic cooperation between enzymes-Factors affecting enzymes, conversion and synergy. Biotechnology Advances. 30, 1458-1480.

Van Dyk, J. S., Sakka, M., Sakka, K. and Pletschke, B. I. (2010). Identification of endoglucanases, xylanases, pectinases and mannanases in the multi-enzyme complex of *Bacillus licheniformis* SVD1. Enzyme and Microbial Technology. 47(3), 112-118.

Van Zyl, W. H., Rose, S. H., Trollope, K. and Gorgens J. F. (2010). Fungal β-mannanases: Mannan hydrolysis, heterologous production and biotechnological applications. Process Biochemistry. 45, 1203-1213.

Vardakou, M., Katapodis, P., Topakas, E., Kekos, D., Macris, B. J. and Christakopoulos, P. (2004). Synergy between enzymes involved in the degradation of insoluble wheat flour arabinoxylan. Innovative Food Science and Engineering Technologies. 5, 107-112.

Várnai, A., Huikko, L., Pere, J., Sikka-aho, M. and Viikari, L. (2011). Synergistic action of xylanase and mannanase improves the total hydrolysis of softwood. Bioresource Technology. 102(19), 9096-9104.

Wang, H., Luo, H., Li, J., Bai, Y., Huang, H., Shi, P., Fan, Y. and Yao, B. (2010). An α-galactosidase from an acidophilic *Bispora sp*. MEY-1 strain acts synergistically with β-mannanase. Bioresource Technology. 101, 8376-8382.

Waugh, D. S. (2005). Making the most of affinity tags. Trends in Biotechnology. 23 (6), 316-320.

Willför, S., Sjöholom, R., Laine, C., Roslund, M., Hemming, J. and Holmbom, B. (2003). Characterization of water-soluble galactoglucumannans from Norway spruce wood and thermochemical pulp. Carbohydrate Polymers. 52, 175-187.

Willför, S., Sundberg, K., Tenkanen, M. and Holmbom, B. (2008). Spruce-derived mannans – A potential raw material for hdrocolloids and novel advanced natural materials. Carbohydrate Polymers. 72, 197-210.

Xiao, Z., Zhang, X., Gregg, D. J. and Saddler, J. N. (2004). Effects of sugar inhibition on cellulases and β-glucosidase during enzymatic hydrolysis of softwood substrates. Applied Biochemistry and Biotechnology. 113-116.

Ximenes, E., Kim, Y., Mosier, N., Dien, B and Landisch, M. (2010). Inhibition of cellulases. Enzyme and Microbial Technology. 46, 170-176.

Ximenes, E., Kim, Y., Mosier, N., Dien, B. and Landisch, M. (2011). Deactivation of cellulases by phenols. Enzyme and Microbial Technology. 48, 54-60.

Yamabhai, M., Sak-Ubol, S., Srila, W. and Haltrich, D. (2014). Mannan biotechnology: from biofuels to health. Critical Reviews in Biotechnology. 1-11.

Yoon, K., Chung, S. and Lim, B. (2008). Characterization of the *Bacillus subtilis* WL-3 Mannanase from a Recombinant *Escherichia coli*. The Journal of Microbiology. 46, 344-349.

Zahura, U. A., Mohammad, M. R., Inoue, A. and Ojima, T. (2012). Characterization of a β-mannosidase from a marine gastropod, Aplysia kurodai. Comparative Biochemistry and Physiology. Part B 162, 24-33.

Zeng, Y., Zhao, S., Yang, S. and Ding, S. (2014). Lignin plays a negative role in the biochemical process for producing lignocellulosic biofuels. Current Opinion in Biotechnology. 27, 38-45.

Zhang, B., Chen, Y., Li, Z., Lu, W. and Cao, Y. (2011). Cloning and functional expression of α-galactosidase cDNA from *Penicillium janczewskii zaleski*. Biologis. 66 (2), 205-212.

Zhang, J., Geng, A., Yao, C., Lu, Y. and Li, Q. (2012). Effects of lignin-derived phenolic compounds on xylitol production and key enzyme activities by xylose utilizing yeast *Candida athensensis* SB18. Bioresource Technology. 122, 369-378.

Zhang, Y. and Lynd, L. R. (2004). Toward an Aggregated Understanding of Enzymatic Hydrolysis of Cellulose: Noncomplexed Cellulase Systems. Biotechnology and Bioengineering. 88 (7).

Zhao, W., Zheng, J. and Zhou, H. (2011). A thermotolerant and cold-active mannan endo-1,4-β-mannosidase from *Aspergillus niger* CBS 513.88: Constitutive overexpression and high-density fermentation in *Pichia pastoris*. Bioresource Biotechnology. 102, 7538-7547.

Appendices

Appendix A: List of chemicals

Acrylamide	Sigma (Cat. No. A8887)
Agarose	Sigma (Cat. No. A9539)
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)
Coomassie Brilliant Blue R250	Merck (Cat. No. 1.12553)
Di-galactosyl-mannopentaose	Megazyme (Cat. No. O-GGM5)
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	Merck (1.09001.0500)
Formic acid	Merck (Cat. No. UN1779)
Furfural	Sigma (Cat. No. W248908)
Galactosyl-mannotriose	Megazyme (Cat. No. O-GM3)
Glacial acetic acid	Merck (Cat. No. 1.00063)
D-Glucose	Saarchem (Cat. No. 2676020)
Glycerol	Saarchem (Cat. No. 2676520)
Glycine	Merck (Cat. No. 1.04169)
Hydroxymethylfurfural	Sigma (Cat. No. W501808)
Imidazole	Merck (Cat. No. 1.04716)
IPTG	Calbiochem (Cat. No. 420322)
Kanamycin (Monosulphate)	Melford (Cat. No. K0126)

Lactose and D-Galactose kit	Megazyme (Cat. No. K-LACGAR)
Levulinic acid	Sigma (Cat. No. L2009)
Lignin, low sulfonate	Sigma (Cat. No. 471003)
Locust bean gum	Fluka (Cat. No. 62631)
D-Mannose	Sigma (Cat. No. M2069)
D-Mannose, D-Fructose & D-Glucose kit	Megazyme (Cat. No. K-MANGL)
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)
Nutrient Broth	Biolab, Merck (C24)
PeqGold protein marker II	peqLab (Cat. No. 27-2010)
Phenol	Sigma (Cat.No. P3653)
Potassium permanganate	Sigma (Cat. No. 223468)
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)
Tryptone	Fluka (Cat. No. 70169)
D-Xylose kit	Megazyme (Cat. No. K-XYLOSE)
Yeast extract	Biolab (Cat. No. BX6)

Appendix B: Standard curves for protein and activity determination

B. 1. Protein standard curve

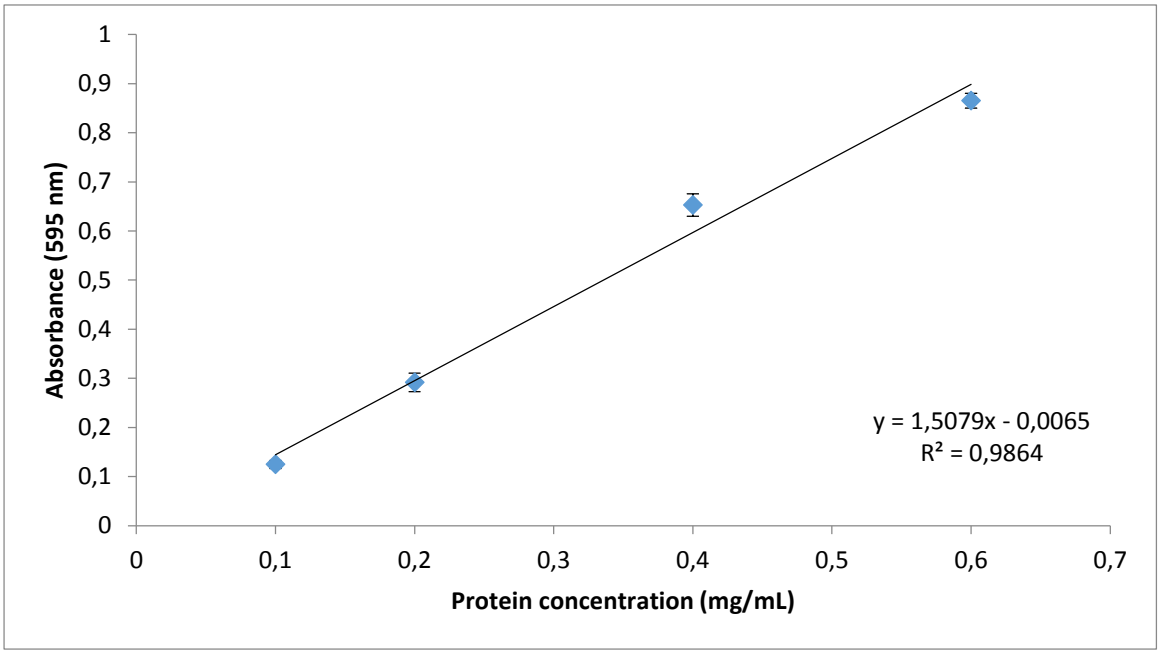

Figure B. 1. Bradford standard curve to determine protein concentration where BSA was used as the protein standard. Values are represented as means ± SD, n=3.

B. 2. Enzyme activity curves

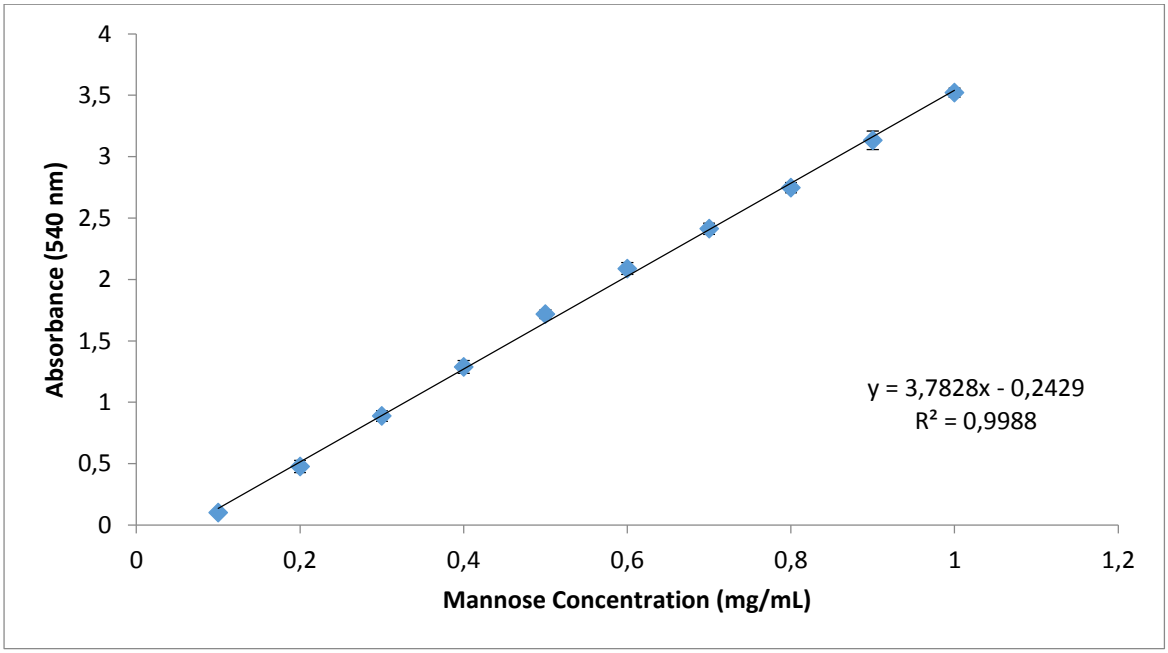

Figure B.2.1. Mannose standard curve using DNS assay. Values are represented as means ± SD, n=3.

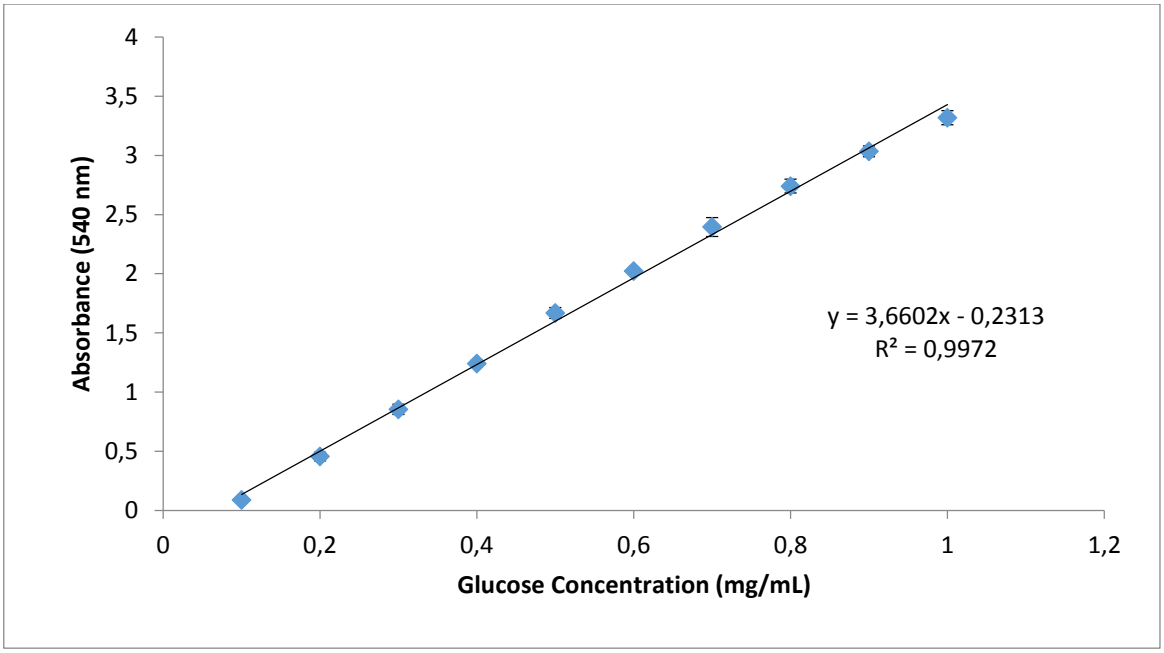

Figure B. 2. 2. Glucose standard curve using DNS assay. Values are represented as means ± SD, n=3.

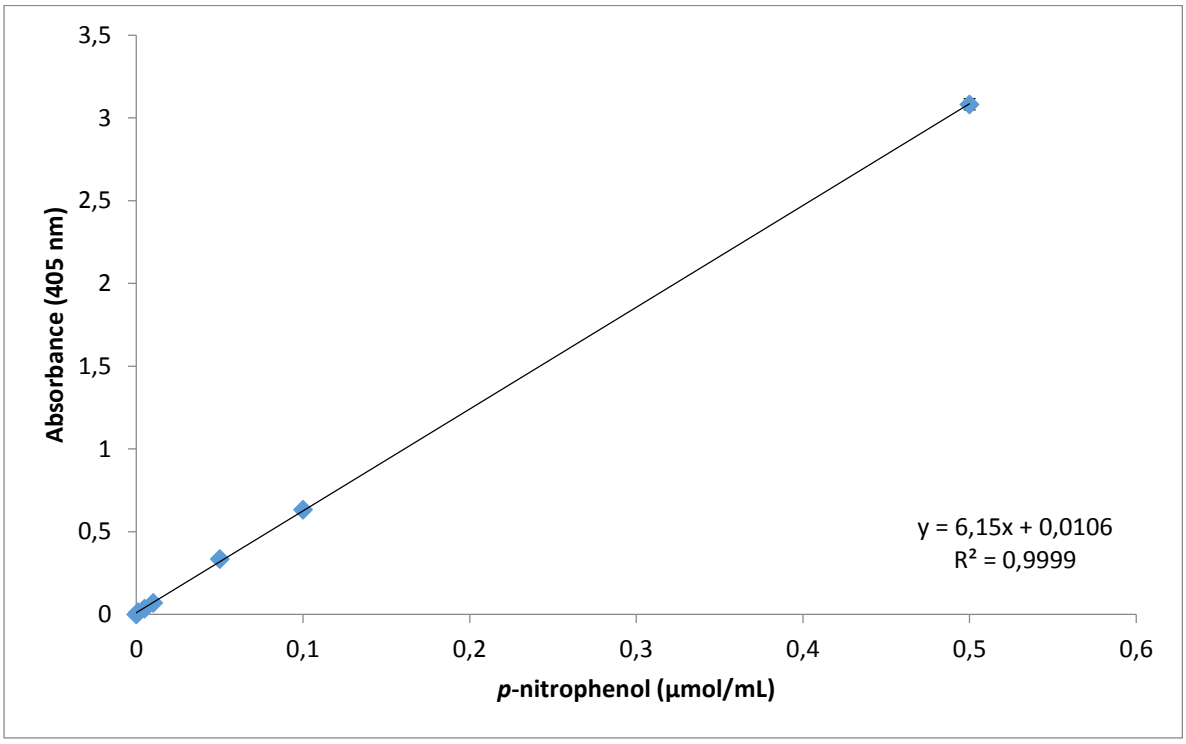

Figure B. 2. 3. *p*-nitrophenol standard curve the the α-galactosidase and β-mannosidase assay. Values are represented as means ± SD, n=3.

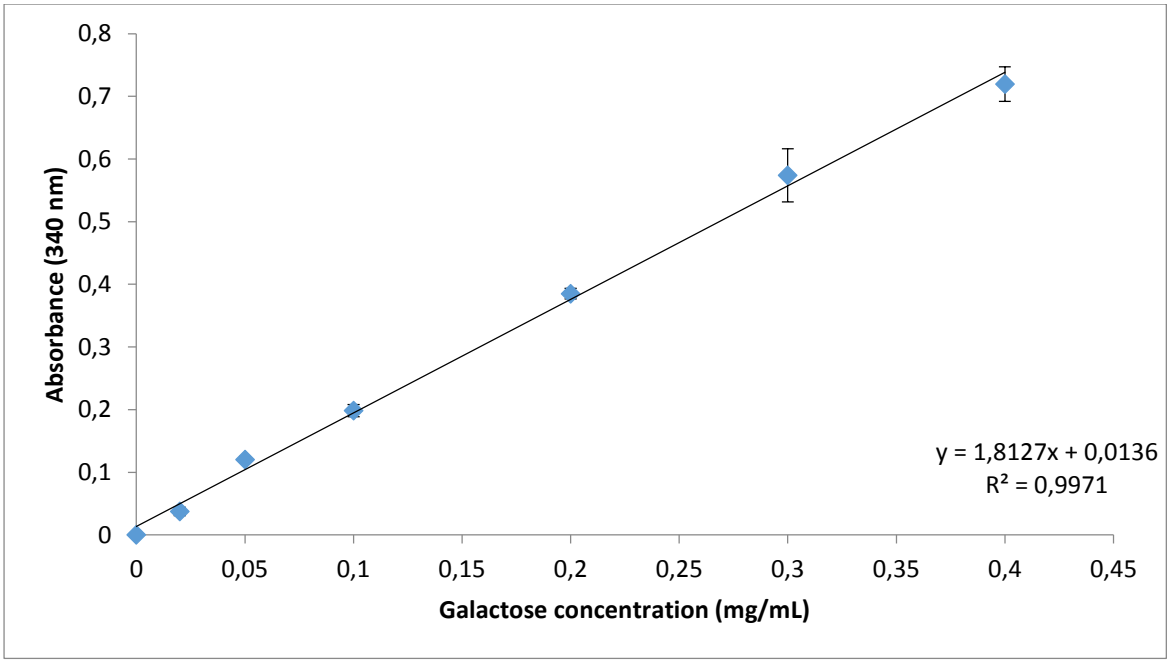

Figure B. 2. 4. Galactose standard curve using AOAC Official Method 984.15 for lactose determination. Values are represented as means ± SD, n=3.

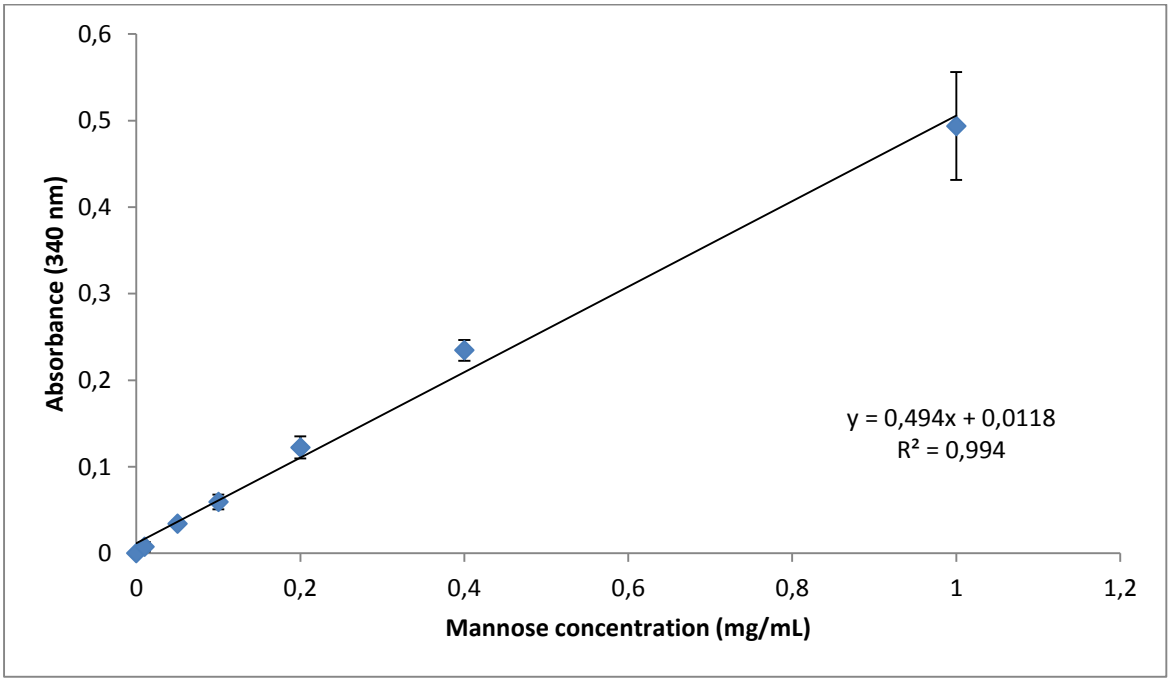

Figure B. 2. 5. Mannose standard curve using Megazyme™ kit for fructose/glucose/mannose detection. Values are represented as means ± SD, n=3.

B.3. Phenolic content curve

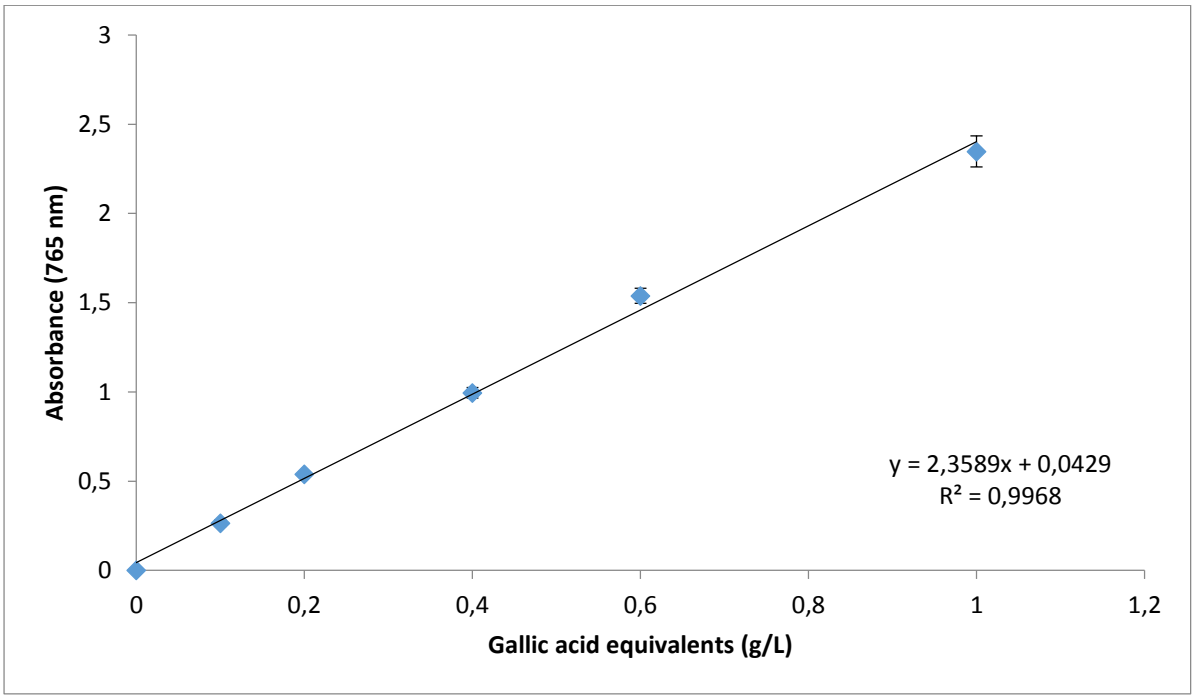

Figure B.3. Phenolics (Gallic acid equivalents) standard curve using Folin-Ciocalteu method. Values are represented as means ± SD, n=3.

Appendix C: Synergy studies

The reaction mixtures for the synergy assays in Chapter 5 were made up to a final volume of 400 µL using 50 mM sodium citrate (pH 5.0); 300 µL of 0.66% (w/v) substrate and 100 µL of enzyme, as shown in Table C.1. Total protein concentration was kept constant in all combinations. The assays were run in triplicate at 37°C for an hour.

Table C.1. Combination of enzymes for synergy assays during galactomannan hydrolysis

Combination #	Mannanase (%)	Auxiliary enzyme (%)
1	100	0
2	75	25
3	50	50
4	25	75
5	0	100

Where mannanase is Man26A or ManA, and auxiliary enzyme can be Aga27A or AglC or Man2A and or Man5A

The reaction mixtures for the synergy assays in Chapter 8 were made up to a final volume of 1200 µL using 50 mM sodium citrate (pH 5.0); 900 µL of 1.33% (w/v) substrate and 300 µL of enzyme, as shown in Table C.2. Total protein concentration was kept constant in all combinations. The assays were run in triplicate at 37°C for 96 hour, with samples withdrawn every 24 hours.

Table C.2. Combination of enzymes for synergy assays during sodium/chlorite pre-treated sugarcane bagasse hydrolysis

Combination #	CTec2 (%)	Auxiliary enzyme (%)
1	100	0
2	75	25
3	0	100

Where auxiliary enzyme can be Man26A or [Man26A 75%: Aga27A 25%]

Appendix D: Map of expression vector

A plasmid map of the pET-29a(+) expression vector system used to express ManA in *E. coli* BL21 (DE3) cells is depicted in Figure D.1.

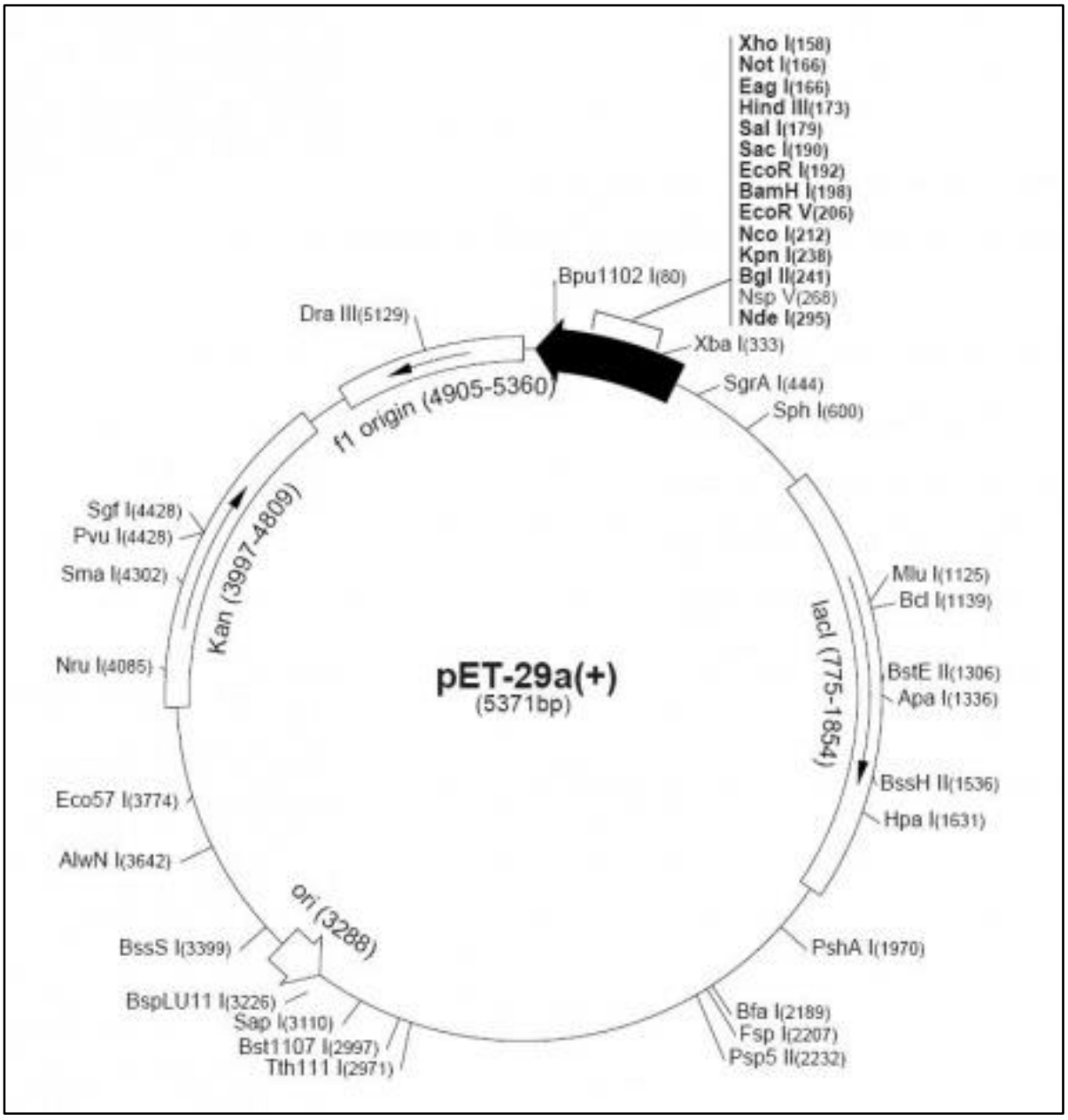

Figure D.1. Plasmid map of the pET-29a(+) Expression vector system used for the expression of *C. cellulovorans* ManA. ManA was inserted between the EcoRI and BamHI restriction sites (Novagen, 1998).