

# Lignocellulosic waste degradation using enzyme synergy with commercially available enzymes and *Clostridium cellulovorans* XylanaseA and MannanaseA

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# Abstract

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The launch of national and international initiatives to reduce pollution, reliance on fossil fuels and increase the beneficiation of agricultural wastes has prompted research into sugar monomer production from lignocellulosic wastes. These sugars can subsequently be used in the production of biofuels and environmentally degradable plastics. This study investigated the use of synergistic combinations of commercial and pure enzymes to lower enzyme costs and loadings, while increasing enzyme activity in the hydrolysis of agricultural waste. Pineapple pomace was selected due to its current underutilisation and the substantial quantities of it produced annually, as a by-product of pineapple canning. One of the primary costs in beneficiating agricultural wastes, such as pineapple pomace, is the high cost of enzyme solutions used to generate reducing sugars. This can be lowered through the use of synergistic combinations of enzymes.

Studies related to the inclusion of hemicellulose degrading enzymes with commercial enzyme solutions have been limited and investigation of these solutions in select combinations, together with pineapple pomace substrate, allows for novel research. The use of synergistic combinations of purified cellulosomal enzymes has previously been shown to be effective at releasing reducing sugars from agricultural wastes. For the present study, MannanaseA and XylanaseA from *Clostridium cellulovorans* were heterologously expressed in *Escherichia coli* BL21 (DE3) cells and purified with immobilised metal affinity chromatography. These enzymes, in addition to two commercially available enzyme solutions (Celluclast 1.5L<sup>®</sup> and Pectinex<sup>®</sup> 3XL), were assayed on defined polysaccharides that are present in pineapple pomace to determine their substrate specificities. The degree(s) of synergy and specific activities of selected combinations of these enzymes were tested under both simultaneous and sequential conditions.

It was observed that several synergistic combinations of enzyme solutions in select ratios, such as C20P60X20 (20% cellulose, 60% pectinase and 20% xylanase), C20P40X40 (20% cellulose, 40% pectinase and 40% xylanase) and C20P80 (20% cellulose, 80% pectinase) with pineapple pomace could both decrease the protein loading, while raising the level of activity compared to individual enzyme solutions. The highest quantity of reducing sugars to protein weight used on pineapple pomace was recorded at 3, 9 and 18 hours with combinations of Pectinex<sup>®</sup> 3XL and Celluclast 1.5L<sup>®</sup>, but for 27 h it was combinations of both these commercial solutions with XynA. The contribution of XynA was significant as

C20P60X20 displayed the second highest reducing sugar production of 1.521 mg/mL, at 36 h from 12.875 µg/mL of protein, which was the second lowest protein loading.

It was also shown that certain enzyme combinations, such as Pectinex<sup>®</sup> 3XL, Celluclast 1.5L<sup>®</sup> and XynA, did not generate synergy when combined in solution at the initial stages of hydrolysis, and instead generated a form of competition called anti-synergy. This was due to Pectinex<sup>®</sup> 3XL which had anti-synergy relationships in select combinations with the other enzyme solutions assayed. It was also observed that the degree of synergy and specific activity for a combination changed over time. Some solutions displayed the highest levels of synergy at the commencement of hydrolysis, namely Celluclast 1.5L<sup>®</sup>, ManA and XynA. Other combinations exhibited the highest levels of synergy at the end of the assay period, such as Pectinex<sup>®</sup> 3XL and Celluclast 1.5L<sup>®</sup>. Whether greater synergy was generated at the start or end of hydrolysis was a function of the stability of the enzymes in solution and whether enzyme activity increased substrate accessibility or generated competition between enzymes in solution.

Sequential synergy studies demonstrated an anti-synergy relationship between Pectinex<sup>®</sup> 3XL and XynA or ManA, as well as Pectinex<sup>®</sup> 3 XL and Celluclast 1.5L<sup>®</sup>. It was found that under sequential synergy conditions with Pectinex<sup>®</sup> 3 XL, XynA and ManA, that anti-synergy could be negated and high degrees of synergy attained when the enzymes were added in specific loading orders and not inhibited by the presence of other active enzymes. The importance of loading order was demonstrated under sequential synergy conditions when XynA was added before ManA followed by Pectinex<sup>®</sup> 3 XL, which increased the activity and synergy of the solution by 50%. This equates to a 60% increase in reducing sugar release from the same concentrations of enzymes and emphasises the importance of removing anti-synergy relationships from combinations of enzymes.

It can be concluded that a C20P60X20 combination (based on activity) can both synergistically increase the reducing sugar production and lower the protein loading required for pineapple pomace hydrolysis. This study also highlights the importance of reducing anti-synergy in customised enzyme cocktails and how sequential synergy can demonstrate the order in which a lignocellulosic waste is degraded.

## **Plagiarism declaration**

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

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Signed: David Morrison

On this \_\_\_\_\_ of \_\_\_\_\_ 20\_\_\_\_



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## List of abbreviations

°C	Degree(s) Celsius
µg	Microgram
µL	Microlitre
µM	Micromolar
µmol	Micromole
AA	Auxiliary Activities
APE	Apple pectin
AVI	Avicel PH101
BG	β-glucosidase(s)
BEX	Beech wood xylan
BWX	Birch wood xylan
C	Celluclast 1.5L <sup>®</sup> <i>Trichoderma reesei</i> cellulase
CAS	Chemical Abstracts Service number
CBH	Cellobiohydrolase(s)
CBM	Carbohydrate binding modules
CMC	Carboxymethylcellulose
CDH	Cellobiose dehydrogenase
CPW	Cellulose powder
Cm	Centimeter
DAFF	Department of Agriculture, Forestry and Fisheries
DNS	Dinitrosalicylic
DS	Degrees of synergy
EC	Enzyme commission number
EG	Endo-glucanase(s)
EngE	Endoglucanase E
g	Gram
<i>g</i>	Gravity
GalA	α-Galacturonic acid
GAX	Glucuronoarabinoxylan
GH	Glycoside hydrolase(s)
h	Hour
HG	Homogalacturonan
His	Histidine
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
ln	Natural log
LBG	Locust bean gum
LMPO	Lytic polysaccharide monooxygenases

M	<i>Clostridium cellulovorans</i> ManA
ManA	Mannanase A
mg	Milligram
min	Minute
mL	Millilitre
mM	Millimolar
MT	Metric tonnes
ng	Nanogram
nm	Nanometer
nkat	Nano katals
NREL	National Renewable Energy Laboratory
OD	Optical density
P	Pectinex <sup>®</sup> 3XL <i>Aspergillus niger</i> pectinase
PMG	Polymethylgalacturonases
PG	Polygalacturonases
PGA	Polygalacturonic acid
rDNA	Recombinant deoxyribonucleic acid
Rha	L-Rhamnose
rpm	Revolutions per minute
SCB	Sugar cane bagasse
SD	Standard deviation
SDS	Sodium docecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	Scanning electron microscopy
TEMED	N,N,N',N'-tetramethylethylenediamine
U	Units of enzyme activity
v	Volume
V <sub>B</sub>	Buffer volume
V <sub>E</sub>	Enzyme volume
V <sub>i</sub>	Incremental enzyme volume
V <sub>S</sub>	Substrate volume
V <sub>T</sub>	Total solution volume
w	Weight
XynA	Xylanase A
X	<i>Clostridium cellulovorans</i> XylanaseA

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## **Dedication and acknowledgements**

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## **List of research outputs**

### **A. Contribution towards a published review article in a peer reviewed journal**

Van Dyk J.S., Gama R., Morrison D., Swart S. and Pletschke B.I., (2013). Food processing waste: Problems, current management and prospects for utilisation of the lignocellulose component through enzyme synergistic degradation. *Renewable and sustainable energy reviews*, 26: 521-531.

### **B. Conference proceedings:**

Morrison D., Van Dyk J.S. and Pletschke B.I. The effect of alcohols, lignin and phenolic compounds on *Clostridium cellulovorans* XynA activity. 17<sup>th</sup> South African Society for Microbiology (SASM) Congress. Southern Sun Cape Sun Hotel, Cape Town, South Africa, 6-9 November 2011.

### **C. Anticipated publication in a peer reviewed scientific journal**

# Chapter 1: Literature Review

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## 1.1 Introduction

Lignocellulose has been identified as the most abundant biomass on the planet (Fry, 2011). Its quantity and global distribution have made it of use to humans throughout the ages. Lignocellulose can be derived from any harvested plant biomass and is composed of tightly spaced and interlinked strands, and layers of polysaccharides, such as cellulose, hemicellulose and pectin (Sørensen *et al.*, 2010). These layers are known to be enclosed within, and can be variably linked with a protective polyphenol layer of lignin. While rich in polysaccharides, the complexity, variable structure and composition between different plant sources have made complete lignocellulose utilisation and its degradation into simple sugars limited (Fry, 2011).

Sugar monomers from agricultural waste lignocellulose can potentially serve as a readily available, sustainable and inexpensive feedstock for the production of biofuels and ecologically degradable plastics and packaging (Brethauer and Wyman, 2010; Department of Minerals and Energy, 2007). The potential these products and fuels offer as a viable substitute for moving away from the current fossil fuel industrial paradigm, has attracted global and national interest, with various governmental biofuel initiatives launched in 2007 and 2009 (Department of Minerals and Energy, 2007; E.U., 2009). Additionally the production of biofuels has been slated as means of developing marginal agricultural land and generating jobs for the rural poor (Department of Minerals and Energy, 2007).

The reason for the investment into biofuels is to reduce the national reliance on plastic and petroleum from imported crude oil. This is in addition to problems of fuel price volatility and other negative externalities, such as pollution and health problems from fossil fuels (Sims *et al.*, 2010). These factors have ensured that alternative liquid fuels are under continuous scientific investigation (Merino and Cherry, 2007). Of further national interest has been the legislated mandatory blending of biofuels with petroleum products set to begin in 2013 (Department of Energy, 2012). National interest has subsequently focused on the production and research of the sustainable and economically feasible biofuels to provide for the demand for biofuels for the prescribed 2% biofuel in petroleum blend (Department of Energy, 2012).

As the technology to produce biofuel has advanced, four distinct generations of technology have been defined. The first generation was based around fermentation of food staples using

unmodified microorganisms and limited pre-treatments (Sims *et al.*, 2010). The production of first generation biofuels have come under heavy scrutiny and condemnation, due to their use of edible food resources and inefficient processing of the total available biomass (Sims *et al.*, 2010). This reduced the available food supply for human consumption, as well as increased the cost of food staples such as maize and wheat, which resulted in sporadic food riots in developing countries (Merino and Cherry, 2007). The use of non-edible lignocellulose waste, generated from the food industry, for the production of second generation biofuels has therefore become an increasingly feasible strategy for the substitution of liquid energy consumption away from fossil fuels (Dien *et al.*, 2003; Sims *et al.*, 2010).

Second generation biofuel production is generally defined as the use of lignocellulosic waste biomass pre-treated with a variety of chemical, mechanical and thermal processes, followed by enzyme hydrolysis through cell free enzyme solutions optimised for lignocellulose degradation (Naik *et al.*, 2010; Sims *et al.*, 2010). The liberated free sugar monomers and simple polysaccharides can subsequently undergo fermentation to produce bio-ethanol, bio-butanol and bio-methane, amongst others (Naik *et al.*, 2010). These include traditional yeast batch fermentation, or novel bacterial continuous bioreactor fermentations (Sims *et al.*, 2010). All biological components used in these processes have undergone genetic modification for improved efficiency and functionality within the biofuel production process (Merino and Cherry, 2007; Sims *et al.*, 2010; van Zyl *et al.*, 2007). The efficient enzymatic degradation of lignocellulosic waste, through enzyme synergy, has increasingly grown to be an important area of scientific and industrial interest (Merino and Cherry, 2007; Sims *et al.*, 2010). The subsequent release of simple sugars can be followed by fermentation, producing biofuels or additional value added products (Merino and Cherry, 2007; van Zyl *et al.*, 2007).

The varied composition and degradation resistant nature of lignocellulose and its polysaccharides, have aided their traditional uses in material and solid fuel industries (Doi, 2008). To date, these properties have made their conversion into liquid fuel and other energy feedstocks impractical and uneconomical (Merino and Cherry, 2007). This has been due to inefficient physical, mechanical, chemical and enzymatic processing methods available with current technology (Merino and Cherry, 2007; Naik *et al.*, 2010). A variety of microbes and fungi have the ability to break down these polymers to their monomer sugars to fulfil their energy requirements (Beg *et al.*, 2001). This is achieved through the use of a variety of unique enzymatic and chemical degradation mechanisms (Sims *et al.*, 2010). The use of these

enzymes within biotechnological processes in a variety of industries can provide an energy solution for this waste resource problem.

The increasing need for biofuel and the desire to beneficiate agricultural waste has emphasised the need to study the enzymes required to degrade lignocellulosic waste for the production of useful sugars and other end products (Dien *et al.*, 2003; van Zyl *et al.*, 2007). The interaction between enzymes in a lignocellulose degradation solution remains a key area of study. A potential enzyme for use in biotechnological application towards this goal could be the cellulosomal xylanase A (XynA) and mannanase A (ManA) from *Clostridium cellulovorans* (Kosugi *et al.*, 2001).

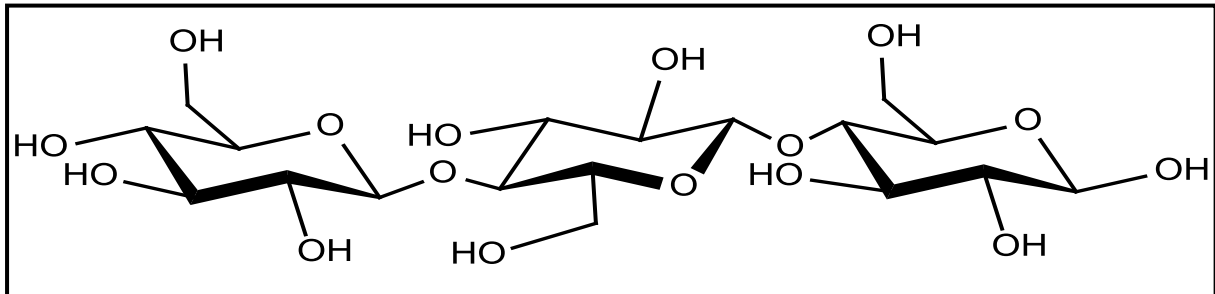
Commercially available enzymes are known to be amongst the main component enzymes used in enzyme solutions of bioreactor fermentations. To obtain the maximum utility from the commercial enzymes, the highest marginal return should be obtained from any enzymes used. This can be ensured through the use of enzyme combinations that generate the maximal yield for the least enzyme quantity utilised. This would entail finding a minimal enzyme cocktail between ManA and XynA from *C. cellulovorans* and selected commercial enzymes (Merino and Cherry, 2007).

## **1.2 Components of lignocellulose and enzymes required for its degradation**

Plant biomass, or lignocellulose, is known to be composed primarily of cellulose, hemicellulose and lignin (Chang, 2007). The structures of the polysaccharides, cellulose and hemicellulose, consist of a variety of sugar moieties, while lignin is a complex polyphenol (Fry, 1988). However, some lignocellulose waste sources can also have a high percentage of pectin, which can form up to 30% of dry mass of the waste, such as in citrus peels. Despite this inherent variability in composition, hemicellulose and cellulose are considered to be the most abundant polysaccharides on Earth and as such, they provide a large, underutilized and readily available energy resource (Sunna and Antranikian, 1997).

### 1.2.1 Cellulose

Cellulose, while being the predominant polysaccharide on earth, accounts for less than half the mass of the dry plant cell wall (Fry, 2011). Cellulose is a relatively homogenous polysaccharide, composed of D-glucose residues, as per Figure 1.1.



**Figure 1.1: A Haworth projection of a cellulose fibril strand structure composed of  $\beta$ -(1 $\rightarrow$ 4) linked D-glucose monomers (Bayer *et al.*, 1998).**

Cellulose consists of long homogenous polysaccharide chains made up of glucose monomers linked via  $\beta$ -1,4-linkages, called glycosidic bonds. These polysaccharides are termed cellulose fibrils, with 500-14 000 linked glucose residues per fibril (Bayer *et al.*, 1998; Chang *et al.*, 2007). Glucose moieties in adjacent cellulose chains hydrogen-bond to one another to form crystalline structures, which in turn provides the tight packing and the strong structural support inherent to crystalline cellulose (Teeri, 1997). This is the primary reason for the degradation recalcitrance inherent in cellulose. Cellulose also contains non-crystalline fractions which are amorphous in nature. The degree of polymerisation of the cellulose strands within plant cells and plant structures can be variable, with increases in polymerisation recorded from samples taken from primary cell walls as compared to secondary cell walls (O'Sullivan, 1997).

The variability of cellulose structure both within an organism and between species have been found to be considerable with six cellulose polymorphs discovered to date: I $\alpha$ ; I $\beta$ ; II; III $\alpha$ ; III $\beta$ ; IV $\alpha$  and IV $\beta$  (O'Sullivan, 1997). Naturally occurring cellulose found under the type I category can be further subdivided between the two polymorphs I $\alpha$  and I $\beta$ . The polymorphs were determined as two distinct forms by the NMR analysis performed by Atalla and Van der Hart (1984). Cellulose I $\alpha$  was found to be the dominant form of cellulose produced in algae and bacteria whilst cellulose I $\beta$  has been predominantly found in secondary cell walls of higher plants (Atalla and Van der Hart, 1984). Two commercial examples of cellulose I $\beta$  are medium length cellulose powder (C6288) and Avicel PH-101 from Sigma-Aldrich. These are identical in chemical structure but differ due to Avicel PH-101 being primarily composed of microparticles of 50  $\mu$ m in length while medium length cellulose powder is composed of

fibrils between 4000 and 10 000 glucose residues per fibril (Bayer *et al.*, 1998; Chang *et al.*, 2007). The exact composition of commercial cellulose products is not consistent between manufacturers or between batches, due to varying starting substrate and differing production methods (Pushpamalar *et al.*, 2006). Cellulose II – IV possess modified structures of type I polymorphs and are formed through various pre-treatment methods such as acid or alkaline, chemical, thermophilic or a combination of the preceding treatments (O’Sullivan, 1997). A commercial example of modified cellulose is carboxymethyl cellulose which consists of medium length cellulose fibrils that possess a carboxymethyl group on either the C-2 or the C-3 carbon (Chang *et al.*, 2007; Pushpamalar *et al.*, 2006). This increases the solubility of the fibrils allowing for higher activities with cellulases not inhibited by the presence of the carboxymethyl residues.

### **1.2.2 Cellulase**

The conventional paradigm of cellulose hydrolysis by bacterial and fungal systems, since its discovery in the 1950’s, has been primarily based around the activities of three types of cellulases; namely  $\beta$ -glucosidases; cellobiohydrolases (or exoglucanases) and endo-glucanases (Horn *et al.*, 2012; Merino and Cherry, 2007; Reese *et al.*, 1950; Teeri, 1997). This conventional system functions with the cellobiohydrolases and endo-glucanases hydrolysing cellulose into cellobiose (dimers of glucose) and  $\beta$ -glucosidases cleaving these into D-glucose monomers (Coughlan, 1985). However, this system has yet to equal the high levels of cellulose conversion documented with crude bacterial and fungal filtrates (Coughlan, 1985). Also the discovery of novel classes of enzymes and the elucidations of their mechanisms has forced the expansion of the conventional system to include accessory and non-hydrolytic enzymes which forms part of the current understandings of cellulose hydrolysis (Horn *et al.*, 2012). The conventional system still forms the basis of, and provides the basic catalytic components for, any complete enzymatic cellulose hydrolysis solution currently in use either for research or industrial purposes (Arantes and Saddler, 2011; Merino and Cherry, 2007).

The majority of the total activity within a cellulase solution is provided by cellobiohydrolases (CBH) which function to cleave cellobiose subunits from the ends of the cellulose chains (Merino and Cherry, 2007). CBHs are considered to be processive enzymes and so the degree of crystallinity of a cellulose substrate does not affect their ability to hydrolyse different structural forms of cellulose (Teeri, 1997). There are two different types of CBH, where the

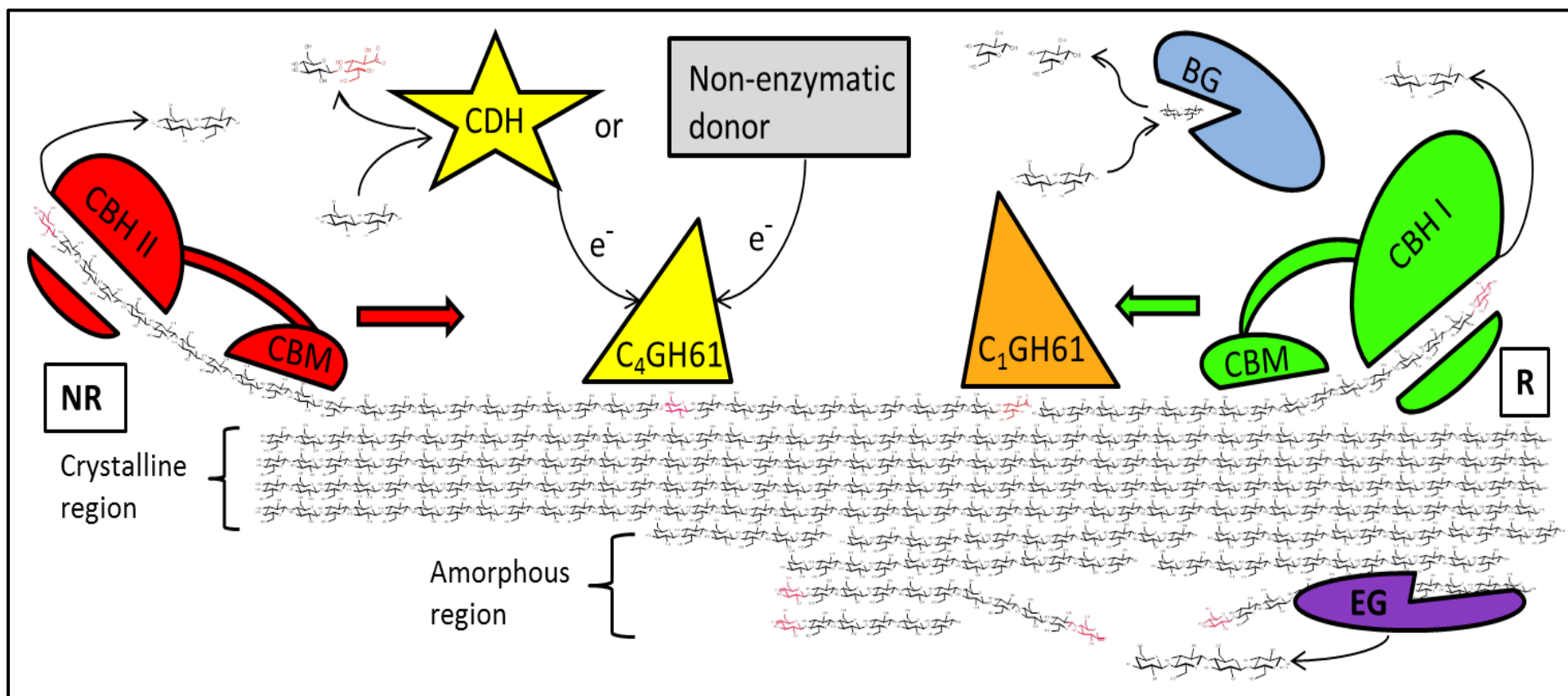
type I hydrolyses from the reducing end while the type II hydrolyses from the non-reducing end of cellulose chains (Teeri, 1997).

Endo-glucanases (EG) differ to CBHs in that they hydrolyse 1,4- $\beta$ -D-glycosidic bonds within cellulose fibrils, thereby reducing the degree of polymerisation of a cellulose fibril, thus providing greater accessibility to scissile bonds for CBHs (Coughlan, 1985; Meyer *et al.*, 2009). This can result in an overall reduction in the crystallinity of a cellulose substrate as well as an increase in the available reducing and non-reducing terminals for hydrolysis with CBH I or CBH II (Arantes and Saddler, 2011). The non-specific hydrolysis of EGs throughout the cellulose fibrils means that activity of any CBHs will not be limited to the initial available reducing ends of the cellulose fibrils (Coughlan, 1985). This synergy between EGs and CBHs has been documented to increase the rate of cellulose degradation of a cellulase solution (Capek *et al.*, 1995). These synergistic relationships have been observed between a variety of cellulases and can be applied to any existing minimal enzyme synergy cocktail (Meyer *et al.*, 2009).

EGs and CBHs are often covalently attached to a carbohydrate binding module (CBM) subunit, which can bind to and assist in the spatial separation of a cellulose chain from a cellulose fibril (Li *et al.*, 2012). In addition to increasing the enzyme activity of an attached enzyme module by proximally binding it to its substrate, they can also influence an enzyme's substrate preference by selectively binding to specific carbohydrate type(s) or structure(s) (Linder and Terri, 1997).

The final process in the production of glucose from cellulose consists of the hydrolysis of the disaccharide cellobiose to glucose through the cleavage of the  $\beta$ -1,4 linkages within cellobiose by  $\beta$ -glucosidases (BG) (Lee *et al.*, 2010). The inclusion of BGs in a cellulase solution can significantly reduce the end product inhibition of both CBHs and EGs caused by high levels of cellobiose (Lee *et al.*, 2010). A schematic diagram of the current model of fungal cellulose hydrolysis, including the conventional cellulase system of CBH, EG and BG is depicted in Figure 1.2 (Coughlan, 1985; Horn *et al.*, 2012).





**Figure 1.2: A schematic diagram of the current model of fungal enzymatic hydrolysis of cellulose. The Haworth projections here represent the organisation and morphology of a cellulose fibril in a typical plant cell wall as well as typical end products from enzymatic hydrolysis. The acronyms CBHI and CBHII represents cellobiohydrolase type I or II, with arrows to indicate their direction of hydrolysis. CBM represents their covalently attached carbohydrate binding domains. EG represents an endo-glucanase in the process of randomly cleaving 1,4- $\beta$ -D-glycosidic bonds in the amorphous region of the cellulose fibril. BG represents a  $\beta$ -glucosidase in the process of the hydrolysis of a cellobiose dimer into D-glucose monomers. CDH represents a cellobiose dehydrogenase. C<sub>4</sub>GH61 and C<sub>1</sub>GH61 represent types of fungal mono-oxygenases, both of which require electron donations from CDH or a non-enzymatic donor for their oxidative activities. The crystalline and amorphous regions of cellulose strands are indicated by brackets, while the reducing terminal side of the strand has been annotated R and the non-reducing terminal side NR. (Horn *et al.*, 2012; Teeri, 1997).**

This conventional cellulase system of CBH, EG and BG has been documented to achieve near complete hydrolysis of some purified types of cellulose (Boussaid and Saddler, 1999; Lin *et al.*, 2011; Van Dyk and Pletschke, 2012). However, the ability of crude fungal and bacterial filtrates to achieve higher levels of cellulose hydrolysis has been noted since the 1950's (Coughlan, 1985; Horn *et al.*, 2012). Recent studies on the activities of bacterial family 33 carbohydrate binding modules (CBM33) and fungal family 61 glycoside hydrolases (GH61) have shown that the conventional cellulase system paradigm has limited potential to effectively degrade lignocellulose without high protein expenditures and harsh pre-treatments (Horn *et al.*, 2012). Current research efforts into CBM33s and GH61s, together with other enzymes in similar classes and non-hydrolytic proteins (such as swollenins and expansins) have demonstrated that all these have important roles in both the degradation of lignocellulose and the increased total activity of the conventional cellulase system, as seen in Figure 1.2 (Horn *et al.*, 2012; Li *et al.*, 2012).

Several studies into the phylogenetics, protein structures and the varied activities of families such as CBM33 and GH61 have revealed that these are actually lytic polysaccharides monooxygenases (LMPO) making their classification within the current families of glycoside hydrolases problematic (Levasseur *et al.*, 2013; Li *et al.*, 2012). It has been proposed that the glycoside hydrolases nomenclature be expanded, with a new class called "Auxiliary Activities" for the LMPOs with the designation AA9 for GH61 and AA10 for CBM33 (Levasseur *et al.*, 2013; Li *et al.*, 2012). As the approval and implementation of the nomenclature changes to AA9 and AA10 has yet to be effected, the current classifications of GH61 and CBM33 will be used in the present study.

Both CBM33 and GH61 have a high degree of structural homology, possessing flat substrate-binding surfaces and a conserved arrangement of their N-terminal amino groups with two histidines that bind a divalent metal ion at their respective active sites (Forsberg *et al.*, 2011; Harris *et al.*, 2010; Vaaje-Kolstad *et al.*, 2010). Both are also capable of cleaving crystalline polysaccharide chains and are classed as copper dependant LMPOs that require an electron donor to function (Harris *et al.*, 2010; Vaaje-Kolstad *et al.*, 2010). These external electron donors are thought to be co-secreted enzymes such as cellobiose dehydrogenase (CDH) or reducing agents such as gallic acid, ascorbic acid and reduced glutathione, as seen in Figure 1.2 (Phillips *et al.*, 2011).

The oxidative cleavage of cellulose fibrils by GH61s is thought to enhance the overall synergy of a cellulase solution by increasing the reducing ends available for CBHs to act upon. Furthermore, the addition of charged groups onto cellulose chains is thought to disrupt cellulose's crystalline packing and to increase enzyme accessibility onto any exposed cellulose fibrils (Horn *et al.*, 2012).

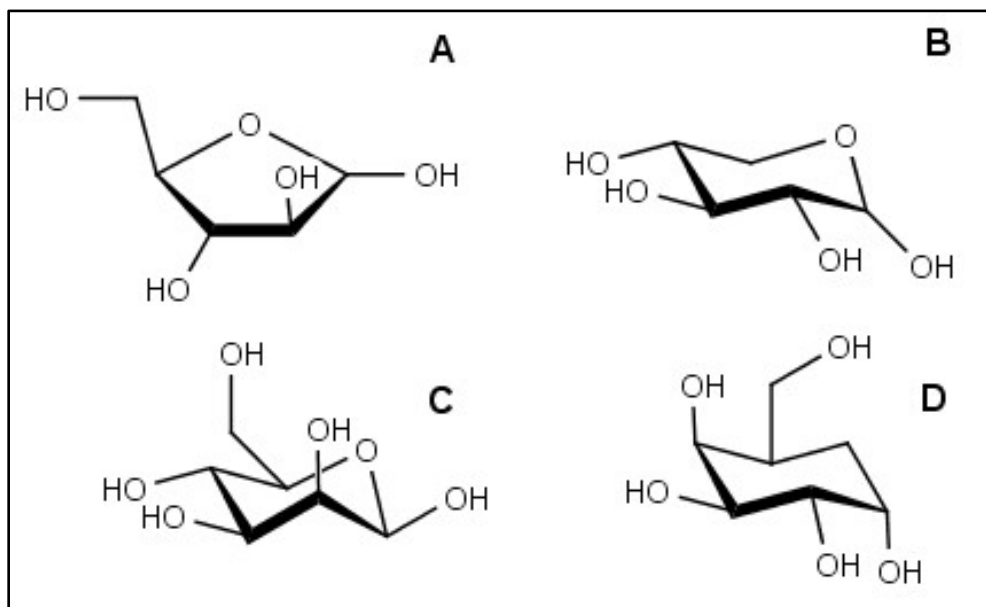
Studies into the enzyme activities of the different GH61s and CBM33s have shown they all utilise similar catalytic mechanisms that function through the oxidative cleavage of cellulose at the C<sub>1</sub> carbon (Phillips *et al.*, 2011). It has also been demonstrated that they may also oxidise at C<sub>4</sub> and C<sub>6</sub> carbons of cellulose (Horn *et al.*, 2012). These activities, in conjunction with those of other co-secreted cellulases, results in the generation of aldonic, gluconic and cellobionic acids (Phillips *et al.*, 2011). Although the exact inhibitory effects of all the potential products of GH61 activity with other cellulases has yet to be determined, the resulting products can be fermented to ethanol and in the case of cellobionic acid it is known to have less of an inhibitory effect on CBH activity than cellobiose (Fan *et al.*, 2012).

The use of these novel GH61s and other non-hydrolytic proteins in commercial enzyme solutions has been reported by multiple authors (Horn *et al.*, 2012). Additionally it has been reported that wild type *Trichoderma reesei* secretes two proteins with high levels of homology to GH61 proteins, when cultured with cellulose containing substrates (Horn *et al.*, 2012). As these commercial mixtures are trade secrets and can be non-purified cellulose induced fungal filtrates, the exact contents in terms of activities and protein composition are not disclosed to the end-user (Horn *et al.*, 2012). Commercially available cellulase solutions, such as Celluclast 1.5L<sup>®</sup> from *T. reesei* are found to have multiple activities on not only cellulose but also a broad range of substrates (Juhász *et al.*, 2005; Van Dyk and Pletschke, 2012). This may be due to enzymes within the solution having undergone genetic modification to increase their substrate ranges (Horn *et al.*, 2012). For instance, certain EGs also possess activity on a form of hemicellulose called xyloglucan, which allows for the enzyme mixture to be used with a greater substrate range (Coughlan, 1985).

### 1.3 Hemicelluloses

Hemicelluloses are defined as a collection of non-cellulosic polysaccharides in plant tissues (Ward, 1989). A definitive property of hemicellulose polysaccharides is their extractability from lignocellulose with aqueous alkaline solutions. Hemicellulose structures can alternatively be composed of either a pentose or hexose residue backbone, depending on the plant species, and may have linear and branched side chains (Ward, 1989). These substrates have a greater variety of structures when compared to cellulose, which can increase the quantity and types of enzymes required to degrade a lignocellulose biomass (Ward, 1989).

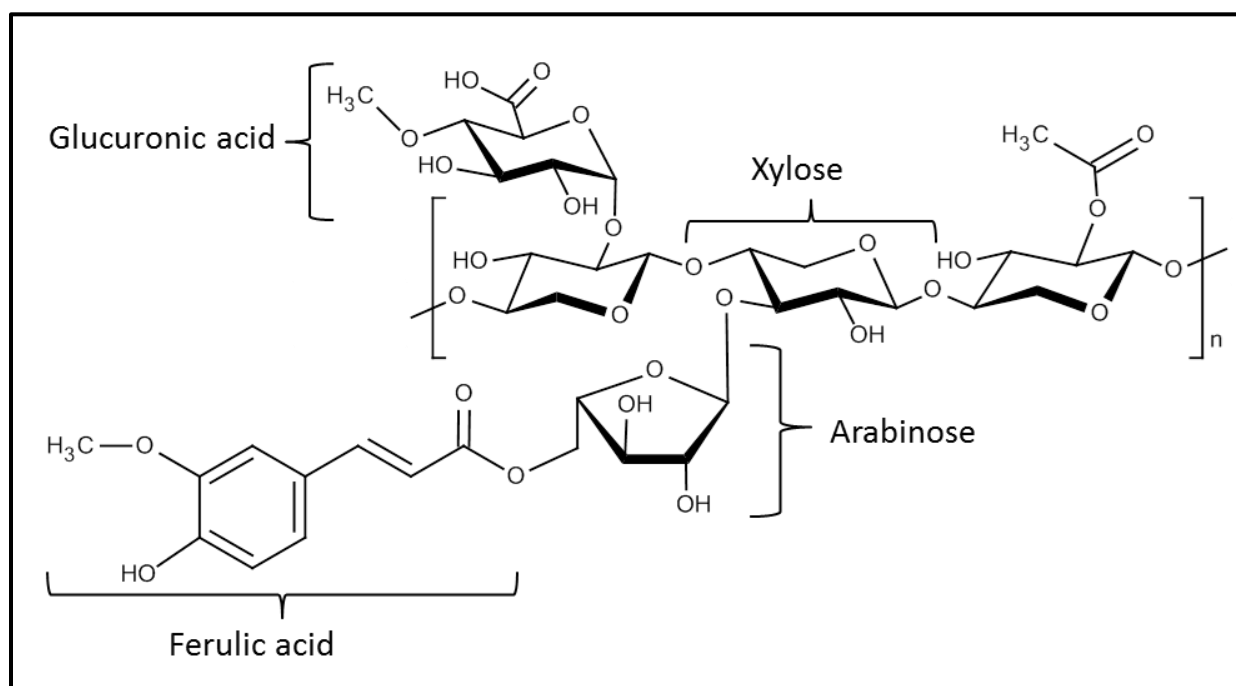
The degradation of both cellulose and hemicellulose is further complicated due to the fact that hemicellulose can hydrogen bond to cellulose. This shared property allows for the tethering of adjacent cellulose fibrils in plant cell walls (Fry, 1989b; Hayashi, 1989). Hemicellulose structures contain heterogeneous matrixes of both linear and branched pentoses (D-xylose, D-arabinose), hexoses (D-mannose, D-glucose, D-galactose) and uronic acids (4-O-methylglucuronic, D-glucuronic and D-galacturonic acid), which surround the cellulose fibrils (Kumar *et al.*, 2009). The structures of the monomers which form the major components of hemicellulose are displayed in Figure 1.3.



**Figure 1.3: A Haworth projections of D-xylose (A), L-arabinose (B), D-mannose (C) and D-galactose (D) structures (Carver, 1991).**

### 1.3.1 Xylan

The major polymer constituent of hemicellulose in plants consists of xylan, such as glucuronoarabinoxylan, the Haworth structure of which is displayed in Figure 1.4 (Wang, 2007). Xylan provides a major structural role in plants by forming an overlaying sheath, by hydrogen bonding to cellulose and by covalent bonds to lignin (Beg *et al.*, 2001). The lignin then forms an outer sheath which reduces mechanical stress to plants. Xylan consists of a backbone of primarily  $\beta$ -D-xylopyranosyl residues linked by 1,4-glycosidic bonds (Beg *et al.*, 2001). The two sugars that generally form major fractions of glucuronoarabinoxylans are either D-xylose or L-arabinose. These pentose sugars have been displayed as constituents in the Haworth structure of glucuronoarabinoxylan as seen in Figure 1.4 (Doi, 2008).



**Figure 1.4: A Haworth projection of glucuronoarabinoxylan structure. The structures of arabinose, glucuronic acid and xylose moieties as displayed in their steric positions have been labelled (Wang, 2007).**

The predominant monomer within xylan, xylose, forms the backbone of the xylan, as displayed in Figure 1.4. Varying amounts of arabinose and glucuronic acid have been found as substituents on the backbone, with ferulic acid linkages attaching the xylan to lignin. The various side-chains and xylan backbone can be cleaved by a number of hemicellulases such as  $\alpha$ -D-glucuronidase; acetyl-xylan esterase; ferulic acid esterase;  $\alpha$ -L-arabinofuranosidase; endoxylanase and  $\beta$ -xylosidase (Wang, 2007).

### 1.3.2 Xylanases

Endo-xylanases have been divided into glycoside hydrolase (GH) family 10 and 11, based on either possessing a high molecular weight (>30 kDa) and low pI (GH10) or a low molecular weight (<30 kDa) and high pI (GH11) (Wong *et al.*, 1988). The active sites of all xylanases have two conserved glutamates which function as the catalytic nucleophile acid/base in hydrolysis of the substrate (Collins *et al.*, 2002). Xylanases occur widely in a variety of organisms, such as algae, fungi, bacteria, snails, protozoa, insects, crustaceans, and seeds of terrestrial plants (Sunna and Antranikian, 1997). Xylan can be degraded through two types of xylanases; either  $\beta$ -1,4-endo-xylanases or exo-xylanases that both hydrolyse the xylan backbone (Sunna and Antranikian, 1997).

XynA is an endo-xylanase from *C. cellulovorans*, and classified as a member of the family 11 glycoside hydrolases (Kosugi *et al.*, 2002). Thus, it possesses narrow substrate specificity and high activity on xylooligosaccharides, as typical for enzymes of this family (Kosugi *et al.*, 2001). It also hydrolyses heteroxylans to a lower degree than family 10 xylanases. XynA has a molecular weight of approximately 57 kDa and associates with the cellulosomal scaffoldin protein of *C. cellulovorans*. XynA has demonstrated synergistic activity with ManA and EngE enzymes, two other cellulosomal enzymes from *C. cellulovorans* (Beukes *et al.*, 2008). A comprehensive review of the cellulosomal enzymes of *C. cellulovorans* has been published by Doi and Kosugi (2004). XynA has been thought to possess limited activity on acetylated xylan, due to the presence of limited amounts of acetate found in solution after hydrolysis of xylan, a typical property of family 10 xylanases (Biely *et al.*, 1986).

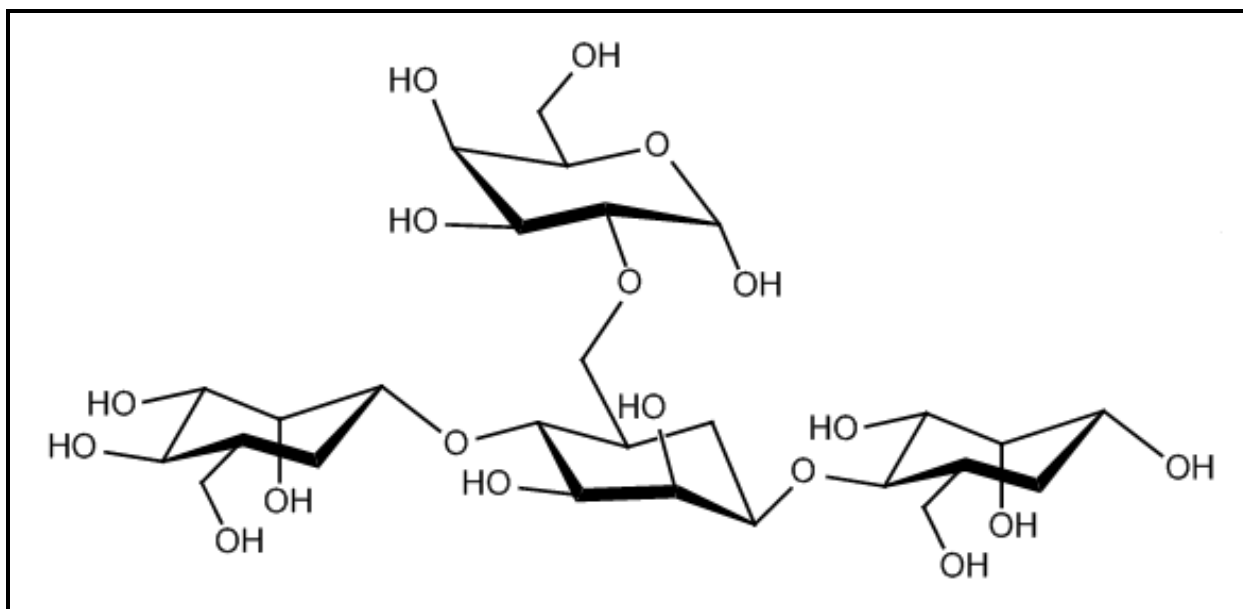
The characterisation of XynA, as reported in literature to date, has demonstrated similar enzymatic properties and narrow substrate specificity, which are similar to the properties of the *Clostridium thermocellum* xylanase (Doi and Kosugi, 2004; Morrison *et al.*, 2011). These characterisations were performed with a recombinant enzyme rXynA which had activity on birchwood and beechwood xylan (Kosugi *et al.*, 2002). Activity was recorded between the pH range of 2 and 7, with the maximum activity observed at 60°C and pH 5 (Kosugi *et al.*, 2002). The specific activity of XynA was 825 U/mg of protein with birchwood xylan, with no activity recorded with xylobiose,  $p$ -nitrophenyl- $\beta$ -D-cellobioside,  $p$ -nitrophenyl- $\beta$ -D-xylopyranoside,  $p$ -nitrophenyl- $\beta$ -D-glucopyranoside and carboxy-methylcellulose (Kosugi *et al.*, 2002). Limited

activity with xylotriose was measured; however, this activity was lower than the activity recorded with longer xylooligosaccharides, which may be due to the greater degree of polymerisation, in comparison with xylotriose (Kosugi *et al.*, 2002; Morrison *et al.*, 2011).

The heterogeneity of the xylan substrates can thus require a variety of enzymes for their degradation. The resultant generation of many degradation products, some of which can inhibit the functioning of other hemicellulose-degrading enzymes, can have deleterious effects on overall lignocellulose degradation (Sunna and Antranikian, 1997). The release of acetic acid by acetyl xylan esterases from the backbone can provide access for endo-xylanase to act upon the backbone due to reduced steric hindrance from acetyl groups (Sunna and Antranikian, 1997).

### **1.3.3 Mannan**

Mannan consists of a backbone of (1-4)-linked  $\beta$ -D-mannopyranose, which sometimes can be acetylated. The backbone of mannan can possess side chains, which can consist of  $\alpha$ -D-galactose attached via a Gal-(1 $\rightarrow$ 6)-Man linkage (Fry, 2011). The overall structure of mannan, while similar to xylan, requires a completely different set of enzymes to degrade mannan into its component constituent sugars (Shallom and Shoham, 2003). The proposed structure of galactomannan is shown in Figure 1.5 (Shallom and Shoham, 2003). The increasing ratio of galactose residues to mannose units on the backbone has been used to differentiate commonly isolated plant gums (Fry, 2011). A commonly used mannan for study has been locust bean gum (LBG), which has one galactose residue or side chain present for approximately every 4 mannose residues on the main chain.



**Figure 1.5: A Haworth projection of a mannan chain with a D-galactose side chain (Fry, 2011).**

Another derivative of mannan, called galactoglucomannan, is known to have a backbone that consists of alternating  $\beta$ -D-mannopyranose units, which sometimes can be acetylated, and alternating  $\beta$ -D-glucopyranose residues with  $\beta$ -1,4-linkages (Fry, 2011). Side chains that are present, consist of alpha-D-galactose attached via a Gal-(1 $\rightarrow$ 6)-Man linkage (Fry, 2011). There have been limited studies into mannans in primary cell walls (Fry, 2011).

### 1.3.4 Mannanases

Mannanases are predominantly members of glycosyl hydrolase family 5 (GH5) (CAZy, 2012). The endo-mannanases function by depolymerising the mannan backbone and releasing oligosaccharides, especially mannobiose. Like the XynA from *C. cellulovorans*, ManA has a catalytic glutamate residue at its active site to reduce the glycosidic bond through a nucleophilic attack (Tamaru *et al.*, 2000). The GH5 mannanases have 8 conserved amino acids located near their active sites, which appear to be highly conserved within this GH family and are involved in substrate binding and stabilisation of the enzyme's active site (Davies *et al.*, 1997, Tamaru *et al.*, 2010).



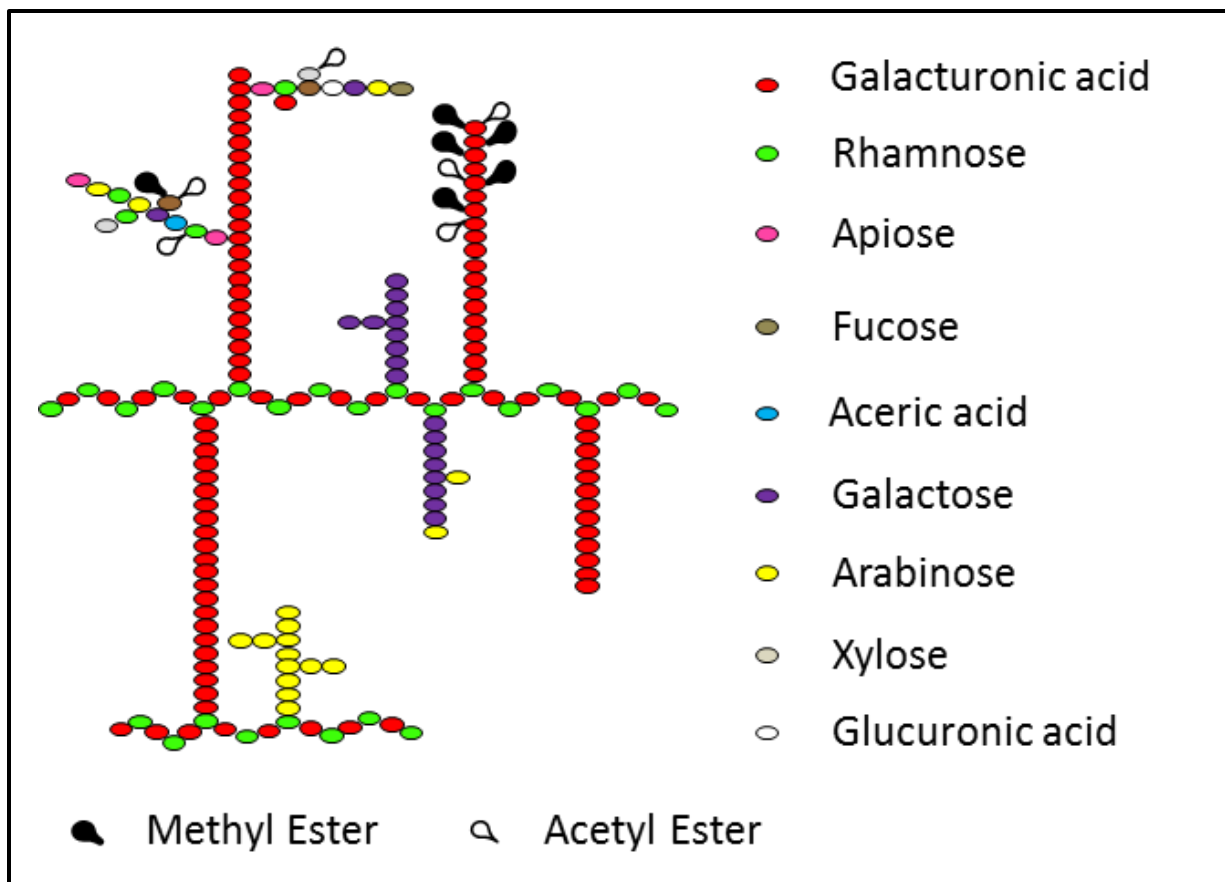
### 1.3.5 Xyloglucan

Xyloglucan polysaccharides can commonly be found in the primary cell walls of higher plants (Zablackis *et al.*, 1995). They consist of a backbone of glucose monomers linked via  $\beta$ -1,4-linkages, with D-xylopyranosyl- $\alpha$ -(1 $\rightarrow$ 6) as a predominant substituent (Fry, 1989a). Other common substituent(s) can include dimers of D-galactopyranosyl- $\beta$ -(1 $\rightarrow$ 2) or L-arabinofuranosyl- $\beta$ -(1 $\rightarrow$ 2) sugars attached to the xylose residue. A trimer of L-fucopyranosyl with an  $\alpha$ -(1 $\rightarrow$ 2)-linkage to the galactose and xylose dimer is the final major type of substituent (Fry, 1989a). Various cellulases have been discovered that also possess activity on xyloglucans, despite the presence of substituents (Coughlan, 1985).

### 1.4 Pectin

Pectins are a family of heterogeneous polysaccharides found in plant cell walls, which have previously been defined primarily by their collective extractability with chelating agents and heating (Willats *et al.*, 2006). The present scientific definition for pectin is that of a polysaccharide which is rich in  $\alpha$ -galacturonic acid (GalA) with GalA levels over 65% (w/v) (FAO, 2010). The main function of pectins has been theorised to adhere adjacent plant cells (Bai *et al.*, 2004). This is due to the high molecular weight and hydrophilic nature of pectin. It thus constitutes most of the middle lamella in plants and is the predominant polysaccharide in the non-lignified primary cell wall of dicotyledonous plants, and the second most predominant polysaccharide in monocotyledonous plants (Harris and Smith, 2006; Jayani *et al.*, 2005).

Pectin consists predominantly of  $\alpha$ -(1 $\rightarrow$ 4) linked GalA as well as a minor amount of (1 $\rightarrow$ 2) linked L-rhamnose (Rha); however, other sugar units, such as D-galactose, L-arabinose and D-xylose, may also be present (Willats *et al.*, 2006). Pectin is an important component of plant cell walls and may constitute more than 35% of the secondary plant cell wall in certain fruits (Fry, 1988). As a biological product it is extremely complex and may be composed of as many as 17 different monosaccharides and over 20 different types of linkages (Figure 1.6) (Mohnen, 1999). The proposed model of pectin has been altered over the decades as linkage analysis, composition determination and structural visualization and modeling efforts have increased, as seen in Figure 1.6 (Willats *et al.*, 2006).



**Figure 1.6: A schematic representation of the proposed model of pectin structure (Willats *et al.*, 2006).**

The conventional model of pectin as a homogalacturonan (HG) polymer formed via  $\alpha$ -(1 $\rightarrow$ 4) linked GalA with various side chains and substituent sugar moieties has been replaced with the proposed model of Figure 1.6 (Willats *et al.*, 2006). The principal difference is that HG polymers are linked with rhamnogalacturonan chains which consist of repeating disaccharides of GalA and rhamnose (Rha) linked via 1 $\rightarrow$ 4,  $\alpha$ -D-GalA  $\alpha$ -1 $\rightarrow$ 2,  $\alpha$ -L-Rha bonds.

### 1.4.1 Pectinases

A wide variety of enzymes have been documented to have activity on pectin due to the variety of sugars and structural arrangements found in pectins (Bai *et al.*, 2004). Amongst the most well studied enzymes are those that act on the homogalacturonan portions of pectin. These include: pectin methyl esterases, polymethylgalacturonases (PMG), polygalacturonases (PG), pectate lyases and pectin lyases. Pectin methyl esterases belong to family 8 of carbohydrate esterases

and allow the action of polygalacturonases through the removal of methyl groups (Pedrolli *et al.*, 2009).

PMG and PG belong to the glycoside hydrolase family 28 and catalyze the hydrolysis of the  $\alpha$ -1,4-glycosidic bonds that form the backbone of the majority of the pectin structures; however, PMG have higher activity on methyl esterified pectin than PG (Pedrolli *et al.*, 2009). Both groups of galacturonases display activity in an endo- and exo- catalytic fashion. The endo-catalytic action cleaves the backbone randomly while the exo-catalytic action will always hydrolyse pectin from the non-reducing end. Pectinases have commonly been isolated from fungi, with the pectinase from *Aspergillus niger* the most commonly used purified pectinase (Jayani *et al.*, 2005). Many pectinases are genetically modified for industrial use, thus a large amount of research has been directed towards finding new pectinases (Beldman *et al.*, 1984).

GalA residues form the pectin backbone and to varying degrees the GalA residues acid groups may have methoxy esters as substitutes for the normal acid groups (Voragen, 2009). The homogalacturonan portions, composed of repeating residues of GalA, are called the smooth regions of pectin. Pectin itself is classified according to its esterification level, with pectin substances having a minimum of 75% of the carboxyl groups methylated and those with levels of methylation lower than this are called pectinic acid (Jayani *et al.*, 2005). Rhamnogalacturonan consists of two types of side chains attached to the homogalacturonan backbone and are collectively called 'hairy regions'. Rhamnogalacturonan I consists of alternating GalA and Rha residues with the GalA having the potential to be acetylated, depending on plant species, as per Figure 1.6 (Willats *et al.*, 2006). Rhamnogalacturonan II has no Rha present and consists only of a homogalacturonan chain with complex sidechains.

More recent models of pectin have incorporated a different backbone of alternating GalA and Rha residues linking the homo GalA backbones, which branches off the hetero backbone, as per Figure 1.6 (Willats *et al.*, 2006). The highly complex and varied structure of pectin, both within a plant and between plant species, indicate that the proposed and conventional models (Figure 1.6) are not definitive of all the pectin structures found to date; but instead are representative of the major groups most commonly encountered (Willats *et al.*, 2006).

In conclusion, there are major classes of polysaccharides associated with plant cell walls. These are primarily defined by their extraction methods which are not an exact means of classifying polysaccharides. Chemically identical hemicelluloses can only be identified through linkage analysis of the varied linkages of pectin and other heterogenous polysaccharides. A brief summary of the polysaccharides composing plant cell walls and the enzymes required for their degradation, as so far discussed in the present study, are represented in Table 1.1.

**Table 1.1: A brief summary of the major classes of polysaccharides in plant cell walls and examples of the enzymes required for their degradation (Fry, 2011).**

<b>Plant cell wall polysaccharides</b>	<b>Enzyme activity required for degradation</b>
<b>Cellulose</b>	CBHI, CBHII, EG, BG, CBM33, GH61, CDH
<b>Xylan</b>	Xylanases from GH10 (XynA) and GH11
<b>Mannan</b>	Mannanase from GH5 (ManA)
<b>Xyloglucan</b>	Xyloglucan endo-transglycosylase (GH16), EG
<b>Pectin</b>	PMG, PG

A comprehensive table of all plant polysaccharides and the enzymes required for their degradation is available in Fry (2011). The polysaccharide classes, listed in Table 1.1, are all encased and variably linked with a protective polyphenol layer of lignin, which encases most fully developed plant cells that are the target of degradation for second generation biofuels.

### **1.5 Lignin**

Lignin is a complex structure of aromatic rings and side chains with various bonds, forming a heterogeneous structure. The highly complex and variable nature of lignin has consequently resulted in the need for its removal through pre-treatment steps rather than degradation to usable end products, such as vanillin (Chang, 2007). The inclusion of lignin degradation products into an enzyme hydrolysis mixture has demonstrated many inhibitory effects and thus should be limited as far as possible (Kaya *et al.*, 2000; Morrison *et al.*, 2011). One source of lignocellulose with low lignin content and a high concentration of pectin is fruit waste from processing.

## **1.6 Fruit Waste Substrates**

Plant cell walls have a varied polysaccharide composition and thus represent a complex substrate for enzyme degradation. Magnoliophyta plant cells possess a relatively thin primary cell wall that is laid down during initial cellular growth and a thicker secondary cell wall is then laid down inside the primary cell wall during cellular maturation (Fry, 1988). The cell walls differ compositionally with the primary walls consisting of ~20-30% cellulose, while the secondary walls are composed of ~40-90% cellulose (Fry, 1988). The differences between the plant cell walls of Magnoliophyta have been generalised down to class level and state of lignification of the cell wall (Smith and Harris, 1995). For both major groupings of magnoliophyta, monocotyledons and dicotyledon, the lignified secondary cell walls share a high degree of polysaccharide composition commonality, with heteroxylan as the major non-cellulosic polysaccharide (Bacic *et al.*, 1988). However, the non-cellulosic polysaccharide composition of unligified secondary cell walls for both varies considerably. The large quantities of lignocellulosic wastes that are produced every year from agricultural processing provide a readily usable source of sugars for use in the production of biofuels (FAOstat, 2010). Of particular interest is the use of fruit wastes from fruit processing facilities.

### **1.6.1 Global Pineapple Waste**

*Ananas comosus*, (common name pineapple) is a monocotyledonous plant and member of the family Bromeliaceae, order *Poales*. With over 19 418 478 million metric tonnes (MT) produced globally in 2010 and between approximately 40-80% of the fruit going to waste, there is a large amount of waste produced (Van Dyk *et al.*, 2013). This potentially equates to an amount of over 7 767 391 MT of waste produced globally each year (FAOstat, 2012). This high quantity is due to processing steps, consisting of canning or drying, which can generate up to 40% of whole pineapple fruit weight as skin wastes, excluding the weight of the crown (Tewari *et al.*, 1987). This mostly consists of the skin (rind) of the pineapple, but may also include the core if cannery processing is involved.

The most commonly grown cultivar variety is the 'smooth cayenne' owing to its cylindrical shape which allows ease of processing in pineapple canning. These canning processes are the major source of pineapple waste in South Africa. The steps towards pineapple processing and an example of a smooth cayenne pineapple are represented in Figure 1.7.



**Figure 1.7: A photograph of a whole smooth cayenne *A. comosus* (left), with equivalent post processing wastes consisting of the crown, rind (middle) and final product of fruit flesh (right).**

### **1.6.2 National Pineapple Waste**

In South Africa, large quantities of pineapple are grown every year and canned for export with 120 000 tonnes having been produced domestically in 2010 alone, according to figures from an annual report of the Department of Agriculture, Forestry and Fisheries (DAFF) of the Republic of South Africa. The production is split between the Eastern Cape and northern Kwa-Zulu Natal, with the former accounting for over 75% of total annual domestic production (DAFF, 2010). Pineapple cultivation remains prominent in the Eastern Cape with increasing yields being recorded in recent years. This was despite a disastrous cadmium contamination of fertilizers in 2006, which led to temporary export bans to the European Union for 2007 (DAFF, 2010).

Historically, over 80% of pineapples cultivated have been processed for export (DAFF, 2010). This equates to approximately 40 000 tonnes of unused lignocellulosic waste, in the form of skins produced, in 2010 and concentrated around several cannery plants in South Africa. The only current use for this waste is feed for dairy cows, and the only conceived potential use was in a bio-methanation plant, for which insufficient funding was procured, leading to project cancellation in 2009 (DAFF, 2010). A large source of lignocellulose, rich in both cellulose and hemicellulose, remains underutilized in the Eastern Cape in the form of pineapple waste.

### 1.6.3 Pineapple Pomace Composition

Composition studies on the unignified cell walls of pineapples have found them to be an intermediate between monocotyledon family *Poaceae* and that of dicotyledonous plants (Smith and Harris, 1995). The primary non-cellulose polysaccharide is highly substituted heteroxylans in the form of glucuronoarabinoxylans (GAX). The presence of ester-linked ferulic acid in unignified pineapple, and approximately half of all monocotyledons, is another indication of the presence of GAX's in significant percentages (Smith and Harris, 1995). This is due to ferulic acid being a common constituent linked via ester bonds to arabinose on GAX. It was found, through linkage analysis, that there was a galactose residue attached to every second mannose residue along a mannan backbone, conferring that galactomannan was present. With a complete linkage analysis and identification of all sugar monomer fractions, a composition table of the polysaccharides in the unignified cell walls of pineapple fruit was constructed in Table 1.1 (Smith and Harris, 1995).

**Table 1.2: Dry weight Composition of the unignified cell walls of pineapple fruit (Smith and Harris, 1995).**

<b>Polysaccharide</b>	<b>Dry weight composition (%)</b>
<b>Cellulose</b>	40%
<b>GAX</b>	28%
<b>Xyloglucans</b>	16%
<b>Pectin</b>	6%
<b>Glucomannan</b>	5%
<b>Ash</b>	5%
<b>Total</b>	100%

The presence of the major polysaccharide groups, identified in Table 1.1, allows for the construction of a synergistic solution of enzymes based on activities required to degrade the constituent polysaccharides.

## **1.7 Enzyme Hydrolysis and Synergy**

In general, carbohydrate degrading enzymes are termed glycoside hydrolases (GH). The sheer number and varied sources of GH's has necessitated a system to organise and classify known and new enzymes with GH activity. This has been achieved through classification and grouping of GH's according to their amino acid sequence similarities into families; which in turn bear a direct relationship to their folding similarities (Henrissat, 1991). This is due to the property of protein structures having a higher conservation rate than protein sequence and has allowed the establishment of families and the grouping of several families into clans based on their common structure (Bourne *et al.*, 2001). The similar structures have, in turn, allowed better prediction of the enzyme characteristics in terms of substrate specificity and activities. This proved superior to the previous classification system based on enzyme activities (Bourne *et al.*, 2001).

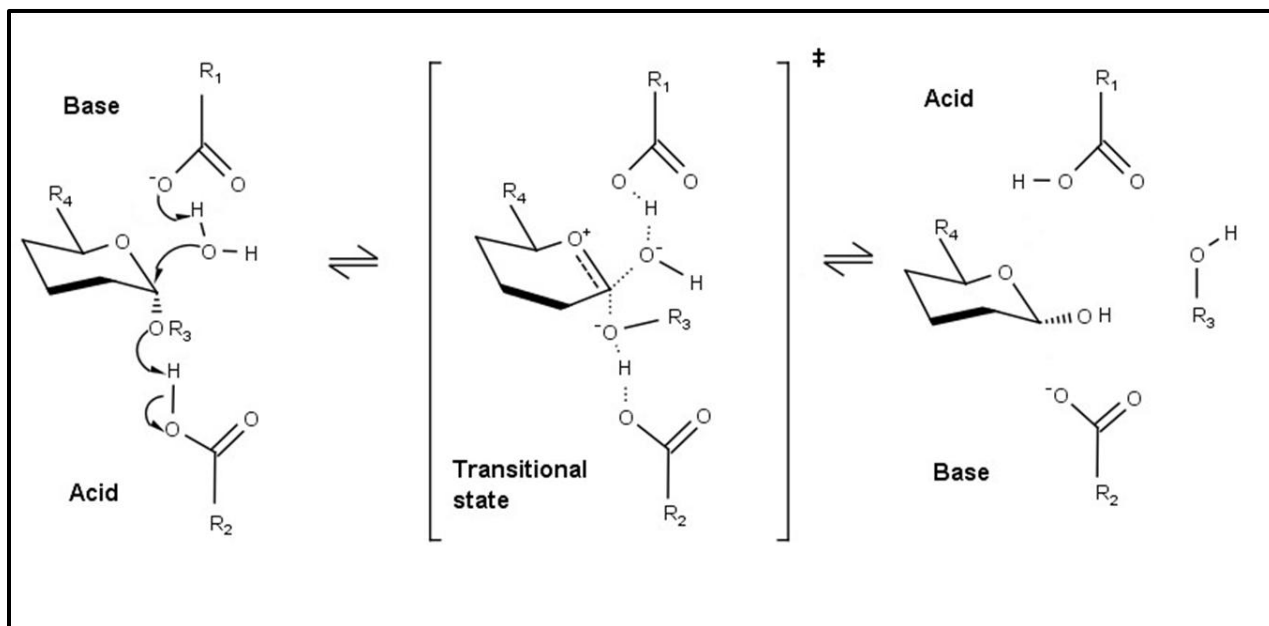
### **1.7.1 Glycoside Hydrolase Reaction Mechanisms**

This classification system has generated 131 families of GH as classified in the Carbohydrate Active Enzymes database (<http://www.cazy.org/>) (CAZy, 2012; Collins *et al.*, 2002; Henrissat, 1991). The structural and AA sequence similarities allowed for the rapid identification of the enzyme catalytic mechanisms inherent to all the families. It was established that the majority of GH's hydrolyse glycosidic bonds through a general acid base catalysis reaction and are divided into two mechanistic classes commonly found amongst bacteria and fungi (McCarter and Withers, 1994). This is determined by whether their reaction mechanisms involve a net retention or inversion of the anomeric configuration of the substrate sugar moiety during the hydrolysis reaction (McCarter and Withers, 1994).

#### **1.7.1.1 Inversion Mechanism**

The GH of family 8, that of XynA (EC 3.2.1.8) from *C. cellulovorans*, follow the inversion mechanism displayed in Figure 1.8, as adapted from McCarter and Withers, (1994) (Kosugi *et al.*, 2002).





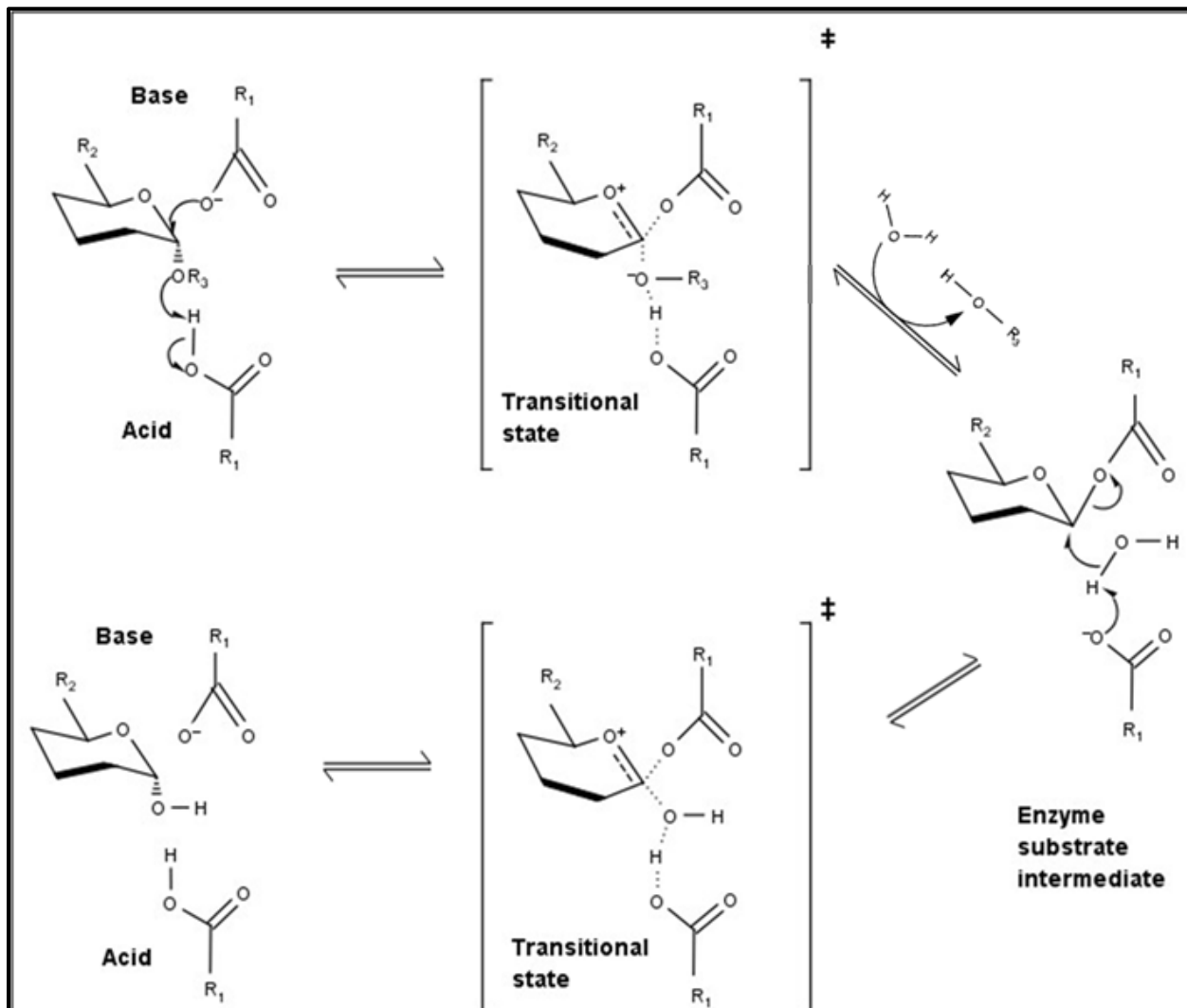
**Figure 1.8: The general acid to base hydrolysis of a glycosidic bond with an  $\alpha$ -inversion mechanism (McCarter and Withers, 1994).**

The hydrolysis of the glycosidic bond commences with a nucleophilic attack by the base hydroxyl moiety upon a proton of an adjacently positioned  $\text{H}_2\text{O}$  (McCarter and Withers, 1994). This allows a nucleophilic attack by the hydroxyl group of the  $\text{H}_2\text{O}$  upon the  $\text{C}_6$  of the substrate and the establishment of an oxocarbenium ion-like transitional state (McCarter and Withers, 1994). This results in a boat conformation and, upon the subsequent transition of the enzyme substrate intermediate to free enzyme and product, the hydroxyl moiety on the sugar residue is inverted from a  $\beta$  to an  $\alpha$  planar orientation (McCarter and Withers, 1994). In the  $\beta$ -inversion mechanism the base residue of the enzyme is in the  $\beta$  planar position relative to the scissile bond as opposed to the  $\alpha$  position, as displayed in Figure 1.8.

### 1.7.1.2 Retention Mechanism

The retention mechanism, also known as the classical Koshland double displacement mechanism for hydrolysis of the glycosidic bond differs from the inversion mechanism, in the lack of any inclusion of an  $\text{H}_2\text{O}$  for the initiation of the reaction mechanism (Koshland, 1953). Instead, the nucleophilic attack of its base glutamic residue on to a proton from the proximal  $\text{H}_2\text{O}$  starts the catalysis and the eventual separation of the glycosidic residue from the enzyme substrate intermediate. This net retention mechanism is common to GH family 5, the family of ManA (EC 3.2.1.78) from *C. cellulovorans* (Kosugi *et al.*, 2001). The net retention mechanism was

proposed by Koshland in 1953, even though the catalytic residues of the subject enzyme were not clear, but was subsequently proved to be correct (Koshland, 1953). The cellobiohydrolase I of *T. reesei* (EC: 3.2.1.91) catalyses its reactions via the retention mechanism. The proposed mechanism has been validated and is represented in Figure 1.9 (McCarter and Withers, 1994).



**Figure 1.9: The classical Koshland retention mechanism for cellobiohydrolase I (McCarter and Withers, 1994).**

This inversion and retention mechanism are amongst the most commonly shared mechanism of the glycoside hydrolase families that hydrolyse cellulose, xylan, mannan, and pectin substrates. However, many families have broad substrate specificities not just limited to these four major constituent polysaccharides found in lignocellulose (Doi and Kosugi, 2004). This has been due to evolutionary pressure to acquire new substrate activity on other types and derivatives of

polysaccharides commonly found in plants. This can lower the amount of overall enzyme expression needed to reduce complex lignocellulose in to simple sugars. The ability to hydrolyse a broad range of substrates can be of use when designing an enzyme cocktail that contains minimal quantities of enzymes. If one enzyme can be active with two or more substrates, it can eliminate the need to include two different enzymes.

### **1.7.2 Lignocellulose Hydrolysis for Fermentation**

In order to achieve the minimal sugar concentrations for successful fermentation, both food-based edible agricultural substrates and lignocellulosic substrates must undergo enzymatic hydrolysis. The enzyme loadings required to produce these concentrations from lignocellulosic substrates can typically be 40-100 times the loadings required for the enzymatic hydrolysis of edible agricultural substrates (Merino and Cherry, 2007). These high levels of enzyme loadings for lignocellulose substrates have meant that cellulosic based biofuels have higher production costs than edible agricultural substrates, such as corn or cane sugar. As a result, most biofuel production has predominantly been from corn and cane sugar fermentations (Sims *et al.*, 2010).

The switch to lignocellulosic biofuel will thus require higher levels of enzyme activity than has been previously achieved, to counteract the high levels of enzyme loadings that are required (Merino and Cherry, 2007; Sims *et al.*, 2010). Achieving high levels of enzyme activity from the minimal possible amount of enzyme is a priority in reducing the overall cost of any enzymatic hydrolysis step(s). This will, in the long term, minimise the impact of biofuel production on global food supplies (Sims *et al.*, 2010). Thus, identifying the best possible candidate carbohydrate degrading enzymes, for use in any minimal enzyme cocktails, is a priority (Meyer *et al.*, 2009; Sims *et al.*, 2010). This is essential when dealing with complex lignocellulosic substrates containing a variety of complex polysaccharides and generating the required levels of simple carbohydrates for successful fermentation (Lynd *et al.*, 2005).

A typical bioreactor or fermentation tank with *Saccharomyces cerevisiae* as a fermentation agent requires a readily available source of carbohydrates in the form of sugar monomers or oligomers which the organism is able to use (Sims *et al.*, 2010). Concentrations of sugar monomers, such as glucose, should be in the range of 80 g/L for *S. cerevisiae* to successfully ferment with an average sugar to ethanol conversion percentage above 95%. Although 60 g/L is considered to be

the minimum sugar concentration required for a successful fermentation, with over 90% of the available sugars converted to ethanol (Pacheco et al., 2010). Two of the key limiting steps in the production of simple polysaccharides and sugar monomers, are the high energy demand and material input required to generate these concentrations (Merino and Cherry, 2007; Naik *et al.*, 2010). These have been in the form of the pre-treatment of lignocellulosic waste, via a combination of thermophilic and/or chemical degradation steps and/or enzyme hydrolysis.

Thus, to expose the component polysaccharides to hydrolysis, lignocellulose has to be separated into its individual components or the overall structural topology has to be degraded sufficiently to allow each enzyme access to its substrate (Sims *et al.*, 2010). This can be achieved through processes such as pre-treatment and degradation by a mixture of enzymes in a ratio that will allow for the maximum release of sugars (Merino and Cherry, 2007).

## **1.8. Enzyme Synergy**

The varied polysaccharide compositions, the variety of inter and intra-polysaccharide bonds and protective topological arrangement of lignocellulose has resulted in a recalcitrant and degradation resistant macromolecule (Fry, 2011). In order to degrade and utilise the energy and material resources contained within lignocellulose, a multitude of enzymes are required to degrade each component. However, the variety of bonds and topological placements of polysaccharides in and around each other hinders and can even prevent the access of individual enzymes to the scissile bonds of their substrate polysaccharide within the lignocellulose ultra-structure (Coughlan, 1985).

### **1.8.1 Synergy evolution and its structural relationship**

For 3.8 billion years, life was composed of unicellular microbes and viruses. This meant ‘pre-treatment’ steps taken by nature on lignocellulose were limited to chemical treatments, in the form of acid and alkali hydrolysis, and not industrial thermal and mechanical treatments, as available today (Merino and Cherry, 2007). Natural degradation of lignocellulose had to rely upon enzymes to provide an effective way for the organism to generate monosaccharides from lignocellulose. As different varieties of lignocellulose evolved, novel enzymes co-evolved to hydrolyse the bonds in these substrates (Fry, 2011). To balance out the energy expenditure of enzyme(s) produced for the sugars released, any organism strives to produce the least amount of enzyme for the maximum amount of sugar. This entailed selecting the corresponding enzymes to hydrolyse available substrates in the accessible lignocellulose and of these the ratio of enzymes that would work best together to generate the highest release of sugars. The ability to degrade multiple substrates, and subsequently utilise their components, with the least expenditure of energy and resources, has always been a driving force of natural selection amongst organisms and their incumbent enzymes (Darwin, 1859).

The enzymes would thus have to perform multiple objectives to achieve this. They would have to depolymerise their particular substrate to a usable form, both to allow product delivery to the organism and generation of polysaccharides that could be further processed into usable sugar monomers (Sims *et al.*, 2007). They would have to do this in a way that did not interfere with the actions of other enzymes on their substrates. It should also assist other enzyme(s) activities by removing its specific substrate, if that is the blocking access of another enzyme(s) to that

enzyme(s) substrate. This would achieve the goal of spatially separating substrates (Bayer *et al.*, 2007). Thus by achieving these objectives the enzymes would be synergistically working together to degrade the maximum amount of substrate with the least amount of enzyme used and thus the least enzyme spent for the amount of sugar released (Bayer *et al.*, 2007).

Individually added enzymes may not have access to the scissile bond on the substrate, which is embedded within the cell wall structure (De Vries *et al.*, 2000). As one enzyme acts on its respective substrate, the degree of complexity and polymerisation is reduced, subsequently allowing access to other enzymes to act on their respective substrates. This enzyme relationship is known as synergy and synergistic enzyme activity occurs when a mixture of enzymes has a higher observed degree of activity than the theoretical sum (total) activity displayed by the individual enzymes on the same substrate (Coughlan, 1985; Koukiekolo *et al.*, 2005). This synergistic relationship between mixtures of enzymes has been documented in nature and is under investigation for *in vitro* replication of these synergistic relationships using pure enzymes (Gilligan and Reese, 1954; Horn *et al.*, 2012).

### **1.8.2 Synergy based on substrate monomer composition**

One approach to establish enzyme synergy on lignocellulose focused on designing enzyme mixtures based on the relative proportions of each polysaccharide in a particular sample of lignocellulose's polysaccharide compositional analysis (Banerjee *et al.*, 2010). These attempts to design and predict enzyme synergy before biochemical assay analysis were not successful (Merino and Cherry, 2007). Current consensus is that biochemical analysis of each enzyme ratio is vital to the establishment of an effective lignocellulose degrading solution (Sims *et al.*, 2010). Thus, despite the numerous assays required for the establishment of any synergistic relationship between enzymes, biochemical assays of each ratio have to be assayed in order to accurately measure the substrate degradation and the sugar release (Banerjee *et al.*, 2010). The sugar monomer composition of a fruit waste should thus only act as a guideline of the potential polysaccharide structures present (Sims *et al.*, 2010).

Pineapple skin is rich in polysaccharides, in particular cellulose (Tewari *et al.*, 1987). The degradation of these into sugar monomers such as glucose, fructose and other hexoses, and even pentoses, could provide a carbohydrate source for local biofuel production in the Eastern Cape

(Harris and Smith, 2006). The use of a GAX degrading enzyme such as XynA is necessary as GAX is present as the predominant polysaccharide in both the non-lignified primary and lignified secondary cell walls of *Poaceae* plants (Harris and Smith, 2006). Secondary in quantity, to GAX in the lignified secondary cell wall are glucomannans, which necessitates the addition of a mannanase in the form of ManA. Pectin is second in quantity to GAX in non-lignified primary cell walls and thus a pectinase is also required (Harris and Smith, 2006). Finally, the high quantity of cellulose in pineapple pulp, up to 40% by dry weight, means that the inclusion of a cellulase solution is beneficial to the degradation of pineapple pulp (Sreenath *et al.*, 1994).

### **1.8.3 Synergistic relationships**

The first demonstration of synergistic relationships was between fractions of culture filtrates from *Trichoderma reesei*, and *Myrothecium verrucaria* on the degradation of a cellulose rich substrate (Gilligan and Reese, 1954). Research in this area was initially slow for the following decade until it was shown that certain individual fractions of the culture filtrate from *T. reesei* displayed synergy with each other. This was demonstrated to be due to the synergistic actions of EGs and CBHs in the mixtures. Since then, research on the synergistic actions of cellulases has become well-established due to the abundant quantity of cellulose in nature, its well defined, and orderly, structure and widespread availability of prepared substrates (Banerjee *et al.*, 2010; Coughlan, 1985). Enzyme mixtures capable of degrading cotton with degrees of synergy (DS) between 3.9 and 7.6 were identified by Zhang and Lynd (2004). However, less crystalline and more amorphous acid treated cellulose-rich lignocelluloses have a DS of less than 1.8 (Zhang and Lynd, 2004).

The crystalline nature of pure cellulose means that the type of synergy displayed is homeo synergy, as the enzymes all have activity on the same substrate, namely the cellulose fibrils (Banerjee *et al.*, 2010). This is due to the activity of main chain cleaving enzymes providing increased access for other enzymes to act on their respective scissile bonds on the backbone of the substrate. This is due to both the creation, and exposure, of both types of reducing terminals on cellulose fibrils. Thus synergy is increased by the action of the enzymes on the same part of the substrate. This would commonly be observed by the actions of an assortment of cellulases on crystalline cellulose (Banerjee *et al.*, 2010). Hetero synergy is the increase in activity due to the actions of enzymes on different substrates that collectively form an overall structure, thus

exposing more substrate for another enzyme to have activity upon (Coughlan and Hazlewood, 1993). Here the enzymes may cleave side chains and thus expose the backbone for other enzymes to work on. This is commonly observed in the action of galactosidases and mannanases on mannan rich substrates and can lead to degrees of synergy above 1.8 (Charrier and Rouland, 2001).

Of importance to synergy analysis is that an assay, appropriate to the objective of the hydrolysis of the lignocellulose, should be used. A reducing sugar assay such as dinitrosalicylic (DNS) or Somogyi-Nelson assay can be used to quantify the reducing sugars in the solution as a result of enzyme action (Miller, 1959; Nelson, 1944). This will give a measure of the depolymerisation of the substrate, due to enzyme hydrolysis. A drawback of these assays are that they do not identify which sugars are being released (Miller, 1959; Nelson, 1944). These assays therefore do not identify which polysaccharides are being degraded, the identity of end products and their degree of polymerisation. However, for the end objective of overall degradation of substrate these methods are sufficient.

#### **1.8.4 Synergy between XynA and ManA**

XynA and ManA are cellulosomal enzymes from *C. cellulovorans* and have demonstrated synergy in the degradation of lignocellulose substrates such as sugar cane bagasse and sugar beet pulp (Beukes *et al.*, 2011; Dredge *et al.*, 2011). The optimal synergy between these enzymes, in a ratio of 3 ManA to 1 XynA, on alkali pre-treated sugar cane bagasse was measured to be 3.5 units of degree of synergy, although the activity was relatively low at ~600  $\mu\text{mol/mL/min}$  of reducing sugar polymers of unknown length (Beukes *et al.*, 2011). The highest activity and second highest degree of synergy, under the same conditions, was at a ratio of 1 ManA to 3 XynA. The activity was 1200  $\mu\text{mol/mL}$  and the synergy was 2.9 units (Beukes *et al.*, 2011). The overall concentration of both enzymes combined were 50  $\mu\text{M}$  in solution. The effect of alkaline pre-treatment of bagasse on enzyme activity was significant with an approximate 5 fold increase in the activity of both of the above-mentioned enzyme ratios.

The recalcitrant nature of the substrate, the high lignin content of 22% and the intertwined lignocellulose structure meant that the inclusion of a pre-treatment step on bagasse was effective at increasing enzyme activity (Beukes *et al.*, 2010). However, on other processed agricultural



waste, specifically fruit waste, pre-treatment may not yield such a high increase in activity, depending on the structure of the substrate.

Synergy between ManA and XynA has also been measured on alkali pre-treated sugar beet pulp (Dredge *et al.*, 2011). It was found that competition was observed for binding on the substrate with negative levels of synergy existing for all ratios except for a ratio of 75% XynA to 25% ManA, which had activity of 1.2  $\mu\text{mol}/\text{mL}/\text{min}$  and a degree of synergy of 1.1 (Olver *et al.*, 2011). The competition was demonstrated from the fact that activity for ManA was at 1.8  $\mu\text{mol}/\text{mL}/\text{min}$  for 100% ManA, but this dropped by 60% to 0.7  $\mu\text{mol}/\text{mL}/\text{min}$  with the reduction of ManA to 87.5% and addition of 12.5% of XynA.

With pineapple fibre waste, individual activity was measured with ManA and XynA on pineapple pulp (Olver *et al.*, 2011). Activity was defined as specific activity with  $\mu\text{mol}/\text{mL}/\text{min}$  of reducing sugars released per mg of protein (U/mg). The activity for ManA and XynA was 15 U/mg and 17 U/mg, respectively (Olver *et al.*, 2011). The highest level of activity was at 39.9 U/mg, with a ratio of 25% XynA to 75% ManA (Olver *et al.*, 2011). Synergy was measured by keeping the total protein concentration for enzymes constant throughout the assays. This optimal level of activity for bi-enzyme synergy also resulted in the highest level of synergy at 2.5 degrees of synergy (Olver *et al.*, 2011).

### **1.8.5 Bi-Synergy between ManA; EngE and XynA**

The synergy between ManA and endoglucanase E (EngE from *C. cellulovorans*), and XynA and EngE was also measured by Olver *et al.* (2011). With these synergy assays, the highest degrees of synergy were again found to correspond to levels of highest activity, both of which were at 50:50 ratios for ManA:EngE and XynA:EngE (Olver *et al.*, 2011). In the study of Olver *et al.* (2011) it was found that the total activity for the 50:50 combination of ManA:EngE was 52.4 U/mg; with 2.8 degrees of synergy; while with the 50:50 combinations of XynA:EngE a total activity of 43.4 U/mg with 2.4 degrees of synergy was generated. These high degrees of synergy demonstrated that high levels of enzyme activity were possible on the pineapple pomace with only a minimal amount of enzyme required. The units of enzyme activity for Beukes *et al.*, (2011) and Olver *et al.*, (2011) have been reported with D-xylose equivalent reducing sugar

measurements generated from DNS assays. This indicated the degree of de-polymerisation of the substrate caused by the enzymes, but did not identify the sugar products released.

### **1.8.6 Synergy between complex commercial mixtures of cellulases and pectinases**

Limited studies in literature on the synergy between cellulases and pectinases have shown that the addition of minimal quantities of pectinases and cellulases can result in increased yield and synergy (Sreenath *et al.*, 1994; Zhang *et al.*, 2013). The degradation of fresh hemp fibres (*Cannabis sativa*) with predominantly cellulase containing solutions was enhanced with the addition of pectinase or xylanase containing solutions, in bi-synergy studies (Zhang *et al.*, 2013). The yield of glucose monomers were increased from 2.94 g/L to 3.72 g/L and 3.97 g/L, respectively, in the presence of either the pectinase or xylanase solutions. There were also minor amounts of galactose (0.14 g/L) and xylose (0.03 g/L) released, depending on whether pectinase or xylanase solutions were also present (Zhang *et al.*, 2013).

Zhang *et al.*, (2013) used crude commercial enzyme solutions for their enzyme activity and thus each of the enzyme solutions were named according to the predominant polysaccharide degrading activity of the enzyme mixture. For pectinase activity the authors used Pectinex Ultra SP-L<sup>®</sup> which contained minor cellulase and xylanase activity. The cellulase and xylanase enzyme mixtures consisted of a mixture of heterogeneously expressed enzymes, with the predominant activity being on cellulose or xylan (Zhang *et al.*, 2013). It was of interest that the addition of all three enzyme mixtures resulted in a negligible increase of glucose released to 4.04 g/L of glucose and no increases in either galactose or xylose released were observed above the amounts from the bi-synergy studies. In addition to this, when all three enzymes were present the standard error increased to 0.46, as opposed to 0.12 and 0.04, when either only the pectinase or xylanase solutions were present, in addition to the cellulase mixture (Zhang *et al.*, 2013).

The use of commercial pectinases and cellulases for the increased juice extraction from pineapple was confirmed by Sreenath *et al.* (1994). A 13% (v/v) increase in juice extracted was achieved with a 0.025% mixture of equal parts of Celluclast 1.5L<sup>®</sup> and Pectinex Ultra SP-L<sup>®</sup> (Sreenath *et al.*, 1994). The measurement of sugar content through specific gravity analysis and spectrophotometric absorbance at 650 nm, in addition to the failure to remove all soluble sugars

from the pomace before the enzyme assay, meant that no statistically significant increases in sugar content were measured (Sreenath *et al.*, 1994). This was due to the known problems of suspended solids interfering with specific gravity and the inability of measurements at 650 nm to quantify the reducing sugars present in solution or decreases in the degree of polymerisation of polysaccharides present.

Further investigation into the depolymerisation and release of reducing sugars from pineapple pomace with the use of reducing sugar assays would allow for the accurate measurements of both synergy and generation of sugar monomers for biofuel production. The lack of current quantifiable data in relation to degradation of pineapple pomace means that accurate data on reducing sugars, through DNS assays, would be of great assistance in pineapple degradation. Incorporation of enzymes to degrade all the component polysaccharides in pineapple pomace would present novel results. The importance of synergy between pure and commercial enzymes is also not well understood when these are combined on pectin rich fruit waste substrates. The current lack of knowledge regarding the activities of mannanases with cellulases and pectinases will also be addressed in this study.

# Chapter 2: Research Motivation and Hypothesis

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## **2.1 Motivation**

It is of increasing importance to study areas of economic significance towards the industrial application of biofuels towards a sustainable and economically feasible means of liquid energy globally and in South Africa (Newman *et al.*, 2012). Of significance in this regard is the use of lignocellulosic wastes. However, the high costs of enzymes required both in terms of per unit cost and total quantity cost required necessitate research in order to find means of reducing overall enzyme loadings (Newman *et al.*, 2012). This has been identified as one of the primary variable input costs in the overall cellulosic ethanol production process (Newman *et al.*, 2012). Thus the use of synergy to reduce the overall enzyme loadings, would act as a significant cost reduction strategy for the establishment of economically viable biofuel plants.

## **2.2 Problem Statement**

The efficient hydrolysis of lignocellulose requires the enzymatic degradation of hemicellulose. Limited studies have been performed to understand the synergy between hemicellulases on the hemicellulose component of lignocellulose, particularly in the presence of pectin.

## **2.3 Hypothesis**

Maximal enzyme activity can be obtained with a minimal quantity of an enzyme cocktail containing XynA, ManA, and other commercial enzymes, used on lignocellulosic waste, by manipulating enzyme concentrations to obtain the highest degree of synergy.

## **2.4 Aims and Objectives**

### **2.4.1 Aim**

To determine an optimal combination of enzymes that will display the optimal synergistic association and sugar production for the degradation of lignocellulosic substrates (with a high pectin content) using XynA, ManA and other commercial enzymes.

### **2.4.2 The objectives:**

- 1) To express, purify and characterise XynA and ManA.
- 2) To perform partial characterisations of the commercial cellulase and pectinase.
- 3) To establish an enzyme mixture for optimal synergy based around XynA and ManA as key enzymes.
- 4) To determine the efficiency of the synergy mixtures by calculating the percentage yield of sugars.
- 5) To establish structural substrate hindrance through simultaneous and sequential enzyme synergy studies.

# Chapter 3: Recombinant protein expression and purification

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## **3.1 Introduction**

Recombinant protein expression has become the method of choice for the production of desired proteins in the forms and quantities that have been used in both industry and research (Swartz, 2001). The use of heterologous host organisms for expression of recombinant DNA (rDNA) products in industry to manufacture economically important products has matured into an established industrial process since the first use of Eli Lilly's rDNA human insulin from *Escherichia coli* (Keefer *et al.*, 1981).

Subsequently, *E. coli* has become one of the most commonly used heterologous host expression system, due to its well understood genetics, ease of genetic manipulation and availability of reliable expression systems. For recombinant protein expression, the most used system has been the T7 based pET expression systems, as commercialised by Novagen (Sørensen and Mortensen, 2005). This plasmid system confers antibiotic resistance to the host cell, includes *lac* repression systems for selective gene transcription and allows for rapid gene transcription via promoter sites for T7 phage RNA polymerase (Appendix D: Figure D1). When combined with an *E. coli*, modified by a DE3 phage fragment, the system can allow for rapid expression of recombinant gene products (Sørensen and Mortensen, 2005). The DE3 fragment allows the expression of a T7 RNA polymerase, but is kept under control by *lac* repression. The high fidelity, ease of handling and culture of *E. coli* BL-21 (DE3) make it a logical choice for producing bacterial recombinant enzymes for kinetic and activity assays.

The modified recombinant proteins produced in this manner can include features for use in later purification procedures. These 'fusion proteins' can include a histidine (His) tag to allow for selective adsorption and elution when used in conjunction with immobilised metal affinity chromatography (IMAC) (Kashlev *et al.*, 1993; Nilsson *et al.*, 1997; Porath *et al.*, 1975). This is achieved through the manipulation of ion content in the enzyme containing solution as it passes through a column matrix with attached Ni<sup>2+</sup> ions on support beads (Kashlev *et al.*, 1993). The affinity of the His tag amino acids to hydrogen bond to the Ni<sup>2+</sup> ions means initially that

extraneous proteins of the host *E. coli* will elute while leaving the protein of interest on the column (Nilsson *et al.*, 1997). The selective elution can be achieved by a low pH to protonate the His residues of the His tag or the inclusion of imidazole to displace the proteins at binding sites through competition, allowing elution (Hochuli, 1990; Kashlev *et al.*, 1993). Resultant protein yields higher than 90% have been recorded, which are ideal for subsequent use in activity assays to establish the enzyme's substrate activity range (Kovacs *et al.*, 2009; Swartz, 2001).

A variety of purified and characterised substrates, also known as defined substrates, are used in the establishment of an enzyme's activity and substrate range (Juhász *et al.*, 2005; Puupponen-Pimiä *et al.*, 2008). There are two major forms of xylan in terrestrial plants, hardwood xylan such as xylan extracted from birchwood or beechwood and softwood xylan, such as xylan extracted from oat spelt (Jacobs *et al.*, 2001). Both forms of xylan share a common backbone of  $\beta$ -(1 $\rightarrow$ 4)-D-xylopyranosyl residues, but hardwood xylans possess methylated glucuronic acid side chains on every 8-20 xylanopyranosyl residues and a higher degree of acetylation (Jacobs *et al.*, 2001). Softwood xylan has a methylated glucuronic acid side chains on every 5-6 xylanopyranosyl units of the xylan main chain. Additionally they have an  $\alpha$ -L-arabinofuranosyl linked to the C-3 carbon of the xylan main chain every 8-9 xylanopyranosyl units (Jacobs *et al.*, 2001). To determine mannanase activity, Locust bean gum (LBG) is commonly used (Shallom and Shoham, 2003).

## **3.2 Aims and Objectives**

### **3.2.1 Aim**

To express and purify ManA and XynA from *C. cellulovorans* in sufficient quantities and at a high level of purity for the purposes of this study.

### **3.2.2 Objectives**

- Express ManA and XynA with a pET-29a expression system.
- Purify ManA and XynA with IMAC.
- Visualise ManA and XynA protein fractions with SDS-PAGE.
- Establish ManA and XynA protein concentration with the Bradford's assay.
- Quantify ManA and XynA enzyme activity through enzyme activity and DNS assays.
- Construct a protein purification table for ManA and XynA.

## **3.3 Methods and Materials**

### **3.3.1 Expression of recombinant protein**

ManA and XynA containing plasmid constructs, pET-29a-ManA and pET-29a-XynA, were previously transformed into competent *E. coli* BL21 (DE3) (Novagen) cells (Beukes *et al.*, 2008). The recombinant proteins were modified to express a 6x his-tag on their respective C-terminals. These pre-inocula cultures were incubated at 37°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. Once cellular optical density, measured at OD 600 nm, had reached 0.8 units, translation of the recombinant protein was induced with the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) at 0.1 mM. The cultures were then incubated at 18°C for 24 hours, with shaking at 150 rpm. After 24 h of expression the intracellular concentration of recombinant protein reached sufficient levels required for subsequent purification procedures.

### **3.3.2 Purification of recombinant protein**

Protein purification was initiated with an initial cellular concentration step using centrifugation, followed by cellular lysis with sonication to release the recombinant proteins into solution. To achieve this, the cultures were transferred to Beckman centrifuge tubes (250 mL) and centrifuged at 8,000 *g* (Beckman Avanti centrifuge) for 10 minutes at 4°C. After centrifugation, the supernatant was removed and the cell pellet suspended in 10 mL lysis/wash buffer (50 mM NaH<sub>2</sub>PO<sub>4</sub>; 300 mM NaCl; pH 8.0) at 4°C. At each step of the purification, 250 µL of sample was removed for later analysis. The solution was then sonicated at 40 Hz (Vibra Cell Sonics Materials) three times for 10 seconds each, with 10 second rest intervals between each sonication. A second centrifugation was performed at 12 000 *g* under identical conditions to that used above. The protein containing supernatant was removed and the pellet, containing cellular debris, was disposed. The supernatants of the cell lysates, for ManA and XynA, were designated as the crude extracts for the remaining steps of the protein purification method.

The crude samples were purified with nickel affinity chromatography with a Ni-TED column (Protino® Ni-TED 2000 packed column). Each elution fraction was separately collected for later analysis. The column was prepared using a pre-wash step with the addition of 10 mL of lysis/wash buffer to remove any remaining ethanol still in the column from the storage solution.



The funnel of the column was then sealed and 5 mL of supernatant was added to the column matrix. The top of the column was sealed and the column incubated at 4°C for 2 h before transfer into the collection support apparatus. For subsequent elution steps, the column was placed in an upright position for the bead matrix to settle into a uniform bed, before elution could begin. All sealing caps were removed and the subsequent flow-through collected. Subsequently, 10 mL of lysis wash buffer was added twice to remove any non-specifically bound proteins. To elute the His-tagged proteins, 5 mL of elution buffer (lysis wash buffer with 250 mM of imidazole) was added. This initial elution fraction formed the initial stock solution for enzyme activity assays. A further 10 mL of elution buffer was added to elute any remaining protein and the column was washed with a further two 10 mL steps of lysis/wash buffer to remove imidazole. For long term storage the column was stored in a 20% (w/v) ethanol solution. To visually assess the proteins present in each protein fraction and thereby ascertain the relative efficacy of the purification procedure, SDS-PAGE was performed on all purification fractions.

### **3.3.3 Discontinuous denaturing SDS-PAGE**

All proteins samples had their purity assessed through analysis of appropriately diluted protein samples and by a modified method from the Mini-Protein® 3 cell protocol (Bio-Rad). All samples were mixed in a 5 to 1 ratio with sodium dodecyl sulphate (SDS) reducing 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 5 min (Labnet AccuBlock™ digital dry bath). To each well, 15 µL of protein sample was added and separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970). A discontinuous gel was used for all analyses, composed of 12% resolving and 4% stacking gels. The 4% stacking gel consisted of 4% (w/v) degassed acrylamide/Bis; 0.125 M Tris-HCl pH 6.8 and 0.1% (w/v) SDS; and the 12% resolving gel consisted of 12% (w/v) degassed acrylamide/Bis; 0.375 M Tris-HCl pH 8.8 and 0.1% (w/v) SDS. The gel solutions were polymerised with the addition of 50 µL of 0.1% (v/v) ammonium persulphate (APS) and 20 µL of pure N,N,N',N'-tetramethylethylenediamine (TEMED). The polymerising 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 1 cm of the gel bottom. The gel was then removed from the glass setting plates for staining in a sealed plastic container. The gels were stained with Coomassie staining solution (0.1% (w/v) Coomassie Brilliant Blue G250; 20% (w/v) methanol and 15% (w/v) glacial acetic acid) for 2 h or overnight. The gels were then destained with destain solution (45% (w/v) methanol and 10% glacial acetic acid). Gels were photographed using a gel documentation system (Uvitech- Uvipro chemi). Any visualized protein could subsequently have their protein concentration measured.

### **3.3.4 Protein concentration determination**

The protein concentrations of the solutions were determined using the Bradford method (Bradford, 1976). A standard curve for protein concentrations ranging from 0.1 to 1.0 mg/mL of protein was constructed with dilutions of bovine serum albumin (BSA), as represented in Appendix B: Figure B1. For protein sample analysis, appropriate dilutions of the solutions were made and 10  $\mu$ L of sample was added to 240  $\mu$ L of Bradford's reagent. After 10 minutes at room temperature the samples' absorbancies were measured at 595 nm with a PowerWave<sub>x</sub> Spectroquant microtiter plate reader using KC Junior software. A dH<sub>2</sub>O control was used as a negative control and blank for each set of samples. The known protein concentrations were then used to determine the appropriate enzyme loadings for the enzyme activity assays.

### **3.3.5 Enzyme activity determination**

Glycoside hydrolase activity assays were performed in triplicate with appropriate positive and negative controls. All methods were standardized as per National Renewable Energy Laboratory (NREL) procedures and guidelines (NREL, 2012).

#### **3.3.5.1 Reducing sugar analysis**

To determine the enzyme activity, as calculated through the quantity of reducing sugars released, a dinitrosalicylic (DNS) assay was used (Miller, 1959). The quantities of reducing sugars in an unknown solution were calculated via a regression equation of an appropriate standard curve. Standard curves of D-glucose, D-xylose, D-galacturonic acid and D-mannose monomers are represented in Appendix B: Figures B2-B5.

All solutions measured for reducing sugar concentrations via the DNS method followed the following standard DNS assay method: A sample of 150  $\mu$ L of prepared supernatant containing

the unknown reducing sugar concentration was added to a sterile Eppendorf tube with 300  $\mu\text{L}$  of 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). Solutions were then heated to 100°C for 5 minutes (Labnet AccuBlock™ digital dry bath). Samples were then cooled on ice and centrifuged again at 8 000 g (Desktop centrifuge-Biofuge Pico Hereaus) for 2 min to further pellet out insoluble substrates. From this, 250  $\mu\text{L}$  of solution supernatant was measured at 540 nm (Power Wave<sub>x</sub> Spectroquant on KC Junior software). Absorbance measurements were converted into reducing sugar concentrations using the appropriate regression equation calculations from the established standard curves. The choice of regression equation was based upon what the predominant monomer in the solution was presumed to be, when a defined substrate such as beech wood xylan or cellulose was used. In the case of complex substrates, such as pineapple pomace, the glucose regression equation was used as literature values are conventionally reported in glucose equivalent units. Sugar concentrations could be calculated from the volumes used in the standard enzyme activity assay.

### **3.3.5.2 Enzyme activity assay**

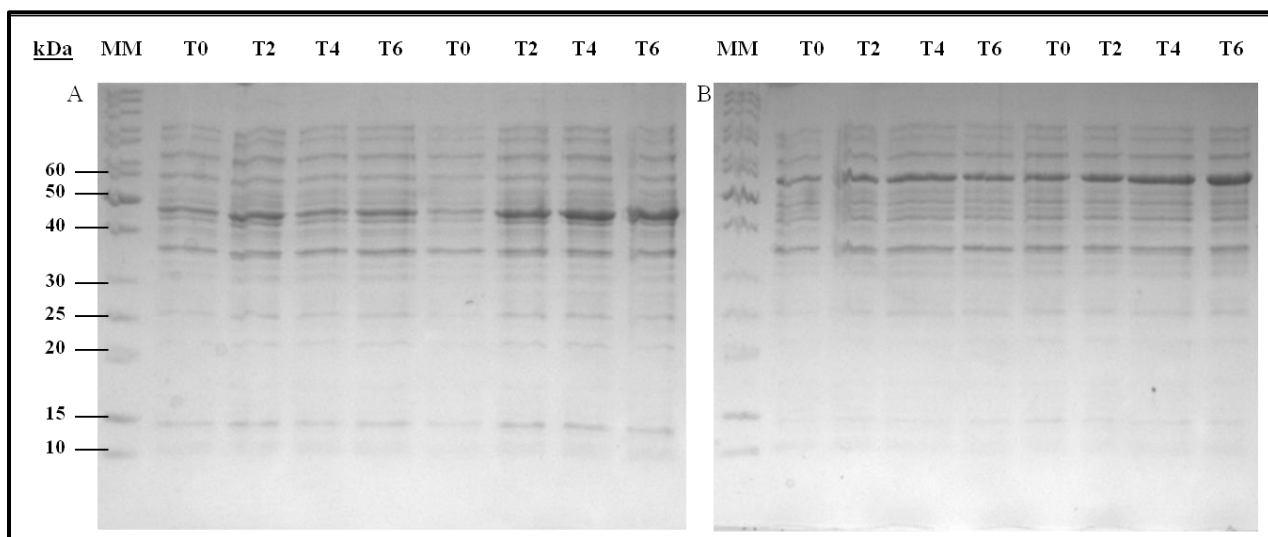
The total solution volume ( $V_T$ ) used in the standard activity assay was 400  $\mu\text{L}$ . This was composed of 50  $\mu\text{L}$  enzyme volume ( $V_E$ ), 100  $\mu\text{L}$  substrate volume ( $V_S$ ) and 250  $\mu\text{L}$  buffer volume ( $V_B$ ). All solutions were buffered at pH 5.0 with sodium citrate buffer (50 mM). A buffer control was used as a negative control and acted as a blank for each sample set. Substrate and enzymes controls were used whereby either, the enzyme or, substrate portion of the mixture volume was not added. The total volume for each control was made up to 400  $\mu\text{L}$  with buffer. To establish activity, defined substrates were used at 2 % (w/v) (0.5% final concentration). All reaction mixtures were kept at 4°C until the addition of any enzyme and were assayed for varying lengths of time at 37°C with tumbling in a rotary shaker at 20 rpm. The assays were terminated by cooling to 4°C before centrifugation at 16060 g (Desktop centrifuge-Biofuge Pico Hereaus) for 2 min to pellet out insoluble substrates. The resulting reducing sugars produced from the inclusion of a particular enzyme or ratio of enzymes were used to calculate the given enzyme(s) activity, as calculated using methods outlined in 3.3.5.1. One unit of activity was defined as 1  $\mu\text{g}/\text{mL}/\text{min}$  of product (reducing sugar) formed from each enzyme or combination of enzymes. Units of  $\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mL}^{-1}$  of glucose were not used, as the polymerisation and

composition of sugars released were unknown, making the use of molar units potentially misleading.

## 3.4 Results

### 3.4.1 Induction Study for ManA and XynA expression

To assess the efficiency of the expression method described in 3.3.1, an induction study was performed on the pET-29a-ManA and pET-29a-XynA competent cultures. To assess the levels and masses of proteins produced over the induction period and to assess the level of IPTG induced protein expressed, 250  $\mu$ L samples were taken every 2 h for 6 hours. The protein content of the cell cultures were visualized with SDS-PAGE as described in section 3.3.3. The SDS-PAGE results of the 6 h induction studies for induced and uninduced pET-29a-ManA and pET-29a-XynA competent cultures are represented in Figure 3.1.



**Figure 3.1: SDS-PAGE gels of ManA (A) and XynA (B) induction studies over six hours.**

#### **Figure legend:**

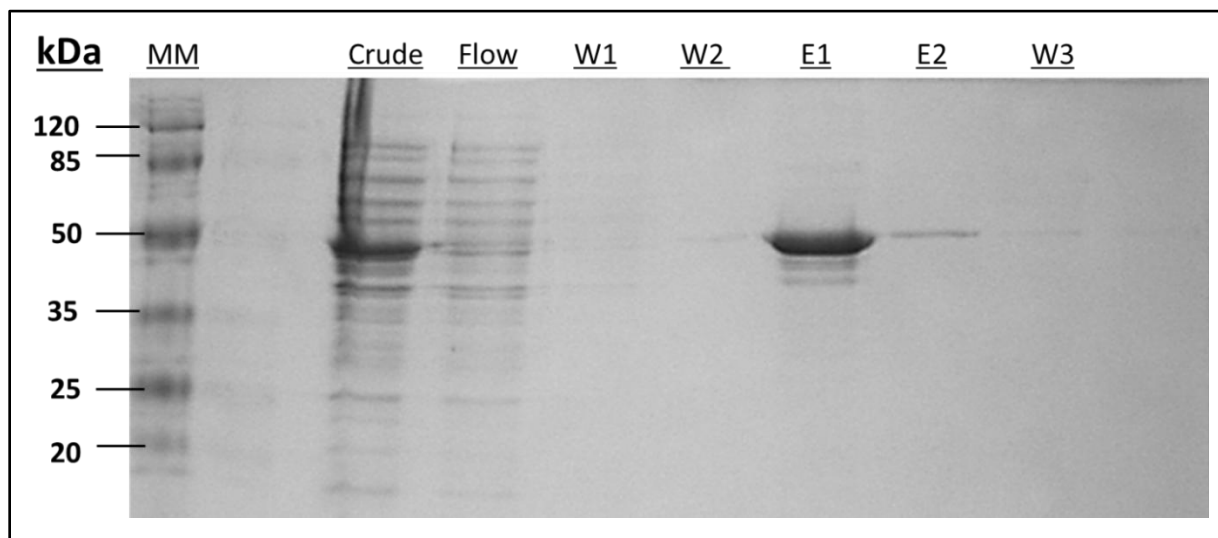
**Gel A:** Lane MM: Molecular weight marker pEqGOLD protein weight Marker II; Lanes T0 to T6 (left): samples of non-induced control pET-29a-ManA culture from time period 0 to 6 h (T0 to T6); Lanes T0 to T6 (right): samples of IPTG induced pET-29a-ManA culture from time period 0 to 6 h (T0 to T6).

**Gel B:** Lane MM: Molecular weight marker pEqGOLD protein weight Marker II; Lanes T0 to T6 (left): samples of non-induced control pET-29a-XynA culture from time period 0 to 6 h (T0 to T6); Lanes T0 to T6 (right): samples of IPTG induced pET-29a-XynA culture from time period 0 to 6 h (T0 to T6).

To assess the relative protein levels of ManA and XynA, induced with IPTG, SDS-PAGE was performed on the control (left) and induced cultures (right), for ManA and XynA, respectively. The expression of ManA, seen at 47.2 kDa, for the control samples were at low levels as the induction study progressed, as seen in Figure 3.1 Gel A. However, the concentration of ManA in the induced culture was at higher levels relative to the control as the study progressed. The expression of XynA, at 57 kDa, also showed higher levels of expression that increased over time in the induced samples relative to the control samples. The demonstration of the selective induction of recombinant protein expression, with IPTG, meant subsequent purification steps could be performed.

### 3.4.2 SDS-PAGE of *C. cellulovorans* ManA and XynA

In order to evaluate the IMAC purification fractions generated from the purification methods in 3.3.2, the ManA and XynA crude protein samples had to be visualised. The relative protein contents and molecular masses of the proteins in each purification step were visualised with discontinuous denaturing SDS-PAGE, performed as detailed in section 3.3.3. A SDS-PAGE gel of the ManA purification fractions is represented in Figure 3.2.



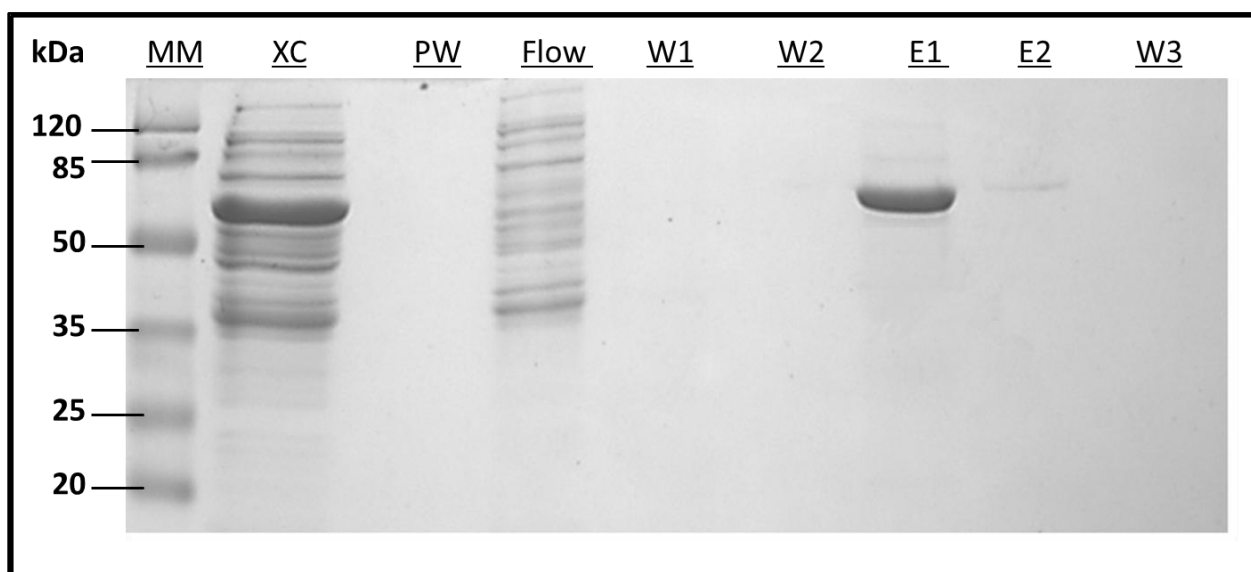
**Figure 3.2: A SDS-PAGE gel of ManA purification with Nickel affinity chromatography.**

#### **Figure legend:**

**ManA Gel:** Lane MM: Molecular weight Thermo scientific® protein molecular marker 26612 ladder; Lane Cru: Sample of crude extract for ManA; Lane Flow: Sample of the flow through elution from ManA crude showing intracellular protein from *E. coli*, No ManA present; Lane W1 and W2: Samples of the wash elution fractions of ManA, no protein was detected; 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 W3 and W4: Samples of the wash elution fractions of ManA, no protein was detected.

The selective elution of ManA, with addition of imidazole containing buffer, allowed for the selective and pure elution of ManA, as indicated in Figure 3.2. A small amount of ManA was observed in lane E2 and may have been due to some residual ManA in the column and/or some spill over from loading Lane E1. The SDS-PAGE results for the XynA purification are represented in Figure 3.3.



**Figure 3.3: A SDS-PAGE gel of XynA purification with Nickel affinity chromatography.**

**Figure legend:**

**XynA Gel:** Lane MM: Molecular weight Thermo scientific<sup>®</sup> protein molecular marker 26612 ladder; Lane XC: Sample of crude extract for XynA; Lane PW: Sample of elution of pre wash fraction from column equilibration; Lane Flow: Sample of the flow through elution from XynA crude showing intracellular protein from *E. coli*, No XynA present; Lane W1 and W2: Samples of the wash elution fractions of XynA, no protein was detected; Lane E1: Samples of the elution fractions of XynA, a protein matching XynA size was detected at 57 kDa; Lane E2: Sample of the elution fraction after additional elution wash step; Lane W3: Samples of the wash elution fraction of XynA, no protein was detected.

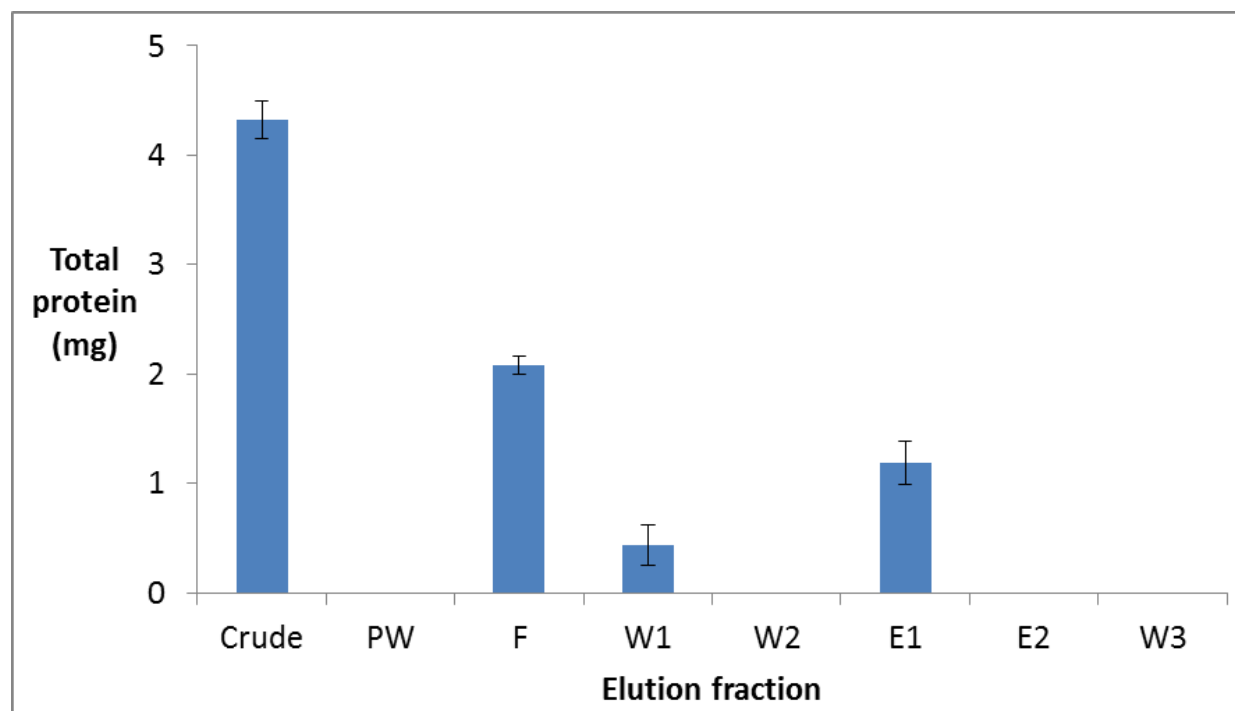
XynA was selectively eluted with addition of imidazole containing buffer and allowed for the selective and pure elution of XynA, as indicated in Figure 3.3. A small amount of XynA was

observed in lane E2 and may have been due to some residual ManA in the column and/or some spill over from loading Lane E1.

The purifications for ManA and XynA showed that the majority of the proteins were selectively eluted from the crude cell lysate relative to the flow-through fractions for ManA and XynA, as per Figures 3.2 and 3.3. Concurrent to any SDS-PAGE analyses of the IMAC purification samples, Bradford assays were also performed to determine the protein concentrations of the fractions obtained.

### 3.4.3 Protein content in ManA and XynA elution fractions

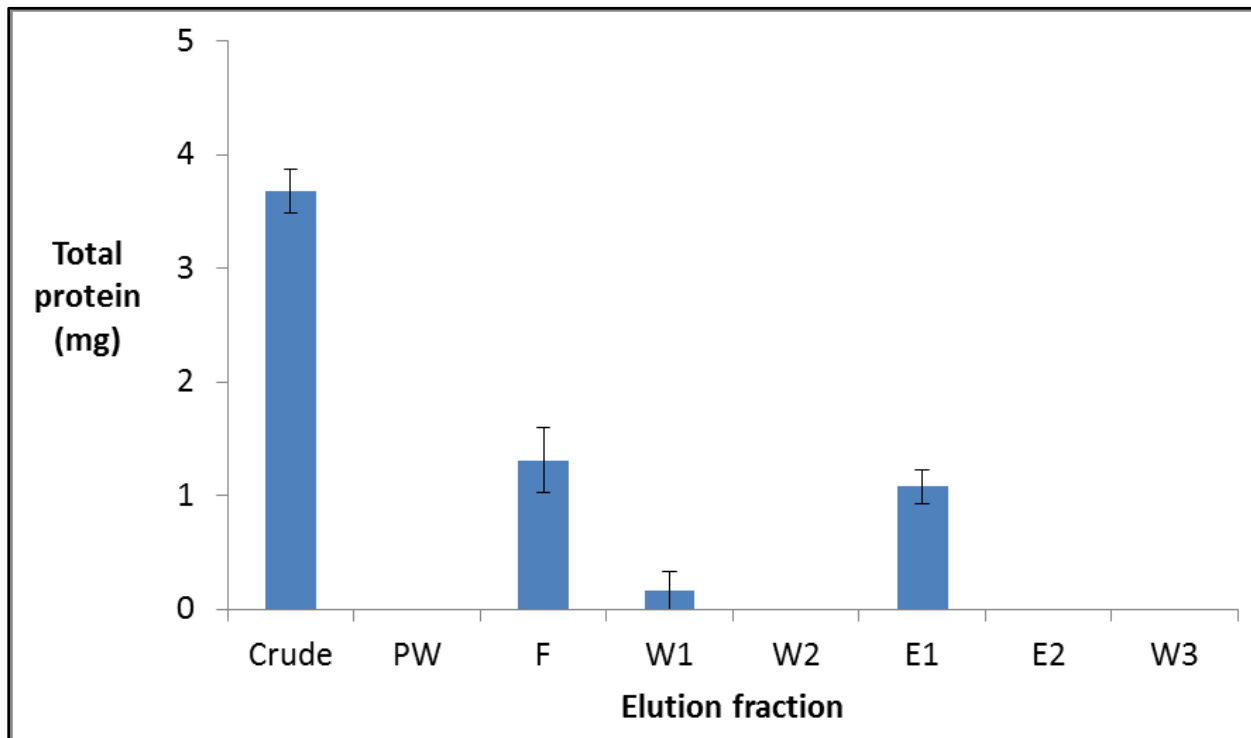
The protein contents in purification fractions can serve as an independent means of assessing the efficiency of an IMAC purification method. Protein concentrations of each of the protein elution samples for ManA purification were determined using the Bradford method (see Figure 3.4).



**Figure 3.4: Protein content in the elution fractions for ManA from the Ni-affinity chromatography purification step. Values represent means  $\pm$  SD, n = 3.**

No significant amounts of protein were detected in the pre wash (PW) fraction of column used to elute ManA, Figure 3.4. To the Ni-affinity column, 5 mL of cell lysate was loaded with the ManA crude (Crude) containing 4.32 mg of protein of which 1.89 mg eluted in the flow-through fraction (F). The initial wash fraction (W1) contained 0.44 mg of protein with the second wash

fraction (W2) having no detectable quantity of protein present. The selective retention and elution of ManA was confirmed with the elution of 1.19 mg protein in fraction E1. No further detectable amounts of protein were measured in the second elution (E2) and third wash fractions (W3). The purified ManA enzyme (in the E1 elution fraction) was used in all subsequent activity assays after appropriate dilution. The same purification procedure was used for the crude XynA fraction and is represented in Figure 3.5.



**Figure 3.5: Protein content in elution fractions for XynA from the Ni-affinity chromatography step. Values represent means  $\pm$  SD, n = 3.**

No significant amount of protein was detected in the pre wash (PW) fraction, as indicated in Figure 3.5. A 5 mL volume of the crude fraction (Crude) which contained 3.67 mg of protein was added to the Ni-affinity column, of which 1.31 mg eluted with the flow-through fraction (F). The initial wash fraction (W1) contained 0.17 mg of protein and the second wash fraction (W2) contained no detectable quantity of protein. The selective retention and elution of XynA was confirmed with the elution of 1.08 mg of protein for the E1 fraction. No further detectable amounts of protein were observed in the second elution (E2) and third wash fractions (W3). The purified XynA enzyme (in the E1 elution fraction) was used in all subsequent activity assays after appropriate dilution.



### 3.4.4 Purification table of ManA and XynA purification

The protein concentrations and activity measurements, recorded in section 3.3.4 and 3.3.5, of purified ManA and XynA allowed the construction of a protein purification table, as indicated in Table 3.I. Defined substrates; 2% (w/v) locust bean gum (LBG) for ManA and 2% (w/v) beech wood xylan (BEX) for XynA, were used under standard enzyme activity assay conditions.

**Table 3.1: Protein purification table for purification fractions of *C. cellulovorans* ManA and XynA. Values represent means, n=3, SD < 0.05.**

<b>ManA</b>	<u>Volume</u>	<u>Total Protein</u>	<u>Activity</u>	<u>Total Activity</u>	<u>Specific Activity</u>	<u>Fold</u>	<u>Yield</u>
<b>Fractions</b>	(mL)	(mg)	(U/mL)	(U)	(U/mg)	Purification	(%)
<b>Crude</b>	10	8.64	472.51	4725.10	54.69	1	100
<b>Ni affinity</b>	10	2.38	446.37	4463.67	187.55	3.429	94.47
<b>XynA</b>	<u>Volume</u>	<u>Total Protein</u>	<u>Activity</u>	<u>Total Activity</u>	<u>Specific Activity</u>	<u>Fold</u>	<u>Yield</u>
<b>Fractions</b>	(mL)	(mg)	(U/mL)	(U)	(U/mg)	Purification	(%)
<b>Crude</b>	10	7.35	341.81	3418.10	39.56	1	100
<b>Ni affinity</b>	10	2.16	329.95	3299.52	138.64	3.504	96.53

Only a single Ni affinity chromatography step was used to purify crude ManA, and XynA, as seen in Table 3.1. ManA was purified with a 94.47% yield after Ni affinity chromatography and a fold purification of 3.43. The yield for XynA was higher at 96.53% with a higher fold purification of 3.5. All data obtained had standard deviations of less than 0.05. These results were used to establish the purity and effectiveness of the purification procedures used for ManA and XynA purification. The elution fractions of ManA and XynA collected after IMAC purification constituted the enzyme solutions used in all subsequent enzyme assays, after appropriate dilution.

### **3.5 Discussion**

In order to prove that a recombinant protein has been produced through heterologous pET-29 system expression, it should be demonstrated that its expression was induced through the relief of inhibition. Confirmation of this would be seen through the introduction of an inducing reagent, such as the IPTG, to alleviate the inhibition of the recombinant protein's expression (Sørensen and Mortensen, 2005). An induction study to prove this would demonstrate the increase of expression of a particular protein as seen in the form of a large protein band present in culture in which the inducer was present, relative to the control. The presence of a relatively large protein fraction expressed at 47 kDa in gel A, with a similar large band recorded at 57 kDa in gel B, for Figure 3.1, indicated the induced expression of the recombinant protein of either ManA in gel A, or XynA in gel B. The induction study of ManA and XynA showed leaky expression of ManA and XynA in control samples, Figure 3.1. The leaky expression was at a baseline level and was also reported previous studies (Beukes *et al*, 2010; Dredge *et al*, 2011). This was expected and ManA and XynA were shown to be induced by the addition of 0.1 mM of IPTG.

The induced expression, of either of the recombinant proteins, required further confirmation through identification. Concurring results were partially provided by the selective elution with IMAC for ManA and XynA, in Figure 3.2 and 3.3. A protein band corresponding to ManA (47 kDa), in Lane E1, and the absence of a corresponding band in the flow through lane, yet present in the crude lane, was observed in Figure 3.2. This demonstrated that a protein corresponding to 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. The same characteristics were true for XynA with the band corresponding to XynA present in relatively large quantity in lane E1 and the crude lane, but absent in the flow through lane, as per Figure 3.3. The selective elution of ManA and XynA was in itself confirmatory as both were fusion proteins with His tags and thus could bind to the Ni<sup>2+</sup> ions on the column matrix. Some faint banding was seen in both of the E1 columns for ManA and XynA purifications, Figure 3.2 and 3.3. These were most likely due to some proteins that nonspecifically bound to the recombinant proteins, for example chaperones that naturally occur in *E. coli* (Hennessy *et al.*, 2005). The selective elution of protein in each

fraction was also supported by the results of the Bradford assays, as displayed in Figure 3.4 and 3.5. High concentrations of protein were recorded in the initial elution columns, with 0.238 mg/mL for ManA in E1 of Figure 3.4 and 0.216 mg/mL for XynA in E1 of Figure 3.5. The final confirmation for the presence of ManA and XynA was the activity generated on each defined substrate. ManA had a specific activity of 187.55 U/mg on 2% (w/v) LBG with 138.64 XynA U/mg for XynA on 2% (w/v) BEX, as recorded in Table 3.1.

Data obtained for protein concentrations, in addition to enzyme activities, allowed the construction of a protein purification table, represented in Table 3.1. The high level of recombinant protein in the starting crude meant that only fold purifications above 3 were possible, with the respective fold purifications of 3.429 and 3.504 calculated for ManA and XynA, respectively. The high yields of 94.47% for ManA and 96.53% for XynA represent a successful purification of ManA and XynA. A purification involving IMAC can be considered a success when a benchmark of above 90% yield and 3 fold purification is achieved (Sørensen and Mortensen, 2005; Swartz, 2001). Thus, a relatively pure solution of enzyme was produced for subsequent enzyme activity assays.

### **3.6 Conclusion**

The initial aim of purifying the recombinant ManA and XynA enzymes was achieved. This could perhaps have benefited from an increase in induction times beyond 24 hours if greater quantities of ManA or XynA were required. The protocols for purification proved successful and the analyses were conclusive and accurate, supporting the validity of the purification procedure employed.

# Chapter 4: Partial characterisation of commercial enzymes

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## **4.1 Introduction**

The use of commercially available enzymes mixtures have become an integral part of the cellulose to biofuel production process (Merino and Cherry, 2007). The efficient industrial degradation of fruit waste requires combinations of enzymes and solutions in high enzyme to substrate loading ratios (Merino and Cherry, 2007). The use of multiple enzyme solutions will equate to higher costs to optimize solutions to any system requirements, and variable costs in the purchase of enzyme solutions (Van Dyk and Pletschke, 2012).

To reduce the enzyme loading costs, commercial mixtures have been under constant research and modification efforts. These have focused on improvements in stability, catalytic efficiency and other industrially important characteristics (Sims *et al.*, 2010). These include efforts from various governmental initiatives, such as the National Renewable Energy Laboratory (NREL), public research laboratories and private business (Merino and Cherry, 2007). A private sector example has been the enzyme manufacturer Novozyme, which was awarded a research sub-contract from the United States of America Department of Energy, to reduce the cost of enzymes for cellulosic ethanol production by ten-fold (Merino and Cherry, 2007). This goal and others were achieved, however, the economic necessity of secrecy in trade knowledge resulted in the non-disclosure of the product information.

The exact contents and enzymatic characterisations of commercial enzyme solutions are not publicly reported by their manufacturers and there is lack of information on their performance in an industrial setting. Some characteristics such as an approximate pH and temperature optima(s) are stated by some manufacturers, however these results are deliberately conservative and in literature are found to be lower than actual optimal assay temperatures and over a broad pH range (Juhász *et al.*, 2005; Sigma-Aldrich, 2012). The present commercial enzyme manufacturing system paradigm has the advantage of many well characterized micro-organisms for the production of the majority of the lignocellulolytic enzymes. Two fungi that are efficient sources of industrial enzymes are *Trichoderma reesei* and *Aspergillus niger* (Sims *et al.*, 2010).

Since its discovery over 70 years ago, *T. reesei* has been the dominant commercial enzyme source for lignocellulose degradation (Merino and Cherry, 2007; Reese, 1976). This was achieved by the constant improvement of the core three enzyme classes secreted by *T. reesei* and have resulted in productivity improvements greater than 20 fold (Reese, 1976). This industrial interest and research has driven development despite the fact that *T. reesei* possesses only a comparatively small set of lignocellulose degrading enzymes (Foreman *et al.*, 2003).

A commonly used commercial solution are cellulases from *T. reesei* (ATCC 26921) (EC: 3.2.1.4) (Sigma C-8546), which is a freeze dried preparation of *T. reesei* cellulases also known as Celluclast 1.5L<sup>®</sup> (Sigma-Aldrich, 2012). The manufacturer stated temperature enzyme for the solution is 37°C, but a temperature optimum of 45°C has been reported (Juhász *et al.*, 2005; Sigma-Aldrich, 2012). No substrate activity range has been issued by the manufacturer, but specific activity assays for a substrate range for Celluclast 1.5L<sup>®</sup> was performed by Juhász *et al.* (2005). A Celluclast 1.5L<sup>®</sup> solution, at a protein concentration of 125 mg/mL, was incubated with common lignocellulose polysaccharides for 7 days and released sugars were analysed with HPLC-RID (Juhász *et al.*, 2005). These were performed under assay conditions of 45°C, 100 mM acetate buffer (pH 5.0) with no shaking, and are represented in Table 4.1 (Juhász *et al.*, 2005).

**Table 4.1: Specific enzyme activities of *T. reesei* Celluclast 1.5L<sup>®</sup> on lignocellulose polysaccharides (Juhász *et al.*, 2005).**

<b><u>Substrate</u></b>	<b><u>Specific enzyme activities (nkat/mg)</u></b>
<b>Hydroxyl-ethyl-cellulose</b>	130
<b>Xylan</b>	100
<b>Locust bean gum</b>	26.1
<b>4-Nitrophenyl-<math>\alpha</math>-L-arabinofuranoside</b>	4.3
<b>4-Nitrophenyl-<math>\beta</math>-D-glucopyranoside</b>	4.2
<b>4-Nitrophenyl-<math>\beta</math>-D-xylanopyranoside</b>	2.3
<b>4-Nitrophenyl-<math>\alpha</math>-L-galactopyranoside</b>	0.1

The highest activity was observed with hydroxyl-ethyl-cellulose with a specific activity of 130 nkat/mg, indicative of endoglucanase activity. The next highest specific activities were of xylanase and mannanase type with 100 and 26.1 nkat/mg, respectively (Table 4.1), but with negligible pectinase activity (data not shown)(Juhász *et al.*, 2005). The lack of pectinase activity could be addressed with the addition of pectinase containing solutions and such industrial and commercial examples include those produced by *A. niger*.

The set of pectin degrading enzymes secreted from *A. niger* have been engineered to be produced in greater quantities and with higher catalytic efficiencies (Beldman *et al.*, 1984). Pectinase solutions from *A. niger*, such as Pectinex<sup>®</sup> 3XL, have been estimated as the largest class of industrially produced enzyme solutions (Jayani *et al.*, 2005). Substrate activity characterization has been performed for Pectinex<sup>®</sup> 3XL, in which 11109 nkat/mL on polygalacturonan (PGA) was reported by Puupponen-Pimiä *et al.* (2008). PGA is a polymer of unmethylated  $\alpha$ -(1→4)-galacturonic acid residues with minimal side chains or substituents and consists of greater than 98% galacturonic acid residues by weight (Willats *et al.*, 2006). The activity level with PGA, at 100 nkat/g, was normalised to represent a 100% level of activity, additional activities with other polysaccharides were represented as a relative percentage of activity. The relative activity levels were assayed at 45°C for 2 h, as depicted in Table 4.1 (Puupponen-Pimiä *et al.*, 2008).

**Table 4.2: Activity of *A. niger* Pectinex<sup>®</sup> 3XL on lignocellulose polysaccharides (Puupponen-Pimiä *et al.*, 2008).**

<b><u>Substrate</u></b>	<b><u>Relative Activity (%)</u></b>
<b>Polygalacturonan</b>	100
<b>4-Nitrophenyl-<math>\alpha</math>-L-arabinofuranoside</b>	27
<b>Esterfied methylated pectin</b>	17
<b>4-Nitrophenyl-<math>\alpha</math>-L-galactopyranoside</b>	11
<b>Birch wood xylan</b>	7
<b>Locust bean gum</b>	5
<b>Hydroxyl-ethyl-cellulose</b>	5

The majority of enzyme activity, by weight, was observed as pectinase and closely related substrates, as per Table 4.2. The relatively low activities on 4-nitrophenyl- $\alpha$ -L-arabinofuranoside at 27%, and lower activity on xylan and mannan at 7% and 5%, indicate that the solution is primarily pectinases. As pectin in complex lignocellulose feed stocks, such as pineapple, would be highly substituted and variable in structure, it is necessary to establish pectinase activity with a complex substrate such as apple pectin (APE). APE contains a higher amount of side chains than PGA and may contain as low as 65% galacturonic acid residues by weight (Willats *et al.*, 2006).

The combination of Pectinex<sup>®</sup> 3XL with Celluclast 1.5L<sup>®</sup> could potentially allow for a “core” enzyme solution to degrade most lignocellulosic biomass. To assess this possibility the protein compositions and activities of each commercial enzyme solutions were determined. This required any uncharacterised solutions to undergo preliminary assays to accurately determine relevant characteristics, such as defined substrate activity, protein concentration, protein purity and substrate activities. These solutions, in turn, had to be selected and optimized for the relevant feedstocks, such as cellulose and/or pectin rich bio mass feedstocks (Sims *et al.*, 2010).

## **4.2 Aims and Objectives**

### **4.2.1 Aim**

To establish the protein content and enzyme activities of *T. reesei* cellulase (Celluclast 1.5L<sup>®</sup>) and *A. niger* pectinase (Pectinex<sup>®</sup> 3XL) commercial mixtures.

### **4.2.2 Objectives**

- To determine the protein content of each commercial enzyme mixture.
- To visualise the protein fractions of each commercial enzyme mixture via SDS-PAGE.
- To visualise the activity of each protein band of *A. niger* pectinase, and *C. cellulovorans* ManA and XynA with activity gels.
- To determine the substrate activity range of each enzyme on common lignocellulosic polysaccharide substrates.

### **4.3 Methods and materials**

Commercially available *A. niger* pectinase and *T. reesei* cellulase enzyme solutions commonly used in the lignocellulosic bioreactors, were purchased for use in this study. The pectinase solution was Pectinex<sup>®</sup> 3XL from *A. niger* (CAS Number 9032-75-1; EC Number: 3.2.1.15) and hereafter referred to as pectinase. The cellulase lyophilised powder from *T. reesei* (ATCC 26921) (EC: 3.2.1.4) (Sigma C-8546) was acquired and hereafter referred to as cellulase. The dominant activity for the cellulase was stated as 1,4-(1,3:1,4)- $\beta$ -glucan-glucano-hydrolase (Sigma-Aldrich, 2012).

#### **4.3.1 Activity gels**

Activity gels were prepared identically to SDS-PAGE gels, as per section 3.3.3, with the exception that the substrate specific to a particular enzyme activity was included into the resolving gel to a final concentration of 0.2% (w/v) for beech wood xylan, 0.1% (w/v) for polygalacturonic acid (PGA) and 0.3% (w/v) for apple pectin (APE). All substrates included in the activity gels were fine powders and allowed for gel setting. The enzyme samples were denatured under identical conditions as per the normal SDS-PAGE procedure with SDS sample buffer, but had variable heating periods at 100°C for either 30 s or 60 s. The protein samples were separated under electrophoresis as per the normal SDS-PAGE conditions.

The proteins were then refolded with renaturation buffer (appropriate assay buffer, 2.5% (v/v) Triton X-100) for 1 h at 4°C rotating at 150 rpm. The gels were then assayed for protein activity by heating at 37°C, for 1 h while rotating at 150 rpm. The gels were then stained with either 0.05% (w/v) Ruthenium Red for pectin containing substrates or 0.05% (w/v) Congo Red for 3 h at room temperature. The gels were then destained with dH<sub>2</sub>O until zones of clearance were visible. Gels were then photographed with a gel documentation system (Uvitech- Uvipro chemi).



## **4.4 Results**

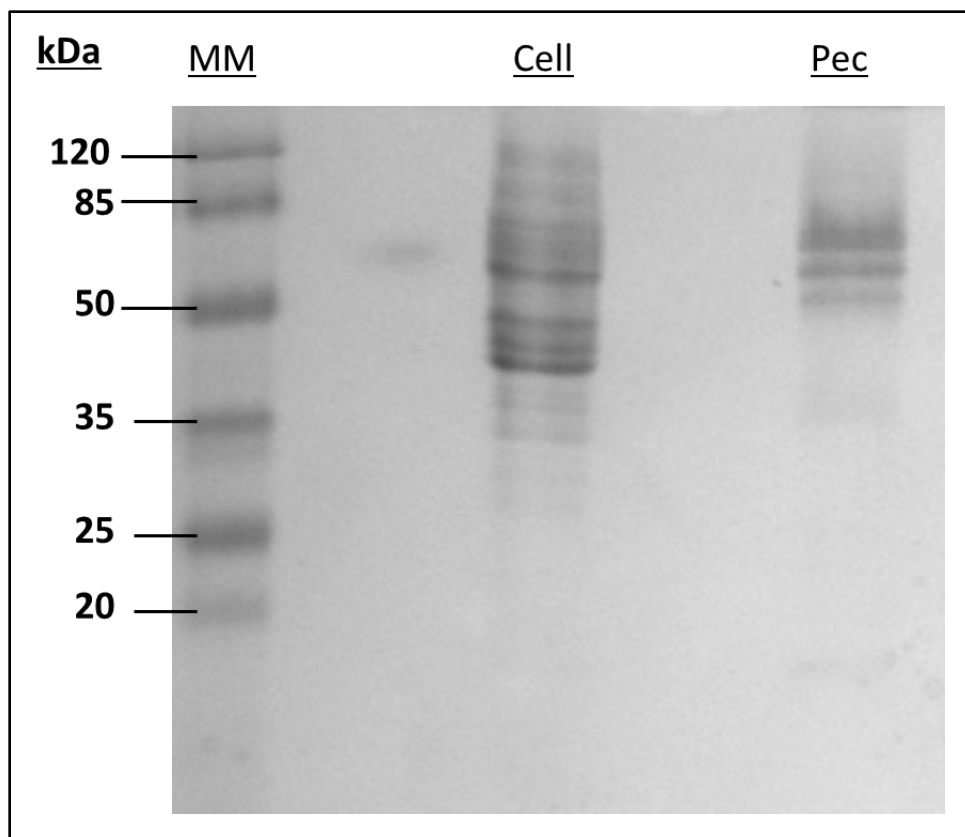
### **4.4.1 Protein concentration and SDS-PAGE analysis of commercial pectinase and cellulase**

To establish the protein concentrations of the cellulase and pectinase solutions, Bradford assays were performed, as per section 3.3.4. Initially, a 2 mg/mL solution of the cellulase powder and a 10% (v/v) of pectinase solution were used. The protein concentrations for the original stock solutions were calculated, as displayed in Table 4.3.

**Table 4.3: Protein concentrations for *T. reesei* cellulase and *A. niger* pectinase using Bradford's method. Values represent means; n=3.**

<u>Enzyme solution</u>	<b>Protein concentration (mg/mg) / (mg/mL)</b>	<b>Standard deviation</b>
<u>Cellulase</u>	0.275	0.031
<u>Pectinase</u>	3.85	0.007

From the protein concentrations for each stock powder/solution it was observed that the cellulase powder only had a protein concentration of 0.275 mg protein/mg powder (Table 4.3). The pectinase stock solution was relatively high at 3.85 mg/mL and thus would require significant dilution before any further analysis. With the protein concentrations established, the visualisation of the protein fractions within each of the solutions was performed with SDS-PAGE. Methods were performed as per section 3.3.3 and an image of the gel is represented in Figure 4.1.



**Figure 4.1: A SDS-PAGE analysis of *T. reesei* cellulase and *A. niger* pectinase.**

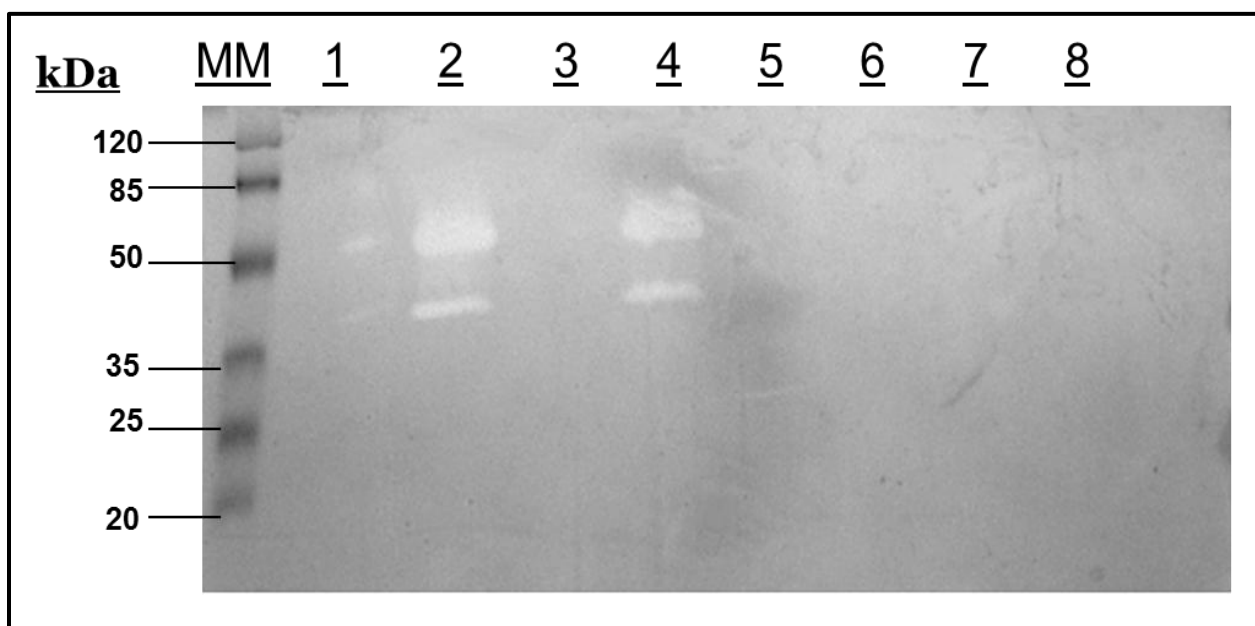
**Figure Legend:**

Lane MM: 4  $\mu$ L Pierce Prestained Protein MW Marker 26612; Lane cell: 8.303  $\mu$ g of cellulase; Lane Pec: 5.775  $\mu$ g of pectinase.

The presence of multiple protein bands in both lanes Cell and Pec indicated that multiple protein fractions were present in both solutions. Regression analysis of the natural log for the migration distance against the known weight of the molecular marker bands established a standard curve of molecular mass versus migration distance, as seen in Figure E1 of Appendix E. The unknown masses of each protein band in Lane cell, Celluclast 1.5L<sup>®</sup>, were calculated from heaviest to lightest at 115.1; 89.7; 77.3; 70.7; 62.3; 53.2; 47.5; 44.0; 40.8; 37.8; 35.1; and 30.0 kDa in Lane Cell for Celluclast 1.5L<sup>®</sup>, in Figure 4.1. For Pectinex<sup>®</sup> 3XL in Lane Pec, Bands were quantified at 73.9; 64.9; 57.5; and 39.3 kDa from heaviest to lightest, in Figure 4.1. The presence of multiple enzymes is expected to confer the ability of either solution to catalyse a variety of substrates. The activities of these proteins/enzymes were unknown and required substrate activity characterisation before use on any lignocellulose substrate could commence.

#### 4.4.2 Pectin activity gel

Although separate substrate specificity assays for ManA and XynA from *C. cellulovorans* indicated no activity on either pectin or PGA, samples of these two enzymes were included for both activity gels. All samples were denatured with SDS-reducing buffer and incubated at 100°C for either 30 s or 60 s (Labnet AccuBlock™ digital dry bath). Different incubation times were employed to allow for analysis of pectinase activity with differing degrees of denaturation. To establish which protein fractions within the commercial pectinase would have activity on either PGA, or pectin, activity gels were conducted, as detailed in section 4.3.1 and are displayed in Figure 4.2 and Figure 4.3, respectively.



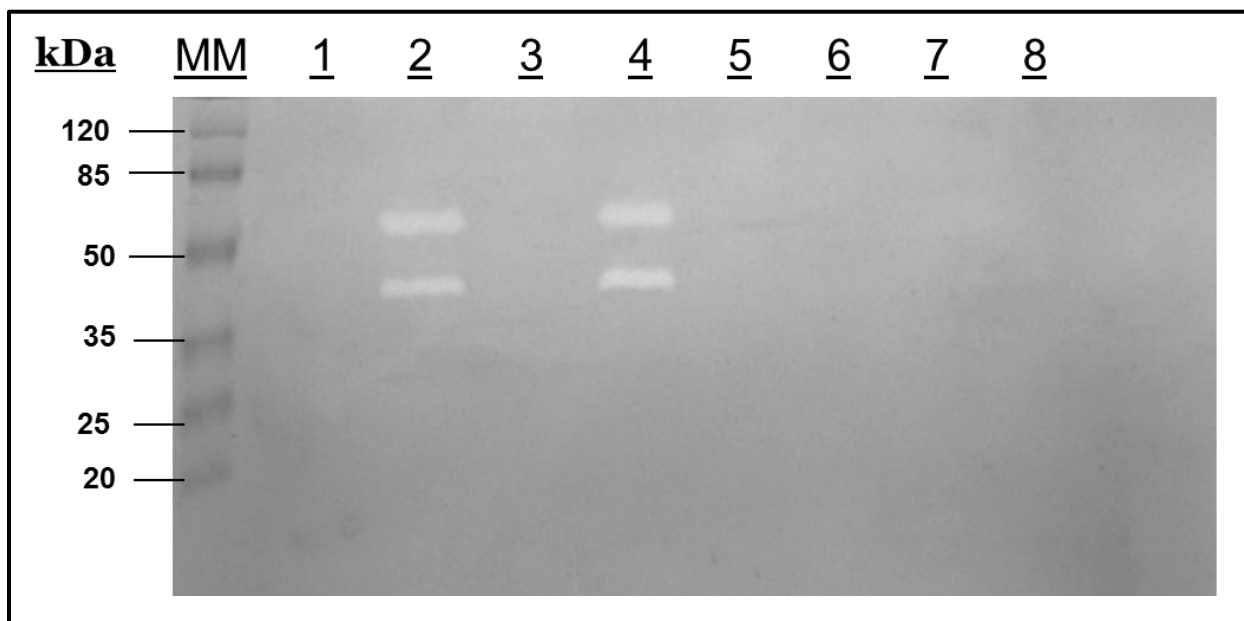
**Figure 4.2: A 0.1% (w/v) PGA discontinuous activity gel of *A. niger* pectinase and *C. cellulovorans* ManA and XynA.**

#### **Figure Legend:**

Lane MM: 4  $\mu$ L Pierce Prestained Protein MW Marker 26612; Lane 1: Some contents of Lane 2 were accidentally placed in lane 1; Lane 2: 28.88 ng Pectinase, incubated for 30 s at 100°C; Lane 4: 28.88 ng Pectinase, incubated for 60 s at 100°C; Lane 6: 450  $\mu$ g ManA, incubated for 60 s at 100°C; Lane 8: 450  $\mu$ g XynA, incubated for 60 s at 100°C.

To calculate the mass of each protein band, the natural log migration distance versus molecular mass was calculated for Figure 4.2, as seen in Figure E2 of Appendix E. The zones of clearance observed in lane 2 and 4 covered the area between 57 and 70 kDa, as seen in Figure 4.2. In

Figure 4.1 there were 3 distinct protein bands within these weights indicating that all three distinct proteins had activity on PGA. An additional zone of clearance at 45 kDa was observed in Figure 4.2 which corresponded with a faint band observed at 45 kDa in Figure 4.1 indicative of another PGA hydrolysing enzyme. No zone of clearance was observed in lanes 6 or 8, in Figure 4.2. To further identify the activities of any proteins within the pectinase solution a pectin activity gel was performed, using the protocols as per section 4.3.1. The activity gel is displayed in Figure 4.3.



**Figure 4.3: A 0.3% (w/v) pectin activity gel of *A. niger* Pectinase and *C. cellulovorans* ManA and XynA.**

**Figure Legend:**

Lane MM: 4  $\mu$ L Pierce Prestained Protein MW Marker 26612; Lane 2: 28.88 ng Pectinase, incubated for 30 s at 100°C; Lane 4: 28.88 ng Pectinase, incubated for 60 s at 100°C; Lane 6: 450  $\mu$ g ManA, incubated for 60 s at 100°C; Lane 8: 450  $\mu$ g XynA, incubated for 60 s at 100°C.

The activities observed in Figure 4.3 were similar to Figure 4.2 in that only two zones of clearance were observed in lane 2 and 4. Molecular masses of all bands were calculated with the natural log migration distance versus molecular mass calibration plots, as seen in Figure E3 of Appendix E. The two zones of clearance observed in lane 2 and 4 formed one large band from 66 to 57 kDa, and again a smaller band at 45 kDa was observed in Figure 4.3. The smaller band at 45 kDa corresponded to the previous bands observed in Figure 4.2 and 4.1. The zone of

clearance between 66 and 57 kDa was observed to be smaller relative to the band between 77 and 57 kDa, of lane 2 and 4 of Figure 4.2. Again no zone of clearance was observed in Lane 6 or 8, for either the ManA or XynA samples as seen in Figure 4.3.

A 0.2% (w/v) for beech wood xylan activity gel was performed with the same enzyme samples under identical denaturing conditions. No zones of clearance were observed, for either the pectinase, or ManA and XynA. To quantify the level of activity of each enzyme, the substrate activity range for each enzyme had to be established.

#### **4.4.3 Defined substrate activity range**

Before the enzymes could be assayed for activity on a lignocellulosic fruit waste solution, an appropriate substrate range for each enzyme had to be established. This was determined, and quantified, through enzyme activity assays on a set of defined substrates which form the majority of polysaccharides in lignocellulose. An initial broad substrate range activity assay was performed with the pectinase and ManA and XynA solutions, which employed the methods described in 3.3.5.

Defined substrates that corresponded to the three major families of purified polysaccharides commonly found in lignocellulose and confirmed as constituents of pineapple pomace were tested (Smith and Harris, 1995). These included for cellulose: Avicel PH101 (AVI), low viscosity carboxymethylcellulose (CMC), and medium length cellulose powder (CPW); hemicellulose: locust bean gum (LBG), beech wood xylan (BEX), and birch wood xylan (BW); pectin: apple pectin (APE), and polygalacturonic acid (PGA); finally lignin was also included. These defined substrates were chosen as they are commonly used for reporting enzyme activity values. The enzyme concentrations were selected to produce between 0.1 mg/mL and 1.0 mg/mL of reducing sugars in 60 minutes on their defined substrates. For ManA and XynA, an enzyme concentration of 4.4  $\mu\text{g/mL}$  and 1.7  $\mu\text{g/mL}$  was used, respectively, with a 0.77  $\mu\text{g/mL}$  enzyme concentration used for the pectinase. The activity, in U/ $\mu\text{g}$ , was calculated for each enzyme, as represented in Table 4.4.

**Table 4.4: Substrate range for *A. niger* pectinase and *C. cellulovorans* ManA and XynA with specific activity on defined substrates. Values represent means; n = 3; ± SD.**

Substrate (w/v)  Enzyme	BWX	BEX	LBG	PGA	APE	AVI	CPW	CMC	Lignin
	3%	3%	2%	2%	1%	6%	6%	6%	1%
<u>ManA</u>	0	0	4.92 ± 0.70	0	0	0	0	0	0
<u>Pectinase</u>	2.10 ± 0.15	2.29 ± 0.12	0	10.55 ± 0.37	4.51 ± 0.23	0	0	0	0
<u>XynA</u>	1.60 ± 0.14	2.54 ± 0.12	0	0	0	0	0	0	0

Considering the relatively high concentration of ManA utilised, the specific activity of ManA was relatively high at 4.92 U/μg, as per Table 4.4, when compared to 2.54 U/μg by XynA on BEX or the 4.51 U/μg from the pectinase on APE. The highest specific activity recorded was pectinase on PGA with 10.55 U/μg, as per Table 4.4. The concentration of ManA and XynA was thus increased for later experiments to produce activity in line with that of the pectinase. The activities of ManA and XynA did correspond to their known substrate ranges and family types, as seen in Table 4.4. Both enzymes displayed no activity outside of their respective defined substrates. The low standard deviations, for all substrates tested, confirmed the validity of DNS methodology used as an accurate measure of enzyme activity. The pectinase had measurable activity on both the birch wood xylan and the beech wood xylan types, with 2.1 and 2.29 U/μg recorded. No activity was recorded on any of the cellulose substrates tested. Positive controls for cellulase activity were subsequently performed with Celluclast 1.5L and AVI, CPW and CMC, to validate the substrate range assays. No positive control for lignin was performed as no enzyme capable of hydrolysing lignin was available in the laboratory at the time of the study.

With the inclusion of the cellulase mixture as part of the enzyme synergy mixtures a substrate activity range assay was performed on one of each of the previously defined substrates

representing each of the three major purified polysaccharides found in lignocellulose. The enzyme concentrations for ManA and XynA were 300 µg/mL, while for cellulase and pectinase the concentrations were 400 µg/mL each. The high protein concentrations, relative to enzyme concentrations typically encountered in bioreactors, allowed for reliable determination of any enzyme activity on a defined substrate without extended incubations of several days (Merino and Cherry, 2007). The activities for each enzyme on specific defined substrates were determined as per section 3.3.5.2. The activity (U/µg) was calculated for each enzyme as per Table 4.5.

**Table 4.5: Substrate range for *T. reesei* cellulase, *A.niger* pectinase, and *C. cellulovorans* ManA and XynA with specific activity on defined substrates. Values represent means; n=3; ± SD.**

<u>Enzyme content</u> \ <u>Substrate (w/v)</u>	<u>BEX 2%</u>	<u>LBG 2%</u>	<u>APE 2%</u>	<u>CMC 2%</u>	<u>Lignin 1%</u>
<u>Cellulase</u>	108.8 ± 0.65	86.10 ± 2.90	54.36 ± 2.92	80.98 ± 3.58	0
<u>ManA</u>	0	185.99 ± 4.71	0	0	0
<u>Pectinase</u>	111.52 ± 1.78	0	300.85 ± 2.65	0	0
<u>XynA</u>	137.48 ± 3.20	0	0	0	0

The activity of cellulase on xylan, mannan and pectin substrates corresponds with the available literature of Celluclast 1.5 L<sup>®</sup>'s substrate range (Juhász *et al.*, 2005; Kovacs *et al.*, 2009). The enzyme activity for pectinase was the highest at 300.85 U/µg. The activity profile for the cellulase mixture had the highest activity with BEX and not CMC, which differs from the results recorded in Table 4.1. This may be due to higher enzyme loadings and decreased incubation times and assay temperature. The highest specific activity with xylan substrates was XynA, at 137.48 U/µg. as per Table 4.5. The high activity of ManA on LBG (186 U/µg) was significantly higher than the only other mannanase activity recorded which was with cellulase at 86.1 U/µg (Table 4.5).

## **4.5 Discussion**

The visualisation of over 12 protein bands for the cellulase sample indicates that the lack of purification and crude nature of the sample, as seen in lane “Cell” of Figure 4.1. The presence of multiple bands explains the multiple activities measured which will most likely be due to individual activities from specific enzymes and not multiple activities from individual enzymes, as seen in Table 4.5. Conversely, the limited activity displayed by the pectinase, as seen in Table 4.4 and 4.5, and the activity by two of the bands within the activity gels, Figures 4.2 and 4.3, indicate the relative purity of the enzyme solution. The activity of three of the protein fractions on PGA can be used to infer that the remaining band at 76 kDa contained the xylanase activities, as per Table 4.4. This most likely was due to the inclusion of a xylanase in the commercial mixture as the polygalacturonase (EC: 3.2.1.15) of *A. niger* belongs to GH family 28. This family has no documented activity on pure xylan, however some members have endo-xylogalacturonan activity (EC 3.2.1.17) (CAZy, 2012). Possibly some xylogalacturonan could have been present in the xylan substrates tested. The most likely explanation for activity was the presence of a xylanase within the pectinase solution.

The activities of both ManA and XynA being selective for only mannan and xylan substrates, respectively, have been confirmed by previous reports in literature (Morrison *et al.*, 2011; Olver *et al.*, 2011). This was due to the shared property of their GH families for high substrate specificity. In addition, there were no contaminating enzyme fractions for ManA and XynA solutions, due to their purification, as demonstrated in section 3.4.2. The purity of these enzymes presents them as ideal reagents for use within synergy assays. The low activity of Celluclast 1.5L relative to xylanase may be due to the use of CMC and not the hydroxyl-ethyl-cellulose used in other cellulase activity assays.

For the cellulase solution, the broad range of activity with all of the pure substrates corresponds to the known activities of the commercial solution, as per Table 4.1 (Juhász *et al.*, 2005). The published results differ from our experimental values in the relatively lower specific activity recorded for xylanase at 108.8 U/ $\mu$ g, but higher specific activity with mannanase at 86.1 U/ $\mu$ g, compared to Table 4.5. These results could be due to higher enzyme/substrate loading and different assay conditions used in Table 4.5. An enzyme loading of 400 mg/mL was used in Table 4.5 compared to 125 mg/mL for Table 4.1. The low level of mannanase activity for the



cellulase would benefit from the inclusion of a high specific activity mannanase such as ManA, when processing mannan rich substrates. The higher enzyme loading with the cellulase demonstrated that the solution had significant pectinase activity which also differed from the results recorded in Table 4.1 (Juhász *et al.*, 2005). The specific activities for ManA and XynA, at 186 and 137.5 U/μg, were the highest of any specific activities recorded with the mannan or xylan substrates and would mandate their inclusion in any enzyme mixtures to potentially increase synergy levels.

#### **4.6 Conclusions**

The inclusion of additional activity gels, on either birch wood or beech wood, would have allowed for the identification of fractions within the pectinase solution with xylanase activity. The use of the pectinase, ManA and XynA solutions in synergy experiments will allow for identification of which polysaccharides could act as steric hindrances for the other enzymes. The use of the cellulase solution will somewhat complicate this due to the multiple activities of the solution. It would, however, demonstrate the effects of specific additions of pure hemicellulases and a relatively pure pectinase solution would have on a mixed commercial enzyme solution.

The use of longer incubation times and lower enzyme concentrations would have allowed for greater differentiation of the levels of activity. Also, the inclusion of other types of the substrates (i.e. to test for activity on modified substrates) would further have demonstrated the substrate specificities of any of the enzyme mixtures.

# Chapter 5: Pineapple pomace preparation and individual enzyme activity

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## **5.1 Introduction**

To prevent the accumulation of fruit wastes while simultaneously promoting their beneficiation, various degradation processes and hydrolysis techniques have been investigated (Laufenberg *et al.*, 2003; Lohrasbi *et al.*, 2010). Two areas, key to cost reduction, are pre-treatment and enzyme hydrolysis. Optimal pre-treatment conditions and techniques are critical as they can represent 20% of total process costs and significant capital investments in process design (Yang and Wyman, 2007). Two common pre-treatment methods of lignocellulose wastes, in scientific and industrial applications, are mechanical milling and thermal pre-treatment (Hendriks and Zeeman, 2009).

Mechanical milling is the reduction in the particle size of lignocellulosic biomass by a mechanical process until a desired particle size is obtained. This has the advantage of reducing the crystallinity and degree of polymerisation of all polysaccharides in the biomass (Palmowski and Muller, 1999). Additional advantages include increasing the surface area to volume ratio of biomass in solution and the specific surface area of the particles, thereby increasing substrate availability to all enzymes, and in particular, substrate terminals (Palmowski and Muller, 1999). Milling does not produce inhibitors, such as furfural or phenolic(s), which can reduce total hydrolysis yields and increase technical digestion times (Beukes *et al.*, 2010; Morrison *et al.*, 2011). Milling can result in higher total hydrolysis yields of between 5% and 25%, depending on the type of biomass and the duration of the milling technique(s) employed (Delgenés *et al.*, 2002).

The greatest advantage of mechanical milling is the reduction in technical digestion times by between 23% and 59%, due to increased hydrolysis rates (Hartmann *et al.*, 1999). Milling particles to below 40 mesh size, in which the maximum diameter of 90% of the particles are less than 0.4 mm, was found to have negligible effects on total hydrolysis yields and hydrolysis rates (Chang and Holtzapple, 2000). Milling therefore offers a proven low cost pre-treatment strategy, but with a high initial capital expenditure on equipment.

Various types of thermal pre-treatment of lignocellulose have been investigated, especially in conjunction with a chemical additive, such as acid, alkali, or oxidative reagents. However, the use of only mild thermal techniques on lignocellulose can reduce both the reagent costs and loss of sugar yield at the end of processing (Kaar and Holtzapfle, 2000). Mild is defined as temperatures below 180°C for less than 30 min, in comparison to steam explosion or liquid hot water pre-treatment. The temperature and duration of the thermal treatment influence the formation of potential inhibitors and overall degradation of various polysaccharides and lignin within the biomass. Temperatures above 180°C for durations longer than 30 min will solubilise hemicelluloses, such as xylan and galactoglucomannan, along with lignin (Garrote *et al.*, 1999). This can prove detrimental as certain genetically modified strains of *Saccharomyces* and bacteria are able to ferment these pentoses into ethanol (Lynd *et al.*, 2005). The removal of hemicellulose can represent a loss of potential yield. This could be due to loss of potential substrate if the sugars are not fully recoverable after pretreatment or if they are denatured, thereby rendering them unsuitable for downstream applications (Garrote *et al.*, 1999; Merino and Cherry, 2007). Additionally the denatured sugars could form inhibitors of later hydrolysis and fermentation stages (Lynd *et al.*, 2005).

The inclusion of reagents for chemical pre-treatment generally incurs both additional monetary costs and loss of substrate yield (Kaar and Holtzapfle, 2000). The loss in yield is due to denatured substrate, from chemical hydrolysis and the creation of inhibitors. The latter can inhibit later enzymatic hydrolysis and/or growth of the fermentative micro-organisms (Delgenés *et al.*, 2002; Morrison *et al.*, 2011). Lime kiln recovery in which alkali lime is recoverable could provide a viable thermo-chemical pre-treatment for pineapple pomace (Kaar and Holtzapfle, 2000). This would be due to the treatment not fully hydrolysing the xylan fraction of lignocellulose, lack of formation of inhibition products, and inexpensive reagent costs of calcium carbonate. There would still be a required investment into alkali resistant equipment and optimisation of alkali pre-treatment for each substrate (Beukes *et al.*, 2008; Dredge *et al.*, 2011). Therefore, only the use of thermal and mechanical pre-treatments aid in cost reductions.

There are limited reports in literature on the activity of Celluclast 1.5L<sup>®</sup> on pineapple pomace in terms of increasing reducing sugars yields from insoluble substrates fractions (Sreenath *et al.*, 1994). However, research on the activity of Celluclast 1.5L<sup>®</sup> with sugar cane bagasse (SCB),

which is also a member of the family *Poaceae*, provides an example for the interaction of this enzyme solution with a lignocellulosic substrate similar to pineapple pomace. It was found that 2% (w/v) steam pre-treated SCB hydrolysed by Celluclast 1.5L<sup>®</sup> resulted in 12.8 g/L sugar, under enzyme hydrolysis conditions of 96 h at 40°C with 0.1 M sodium acetate buffer (pH 4.8) (Kovacs *et al.*, 2009).

Previous studies related to improvements in juice recovery from pineapple pulp have been performed with Celluclast 1.5L<sup>®</sup> and Pectinex<sup>®</sup> 3XL, but only with 30 min room temperature assays (Sreenath *et al.*, 1994). This resulted in each enzyme only generating a negligible increase in sugar of 0.2° and 0.3° Brix, respectively, relative to the untreated controls (Sreenath *et al.*, 1994). This was most probably due to the use of short assay times and the lack of substrate processing. Further research on the abilities of these two enzyme solutions to degrade pineapple pomace insoluble fractions should be performed.

The activities of ManA and XynA from *Clostridium cellulovorans* have been reported for several members of the *Poaceae* family, including pineapple pomace (Beukes *et al.*, 2008; Olver *et al.*, 2011). It was reported that ManA and XynA had activities of 15 U/mg and 17 U/mg, respectively, on homogenised, dried and autoclaved pineapple pomace (Olver *et al.*, 2011). Activity was defined as the quantity of enzyme required to release 1 µmol of reducing sugar per minute, with reducing sugars recorded in glucose equivalents. The reaction conditions were 50°C for 7 days with acetate buffer (pH 5.0).

With the overall goal of reducing the costs involved in the production of second generation biofuels, measures should be taken to optimise process systems towards lower costs. To this end, hydrolysis and assay conditions at comparatively low temperatures of 37°C with tumbling, limited mechanical and thermal pre-treatments of pomace, with no chemical pre-treatments should be performed.

With literature of enzyme activity from all four enzyme solutions with pineapple pomace it was necessary to establish the activity levels of each enzyme with this study's assay procedures (Beukes *et al.*, 2008; Sreenath *et al.*, 1994). This would establish baseline activities for each enzyme and allow for later calculation of degree(s) of synergy.

## **5.2 Aims and Objectives**

### **5.2.1 Aims**

- To produce pineapple pomace approximately equivalent to cannery waste, in terms of minimal pre-treatment, for enzyme assay purposes.
- To determine the specific enzyme activity for Celluclast 1.5L<sup>®</sup> (*T. reesei* cellulase) and Pectinex<sup>®</sup> 3XL (*A. niger* pectinase) and *C. cellulovorans* ManA and XynA on pineapple pomace.

### **5.2.2 Objectives**

- To generate pineapple pomace representative of cannery waste.
- To perform limited processing and pre-treatment of generated pineapple pomace to allow for ease of handling.
- Perform individual enzyme specific activity assays to establish baseline enzyme activities.

## **5.3 Methods and materials**

### **5.3.1 Substrate preparation and electron microscopy analysis**

To accurately assess the potential use of enzyme hydrolysis with cannery processing generated pineapple pomace, limited pre-treatments were performed. This was to negate the cost of expensive pre-treatment steps, while assessing the enzyme activity on pomace with no chemical pre-treatments to alter the substrate chemical composition. Pre-treatment steps were limited to mechanical, desiccation and thermal methods. This was to ensure the substrate was homogenised to allow for accurate and reproducible enzyme activity measurements between samples, long term storage and sterility to minimise microbial contamination. These were performed as per the methods stated in section 5.3.1.1.

#### **5.3.1.1 Physical processing, homogenisation, lyophilisation and thermal treatment**

Preparation of pineapple pomace commenced with the removal of the crown and skins of the pineapple. The skins were then collected, mixed with an equal volume of dH<sub>2</sub>O and homogenised in a blender until a fine pulp was produced (Salton Food processor). The pulp was

filtered through a colander, layered with cheese cloth, and periodically removed and hand pressed to remove any liquids present. The pulp was then mixed with 3 times volume of dH<sub>2</sub>O and then stirred at 200 rpm for 30 minutes before an additional filtration and pressing step. The pulp was then spread onto the inner walls of a 500 mL beaker and frozen at -20°C overnight. The generation and processing steps used to create the pineapple pomace have been represented in Figure F1 of Appendix F. The pineapple pomace was placed into a freeze dryer and lyophilised until dry. The substrate was then transferred to a 500 mL Schott bottle and autoclaved at 121°C for 20 min. The substrate was then blended further, for 30 min, to reduce particle size below 40 mesh.

To measure the particle size and image the powder produced, electron microscopy was performed, as per section 5.3.1.2.

### **5.3.1.2 Electron-microscopy**

To visualise the pineapple pomace powder, a 50 mg sample of fine powder was fixed to double-sided tape on a sample platform for sample preparation. The sample was further lyophilised to remove any moisture and subsequently coated with gold particles. The sample was imaged with a scanning electron microscope (Vega<sup>®</sup> Tescan). Images of the pomace powder were captured at magnifications ranging from 33x to 7460x magnification.

### **5.3.2 Enzyme complex substrate activity determination**

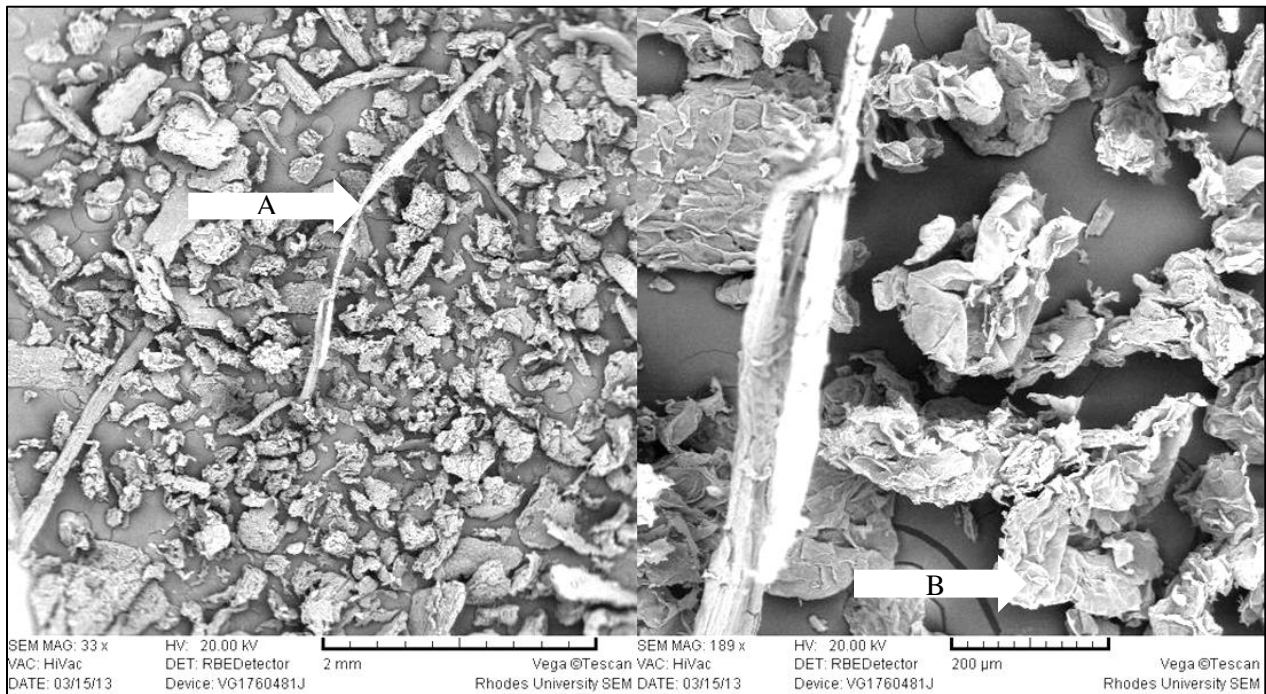
For assaying the enzyme activity on complex lignocellulose substrate, pineapple pomace was dissolved in assay buffer overnight before any assays were performed. This was done to allow the pomace to suitably rehydrate. In order to load uniform concentrations of substrate when pipetting, the buffer and substrate volumes of the standard assay solutions were combined. The insoluble fractions were kept suspended in substrate solution with a magnetic stirrer bar at approximately 200 rpm while 350 µl aliquots of solution were added to each assay tube. For ease of use, the following enzyme solutions have been abbreviated to single letters; Celluclast 1.5L<sup>®</sup> *Trichoderma reesei* cellulase (C), Pectinex<sup>®</sup> 3XL *Aspergillus niger* pectinase (P), and *Clostridium cellulovorans* ManA (M) and XynA (X).

## 5.4 Results

The physical processing, homogenisation and lyophilisation of the pineapple skins resulted in a fine powder of pineapple pomace. To visualise the effects of the mechanical and thermal pre-treatments on the pineapple pomace, scanning electron microscopy (SEM) was performed on the powder.

### 5.4.1 Substrate preparation

SEM imaging was performed on the pomace powder, as per section 5.3.1.2. Suitable images, that were representative of the overall particulate size and physical characteristics, were recorded, as shown in Figure 5.1.



**Figure 5.1: A scanning electron microscopy image of *A. comosus* pineapple pomace powder at magnifications of 33x (left) and 189x (right).**

The homogenisation and lyophilisation of the pineapple pomace created substantial quantities of pomace particles with diameters of between approximately 200  $\mu\text{m}$  and 400  $\mu\text{m}$ , as seen in Figure 5.1. A mean diameter of particle size was calculated as 281  $\mu\text{m}$  with a standard deviation (SD) of 68  $\mu\text{m}$ . Despite the homogenisation of pomace, particles in sizes ranging from several millimeters to micrometer diameters were generated with no standard diameter, in addition to fibers of several mm in length, as indicated by the arrow A in Figure 5.1. Due to the mechanical

shearing and thermal treatments, the surface area of the particles represented a relatively smooth and undisrupted surface area, as indicated by arrow B in Figure 5.1. Further images of the pomace powder are represented in Figure F2 of Appendix F.

With the pomace processed into an appropriate form for enzyme activity assays, the individual specific activities of both commercial and heterologously expressed enzymes with pineapple pomace could be determined, utilising methods described in section 5.4.2.

### **5.4.2 Individual enzyme activity with pineapple pomace**

To establish the optimal time period for enzyme activity, activity assays were performed over time periods ranging from 1 to 168 h (1 week). The activity was determined through measuring the increases in reducing sugar concentrations, as per the methods described in 3.3.5 and 5.3.2. The majority of enzyme activity with pineapple pomace was found to occur within 36 h of enzyme addition, with longer incubations at 37°C increasing the risk of inaccurate measurements of activity due to enzyme denaturation, fungal/microbial contaminations, and substrate auto-degradation.

#### **5.4.2.1 Cellulase and pectinase activity**

In order to establish the enzyme activities for subsequent use in determining the enzyme synergy and design of enzyme synergy experiments, the individual enzyme activities on the pineapple pomace were determined. The controls for positive activity with pineapple pomace were conducted under identical conditions and were performed concurrently to their respective synergy assays. Enzyme concentrations for cellulase, pectinase, ManA and XynA were 50; 0.625; 12.5 and 12.5 µg/mL, respectively. These were treated as the “100%” concentrations of each enzyme for the synergy ratios.

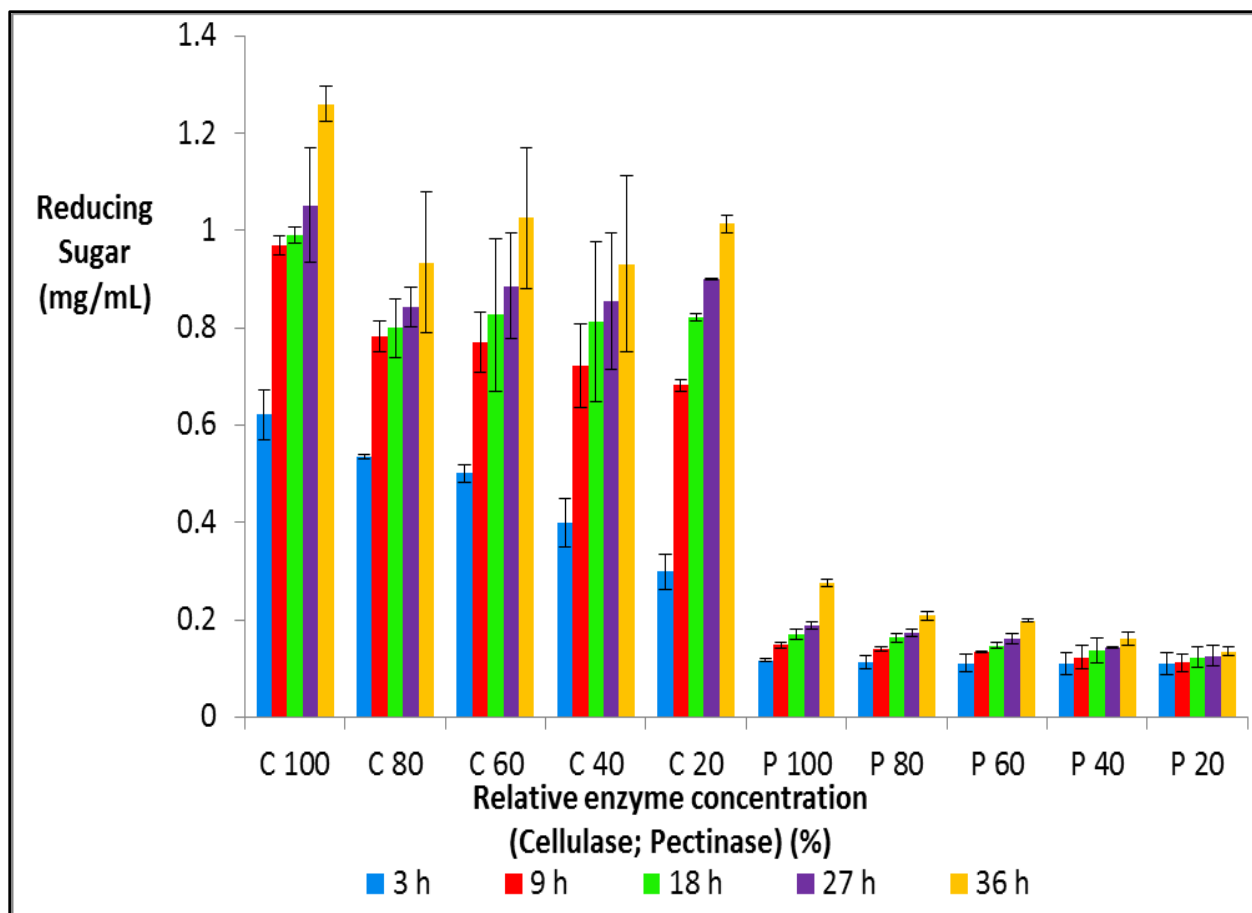
The protein concentration for each of the enzymes used was determined by each enzyme’s activity on its defined substrate, so that each enzyme at 100% protein loading would have 30 µg/mL/min of activity, as per section 3.3.5. Thus, for synergy studies, the total activity of the enzyme solution added to each tube, when assayed with each enzyme’s defined substrate, was kept constant at 30 µg/mL/min of activity, thereby allowing the total protein loadings different tubes to vary. This approach would allow for the minimisation of protein loadings while



simultaneously allowing for the selection of an enzyme combination with the appropriate levels and types of substrate activities required to produce the highest concentration of reducing sugars.

The choice of a high level of enzyme activity on defined substrate, relative to the activity generated with the pineapple pomace, was to ensure that the free enzyme was always in excess to available substrate. This would lessen the effect of gradual losses in total enzyme activity over time and minimise any enzyme rate limiting steps in the degradation of the substrate due to limited enzyme activity. This would also ensure that any changes in reducing sugar concentrations measured within solutions were due to increased enzyme activity from increased substrate availability and thus these changes in availability and accessibility would not be masked by the lack of enzyme catalytic capacity. A potential weakness in this approach is that high concentrations of cellulases and other lignocellulosic enzymes can produce minimal levels of synergy (Woodward, 1991). This is due to all substrate binding sites, including common binding sites such as pores within the plant cell walls, becoming oversaturated with enzymes leading to competition for binding sites (Woodward, 1991).

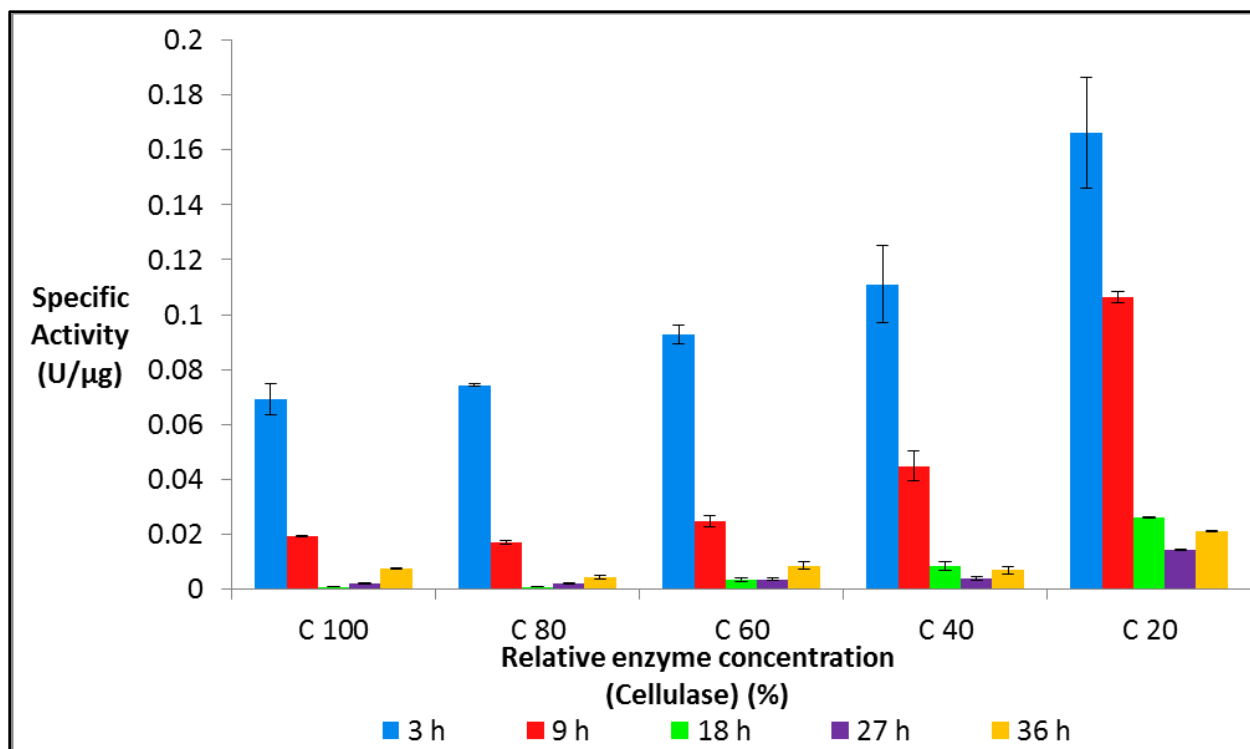
The total volume used for each of the assays for the 36 h synergy study was increased to 1.5 mL as a total volume of 750  $\mu$ L of supernatant was required from each combination to measure the reducing sugar concentrations. The concentrations of reagents and enzymes used in these 1.5 mL assays were identical to the reagent concentrations as described in section 5.3.2. Samples of 150  $\mu$ L of supernatant were taken after centrifugation to avoid inclusion of any insoluble substrate, as per section 3.3.5. The use of a larger total volume allowed for the minimisation of any inter-sample variability, due to handling errors and variability in substrate composition due to the use of a complex lignocellulose substrate. These individual enzyme activities for use with the quad synergy study are provided in section 6.3.1. The reducing sugars, produced by individual enzymes with 1% pineapple pomace (w/v) over 36 h were sampled at 3, 9, 18, 27 and 36 h (see Figure 5.2).



**Figure 5.2: Reducing sugar concentrations produced by *T. reesei* cellulase [50 µg/mL] or *A. niger* pectinase [0.625 µg/mL], with 1% (w/v) of pineapple pomace, at relative percentage enzyme concentrations to those used for synergy assays. Reducing sugar concentrations were measured at time periods of 3, 9, 18, 27 and 36 hours. Values represent means ± SD, n=3.**

With a 1% (w/v) concentration of pineapple pomace, both ManA and XynA had no detectable activity within the 36 h period measured. The concentration of reducing sugars produced by cellulase with the 100% loading (C100) after 36 h was 1.26 mg/mL, which represents the highest production of reducing sugars, for any of the individual enzyme assays, as per Figure 5.2.

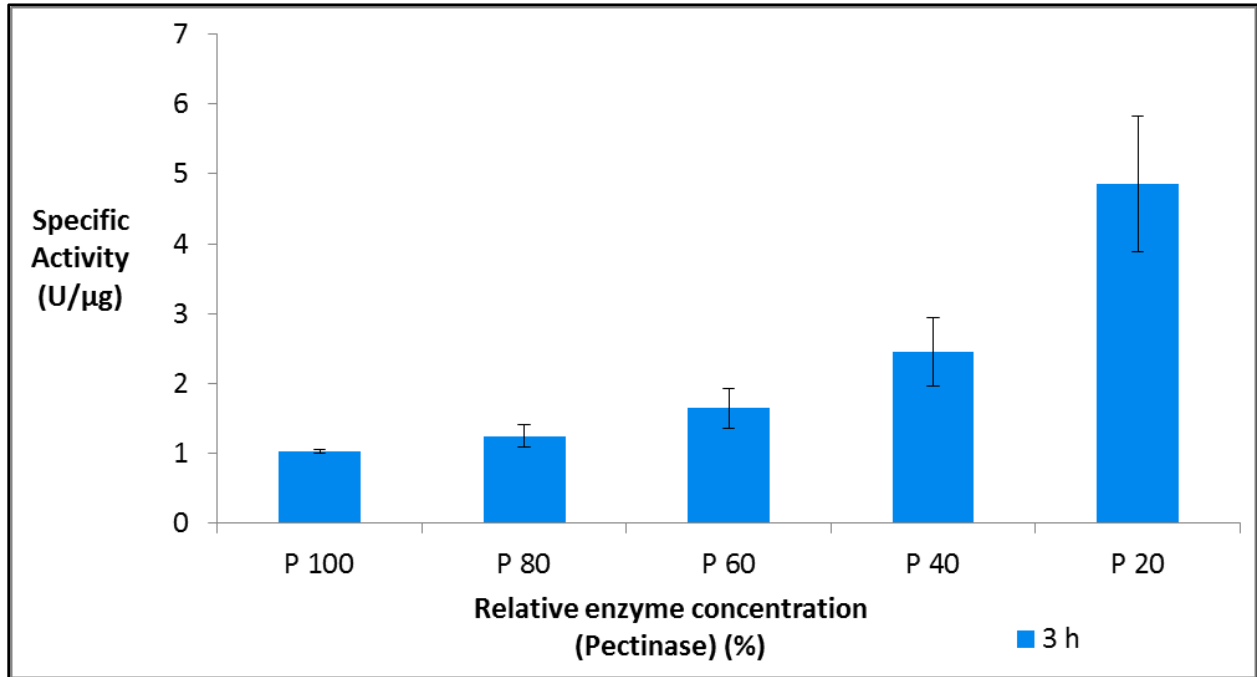
For pectinase the highest sugar concentration was at 0.276 mg/mL after 36 h. For all pectinase and cellulase concentrations at 3 h, there was a direct relationship between enzyme concentration and changes in the concentration of reducing sugars produced. Thus an increase of enzyme concentration would result in an increase of the reducing sugar concentration. To visualise the specific activity of the cellulase and pectinase, the specific activity of each relative enzyme concentration was plotted for the measurements over the 36 h, as per Figures 5.3, 5.4 and 5.5.



**Figure 5.3: Specific activities for *T.reesei* cellulase, with 1% (w/v) pineapple pomace, at relative percentage enzyme concentrations to those used for synergy assays. Reducing sugar concentrations were measured at time periods of 3, 9, 18, 27 and 36 hours. Values represent means  $\pm$  SD, n=3.**

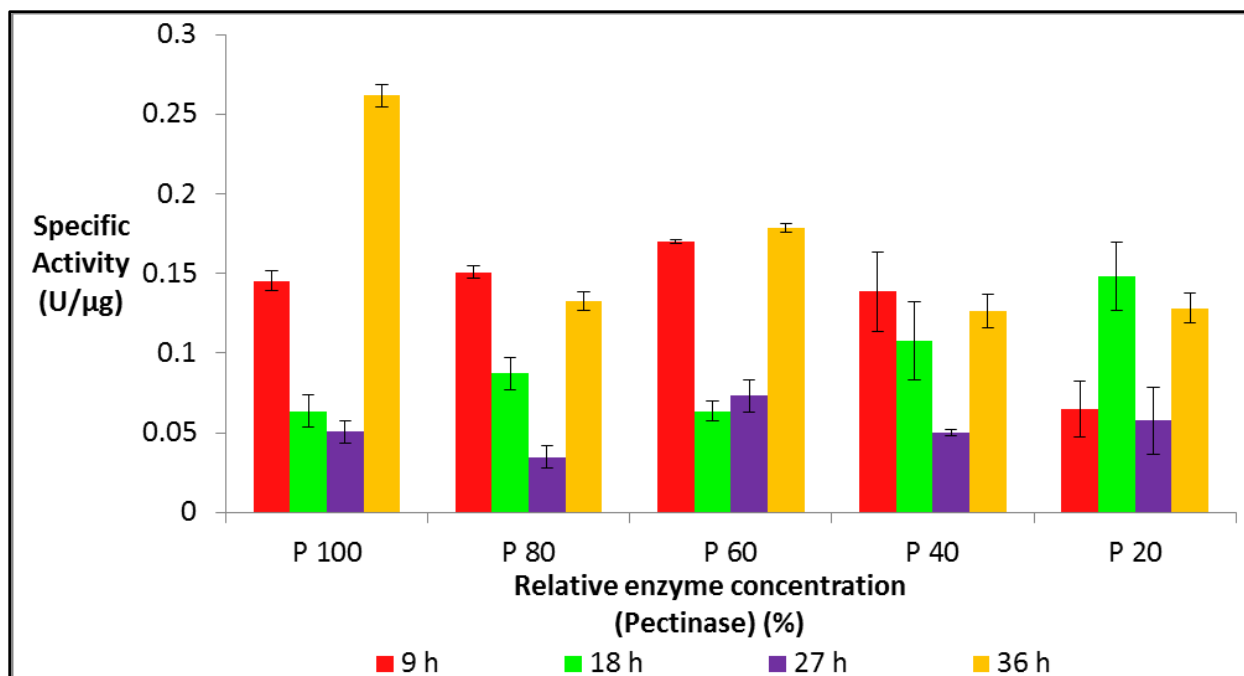
The highest level of specific activity for the cellulase activity assays were recorded with C20, the 20% enzyme loading of cellulase, at 0.166 U/ $\mu$ g which was equivalent to a 10  $\mu$ g/mL protein concentration in the assay solution. At this protein concentration the enzyme to substrate loading was low enough to allow for high specific activity throughout the 36 h period, as per Figure 5.3. This was due to the lack of enzyme to hydrolyse the pomace to the same level as seen with C100, which had an enzyme concentration of 50  $\mu$ g/mL, as per Figure 5.2. It can also be seen that the majority of enzyme activity for cellulase, regardless of enzyme concentration, occurred within the first 3 h.

The specific activities for pectinase, in the initial 3 h, were significantly higher than the cellulase activity levels recorded over the same time period and have been represented separately, in Figure 5.4.



**Figure 5.4: Specific activities for *A. niger* pectinase, at relative percentage enzyme concentrations used for synergy assays, after 3 hours with 1% (w/v) of pineapple pomace. Values represent means  $\pm$  SD, n=3.**

Again, the highest specific activity, at 4.86 U/μg, was observed with the lowest concentration of enzyme, P20, with a protein loading equivalent to 0.125 μg/mL. The specific activity of pectinase was the highest of all the enzymes assayed over all time period. After the initial 3 h the specific activities for all the pectinase concentrations decreased to below 0.3 U/μg as seen with the time periods of 9; 18; 27 and 36 hours displayed in Figure 5.5 below.



**Figure 5.5: Specific activities for *A. niger* pectinase, with 1% (w/v) of pineapple pomace, at relative percentage enzyme concentrations used for synergy assays. Reducing sugar concentrations were measured at time periods of 9, 18, 27 and 36 hours. Values represent means  $\pm$  SD, n=3.**

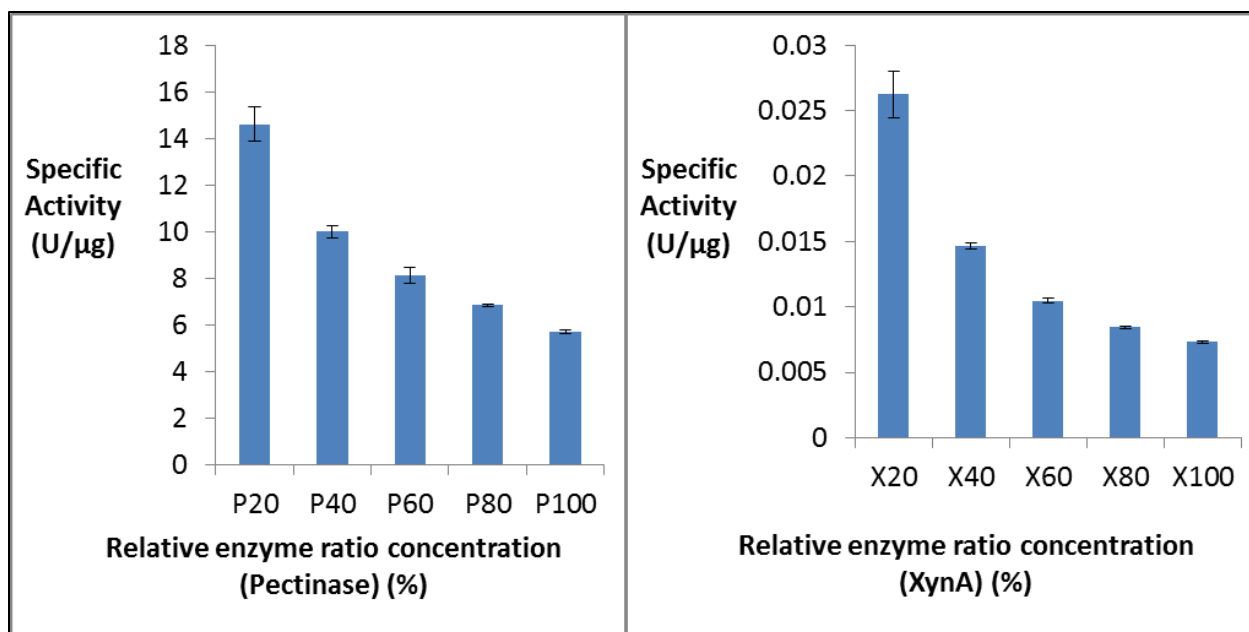
There was a general trend with all enzyme concentrations of decreasing specific activity until 27 h, with minimum specific activities at 27 h, as per Figure 5.5. The exceptions to this trend were with P60 and P20, which had specific activities that were lower at 18 h or too high at 18 h relative to 9 h measurements. The specific activity for P20 at 9 h was lower than that at 18 h, which may have been due to delayed degradation of substrate due to low enzyme concentration. For P60 to P20, the specific activities at 9 h decreased with enzyme concentration, while for the 18 h measurements the specific activity increased with decreased substrate concentration.

It was observed, that at 36 h, the specific activity of all enzyme concentrations had increased relative to both 18 and 27 h measurements for each enzyme concentration, in Figure 5.5. The specific activities for the 36 h measurements were approximately equivalent to the 9 h measurements, except for the P100, at 0.225 U/ $\mu$ g, as seen in Figure 5.5. This would indicate that enzyme activities with the substrate had begun to expose additional substrates for degradation. With the specific enzyme activities for individual enzyme concentrations over 36 h established, it would be possible to calculate the degrees of synergy generated by selected enzyme combinations.

#### 5.4.2.2 Pectinase and XynA activity

To establish enzyme activity levels for ManA, pectinase, and XynA solutions within the linear range of detection for the DNS assay, the substrate and enzyme concentrations were varied so as to produce over 0.1 mg/mL of reducing sugars after 3 hours. This would also establish the single enzyme activity controls for synergy studies. The protein concentration for pectinase was decreased to 0.08 µg/mL, with the XynA and ManA protein concentration increased to 26.375 µg/mL. These represent a change in protein concentrations from those used in section 5.4.2.1. The substrate concentration was 4% (w/v) pineapple pomace, representing an increase from section 5.4.2.1. The modified enzyme and substrate concentrations were chosen to ensure that XynA and ManA individual activities with pineapple pomace could be measured. This was also done to minimise the level of xylanase activity within the Pectinex<sup>®</sup> 3XL solution, so that when both XynA and Pectinex<sup>®</sup> 3XL were in solution the majority of xylanase activity would be due to XynA.

The specific activity of each of the enzyme concentrations relative to those found in the synergy ratio solutions were assayed for 18 h. This time period was chosen as approximately 80% of all the reducing sugars had been produced with pectinase by 18 h, relative to the 36 h measurements. The activities of ManA and XynA on their respective defined purified substrate were standardised at 60µg/mL/min, while pectinase activity was reduced to 3.85 µg/mL/min. The specific activities for each individual enzyme concentration are displayed in Figure 5.6.



**Figure 5.6: Specific activities for *A. niger* pectinase [0.08 μg/mL] (left) and *C. cellulovorans* XynA [26.375 μg/mL] (right), with 4% (w/v) pineapple pomace, at relative percentage enzyme concentrations used for synergy ratios. Reducing sugar concentrations were measured after 18 hours. Values represent means ± SD, n=3.**

It was observed that the lowered protein concentration for pectinase resulted in an increase in the specific activity of pectinase by over a 1000 fold relative to Figure 5.5. This would have been due to the increased substrate to enzyme ratio from the lowered amount of pectinase used in Figure 5.6. There was no activity observed with ManA after 18 h, despite the increased enzyme concentrations and positive control assays with defined substrates. The increased substrate and enzyme loading for XynA resulted in detectable levels of reducing sugar for all the concentrations of XynA used, as per Figure 5.6. The 100% concentration of XynA produced 0.208 mg/mL of reducing sugar, with X20 producing 0.15 mg/mL of reducing sugar.

## **5.5 Discussion**

### **5.5.1 Physical and thermal pre-treatments**

The physical and thermal treatments of the pineapple pomace succeeded in reducing the pineapple pomace to a dry powder with particle sizes of approximately 280 µm diameter, as seen in Figure 5.1. This allowed for ease of use and helped to minimise microbial contamination through the use of autoclaving and lyophilisation. The reduced particle size, in the form of pomace powder, allowed for uniform substrate loadings, reproducible results, lowered standard deviations and for maximum activity from particle size reduction (Chang and Holtzapfle, 2000).

The overall lack of homogeneity in particle size could be further reduced by both steel ball milling and chemical pre-treatments. The increased uniformity of particle size and any further reductions of particle sizes below the 40 mesh would only result in negligible increases in enzyme activities and would add to the overall substrate pre-treatment and processing costs (Chang and Holtzapfle, 2000). The exposure of additional substrate sites from chemical pre-treatment, as well as the removal of lignin, would prove beneficial (Beukes *et al.*, 2011; Dredge *et al.*, 2011). However, the use of alkali pre-treatments such as lime kiln recovery technology would be advantageous in the exposure of additional hemicellulose fibers and the disruption of particle surface topology.

### **5.5.2 Individual enzyme activity on pineapple pomace**

It was observed that with all the individual enzyme components, assayed with pineapple pomace, that the reducing sugar concentrations increased with longer incubation times, as per Figure 5.2. This confirmed the activity of cellulase and pectinase on the pineapple pomace and these values were used as positive controls for the simultaneous synergy studies. The quantity of reducing sugar produced after only 3 hours of 0.62 and 0.12 mg/mL, for Celluclast 1.5L<sup>®</sup> and Pectinex<sup>®</sup> 3XL, are significantly higher than the amounts recorded by Sreenath *et al.* (1994). This illustrates the importance of substrate preparation and sufficient assay time to establish quantifiable levels of activity. The decreasing specific activities over time from 3 h to 27 h, for both cellulase and pectinase, are indicative that the enzymes rapidly degraded the available surface polysaccharides, as seen in Figures 5.3, 5.4 and 5.5. The increase in specific activity at 36 h may be due to a combination of auto-degradation of the pomace, acid hydrolysis due to the low pH of 5.0 used and increased surface area through pore formation due to hydration (Aguilar



*et al.*, 2002; Conner *et al.*, 1986). This would be in addition to the unknown activities of the unidentified protein fractions within both the pectinase and cellulase solutions, as shown in Figure 4.1.

The use of a higher concentration of XynA, at 26.375 µg/mL, with 4% (w/v) pineapple pomace, allowed for suitable levels of enzyme activity over a range of protein concentrations, as seen in Figure 5.6. The high protein loadings required for measurable activities and the low levels of activities that resulted, would most likely be prohibitively expensive for any commercial use of the XynA on an individual basis (Sørensen and Mortensen, 2005). The enzyme would therefore have to be part of a minimal enzyme synergy cocktail in order to be cost effective (Meyer *et al.*, 2009).

The lack of activity of ManA with pineapple pomace is of concern, but can be explained by both the low concentrations of mannan residues within pineapple pomace and the likelihood that these were shielded by cellulose fibrils. This does not exclude the possibility that ManA can play a vital role within pineapple degradation. The same cost limitations that apply with the high protein loadings of XynA would also apply to ManA and thus it would also have to be used as a part of a minimal enzyme synergy cocktail (Meyer *et al.*, 2009).

## **5.6 Conclusions**

The use of lime pre-treatment and steel ball milling may significantly increase the homogeneity and cell wall degradation of pineapple pomace and should be considered in future work on pineapple pomace. This must be within the scope of economic feasibility for later industrial cost efficiency. This would have the added benefit of increased surface area to volume ratio of the particles and decreased lignin content, both of which could dramatically increase enzyme activities.

Celluclast 1.5L<sup>®</sup> may be used independently to generate reducing sugars from pineapple pomace. However, whether this would be viable at protein loadings of 50 µg/mL of 1% substrate is unlikely. A pilot bioreactor utilising Celluclast 1.5L<sup>®</sup> under these conditions would have to be performed to generate values for running cost and capital outlays. In addition, the low levels of reducing sugars, at 1.26 mg/mL, generated after 36 h with C100, would be insufficient to achieve a successful fermentation (Pacheco *et al.*, 2010).

It can also be assumed that, as pectinase, ManA and XynA produced lower quantities of reducing sugars, they would also be excluded from individual use within a bioreactor system. Even though pectinase had a much higher specific activity than cellulase, as seen in Figures 5.2-4, it would still not be individually viable as it possessed no cellulase activity, as seen in Table 4.4. Thus it would not degrade the polysaccharide that constitutes over 40% of the unligified cell walls of pineapple pomace (Smith and Harris, 1995).

Another unknown factor is whether the reducing sugars generated would be in a usable form for any fermentative agent. These could be identified with HPLC-MS and from there a decision as to whether more hydrolysis, of any of the oligosaccharides present, to sugar monomers would be required, or could be cost effective. In addition, it should be investigated whether fermentation could be attempted with the reducing sugars generated from each of the individual enzymes activities should the sugars be concentrated to a suitable concentration.

The problem of high protein loadings and limited substrate ranges with each of the individual enzymes remains. The effects of both additive and synergistic activities generated from combinations of enzymes pose a potential solution to these problems. Thus, further investigation into the generation of synergy between these four enzyme solutions is warranted and is the subject of the next chapter, Chapter 6.

# Chapter 6: Simultaneous and sequential synergy studies

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## **6.1 Introduction**

Since the first demonstration of enzyme synergy in the 1950's, the majority of synergy studies have been performed on cellulose degradation to the neglect of other polysaccharides in lignocellulose, such as xylan, mannan and pectin (Banerjee *et al.*, 2010; Gilligan and Reese, 1954). With glucuronoarabinoxylan, pectin and glucomannan representing 28%, 6% and 5% (w/v) of the unignified cell walls in pineapple pomace, these would (in addition to cellulose) constitute the major sources of sugars for fermentation (Smith and Harris, 1995; Yapo, 2009). As pre-treatment and fermentation technologies have advanced, so has the economic necessity of utilising all available sugars within lignocellulosic biomass (Merino and Cherry, 2007).

The use of commercial enzyme solutions for the hydrolysis of pineapple waste has been investigated in literature, but to a limited extent. It was reported that a combination of Celluclast 1.5L<sup>®</sup> and Pectinex<sup>®</sup> 3 XL with crushed pineapple pulp resulted in only a 0.3° Brix increase of potential sugars relative to negative controls (Sreenath *et al.*, 1994). These assays were only run for 30 min and the free sugars, which would still have been present after juicing, were not removed. The use of only the specific gravity would thus not have been sufficiently accurate to detect any changes in sugar concentration due to enzyme activity. The assay of pre-treated pineapple pomace insoluble fractions, with an accurate measure of reducing sugars and over a longer period, would have been able to overcome these shortcomings.

Synergy studies with ManA and XynA from *C. cellulovorans* with pineapple waste have demonstrated synergistic interactions between these enzymes (Beukes *et al.*, 2008; Olver *et al.*, 2011). The optimal combination for both specific activity and synergy on pineapple pulp was reported by Olver *et al.* (2011) to be a molar ratio of 75% ManA and 25% XynA, which had activity of 39.9 U/mg and degree(s) of synergy (DS) of 2.5. This combination also displayed the highest specific activity of all the bi-synergy combinations tested between ManA, XynA and Endoglucanase E from *C. cellulovorans*. Beukes *et al.* (2008), who conducted assays on pineapple leaf fibre using the same enzymes and similar assay conditions, reported that the

optimal combination for highest specific activity and DS was at a 50% ManA to 50% XynA molar ratio. This resulted in a specific activity of 55.71 U/mg and 2.36 DS (Beukes *et al.*, 2008). Both of these synergy studies were run for 7 days at 50°C, with 2% (w/v) of either pineapple pulp or leaf fibre that had been oven dried and autoclaved. The higher assay temperatures, different pineapple tissue waste types and the longer assay periods used in previous studies makes comparison with this present study difficult.

The use of only the skin wastes from pineapple processing represent a desirable target for biofuel research, as this will not have an impact on the human food supply (Van Dyk *et al.*, 2013). The well characterised *Poales* lignocellulosic structure offers an ideal substrate for use in enzyme synergy research for cost reductions and productivity improvements (Smith and Harris, 1995). This will allow for novel research as there is a current lack of research in both simultaneous and sequential synergy studies with commercial enzyme combinations along with purified hemicellulases. The use of these combinations on a hemicellulose rich substrate containing minor amounts of pectin, such as pineapple skin pulp, will also provide novel findings.

Celluclast 1.5L<sup>®</sup> was used as it is derived from a model lignocellulose degrading organism, *T. reesei*, which is a mainstay of current lignocellulose hydrolysis. It provides a suitable core cellulase mixture for use with hemicellulases under the minimal enzyme cocktail concept (Meyer *et al.*, 2009; Yang *et al.*, 2011). For an industrial process, the logistics of having to use only purified enzymes would not be cost effective, and commercial enzymes will form a dominant part of any enzyme cocktail used (Sills and Gossett, 2011).

As multiple lignocellulosic feedstocks will be used in a commercial plant, the addition of only selected purified enzymes to fill in for the lack of activities in a core enzyme mixture, such as Celluclast 1.5L<sup>®</sup>, would reduce operating costs and protein loadings (Sills and Gossett, 2011). These solutions could be produced according to set ratios for a particular feedstock under optimised operating conditions for a particular bioreactor. It has been reported that commercial enzyme mixtures have limited xylanase and mannanase activities and thus supplementation with XynA and ManA from *Clostridium cellulovorans* could help improve the overall hydrolysis of pineapple pomace (Juhász *et al.*, 2005; Qing and Wyman, 2011). Further work could include the determination of the composition of substrates such as pineapple skin waste and if significantly different enzyme cocktails are required for various tissue types from the same species.

## **6.2 Aims and Objectives**

### **6.2.1 Aim**

To determine the degree(s) of synergy generated from enzyme ratios in bi-, tri-, and quad-synergy studies with Celluclast 1.5L<sup>®</sup> (*T. reesei* cellulase), Pectinex<sup>®</sup> 3 XL (*A. niger* pectinase) and *C. cellulovorans* ManA and XynA on pineapple pomace.

### **6.2.2 Objectives**

- To perform bi-synergy assays with all potential combinations of each enzyme under simultaneous conditions.
- To perform tri-synergy assays with combinations of either pectinase, ManA or XynA paired with cellulase under simultaneous conditions.
- To perform quad-synergy assays with all four enzymes under simultaneous conditions.
- To perform sequential bi- and tri-synergy assays with pectinase, ManA and XynA.
- To perform sequential bi-synergy assays with cellulase and pectinase.

## **6.3 Methods and materials**

### **6.3.1 Synergy studies on complex substrates**

Enzyme activity assays were run in triplicate, under conditions identical to section 3.3.5, unless stated otherwise. For synergy studies, the total volume of enzyme ( $V_E$ ) added was kept constant at 50  $\mu$ L or 12.5% (v/v) of the assay solution. The volumes of the various enzyme solutions were added ( $V_i$ ) so that their sum would add up to 50  $\mu$ L. Thus the ratios of enzyme solution volumes were adjusted in set 12.5  $\mu$ L or 2.5% (v/v) volume increments ( $V_i$ ) for the relative enzyme loading ratios. Thus the  $V_E$  of 50  $\mu$ L was always kept constant as  $\sum V_i(n) = V_E$  with  $n=4$  maximum. The protein concentration of a solution was varied as the ratios varied, but the sum of the activities for all enzymes in solution was kept constant, unless otherwise stated. The ratio of the enzyme volumes added would vary according to set ratios, as displayed in Appendix C. All protein concentrations of the 100% enzyme loading used for each study are detailed before each assay.

Positive controls for each enzyme at  $V_E$ , equivalent to 100% protein loadings with defined substrates (purified model substrate), were performed. These were carried out to confirm enzyme activity and standardise the enzyme concentrations according to a set level of enzyme activity on

their defined substrate, if a standard level of enzyme activity was used. Each of the incremental increases in an enzyme's concentration ( $V_i$ ) were assayed with pineapple pomace to obtain levels of individual enzyme activity, to use in calculating DS for an enzyme combination.

For the sake of convenience, the following enzyme solutions have been abbreviated to single letters or named after the dominant enzyme activity as enclosed in brackets; Celluclast 1.5L<sup>®</sup> *Trichoderma reesei* (cellulase) (C), Pectinex<sup>®</sup> 3XL *Aspergillus niger* (pectinase) (P), and *Clostridium cellulovorans* (ManA) (M) and (XynA) (X). These enzyme solutions were the same as those produced and described in chapters 3, 4 and 5.

### **6.3.2 Calculation of degree of synergy**

For the calculation of degrees of synergy produced by a certain combination of enzymes, the total concentration of reducing sugar was divided by the theoretical sum of the reducing sugars produced by the individual enzyme controls for the each concentration of enzyme used in a particular ratio.

#### **6.3.2.1 Simultaneous synergy**

For simultaneous synergy studies all the enzyme components were added together at the commencement of the assay. The enzyme components were kept at 4°C until addition to the assay solution, with the hemicellulases added first before the pectinase or cellulase. Synergy studies were run at 37°C and over sufficient time periods, all of which were greater than 1 hour, to generate measurable quantities of reducing sugars. The total volume of all the enzyme samples added was kept at or below 50  $\mu$ L. If below 50  $\mu$ L, the remaining volume was made up with assay buffer. Enzyme activities were measured as per section 3.3.5, unless stated otherwise.

#### **6.3.2.2 Sequential synergy**

Sequential synergy was initiated by the addition of a particular enzyme in a pre-determined order. The assays were performed, under the same conditions as simultaneous synergy, and reactions were terminated by heating to 100°C for 5 min. The solutions were centrifuged at 10 000 g (Desktop centrifuge-biofuge pico; Hereaus) for 2 min to pellet out insoluble fibres and cooled to 4°C. Then the next enzyme in the order was added and the assay process repeated until all the selected enzymes had been added. Appropriate controls were included measure any sugars released from boiling steps in the experiment.

## **6.4 Results**

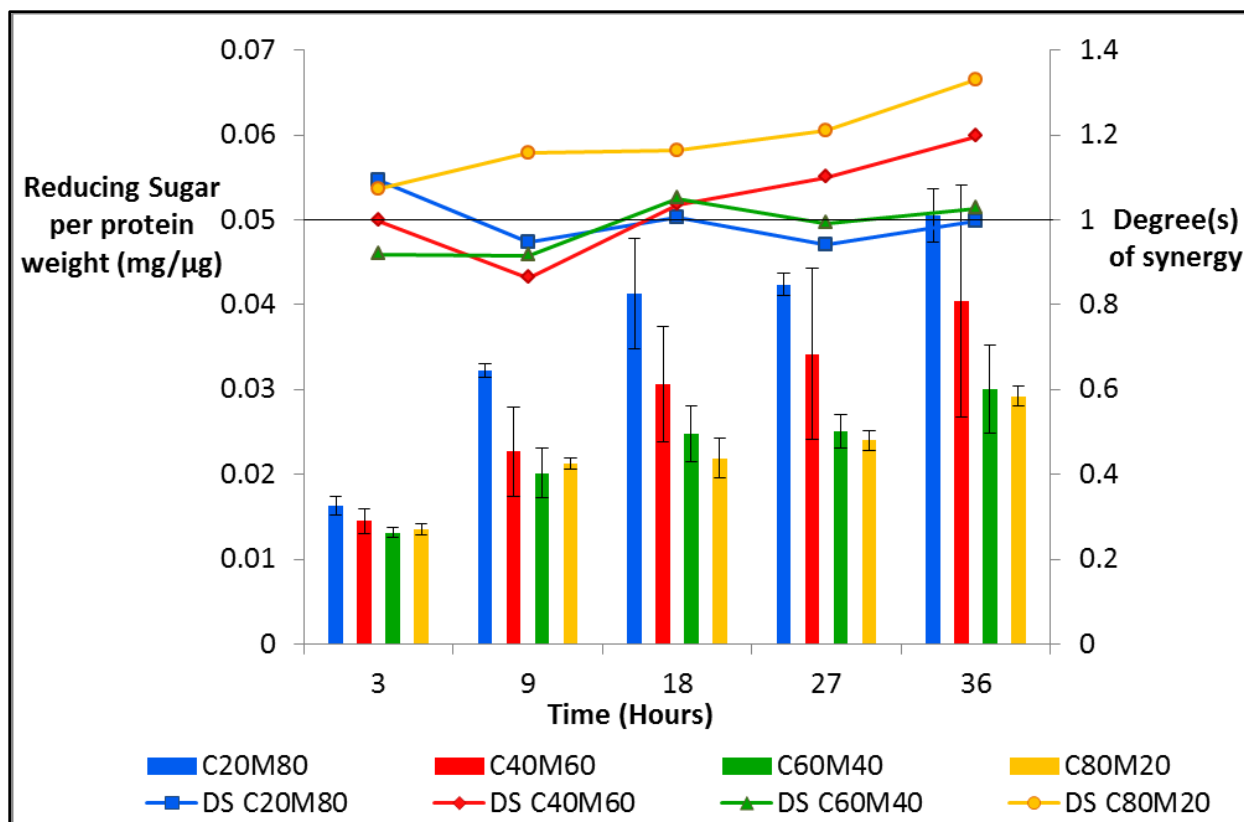
Synergy studies were performed using the hydrated powder of pineapple pomace, as generated in section 5.3.1. With the specific enzyme activities for individual enzyme concentrations established over 36 h, as seen in section 5.4.2, the degrees of synergy (DS) generated by selected enzyme combinations were calculated, using methods detailed in section 6.3.1 and 6.3.2.

### **6.4.1 Simultaneous synergy studies**

The enzyme concentrations used were chosen on the basis that all enzymes had approximately equivalent activity on their respective defined substrates of 30  $\mu\text{g}/\text{mL}/\text{min}$ . The reducing sugar per protein weight ( $\text{mg}/\mu\text{g}$ ) and degree(s) of synergy for simultaneous bi-, tri-, and quad-synergy assays were measured and are represented in sections 6.4.1.1-3.

#### **6.4.1.1 Bi-Synergy**

The levels of synergy between dual enzyme mixtures were established with cellulase as a constant component in the enzyme mixture. Cellulase was chosen due to the high concentrations of cellulose present in pineapple pomace and the high levels of activity it had generated on this substrate with a maximum cellulase protein concentration of 50  $\mu\text{g}/\text{mL}$  (C100), as per Figure 5.3. Mannan is a minor constituent of lignified pineapple cells but could potentially hinder substrate access for cellulase and other lignocellulose degrading enzymes. A maximum protein concentration (M100) at 12.5  $\mu\text{g}/\text{mL}$  of ManA was combined with cellulase in pre-selected ratios. The weight of reducing sugar per protein and degree(s) of synergy generated were measured for set bi-synergy combinations of cellulase with ManA, as represented in Figure 6.1.

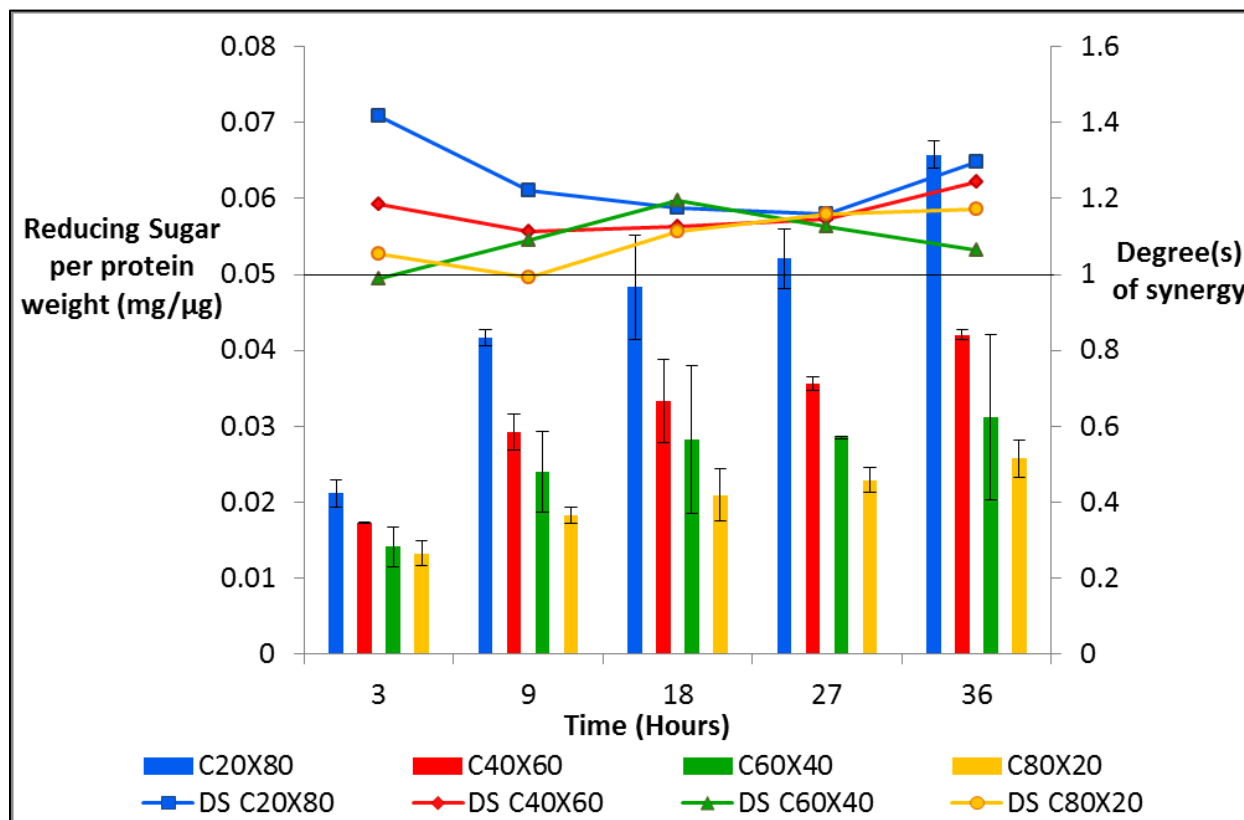


**Figure 6.1: Bi-enzyme synergy assays between *T. reesei* cellulase and *C. cellulovorans* ManA with reducing sugar per protein weight (bars) and degree(s) of synergy (line) at 37°C, pH 5.0, between 3 and 36 hours with 1% (w/v) pineapple pomace. Values represent means  $\pm$  SD, n=3.**

The reducing sugar per protein weight values increased over each period for all combinations of cellulase with the combination of C20M80 having generated the highest quantity of reducing sugar for protein weight used, as per Figure 6.1. This was due to its low total protein concentration of only 20  $\mu\text{g/mL}$  of protein used to generate 1.01 mg/mL of reducing sugar, as compared to the 42.5  $\mu\text{g/mL}$  of total protein used for C80M20 that produced 1.24 mg/mL of reducing sugar. This was despite C80M20 producing synergy above 1 for all time periods and having a maximum of 1.33 DS produced at 36 h. It was observed for all cellulase and ManA combinations that the reducing sugar per protein weight decreased with increasing protein concentrations. This was due to the high protein concentrations of cellulase used to equal the constant level of activity used to determine the enzyme ratios. There was no observed correlation between the enzyme combinations and changes in synergy over time with C80M20 and C40M60 having an overall trend of DS increases to greater than one DS, while both C20M80 and C60M40 both varied over time and had no significant synergy by 36 h.



As xylan forms the second predominant polysaccharide constituent in pineapple pomace and many industrial lignocellulose degrading mixtures have been documented to lack sufficient xylanase activity, synergistic combinations of cellulase and XynA were assayed for activity. These were combined in the same ratios and at the same protein concentrations as the cellulase and ManA assays of Figure 6.1. The quantity of reducing sugar produced per protein weight for combinations of cellulase and XynA are displayed in Figure 6.2.

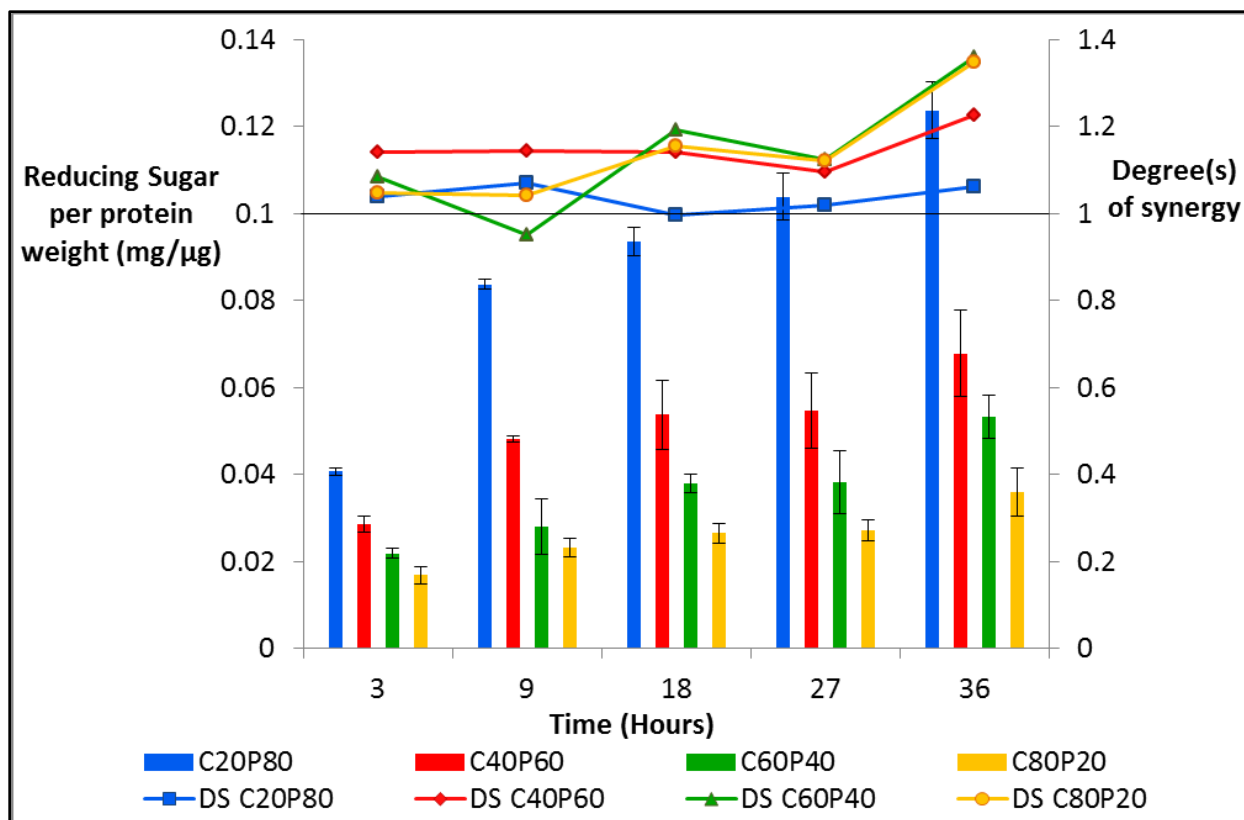


**Figure 6.2: Bi-enzyme synergy assays between *T. reesei* cellulase and *C. cellulovorans* XynA with reducing sugar per protein weight (bars) and degree(s) of synergy (line) at 37°C, pH 5.0, between 3 and 36 hours with 1% (w/v) pineapple pomace. Values represent means ± SD, n=3.**

The highest level of reducing sugar per protein weight was observed with C20X80 for all time periods (see Figure 6.2). Significantly, this combination also displayed the highest DS throughout the assay period. The significance of the high quantity of reducing sugar produced by the minimal quantity of protein is contrasted when C80X20 is compared to C20X80. At 36 h C80X20 had produced 1.1 mg/mL of reducing sugar compared to the 1.32 mg/mL of C20X80, with C20X80 only requiring 20 μg/mL of protein equalling 47% (w/w) of the protein used for

C80X20. It was observed that at 3 h and 36 h, the combinations with less cellulase (C20X80 and C40X60) had higher DS. It was also seen that when the DS decreased for both combinations that their reducing sugar to protein weight had their lowest increases, but when DS increased the reducing sugar to protein weight for the combinations had the largest increases. The higher DS were generated with combinations containing higher levels of xylanase activity. Although only C20X80 initially had synergy significantly above 1 DS, by 36 h all combinations were above 1 DS. However, from 3 to 27 h all synergy values increased or decreased to between 1.15 and 1.2 DS. Thereafter combinations with high levels of xylanase activity (C20X80 and C40X60), had increased synergy by 36 h, while those where the majority of activity was cellulase, had their synergy decrease.

The ability of pectin to non-specifically absorb enzymes in solution and prevent the access of enzymes such as cellulase to their substrates indicates that the degradation of pectin within lignocellulosic waste could offer improved levels of total activity and substrate degradation from designer enzyme cocktails (Zhang *et al.*, 2013). As the pectinase solution had high levels of specific activity on its defined substrate and some xylanase activity, a low protein concentration with a maximum of 0.625 µg/mL was used. The reducing sugar per protein weight produced by selected combinations of cellulase and pectinase was assayed over 36 hours and is represented in Figure 6.3.

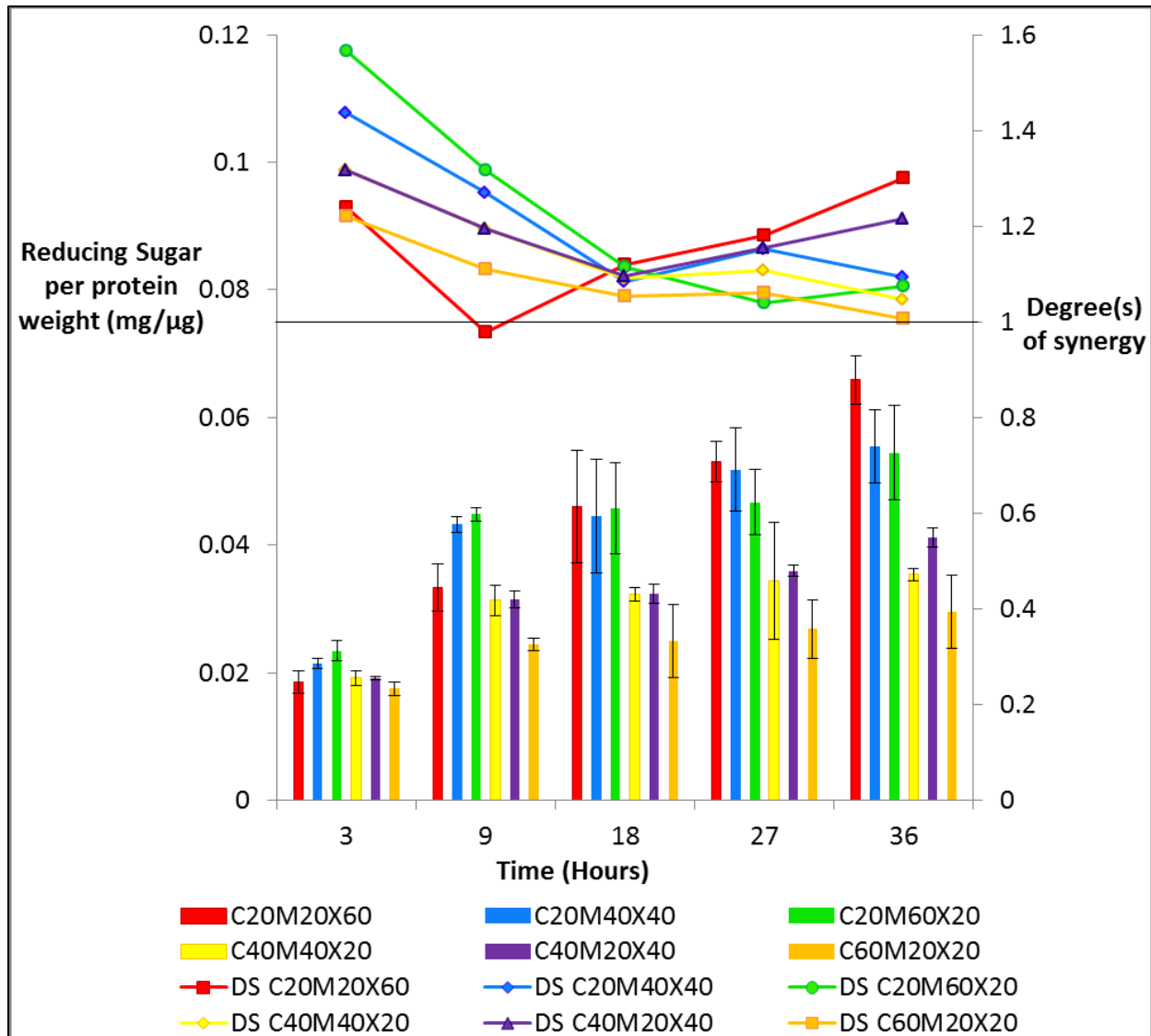


**Figure 6.3: Bi-enzyme synergy assays between *T. reesei* cellulase and *A. niger* pectinase, with reducing sugar per protein weight (bars) and degree(s) of synergy (line) at 37°C, pH 5.0, between 3 and 36 hours with 1% (w/v) pineapple pomace. Values represent means  $\pm$  SD, n=3.**

The combination of C20P80 showed the highest levels of reducing sugar per protein weight for all combinations of cellulase and pectinase, but also possessed the lowest DS for all cellulase and ManA combinations from 18 h onwards, as per Figure 6.3. The combination of C60P40 had produced the most reducing sugar at 1.61 mg/mL and the highest synergy at 1.36 DS, but required 30.25  $\mu$ g/mL of protein, as compared to C20P80 which produced 1.3 mg/mL of reducing sugar with a total protein concentration of 10.5  $\mu$ g/mL. All the combinations had synergy greater than 1 DS but these were all below 1.2 DS from 3 h to 27 h. Only at 36 h was synergy greater than 1.2 DS recorded which also coincided with large increases in reducing sugar per protein weight for all the combinations, which had displayed marginal levels of activity between 9 and 27 h. This would seemingly indicate that synergy had an important role at increasing activity over long time periods. All combinations displayed greater DS as time progressed, with the combinations containing more cellulase activity generating higher DS than those combinations with lower levels of cellulase activity.

### 6.4.1.2 Tri-Synergy

To potentially increase reducing sugar production through additional synergy, tri-enzyme synergy studies were performed. These were performed concurrently and with identical protein concentrations to the bi-synergy assays of section 6.4.1.1. The cellulase solution was a constant component in all the enzyme combinations used. The reducing sugars per protein weight of the tri enzyme synergy combinations from cellulase with both ManA and XynA were calculated over 36 h, as displayed in Figure 6.4.

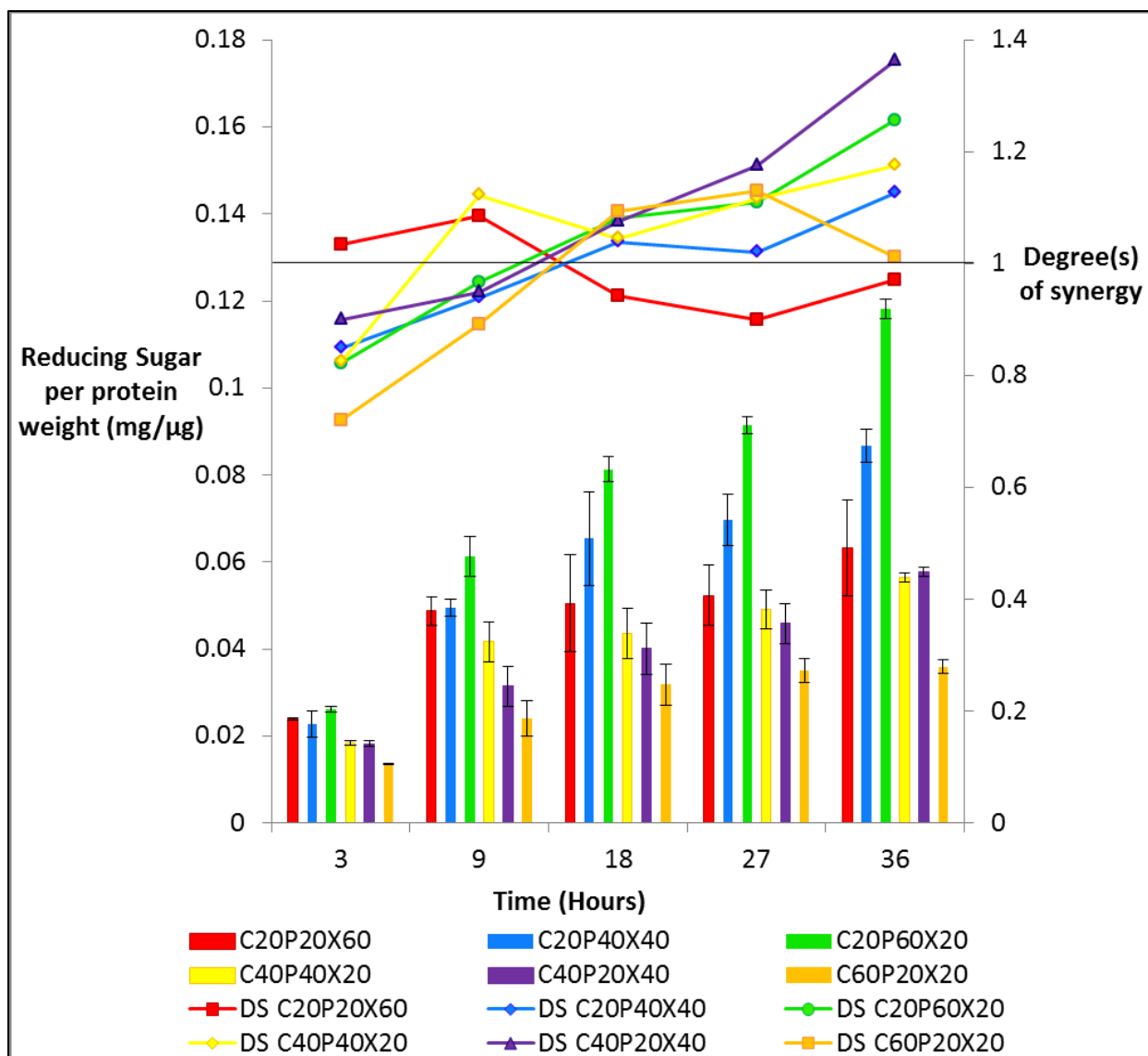


**Figure 6.4: Tri-enzyme synergy assays between *T. reesei* cellulase, and *C. cellulovorans* ManA and XynA with reducing sugar per protein weight (bars) and degree(s) of synergy (line) at 37°C, pH 5.0, between 3 and 36 hours with 1% (w/v) pineapple pomace. Values represent means  $\pm$  SD, n=3.**

All the tri-synergy combinations with C20 had higher reducing sugar to protein values than those with either C40 or C60, with C20M20X60 producing the most reducing sugar at 1.32 mg/mL at 36 h, as per Figure 6.4. This combination had a low total protein concentration of 20 µg/mL, but it also displayed the highest synergy of 1.30 DS at 36 h, despite a lack of synergy at 9 h. This constant increase in synergy with time from a minimum period was opposite to that observed with C60M20X20 which initially had synergy levels equal to C20M20X60, but thereafter decreased to approximately 1 DS at 36 h and only generated 1.03 mg/mL of sugar. This was the second lowest concentration of reducing sugars produced by the CMX combinations, despite it possessing 75% (w/w) more protein than C20M20X60. The combination C20M60X20 initially had the highest reducing sugar to protein weight ratio, and synergy at 1.56 DS, of all the CMX combinations, but the activity of this combination slowed significantly after 9 h, with no significant increase in reducing sugar to protein weight and a drop in synergy to 1.07 DS by 36 h.

It was observed that similarly to the bi-synergy assays with cellulase and xylanase, that the combinations with higher levels of xylanase activity initially showed high DS before decreasing at 18 h and then increasing at 36 h. The combinations with higher levels of xylanase activity (either X60 or X40) all generated higher DS and reducing sugar to protein weight values than other combinations by 36 h. The combinations with high levels of mannanase activity all had their highest DS at 3 h and their lowest DS and reducing sugar to protein weight at 36 h. C60M20X20, despite having a high concentration of Celluclast 1.5L<sup>®</sup>, which contained multiple activities, as per section 4.4, only benefitted from the addition of minor amounts of ManA and XynA in the first 9 hours with synergy above 1.1 DS, but this decreased from 9 h onwards.

As high activity with pectinase was observed with pineapple pomace and there is a documented lack of sufficient xylanase activity in commercial enzyme solutions, tri-synergy combinations of cellulase, pectinase and XynA were assayed at identical protein levels to those used in section 6.4.1.1 (Qing and Wyman, 2011). The reducing sugar per protein weight was calculated for each of the time periods in the 36 h assay, as displayed in Figure 6.5.



**Figure 6.5: Tri-enzyme synergy assays between *T. reesei* cellulase, *A. niger* pectinase and *C. cellulovorans* XynA with reducing sugar per protein weight (bars) and degree(s) of synergy (line) at 37°C, pH 5.0, between 3 and 36 hours with 1% (w/v) pineapple pomace. Values represent means  $\pm$  SD, n=3.**

Although all combinations with C20 initially had reducing sugar per protein weight values below 0.024 mg/μg at 3 h, the activity after this period significantly differed, as per Figure 6.5. The combinations containing higher pectinase generated greater DS and reducing sugar per protein weight values than those with higher xylanase activities by 36 h. It was observed with C20P20X60 that the reducing sugar per protein weight did not significantly increase after 9 h, which coincided with its loss of synergy from 9 h onwards. This was unlike C20P60X20 which had consistent increases in both reducing sugar per weight and DS from 3 h onwards and

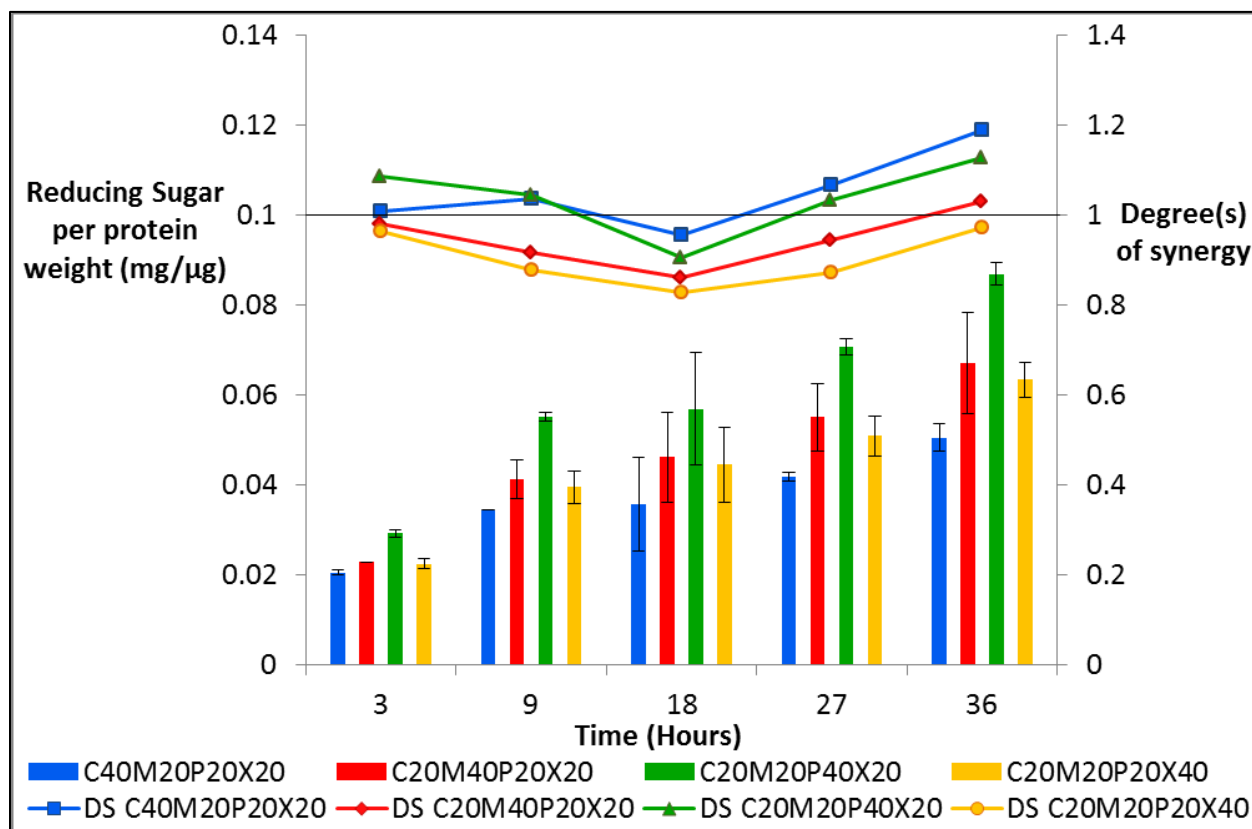
produced the highest concentration of reducing sugar at 1.52 mg/mL for these CPX combinations by 36 h. The difference in synergy is related to this, as C20P20X60 initially had synergy above 1 DS until 9 h, but below 1 DS from 18 h onwards. This differs from C20P60X20 which had synergy below 1 DS at 3 h and thereafter had constant increases through to 36 h where it had the second highest synergy level at 1.26 DS.

The combinations with C40 and either P40 or X40 displayed similar levels of reducing sugar per protein weight values and DS. The importance of increased synergy over time on enzyme activity, was observed with C40P20X40. This combination also initially had synergy below 1 DS but thereafter displayed increases in synergy until 36 h where it had the highest synergy of 1.36 DS and the second highest level of reducing sugar at 1.45 mg/mL.

As many of the bi- and tri-synergy combinations had displayed synergy and increased activity together, four combinations with all the enzymes present were assayed. Each combination had one of the enzyme solutions protein concentrations increased to a higher level, to make it 40% of the solution's total activity. To assess the levels of synergy and reducing sugar per protein weight, combinations of all four enzymes were assayed with quad-synergy conditions, in section 6.4.1.3.

### 6.4.1.3 Quad-Synergy

To investigate the potential for increasing the activity and overall release of reducing sugars from an enzyme mixture, a quad-synergy study with all the enzyme solutions was performed. These were performed concurrently under the same conditions and identical protein concentrations as to those used for the bi- and tri-synergy studies. The reducing sugar per protein weight and DS for different ratios of the quad enzyme combinations after 36 h of incubation are presented in Figure 6.6.



**Figure 6.6: Quad-enzyme synergy assays between *T. reesei* cellulase, *A. niger* pectinase, and *C. cellulovorans* ManA and XynA with reducing sugar per protein weight (bars) and degree(s) of synergy (line) at 37°C, pH 5.0, between 3 and 36 hours with 1% (w/v) pineapple pomace. Values represent means  $\pm$  SD, n=3.**

Despite possessing all the substrate specific activities used in the present study, the reducing sugars per protein weight levels and DS for the quad combinations at 3 h were relatively low when compared to the appropriate values in section 6.4.1.1-2, as seen in Figures 6.3, 6.4 and 6.5. Despite this, the reducing sugar per protein weight did display increases from 3 to 9 h for all combinations despite a general decrease in DS. The reducing sugar per protein weight remained



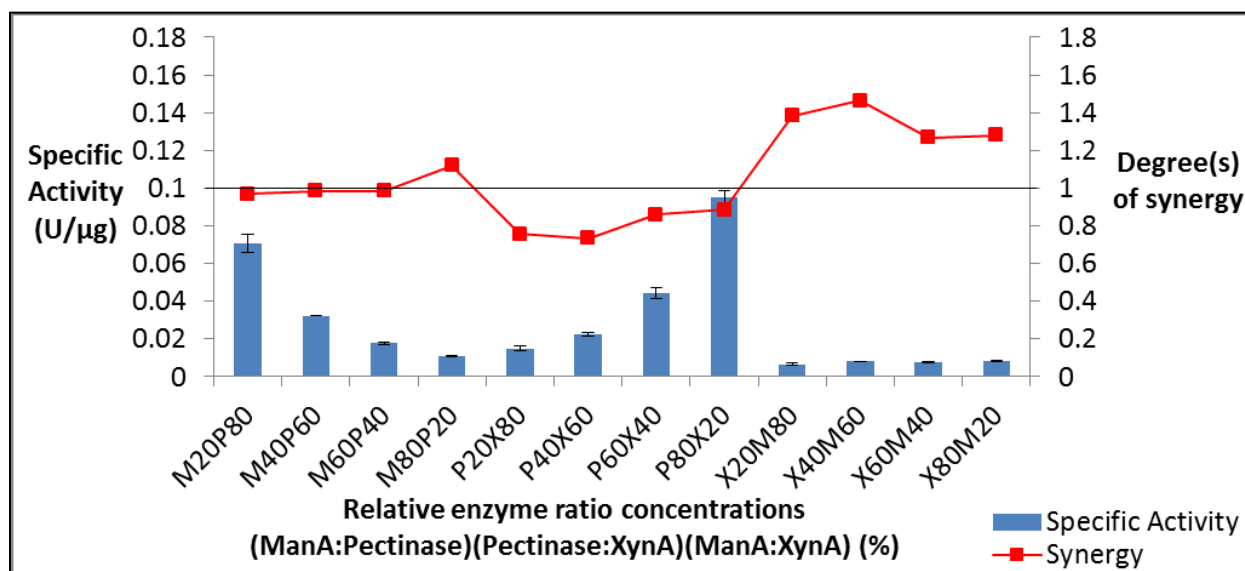
at approximately this level from 9 h to 18 h and only increased from 27 h onwards, which coincided with increases in the DS for all combinations.

The highest reducing sugar per protein weight values were recorded with the higher level of pectinase activity (C20M20P40X20), while the lowest was observed with (C40M20P20X20), which was as a result of significantly different protein levels of 15.25 and 25.125  $\mu\text{g}/\text{mL}$  respectively. Despite the protein concentration difference C40M20P40X20 had the highest DS and reducing sugar at 1.32  $\text{mg}/\text{mL}$  for the quad-synergy assays.

The presence of multiple enzyme activities within the cellulase solution would create redundant enzyme activities that could potentially mask the synergistic effects from the addition of ManA and XynA. To limit the overall activities within a combination, simultaneous synergy studies were performed between ManA, pectinase and XynA on pineapple pomace. These enzyme solutions were selected based on their narrow substrate activity ranges (See Table 4.4).

### 6.4.2 Simultaneous synergy with pectinase, ManA and XynA

To determine the synergy between ManA, pectinase and XynA solutions, activity assays were performed under standard conditions. The protein concentration for pectinase was 0.08  $\mu\text{g}/\text{mL}$ , but with XynA and ManA the protein concentration was 26.375  $\mu\text{g}/\text{mL}$ . These represented a respective decrease and increase from the enzyme concentrations used in section 6.4.1. The substrate concentration was also increased to 4% (w/v) pineapple pomace relative to conditions used in section 6.4.1. The alternate enzyme and substrate concentrations were selected to ensure that XynA and ManA individual activities with pineapple pomace could be measured, and to minimise the xylanase activity within the pectinase solution relative to XynA, when both were present in solution. Concurrent to the individual enzyme assays the bi-synergy assays were performed and the specific activities (mg/mL/min of reducing sugar per  $\mu\text{g}$  of protein) and DS are shown in Figure 6.7.



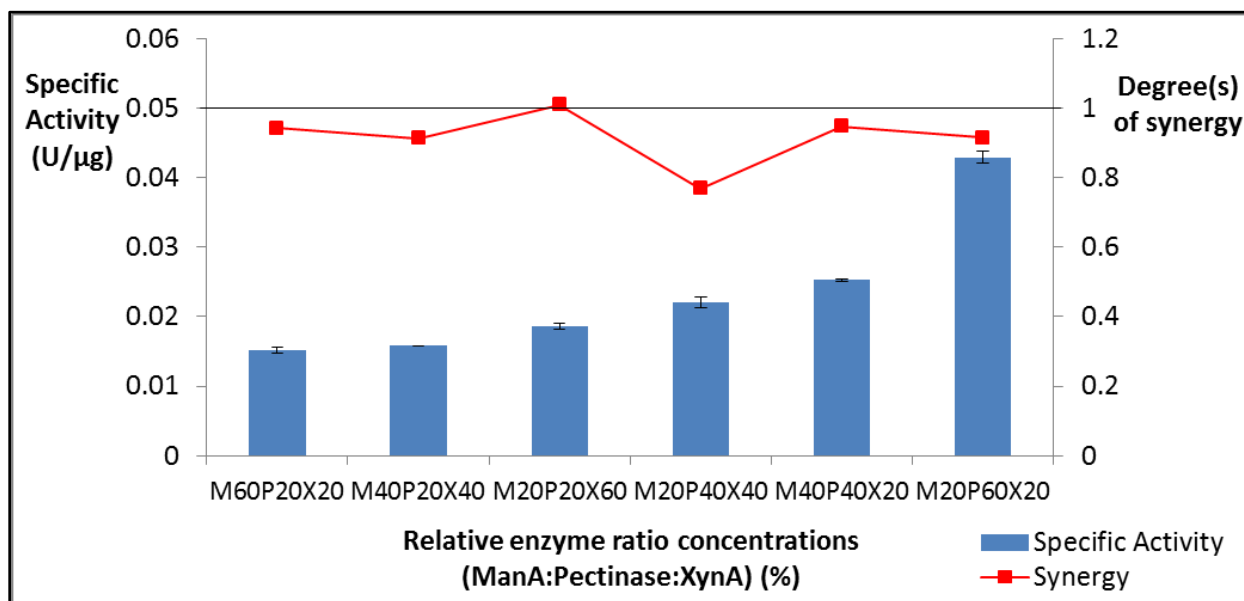
**Figure 6.7: Bi-enzyme synergy assays between *A. niger* pectinase and *C. cellulovorans* ManA and XynA with specific activities (bars) and degree(s) of synergy (line) at 37°C, pH 5.0, after 18 hours with 4% (w/v) pineapple pomace. Values represent means  $\pm$  SD, n=3.**

For the combinations of ManA and pectinase, there was a single point of synergy at 1.12 DS, generated for the combination of 80% ManA and 20% pectinase. This was despite the lowest specific activity for the combinations, at 0.011 U/ $\mu\text{g}$ , as per Figure 6.7. The synergy generated by the increased ManA concentration would have been due to the removal of any mannan preventing substrate access for the pectinase and xylanase enzymes in the pectinase solution. The

highest specific activity was M20P80 with 0.07 U/ $\mu$ g, which would have been due to the high specific activity of the pectinase. The specific activity of each combination decreased with decreases in pectinase concentrations.

For all the combinations of pectinase with xylanase there was no synergy observed, as per Figure 6.7. This could have been due to the xylanase in the pectinase solution, which would have contributed to overall xylanase activity and made any increase in xylanase activity potentially redundant. The specific activity of each combination increased with increases in pectinase concentrations.

The synergy for combinations of ManA and XynA were higher than for the all the other bi-synergy combinations at 1.38, 1.46, 1.27, and 1.28 DS for X20M80; X40M60; X60M40; and X80M20, in Figure 6.7. This occurred despite the relatively low specific activities compared to combinations of ManA or XynA with pectinase. To investigate any possible synergistic relationships between pectinase, ManA and XynA, a tri-synergy assay was conducted under the same conditions as section 6.4.1.2, with the results for the tri-synergy study shown in Figure 6.8.



**Figure 6.8: Tri-synergy assays between *A. niger* pectinase and *C. cellulovorans* ManA and XynA with specific activities (bars) and degree(s) of synergy (line) at 37°C, pH 5.0, after 18 hours with 4% (w/v) pineapple pomace. Values represent means  $\pm$  SD, n=3.**

All enzyme combinations failed to generate synergy above 1 DS, as per Figure 6.8. The specific activities of all the tri-synergy combinations investigated in Figure 6.7 were comparable to bi-

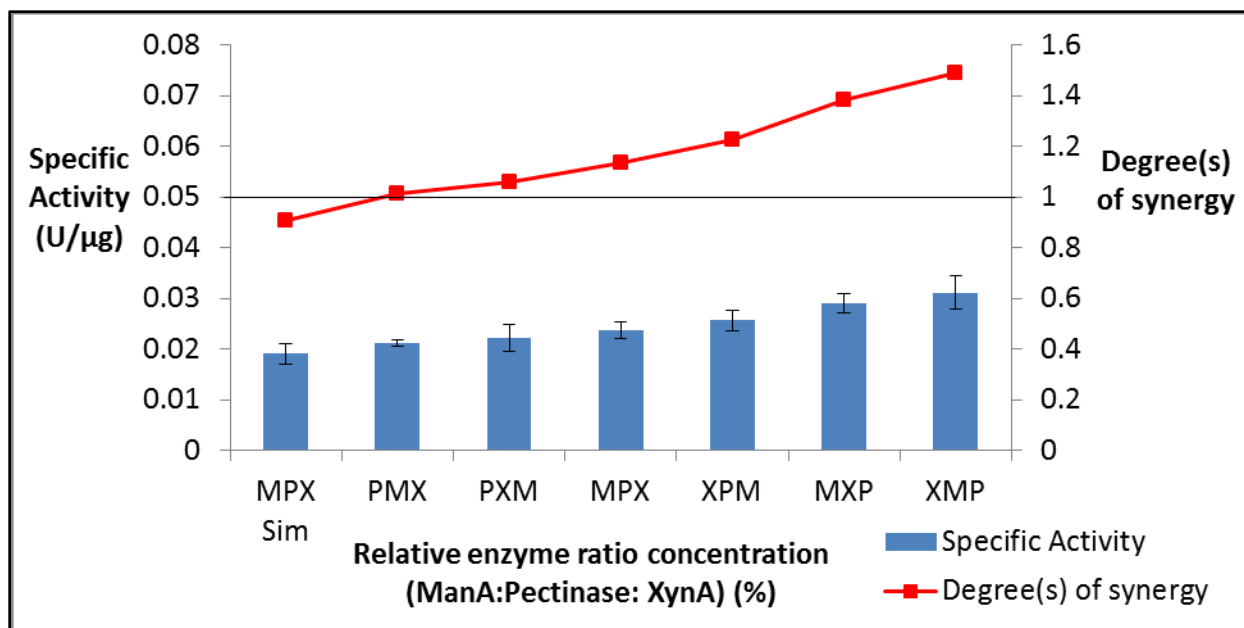
synergy ratios where the concentration of pectinase was the same but only either ManA or XynA were present, as per Figure 6.7. Despite the use of higher enzyme concentrations for ManA and XynA and increased pineapple pomace concentrations, the lack of synergy suggested a need for pre-treatment or the inclusion of a solution containing cellulase activity to complement the ManA, XynA and pectinase activities.

In order to determine the relationship between the different enzymes within the ratios that displayed high levels of synergy, sequential studies were performed. This would allow the determination of which enzyme activities are necessary to remove specific polysaccharides to expose additional scissile bonds for later enzymes to act upon.

### **6.4.3 Sequential synergy**

The multiple activities within the cellulase solution meant that the synergistic effects with only pectinase, ManA and XynA would offer a more detailed analysis of the hemicellulose topology within the pineapple pomace. Sequential synergy assays with a set ratio of 60% pectinase, 20% ManA and 20% XynA were performed, using methods as described in section 6.3.2. This ratio was selected due to the generation of high specific activity in tri enzyme simultaneous enzyme studies, but with a lack of synergy. All sequential synergy assays had appropriate substrate controls to quantify any additional sugar released from heat treatments for enzyme inactivation.

The protein loading was 11.6  $\mu\text{g/mL}$  for ManA, 0.36  $\mu\text{g/mL}$  for pectinase and 7.3  $\mu\text{g/mL}$  for XynA, which represented a 95 fold increase in protein concentration for pectinase. This increase was chosen to compensate for the shorter incubation period of 9 h, which was chosen to minimise the overall period of the assay and still achieve high activity levels. Pectinase was chosen as the major activity constituent due to its high specific activity. The activity for ManA and XynA was standardised at approximately 0.6  $\mu\text{g/mL/min}$ . The placement of an enzyme's letter in its combination abbreviations, indicate when an enzyme's was loaded into a solution. Thus if an enzyme's abbreviation is the first letter it was the first loaded, while if it was the second abbreviation it would have been the second loaded, etc. The DS and specific activities that resulted from these sequential synergy combinations are represented in Figure 6.9.

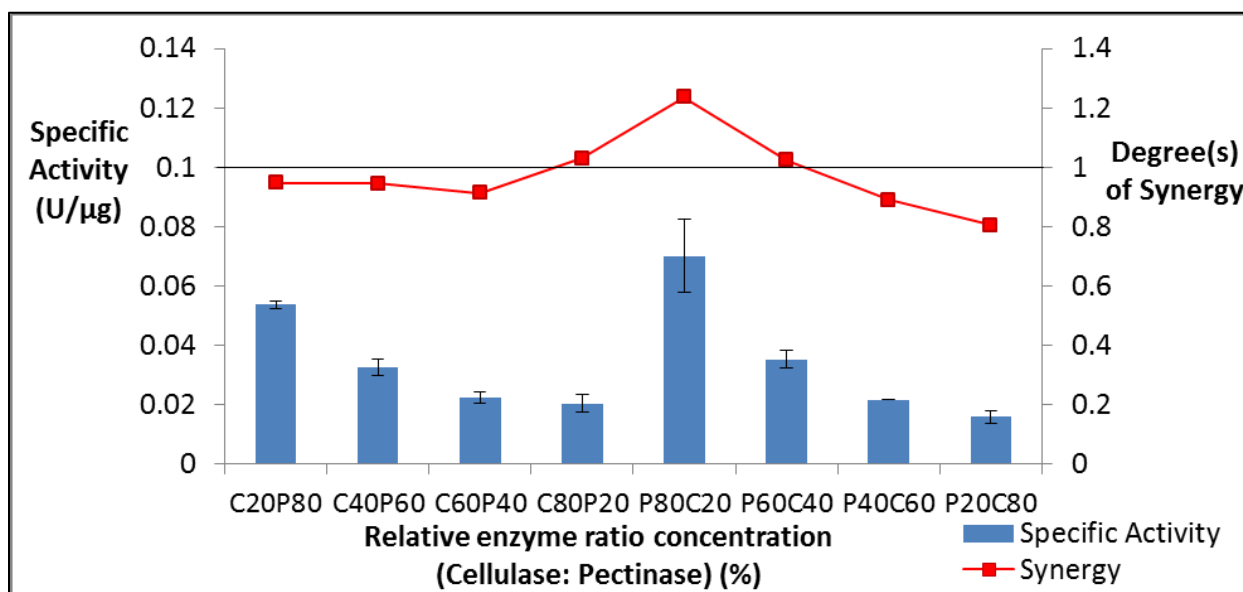


**Figure 6.9: Sequential synergy between *A. niger* pectinase, and *C. cellulovorans* ManA and XynA with specific activities (bars) and degree(s) of synergy (line) at 37°C, pH 5.0, after 9 hours with 1% (w/v) pineapple pomace. Values represent means  $\pm$  SD, n=3.**

The simultaneous synergy control of the enzyme ratio (MPX Sim) displayed no synergy and may have experienced anti-synergy or competition, as per Figure 6.9. Two enzyme loading sequence combinations, PXM and PMX, showed low DS, at 1.01 and 1.06 DS, respectively. This was higher than for the simultaneous control and could indicate that chronologically separating enzyme activities could reduce the anti-synergy effect of having all three enzymes active on the substrate simultaneously.

Two points, MPX and XPM, generated synergy at 1.14 and 1.23 DS and both had pectinase as the second enzyme in the loading sequence. These assays also generated higher specific activities at 0.024 and 0.026 U/μg, relative to samples where pectinase was first in the enzyme loading order. The highest DS and specific activities were measured when pectinase was the last enzyme solution in the enzyme loading order, with 1.49 and 1.39 DS; and 0.031 and 0.029 U/μg of specific activity recorded for XMP and MXP, respectively. The specific activity increase for XMP represented a 63.8% increase in specific activity relative to the simultaneous synergy control. With synergy having been generated through sequential additions of hemicellulases and the pectinase, the effect of sequential synergy with pectinase and cellulase additions were investigated.

The high levels of synergy recorded after 18 h with the bi-synergy ratio of cellulase and pectinase, between 1.0 and 1.2 DS in Figure 6.3, were investigated with sequential synergy studies. These were performed with the same enzyme concentrations, as per Figure 6.3, and measured after 18 h, as seen in Figure 6.10. The placement of the enzyme abbreviations indicates enzyme loading order, using methods as described in section 6.3.2. The four ratios, in which the cellulase was the first enzyme added, have been grouped on the left and those with pectinase as the first enzyme added to the solution were grouped on the right.



**Figure 6.10: Sequential synergy assays with *T. reesei* cellulase, *A. niger* pectinase, with specific activities (bars) and degree(s) of synergy (line) at 37°C, pH 5.0, after 18 hours with 1% (w/v) pineapple pomace. Values represent means  $\pm$  SD, n=3.**

For samples in which cellulase was added initially, only C80P20 displayed marginal synergy of 1.03 DS, as per Figure 6.10. The lack of synergy for these combinations differed from the simultaneous synergy results for Figure 6.3. When the simultaneous synergy study values were calculated for the whole time period of 18 h, they had synergies of 1.07, 1.14, 1.20, and 1.08 DS with specific activities of 0.087, 0.050, 0.035, and 0.025 U/μg, for C20P80, C40P60, C60P40, and C80P20, respectively. Thus the activity and the synergy values generated under sequential synergy conditions, when cellulase was the initial enzyme, demonstrated decreased synergy and specific activity for all ratios when compared to simultaneous conditions.

The largest synergy measured under sequential conditions, at 1.24 DS, was observed with initial pectinase additions for the ratio of P80C20, as seen in Figure 6.10. The specific activity, at 0.07

U/ $\mu$ g, was lower than for the equivalent simultaneous synergy ratio value of 0.087 U/ $\mu$ g. Even though the activity was lower for the sequential synergy method, an additional 0.15 mg/mL of reducing sugar was produced relative to the single enzyme controls. The total reducing sugar from the sequential synergy method was 0.794 mg/mL, which was lower than the 0.983 mg/mL produced by the simultaneous synergy method. The specific activity values obtained for the remaining combinations where pectinase was the first enzyme added were lower than the specific activity values for the simultaneous synergy measurements and failed to produce significant amounts of synergy.

## 6.5 Discussion

The simultaneous synergy studies performed in section 6.4.1 allowed for the observation of changes in the DS and reducing sugar per protein weight generated by various combinations of commercial and purified enzyme solutions at time periods between 3 and 36 hours. This had the advantage of mimicking potential industrial applications where individually tailored combinations of enzymes are used to degrade lignocellulose (Banerjee *et al.*, 2010). To lower enzyme costs biorefineries use commercial enzyme mixtures, that contain core sets of enzymes, and combine these with select pure enzymes, to add necessary enzyme activities, depending on the biomass to be degraded (Banerjee *et al.*, 2010; Merino and Cherry, 2007; Sills and Gossett, 2011).

### 6.5.1 Summary of optimal enzyme combinations from synergy studies

A summary of the optimal enzyme combinations from section 6.4.1, for either maximum reducing sugar production or reducing sugar per protein weight is represented in Table 6.1. The middle column (maximum reducing sugar) represents the enzyme combinations that produced the maximum reducing sugar concentrations for each time period. The right hand column (reducing sugar per protein weight) represents the enzyme combinations that produced the maximum reducing sugar per protein weight as calculated for each time period.

**Table 6.1: A table of optimal enzyme combinations for either maximum reducing sugar production or maximum reducing sugar per protein weight for the time periods 3, 9, 18, 27 and 36 hours. Values represent means  $\pm$  SD, n=3.**

<b><u>Time</u></b>	<b><u>Maximum reducing sugar</u></b> <b>Enzyme combination</b> <b>[Protein loading in <math>\mu\text{g/mL}</math>]:</b> <b>Reducing sugar concentration</b> <b>(mg/mL)</b>	<b><u>Reducing sugar per protein weight</u></b> <b>Enzyme combination</b> <b>[Protein loading in <math>\mu\text{g/mL}</math>]:</b> <b>Reducing sugar concentration</b> <b>(mg/mL)</b>
<b>3</b>	C80P20 [40.125]: 0.676	C20P80 [10.5]: 0.428
<b>9</b>	C40P60 [20.375]: 0.981	C20P80 [10.5]: 0.879
<b>18</b>	C60P40 [30.25]: 1.149	C20P80 [10.5]: 0.983
<b>27</b>	C20P60X20 [12.875]: 1.178	C20P80 [10.5]: 1.091
<b>36</b>	C60P40 [30.25]: 1.613	C20P80 [10.5]: 1.299



With pectinase and cellulase combinations representing nine of the ten optimal ratios for either maximizing yield or reducing sugar production, it is apparent that they are essential in enzyme cocktails for use with pineapple pomace, as per Table 6.1. This demonstrates the importance of combining commercial solutions of pectinase and cellulase in set ratios, to allow for both the increase of specific activity and minimisation of protein loading. Two factors that made these solutions partners in the ideal combinations were that pectinase had a high specific activity, thereby minimising its protein loading, while Celluclast 1.5L<sup>®</sup> possessed a core mix of cellulases (Juhász *et al.*, 2005). It was also observed that the inclusion of XynA can, in combination, also assist in raising the activity of a solution while minimizing protein loadings for hydrolysis periods of 27 h or longer.

For any industrial process that utilises a 36 h hydrolysis step, either C60P40 or C20P60X20 would be highly beneficial as these represent only protein loadings of 30.25 µg/mL and 12.875 µg/mL and generated over 1.5 mg/mL of reducing sugars. This does, however, fall below the threshold for a successful fermentation with *Saccharomyces cerevisiae* of 40 g/L (Pacheco *et al.*, 2010). Thus, a higher concentration of pineapple pomace should be used, in conjunction with longer hydrolysis times and a sugar concentration step, in order to raise sugar levels for a successful fermentation. Alternatively, additional non-essential enzymes could be added to the optimal combinations to further increase specific activity of a solution. ManA and XynA could be referred to as non-essential enzymes, as at the protein concentrations used in section 6.4.1; they did not individually generate measurable activity with pineapple pomace.

### **6.5.2 Benefits of adding ManA and XynA to a commercial enzyme solution**

The benefits of including purified non-essential enzymes into a commercial enzyme solution include the raising of the enzyme activity level through synergy and additional activity, while simultaneously maintaining low protein concentrations. This was demonstrated by the combination C80M20 at 9 hours, as per Figure 6.1. The inclusion of 2.5 µg/mL of ManA with 40 µg/mL of cellulase generated an additional 0.12 mg/mL of reducing sugar. This warrants the addition of ManA with Celluclast 1.5L<sup>®</sup> when preparing a minimal enzyme cocktail for use with mannan containing substrates, such as pineapple pomace (Meyer *et al.*, 2009). This would only apply in situations where the total incubation time was less than 12 hours as ManA has been documented to have lost over 60% of its initial activity by 9 hours when in solution at 45° C

(Dredge *et al.*, 2011). This could be acceptable under circumstance in which there are short bioreactor incubation times, minimal inputs of inexpensive ManA and higher sugar yields are desired (Aden *et al.*, 2002). Calculations from NREL have estimated the cost of enzymes towards the overall retail price of bio-ethanol as low as 10%, when capital and equipment costs are included, which they advise would allow for the use of minor quantities of expensive accessory enzymes (Aden *et al.*, 2002).

Despite the limited stability of ManA, the inclusions of high levels of mannanase activity in solution with cellulase (C20M80) lead to increased DS and activity from 27 to 36 h, as per Figure 6.1. This was despite the probability that ManA would have had significantly reduced activity at this point, despite the use of lower assay temperature of 37°C, but the activities of ManA in the initial 9 hours of incubation would have degraded the mannan in the pineapple pomace to expose the additional substrate for the cellulase enzymes. The ability of ManA to significantly contribute to yield and prolonged enzyme cocktail activity (of up to 168 hours assays) with the cell walls of the *Poales* order of plants has been documented (Beukes *et al.*, 2011; Olver *et al.*, 2011).

Support for including XynA, in addition to ManA, was observed with combinations of cellulase, ManA and XynA after 3 h incubation. These all generated high levels of synergy above 1.22 DS, and the combination of C20M60X20 had the highest DS at 1.57 degrees and a specific activity of 0.13 U/μg as per Figure 6.4. This combination only required 20 μg/mL of protein to produce 0.469 mg/mL of reducing sugar, and supports the results of Qing and Wyman (2011), who reported that commercial enzyme mixtures require additional xylanase supplementation to increase specific activity.

With Figures 6.2, 6.4 and 6.5, the combinations containing greater than 20% XynA, the synergy and activity values were higher at 36 h than those containing less xylanase activity. This indicates that XynA functions to synergistically increase the activity in the longer term with these combinations, as XynA has been documented to have substantial stability in solution with over 50% activity retained after 48 hours in solution (Dredge *et al.*, 2011; Morrison *et al.*, 2011). XynA would thus be functioning to remove newly exposed xylan uncovered by prolonged cellulase activity and which would be inhibiting or reducing further cellulase activity through steric hindrance or shielding.

It was observed at all time periods measured, that a combination which generated the highest reducing sugar per protein weight did not always have the highest DS for a set of enzyme combinations, as per section 6.4.1. However, throughout all time periods and combinations measured, the highest reducing sugar concentrations were produced by combinations that had synergy above 1 DS. This demonstrates that synergistic relationships are a primary factor for increasing the reducing sugar yield and reducing sugar per protein weight in a solution. This supports the inclusion of enzymes, such as ManA and XynA that have demonstrated synergistic relationships into enzyme cocktails (Beukes *et al.*, 2011; Dredge *et al.*, 2011). This was observed with combinations of cellulase, ManA and XynA, as they significantly reduced protein loading levels and consistently generated synergy above 1 degree, as per Figure 6.4. The quantities of ManA and/or XynA used will be dependent on the time period of an enzyme hydrolysis step as it was observed that specific activities of all combinations varied over time.

### **6.5.3 Changes in solution activity over time**

For all the synergy studies it was calculated that over 50% of the reducing sugar concentration at 36 h, had been produced by 9 h. Consequently the majority of activity for all combinations tested had occurred within solution by 9 h. The anti-synergy recorded with the CPX combination in this time period was not expected based on the bi- and tri-synergy assays of cellulase with pectinase and/or XynA, which displayed high synergy between 1 and 1.4 DS, as per Figures 6.2, 6.3 and 6.5. This would imply that the activities or presence of active pectinase and XynA in solution together had to some degree inhibited the activity of each other. Despite this, solutions containing high levels of cellulase and pectinase activity (C40P40X20; C20P60X20) or high levels of xylanase (C40P20X40; C20P40X40), with the exception of C20P20X60, had overcome the anti-synergy and produced synergy above 1.15 DS by 36 h, seen in Figure 6.5. This synergy was significant as these combinations all had nearly half of their activity after 9 hours and had produced high levels of reducing sugar, above 1.3 mg/mL, by 36 hours. This affirms that, for high levels of reducing sugars and prolonged activity from an enzyme solution, synergy is a vital component and that appropriate levels of accessory enzymes are required to fully degrade a complex lignocellulose substrate, in this case pineapple pomace, with limited pre-treatment.

With all the bi-; and tri-enzyme combinations tested in section 6.4.1, a trend was observed of a period of minimal activity at 27 h, as seen in Figures 6.1-5. From this period onwards, the

activity of the solutions returned and the reducing sugar per protein weight values then all increased by 36 h, as per Figure 6.1-5. This minimum period for bi-enzyme combinations and tri-enzyme combinations of cellulase with ManA and XynA, also coincided with the termination of a direct linear relationship between DS and specific activity that had been generated by these combinations before 18 h. This may have been due to the depletion of accessible substrate and the loss of enzyme activity. Dredge *et al.* (2011) reported that ManA and XynA could lose up to 60% and 35% of their respective activities after 12 hours at 45°C, while the stability of all enzymes within Celluclast 1.5L<sup>®</sup> and Pectinex<sup>®</sup> 3XL are unknown (Juhász *et al.*, 2005; Puupponen-Pimiä *et al.*, 2008).

For the quad-enzyme combinations, the period of negligible activity was observed at 18 h with increases measured thereafter, as displayed in Figures 6.6. The increases in both DS and reducing sugar per protein weight after each period of minimum activity section in 6.4.1 may have been due to reduced competition between enzymes for binding sites as competing enzymes became denatured. Also, the activities of any  $\beta$ -glucosidases and  $\beta$ -xylosidases present in the commercial solutions would have increased the quantity of reducing sites present by continued processing of any released oligosaccharides (Juhász *et al.*, 2005; Puupponen-Pimiä *et al.*, 2008). While reduction to monomers is important for any eventual end product use, such as bio-fuel production, this could mask the actual level of total substrate degradation. Enzyme solutions with high levels of hydrolysis of a minor fraction of the total oligosaccharides to monosaccharides, may not generate high yields but would still possess high levels of activities in short period hydrolysis. Thus if a greater variety were degraded over a longer time period and subsequently utilised it would achieve a greater overall degradation of the substrate, than if a minor proportion of the total polysaccharide were reduced completely to monomers. End product analysis to identify sugar monomers from each time period would be necessary to confirm the increase in reducing sugars due to activities of  $\beta$ -glucosidases and  $\beta$ -xylosidases. It would also assist in quantifying an enzyme's specific activity at a time period and the identification of synergistic relationships in a solution.

A full techno-economic analysis within the parameters of a modern cellulosic ethanol production plant is required to answer the issues relating to synergy that have been generated by this study. An excellent analysis of related biochemical scenarios is elaborated upon by Kazi *et al.*, (2010).

#### 6.5.4 The relationship between synergy and time

There are two conflicting opinions in literature on the relationship between synergy and time (Andersen *et al.*, 2008; Boisset *et al.*, 2001; Jung *et al.*, 2008). These are classified as to whether synergy is highest at the initiation of hydrolysis or whether synergy will gradually increase over time due to hydrolysis of a substrate (Andersen *et al.*, 2008; Jung *et al.*, 2008). The theory of Andersen *et al.* (2008) proposes a direct negative relationship between synergy and time. Therefore the highest DS and specific activity both occur in a short period after the commencement of enzyme hydrolysis.

In bi-synergy studies, with cellulase and XynA, this direct negative relationship was observed with C20X80 which generated its maximum synergy (1.42 DS) at 3 h, and lower DS thereafter, as per Figure 6.2. A similar relationship was observed in the tri-synergy studies with combinations of cellulase, ManA and XynA, as per Figures 6.4. These combinations generated their highest DS (between 1.22 and 1.57 degrees) at 3 h and consequently produced lower DS as time progressed, as per Figures 6.3-4. These results agree with Andersen *et al.* (2008), who argued that the highest concentration of accessible substrate available for enzyme activity occurs at the start of hydrolysis and diminishes thereafter (Andersen *et al.*, 2008; Boisset *et al.*, 2001). This would subsequently result in less accessible substrate as hydrolysis proceeds, with lower DS and activity levels as time elapsed.

In contrast to the theory and results of Andersen *et al.* (2008), the highest DS for the bi-synergy studies of cellulase with ManA or pectinase was observed at 36 h, as per Figure 6.1 and 6.3. This theory concurs with the results of Jung *et al.* (2008), who postulated that DS increases over time as the substrate is hydrolysed, with synergy and time sharing a direct positive relationship (Jung *et al.*, 2008). These results could be explained by initial enzyme activities exposing novel accessible substrates for the enzymes to act upon. With the cellulase and pectinase combinations, the initial low DS could be due to the presence of xylanase activity in both solutions, as recorded in Table 4.5. Thus an excess of xylanase activity to the accessible xylan at 3 h could have led to competition for enzyme binding sites. However, as hydrolysis progressed more xylan would become accessible, thus increasing both the DS and specific activity of the solution.

Complicating the use of these results to support either theory are the levels of enzyme stability and enzyme competition in a solution. Both of these can be quantified and their impact reduced through novel enzyme discovery and genetic engineering of enzymes. The relationship of synergy over time in a solution is an important parameter for designing a bio-fuel refinery. It will influence the choice of enzymes used, immobilisation and/or recovery of enzymes from solution, total protein loadings and the length of incubation times required for enzymatic hydrolysis (Newman *et al.*, 2012). The presence of multiple protein fractions within Celluclast 1.5L<sup>®</sup> complicates synergy studies and immobilisation (Juhász *et al.*, 2005). Therefore the studies of synergy between enzyme solutions which have fewer protein fractions can identify synergistic relationships and ideal combinations that later could be used in cocktails containing Celluclast 1.5L<sup>®</sup>.

#### **6.5.5 Simultaneous synergy studies with pectinase, ManA and XynA**

It was thought that bi-synergy studies with ManA and pectinase would generate synergy as Pectinex<sup>®</sup> 3XL contained no mannanase activity, however, only one combination (M80P20) generated synergy at 1.12 DS, as per Figure 6.7. This may be due to the higher substrate concentration at 4% (w/v) and insufficient hydrolysis capacity from the lowered pectinase concentration. It may, however, be due to the topographic isolation of pectin from mannan, whereby either substrate is degraded independently of the other and therefore the degradation of the one would not increase the quantity of accessible substrate for the other enzyme. There was also a lack of synergy between pectinase and XynA combinations, which may have been due to Pectinex<sup>®</sup> 3 XL also containing xylanase activity and the lack of association between pectin and xylan within the plant cell wall.

There were high degrees of synergy observed with ManA and XynA, with the maximum DS and specific activity measured with combinations where ManA was in excess of XynA, as per Figure 6.7. The synergistic relationship between ManA and XynA provides strong evidence that mannan and xylan are closely topologically associated within pineapple pomace (Smith and Harris, 1995). This would allow mutual shielding of each substrate from degradation and would help protect any cellulose fibrils encased within this hemicellulose layer from potential cellulase degradation (Várnai *et al.*, 2011). Therefore, removal of mannan and xylan would increase the specific activity of a cellulase mixture through synergy (Várnai *et al.*, 2011).

This lack of synergy between pectinase and ManA or XynA was also observed with the tri-synergy combinations of ManA, pectinase and XynA, in Figure 6.8. This was unexpected as ManA and XynA have demonstrated synergy in bi-synergy assays, as per Figure 6.7. This indicated that, either through competition, inhibition, or protein aggregation, the presence of an active pectinase had resulted in anti-synergy, as per Figure 6.8. The use of sequential synergy studies would help elucidate the causes of this anti-synergy relationship.

### **6.5.6 Sequential synergy**

The change to sequential conditions with the tri-synergy combination of ManA, pectinase and XynA, generated synergy above 1 degree and removed the anti-synergy observed under simultaneous conditions, as per Figure 6.9. While the incubation times and protein concentrations differed to those in sections 6.4.2, it was observed that 0.91 DS were generated by both combinations of M20P60X20 in either sequential or simultaneous conditions.

The confirmation that the anti-synergy was partially due to non-denatured XynA and pectinase competing in solution could be seen by the lack of anti-synergy in the loading orders PXM and PMX, which generated 1.06 and 1.02 DS respectively, as per Figure 6.9. This indicated that the products formed by, or the changes effected on the pomace by the pectinase solution, probably inhibited later XynA activity. Additionally, competitive binding under simultaneous conditions by Pectinex<sup>®</sup> 3 XL xylanases may have further inhibited XynA activity (Boussaid and Saddler, 1999). The rapid adsorption of enzymes onto lignocellulose substrates and its effect of reducing hydrolysis levels have been reported with enzymes hydrolysing lignocellulose (Boussaid and Saddler, 1999; Coughlan, 1985). This might explain the later appearance of synergy when both pectinase and XynA were present in simultaneous synergy conditions in section 6.4.1.

It was observed that the DS and specific activity increased in the tri-enzyme combinations when XynA or ManA were added first and pectinase was second in the enzyme loading order as per Figure 6.9. These increases may have resulted from XynA having none of the inhibiting effects of pectinase hydrolysis and sufficient time for hydrolysis, while for ManA the removal of mannan may have increased the quantity of accessible substrate for later enzymes. It was also observed that, for all points, when XynA was allowed to hydrolyse before ManA, the DS and

specific activities were greater than when the order was reversed. This was demonstrated with XMP, MXP, PXM and PMX, as per Figure 6.9.

The highest specific activities and DS were seen with the XMP loading order. This suggests that the activity of XynA modifies the structure of pineapple pomace or releases products that support the synergistic relationship between itself and ManA and pectinase. It also proves that the anti-synergy relationship between XynA and pectinase is not entirely due to the presence of active XynA and pectinase in solution together. For industrial applications where total activity is a priority, XynA and/or ManA should be amongst any enzymes added initially and additionally be given time to hydrolyse before other enzymes are introduced in an enzymatic hydrolysis stage. The practicality and cost effectiveness of an additional processing step such as this would have to be further investigated to establish its feasibility.

For the sequential synergy studies with cellulase and pectinase, no synergy was recorded where cellulase was added first, which differed from the simultaneous synergy studies where the same combinations generated synergy above 1 DS, except for C80P20, as seen in Figure 6.3 and 6.10. This could be due to a combination of the cellulase requiring the activities in Pectinex<sup>®</sup> 3 XL to increase substrate accessibility and potentially the cellulase activities rendering the pectin and xylan unusable for pectinase. With the combinations where pectinase was the initial enzyme loaded, only one combination (P80C20) displayed synergy, unlike under simultaneous conditions in which no synergy was observed at 18 h, as per Figures 6.3 and 6.10. The opposite effect was observed for P60C40, P40C60 and P20C80, which displayed synergy under simultaneous, but not sequential conditions. This could be due to the redundant xylanase activities and/or the generation of inhibitory products by both solutions, but end product analysis would be required to confirm this. Ultimately, the presence of multiple enzyme activities, lack of purity and lack of enzyme kinetics for the commercial solutions used, increase the difficulty of interpreting both overall enzyme contributions towards hydrolysis and any synergistic relationships (Banerjee *et al.*, 2010).



## **6.6 Conclusions**

Synergy studies were successfully performed with bi-, tri-, and quad-synergy combinations of Celluclast 1.5L<sup>®</sup>, ManA, Pectinex<sup>®</sup> 3 XL and XynA. It was found that it was possible to generate and measure the synergy of various combinations of these commercial and heterologously expressed enzymes. It was found that ManA and XynA enzyme loadings, at concentrations where their individual activities were not detectable on the pineapple pomace, were beneficial for enzyme cocktails in terms of synergy, lowered enzyme loadings and increased reducing sugar production. The inclusion of pure ManA or XynA did not consistently produce higher reducing sugar concentrations, but it did lower the overall protein loadings significantly. It can be concluded that quad enzyme combinations, with the enzyme solutions used, are not effective for minimizing protein loadings while maintaining high reducing sugar levels, or maximizing reducing sugar production regardless of protein loadings.

The ability of selected enzyme combinations to produce over 1.5 mg/mL of reducing sugar after 36 h with only 12.875 µg/mL of protein demonstrates the effectiveness of selecting enzyme ratios based on their own model substrate activities. This differs from other methods which keep protein loadings or enzyme activity on the chosen lignocellulose substrate constant. With both methods, the goal of increasing the production of reducing sugar may be achieved. However, only when the total protein loadings are adjusted would the goal of reducing total protein loading be achieved. Thus, accurately reporting the activities, protein loadings and protocols used for enzyme combinations may be crucial for optimisation of both enzyme ratios and protein loadings (Van Dyk and Pletschke, 2012). This is especially important when higher protein concentrations have to be used to generate detectable activity, as was the case with ManA and XynA. In lieu of this, assay times may have to be increased to detect activity and this will incur significant operating costs from a heating and suspending perspective. Both of these considerations may be cost prohibitive (Aden *et al.*, 2002).

Although an optimal set of enzymes was used, the reducing sugar concentration after 36 h was only 1.61 mg/mL (produced by C60P40, as per Table 6.1). This would only equate to 16% of the 10 g/L dry weight pineapple pomace substrate having been hydrolysed to oligosaccharides of various lengths. This would not include the percentage of lignin present in the lignified cell walls that form the skin fraction of the pineapple peel waste. The inclusion of the 15% (w/v) lignin

portion of the dry weight would increase the reducing sugar yield to 18.94% (w/v) on a dry weight basis (Cordenunsi *et al.*, 2010).

Thus it can be recommended that further pre-treatment processes should be used with the pineapple pomace to increase sugar yields. A more active cellulase with a higher purity level and fewer enzyme components should be used to reduce redundant enzyme activities and lower total protein loadings. Higher protein loadings of pectinase should be used as the high specific activity and synergy potential is industrially relevant. However, care should be taken with using a combination of pectinase and XynA as anti-synergy has been observed. Additional ideas and conclusions on improving enzyme activities and DS with commercial and purified enzymes combinations are the subject of the next chapter, Chapter 7.

# Chapter 7: General discussion, conclusions and future recommendations

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## 7.1 General discussion

The reduction of protein loading levels and the increase of enzyme activity levels in customised enzyme solutions used on lignocellulosic wastes are of great importance and significance for the commercialisation and implementation of second generation biofuel production (E.U., 2009; Merino and Cherry, 2007). These combinations could consist of commercial enzyme mixtures supplemented with additional enzymes to increase the total activity, synergy and required specific activities of a solution for a particular lignocellulosic substrate. This should be performed within the framework of a minimal enzyme cocktail concept, that maximizes synergy, in order to reduce enzyme costs which are a major economic barrier to entry for the widespread commercialisation of second generation biofuels (Merino and Cherry, 2007; Meyer *et al.*, 2009).

The beneficiation and/or reduction of wastes from the cultivation and processing of crops will offer economic benefits for industry, farmers and processors, as well as to the public in general. In an industrial setting, enzyme combinations producing the highest quantity of reducing sugars may initially be selected in demonstration pilot plants for their ability to prove the feasibility of sustainable sugar and consequently biofuel production (Aden *et al.*, 2002). However, in the pursuit of economic viability enzyme combinations with greater yield per mg will be selected for, to reduce costs (Aden *et al.*, 2002).

The commelinids clade, which includes all *Poales* and *A. comosus*, differs from all other plant clades in that they contain glucuronoarabinoxylans as the primary constituent of lignified secondary cell walls and non-lignified primary cell walls (Harris and Smith, 2006). They also contain glucomannans in minor amounts in the secondary cell wall and pectin in the non-lignified primary cell wall (Harris and Smith, 2006). This represents a unique cell wall structure and any improvements of the enzyme solutions that saccharify pineapple pomace may aid research into the saccharification of other commelinids crops. There are 32 economically important crops which are part of the same *Poales* order as pineapple, and they include sugar cane, bamboo, wheat, maize, oats, rice and sorghum amongst others (FAO, 2010). Annually the

*Poales* order produces the most agricultural waste by weight of all the globally cultivated crops (FAO, 2010; Van Dyk *et al.*, 2013).

The expression of ManA and XynA in this study was successful and comparable to previous studies expressing these constructs using the same host (Beukes *et al.*, 2011; Olver *et al.*, 2011). However, the quantities of recombinant protein produced in this study are approaching the expression limits of the *E. coli* cells, before translation inhibition and aggregation can become problematic (Beukes *et al.*, 2011; Jensen and Hammer, 1998; Lin Cereghino *et al.*, 2002). Additionally, for any industrial concerns, the limited yields from *E. coli* and the need for further in-house purification is costly and time consuming, thereby rendering it an unattractive method (Aden *et al.*, 2002). The recombinant expression of these enzymes in yeasts, such as *Pichia pastoris* or *S. cerevisiae*, offers attractive novel methods for simultaneous saccharification and fermentation, especially as certain strains have been modified to utilise pentoses, such as xylose, produced by their recombinant proteins (Lin Cereghino *et al.*, 2002; Lynd *et al.*, 2005). This would allow for the use of only minimal quantities of commercial enzymes with the fermentative organism, as they ferment any locally produced agricultural wastes, such as *A. comosus*.

Following the production of the enzymes, they were combined with Celluclast 1.5L<sup>®</sup> and Pectinex<sup>®</sup> 3 XL in different ratios and combinations in bi-, tri- and quad-synergy ratios experiments. The highest reducing sugar production, with simultaneous synergy combinations tested in section 6.4, was 1.61 mg/mL from C60P40 at 36 h using 30.25 µg/mL of protein. The use of a combination like C20P80, which only had a protein concentration of 10.5 µg/mL, yet produced 1.30 mg/mL of reducing sugar, would be of greater industrial interest. However, other combinations may also be of interest depending on the economic constraints, such as C20P60X20 which produced 1.52 mg/mL of reducing sugar at 36 h with 12.875 µg/mL of protein.

This demonstrated the importance of including pectinase and xylanase activity with cellulases and that the removal of pectin and xylan substantially increased reducing sugar production. This is similar to studies with sugar beet pulp and hemp in which it was found that the addition of a commercial Pectinex solution to Celluclast 1.5L<sup>®</sup> increased the hydrolysis yield of cellulose to 64% (Pakarinen *et al.*, 2012). While pectin is not directly associated or covalently linked in any way to cellulose, its removal or depolymerisation has been reported to destabilise the plant cell

wall and increase substrate accessibility for other enzymes (Caffall and Mohnen, 2009). The degradation of pectin also decreases the cohesion between plant cell wall remnants in lignocellulose as pectin is primarily located in the middle lamella between plant primary cell walls (Caffall and Mohnen, 2009; Jayani *et al.*, 2005). The degradation of pectin along with the additional xylanase activities in Pectinex<sup>®</sup> 3XL, allowed for the exposure of new fibrils by reducing the intermolecular cohesion of fibrils and allowed for the creation and enlargement of pores within the lignocellulose structure (Buchert *et al.*, 2005; Pakarinen *et al.*, 2012). This is the most probable explanation for the synergy above one DS with Celluclast 1.5L<sup>®</sup> and Pectinex<sup>®</sup> 3 XL solutions in the simultaneous and sequential synergy assays.

An extension to this synergy explanation was seen with the sequential synergy studies, where the initial xylanase hydrolysis was followed by mannanase and then pectinase activity. The theory to explain the activity for the sequential XMP loading order, which had the highest DS, was that the initial xylanase enzymes would have diffused into the space formerly occupied by cytoplasm of the plant cells. This would have been via the plant's former xylem, phloem and plasmodesmata network, in addition to the newly exposed fissures and holes created by the pretreatment (Caffall and Mohnen, 2009; Fry, 2011; Hendriks and Zeeman, 2009). XynA would then have hydrolysed the accessible xylan of the secondary cell walls in addition to any xylan exposed from the primary cell wall due to the pretreatment procedures. Then ManA would enter the cell cavity and hydrolyse the mannan of the secondary cell wall, including that exposed after the xylanase activity (Harris and Smith, 2006). Finally, with the secondary cell wall partially degraded, the pectinase would enter the cell cavity and degrade the pectin within the exposed primary cell wall. As Pectinex<sup>®</sup> 3 XL contained minor levels of xylanase activity, it would also continue to hydrolyse the remaining secondary cell wall and work synergistically with the pectinase fraction to hydrolyse the primary cell wall, as well as increasing the access of the pectinase to the primary cell and the pectin-rich middle lamella, by hydrolysing the secondary and primary cell wall (Caffall and Mohnen, 2009; Harris and Smith, 2006). The effect of this order was significant as it increased the combinations' total activity by 50% relative to the individual controls and by 60% compared to the simultaneous synergy control which displayed anti-synergy at 0.91 DS.

The anti-synergy relationship observed between XynA and pectinase demonstrated that, if synergy studies are neglected, then both specific activity and reducing sugar yield may be

significantly reduced, as seen in section 6.4.3. The incorporation of XynA also demonstrated the importance of raising xylanase activity levels in commercial cellulase solutions, as suggested by Qing and Wyman (2011). Xylanase would remove the xylan sheath surrounding the cellulose fibres, which would have increased access to the major polysaccharide fraction of pineapple pomace, cellulose.

As novel enzymes are discovered and substrate-optimised enzyme cocktails are improved or developed, synergy studies will remain an important method in this process (Van Dyk *et al.*, 2012; Merino and Cherry, 2007). These will not only minimise anti-synergy relationships with a newly-introduced enzyme into a commercial solution like those used, but will also boost overall yield and activities, as was seen in sequential studies. It should also be noted that the enzyme solutions used came from three distinct micro-organisms which could be in competition in their natural environments. Therefore the enzyme combinations produced by each microbe for lignocellulosic degradation may also possess activities designed to inhibit the enzymes of the microbe's competitors. However, industrial bioreactor designs and enzyme immobilisation methods can incorporate features to negate anti-synergy and generate maximum DS, higher specific activities and greater yields.

The release of less than 20% of the reducing sugars from the optimal enzyme combination (C60P40 at 36 h) with pineapple pomace is considered low when compared to studies with other agricultural waste from the *Poales* order, such as corn stover and sugar cane bagasse, where over 80% sugar yields were recorded (Gottschalk *et al.*, 2010; Gao *et al.*, 2010). These studies also benefited from the use of various pretreatment conditions, including ammonium fibre explosion, steam pretreatment as well as prolonged incubation at higher temperatures (Gottschalk *et al.*, 2010; Gao *et al.*, 2010). It has been recommended that a form of mild pretreatment and hydrolysis of two or more days is required for high yields from enzyme hydrolysis instead of the 36 hours of hydrolysis used in the present study (Beukes *et al.*, 2011; Olver *et al.*, 2011; Tenborg *et al.*, 2001).

In terms of increasing synergy the same cellulosomal enzymes with various forms of pineapple waste, Beukes *et al.* (2011) and Olver *et al.* (2011) were able to obtain synergy above 2.8 DS. These studies were performed with five days incubations at 50°C, alkali pretreatments, which resulted in significantly higher levels of specific activity which would have been related to the

high DS obtained. Both of these studies did, however, have high levels of enzyme denaturation due to the high assay temperatures used. The use of hydrolysis temperatures below 40°C, with ManA and XynA, has been recommended by Dredge *et al.*, (2011), and was the reason 37°C was used for activity assays in the present study. The use of longer incubation times, higher temperatures and mild pretreatment is therefore recommended to boost both the yield and synergy from the current enzyme combinations used in this study (Dredge *et al.*, 2011).

Alternatively, other mannanases and xylanases with greater stability and higher levels of activity could be used instead of ManA or XynA. As ManA only displayed its activity, through its raising of DS when in combination with XynA, it should only be used when XynA is included as part of a lignocellulose hydrolysis solution and where there is prolonged incubation so as to achieve the high DS reported by Olver *et al.* (2011). Another mannanase with a higher specific activity and greater enzyme stability could prove more beneficial under those circumstances. The degradation of glucomannan is important for overall pineapple degradation, as glucomannan forms 5% (w/v) of the plant secondary cell wall, which is only 1% less than the pectin.

XynA could be recommended for inclusion within an enzyme cocktail for use with pineapple as most lignocellulose degrading enzyme solutions, including Celluclast 1.5L<sup>®</sup> and Pectinex<sup>®</sup> 3 XL, have insufficient xylanase activity and glucuronoarabinoxylan forms approximately 29% of pineapple cell walls (Qing and Wyman, 2011; Smith and Harris, 1995). For the operation of any commercial bioreactor system, cost will be a major concern and the decision to include any enzyme solution would be based on a function of its ability to increase yield or specific activity, and to reduce the overall cost of a custom enzyme cocktail (Aden *et al.*, 2002; Merino and Cherry, 2007; Meyer *et al.*, 2009). To further establish the effect the activities of enzyme combinations have on a substrate and the synergistic relationships that exist between them, more specific end-product analysis is required.

The use of the DNS assay in the present study made the identification of the sugars produced impossible as the DNS assay cannot distinguish between individual sugars. Identification of sugar moieties and structures produced by enzyme activities with complex polysaccharides would differ from the standard used (glucose). This would aid in establishing a more accurate level of substrate degradation and may reveal that certain enzyme combinations have under-reported activities due to their limited ability to degrade their products to monomers. Accurate

identification of the sugars produced will also reveal whether further processing is required before their end use, e.g. fermentation.

The use of HPLC-MS would help elucidate the exact contribution of each enzyme activity to overall sugar release. In addition, the increase in specific sugar products would indicate which enzymes have synergistically benefited from specific enzyme combinations or the inclusion of enzymes with novel substrate activities. However, HPLC-MS has low resolution of sugar polymers larger than dimers, and as such, would require any sugar samples to be reduced to monomers. This would require the inclusion of monomer producing enzymes into an enzyme cocktail for the purposes of synergy and activity research. However, the costs from including these could be negated by the use of genetically modified fermentative organisms within an industrial setting. The inclusion of  $\beta$ -glucosidases and  $\beta$ -xylosidases and potentially other short chain cleaving enzymes into the expression systems of these fermentation vectors would reduce the overall enzyme costs of a hydrolysis step (Lynd *et al.*, 2005, Van Zyl *et al.*, 2007). This would also improve the total yields of biofuels or other value added products by reducing end product inhibition.

To establish the cost effectiveness of the enzyme solutions and their combinations used in the present study further techno-economic investigation would be necessary. This could eventually be expanded to a pilot plant, which would allow for the establishment of the cost effectiveness of the enzyme combinations used. Thus, the data generated from the present study would be important towards establishing a potentially economically viable method of pineapple pomace beneficiation instead of the current disposal methods used for this waste.



## **7.2 Conclusions**

This study was successfully able to heterologously express, purify and perform limited substrate activity assays with ManA and XynA from *C. cellulovorans*. Additionally, limited substrate activity assays and protein identification with SDS-PAGE were performed with Celluclast 1.5L<sup>®</sup> and Pectinex<sup>®</sup> 3 XL. Limited activity was observed with each enzyme individually on pineapple pomace (except ManA) over 36 hours as compared to activity on their defined substrates. Through simultaneous bi-, tri-, and quad-synergy studies with each enzyme it was found that Celluclast 1.5L<sup>®</sup> and Pectinex<sup>®</sup> 3 XL combinations constituted the majority of the optimal enzyme combinations for either maximum sugar production or reducing sugar per mg protein, over the 36 hours tested. It was observed that these combinations displayed increasing levels of synergy above one DS over time which contributed to the combination C60P40 producing the highest reducing sugar combination after 36 hours, at 1.61 mg/mL. The highest reducing sugar per protein weight was produced with C20P80 for each measurement over the 36 hour assay with 1.3 mg/mL of reducing sugar produced from 10.5 µg/mL of protein at 36 hours. Celluclast 1.5L<sup>®</sup>, Pectinex<sup>®</sup> 3 XL and XynA combinations could also form an optimal enzyme combination and demonstrated the need for additional xylanase activity in custom enzyme cocktails. Pectinex<sup>®</sup> 3 XL displayed anti-synergy in certain combinations with XynA, Celluclast 1.5L<sup>®</sup> and to a lesser extent ManA, as seen with simultaneous and sequential synergy studies. Celluclast 1.5L<sup>®</sup>, ManA and XynA possess a simultaneous synergistic relationship together and it is recommended that they are added to a substrate and given sufficient time to hydrolyse it, before Pectinex<sup>®</sup> 3 XL is placed in solution. Further work on combinations of these enzymes in the form of additional synergy studies and end product analysis would prove beneficial for bioreactor design and operation in the production of biofuels from pineapple pomace.

### **7.3. Future recommendations**

Further work should be performed with the cellulosomal enzymes of *C. cellulovorans* and commercial enzyme solutions with regards to the degradation of fruit wastes such as pineapple pomace. The use of a scaffoldin protein (CbpA) with XynA and ManA to create mini cellulosomes would increase the stability and activity of these enzymes and should be used in any synergy studies containing these enzymes (Doi, 2008). The use of synergistic combinations of cellulosomal enzymes in a cellulosome complex to increase the enzyme stability and yield from a substrate has been demonstrated with both wheat straw and corn fiber (Koukiekolo *et al.*, 2005; Moraïs *et al.*, 2010). Sequential synergy studies with both ManA and XynA, and potentially arabinofuranosidaseA in solution simultaneously, or as part of a mini-cellulosome complex, sequentially followed by high levels of Pectinex<sup>®</sup> 3 XL and Celluclast 1.5L<sup>®</sup>, should be investigated to determine whether this will further raise activity and DS beyond those established in this study. Incubation temperatures with ManA, XynA and ArfA mini-cellulosomes should be raised to 45°C for over 72 hours and after inactivation followed by Pectinex<sup>®</sup> 3 XL and Celluclast 1.5L<sup>®</sup> combinations incubated at 55°C.

Due to the high cost and enzyme loadings used in cellulosic ethanol production and the relatively low cost of the product produced in bulk (USD \$1.69/gallon as of 4<sup>th</sup> October 2013), methods of recovery and/or immobilisation are vital to increase enzyme stability and reduce costs through recycling of enzyme solutions (NASDAQ, 2013; Tu *et al.*, 2006; Twala *et al.*, 2012). A novel method of immobilisation that allows increased enzyme to substrate accessibility and maintains enzyme recoverability is magnetic microspheres such as those offered by MagReSyn<sup>™</sup> (Qui and Li, 2000; Twala *et al.*, 2012). Investigation into the use of these magnetic microspheres with cellulosomes could offer a method for significantly increasing enzyme stability and activity while lowering enzyme costs through enzyme recycling and rejuvenation. The demonstration of sequential synergy with this study's enzymes attached to magnetic microspheres, would provide an important proof of the enzyme synergy and reuse concept for the biofuel industry, even with the limited activity displayed by these enzymes.

Mild alkaline pre-treatments on pineapple pomace would likely prove beneficial in raising enzyme activity and sugar yield and should be investigated for use with minimal enzyme cocktails, similar to those used in the present study. Pretreatment is considered a vital component in bioreactor operations (Merino and Cherry, 2007). The use of lime pre-treatment and steel ball milling would significantly increase the homogeneity and cell wall degradation of pineapple pomace and as such should be used with future work on pineapple pomace, as it proved highly beneficial with the closely phylogenetically related sugar cane and beet (Beukes *et al.*, 2011; Dredge *et al.*, 2011). This would have the added benefit of increased surface area to volume ratio for the substrate particles and decreased lignin shielding, both of which could drastically increase enzyme activities (Sills and Gosset, 2011). A detailed chemical analysis of laboratory generated and commercially generated pineapple pomace, the effects of freeze drying with rehydration and the use of more severe pretreatment methods would all assist any future studies of pineapple pomace hydrolysis and beneficiation.

The use of a full factorial design approach should be considered with regards to quantifying the contributions and relative importance of various enzymes in solution. Simultaneous synergy studies with combinations of Celluclast 1.5L<sup>®</sup>, Pectinex<sup>®</sup> 3 XL and ManA should be investigated, as ManA and Celluclast 1.5L<sup>®</sup> each individually demonstrated a limited anti-synergy relationship with Pectinex<sup>®</sup> 3 XL, under both simultaneous and sequential synergy assays. Longer assays, of more than 36 hours, should be performed to study the effects that further substrate degradation and prolonged hydrolysis may have on enzyme synergies.

Additional sequential synergy studies would help determine the nature of the synergistic relationships between these enzymes. Additional investigations should take place with commercial cellulase and pectinase cocktails with defined substrate specificities, to exclude the effects of unknown proteins and activities present within the Celluclast 1.5L<sup>®</sup> and Pectinex<sup>®</sup> 3 XL cocktails. This will illustrate the effects of anti-synergy in minimal enzyme cocktails and will assist in optimising custom enzyme cocktails in relation to changes in synergy over time. Further work should be performed with the end products produced by the enzyme combinations used in this study to identify any inhibitory effects these products may have on later fermentation and enzymatic hydrolysis stages.

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# Appendices:

## Appendix A: Reagents list

All the reagents used to generate the results reported in the current study are recorded in Table A1. The left hand side column contains the name of the reagent used. The right hand side column contains the supplier's name along with the relevant catalogue or product number, as stated either by the supplier or listed on the relevant container.

**Table A1: A list of all reagents used in the present study with the reagent name, supplier name and catalogue number.**

<b><u>Name of reagent</u></b>	<b><u>Supplier (Catalogue number)</u></b>
2-mercaptoethanol	Fluka (Cat. No. 63700)
3,5-Dinitrosalicylic acid	Sigma (Cat No. D0550)
Acetone	Merck (8.22251.2500)
Acrylamide	Sigma (Cat No. A8887)
Ammonium persulphate	Sigma Aldrich (Cat no A3678)
Avicel PH-101	Fluka (11365)
Birchwood xylan	Fluka (Cat. No. 95588)
Beech wood xylan	Sigma (Cat no. X4252)
Bovine serum albumin (BSA)	Sigma (Cat no. A7906)
Bradford reagent	Sigma (Cat. No. B6916)
Bromophenol blue	Sigma (Cat. No. B8026)
Cellulose powder	Sigma (Cat. No. C6288)
Carboxymethyl cellulose	Calbiochem (Cat. No. 217277)
Congo Red	Sigma (Cat no. C6767)
Coomassie Brilliant Blue R250	Merck (Cat. No. 1.12553)

D-(+)-Xylose	Sigma (Cat no. X-3877)
Dextran Blue from <i>Leuconostoc</i> ssp.	Fluka (Cat no. 31393)
3,5-Dinitrosalicylic acid	Sigma (Cat No. D0550)
Di-potassium hydrogen phosphate	Merck (1.05104.1000)
Di-sodium hydrogen orthophosphate	Saarchem (Cat. No. 5822860)
Ethanol	Merck (Cat. No. 8.18700)
D-Galacturonic acid	Supplier and Cat. no. not listed on container
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)
Imidazole	Merck (Cat. No. 1.04716)
IPTG	Calbiochem (Cat. No. 420322)
Kanamycin (Monosulphate)	Melford (Cat. No. K0126)
Locust bean gum	Fluka (Cat. No. 62631)
D-Mannose	Sigma (Cat. No. M2069)
Methanol	Merck (Cat. No. 8.22283)
N,N-methylenebisacrylamide	Sigma (Cat. No.M7279)
Nutrient Broth	Biolab, Merck (C24)
Oatspelt xylan	Fluka (95590)

Orcinol monohydrate	Sigma-Aldrich (Cat no. O1875)
Pectin (Apple)	Sigma (P8471)
Peptone	Fluka ( 70169)
PeqGold protein marker II	peqLab (Cat. No. 27-2010)
Pierce Prestained Protein M W Marker 26612	Thermo scientific (Cat. No. 26612)
Phenol	Sigma (Cat.No. P3653)
Polyethylene glycol (PEG) 20,000	Merck (Cat. No. 8.18897)
Polygalacturonic acid (PGA)	Sigma (P3850)
Ponceau S	Fluka (81460)
Potassium hydrogen phosphate	Merck (1.04877.1000)
Sodium azide	Merck (Cat. No. 8.22335)
Sodium chloride	Saarchem (Cat. No. 5822320)
Sodium hydroxide	Saarchem (Cat. No. 5823200)
Sodium metabisulfite	Sigma-Aldrich (Cat. No.255556)
Sodium Potassium tartrate	Merck (Cat. No. 1.08087)
Sodium dodecyl sulphate (SDS)	BDH biochemicals (Cat. No. 301754)
Sulfuric acid	AECI
Tris (hydroxymethyl) aminomethane	Merck (Cat. No. 1.08382)
Tri-sodium citrate dehydrate	Merck (Cat. No. 1.06448)
Tryptone	Fluka (Cat. No. 70169)

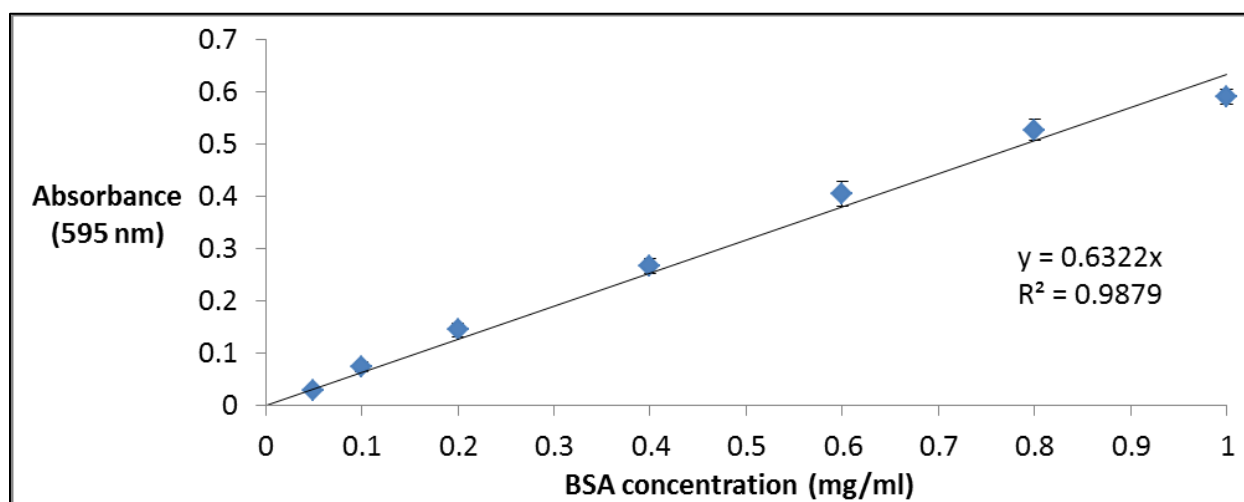
Triton X-100	Merck (1.08603.1000)
Yeast extract	Biolab (Cat. No. BX6)
D-(+)-Xylose	Sigma (Cat. No. X-3877)

## **Appendix B: Standard curves for protein concentration and enzymatic activity**

To determine the concentration of specific analytes in a solution, the appropriate standard curves, for referring experimentally obtained absorbance values to, were constructed. These were generated using known concentrations of the monomer of a relevant analyte.

### **B.1 Protein concentration standard curve**

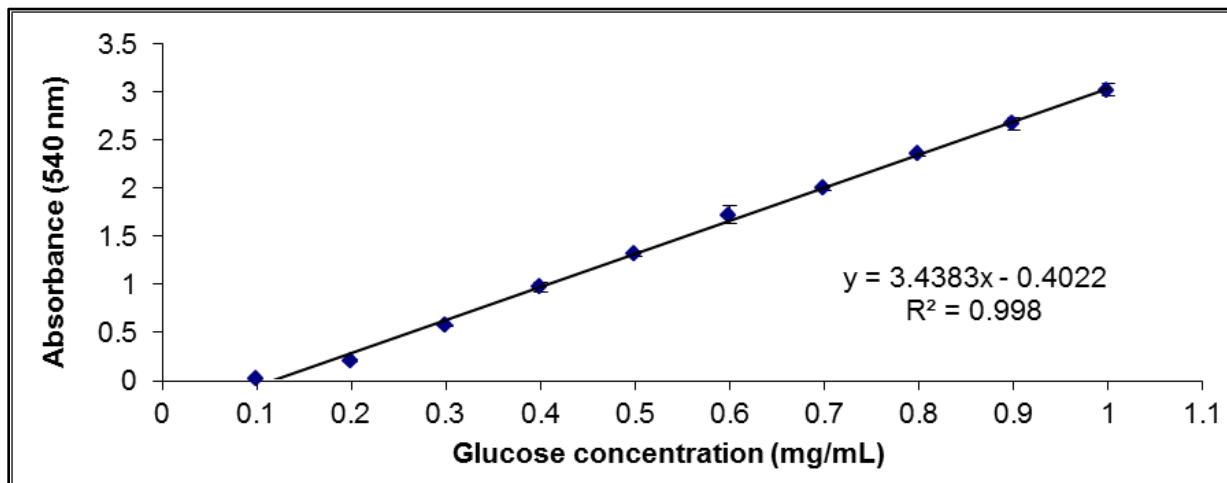
To determine the protein concentration of an unknown solution a standard curve of known protein concentrations was constructed using the Bradford's method of protein determination, as described in section 3.3.4. The absorbencies at 595 nm for known protein concentrations between 0.1 and 1.0 mg/mL, is depicted in Figure B1.



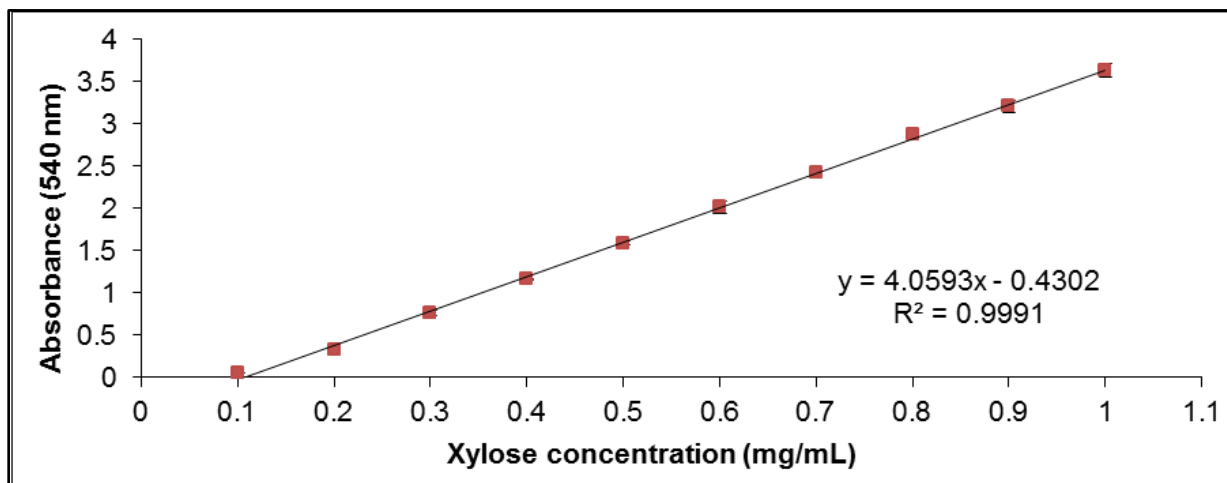
**Figure B1: Protein standard curve for concentrations of bovine serum albumin between 0.1 and 1.0 mg/mL, generated with Bradford's assay with absorbance measured at 595 nm. Values represent means,  $\pm$ SD, n = 3.**

## B.2 Reducing sugar standard curves

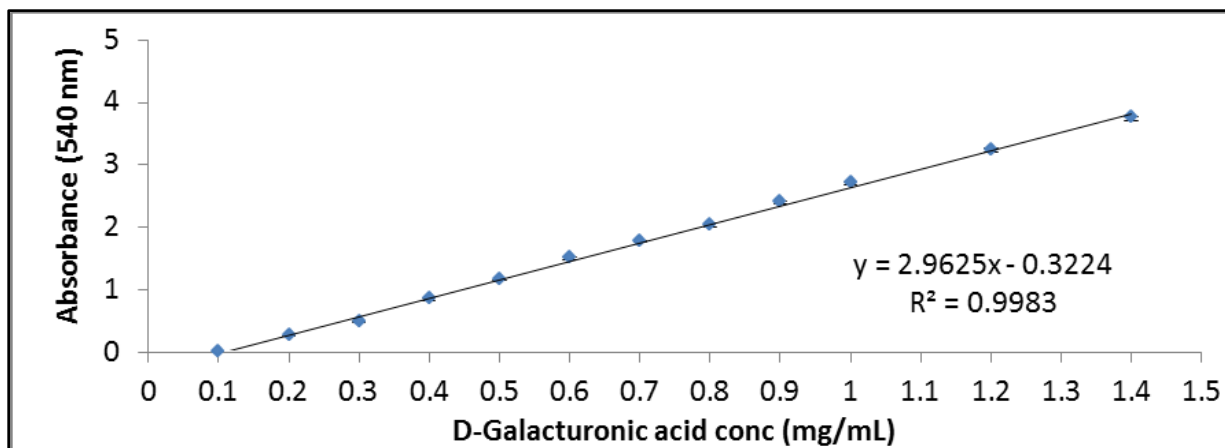
To determine the reducing sugar concentration of an unknown solution a standard curve of known reducing sugar concentrations were constructed for D-glucose, D-xylose, D-galacturonic acid and D-mannose using the DNS method, as described in section 3.3.5.1. The absorbance values at 540 nm for the known reducing sugar concentrations for each sugar, are depicted in Figures B2-5.



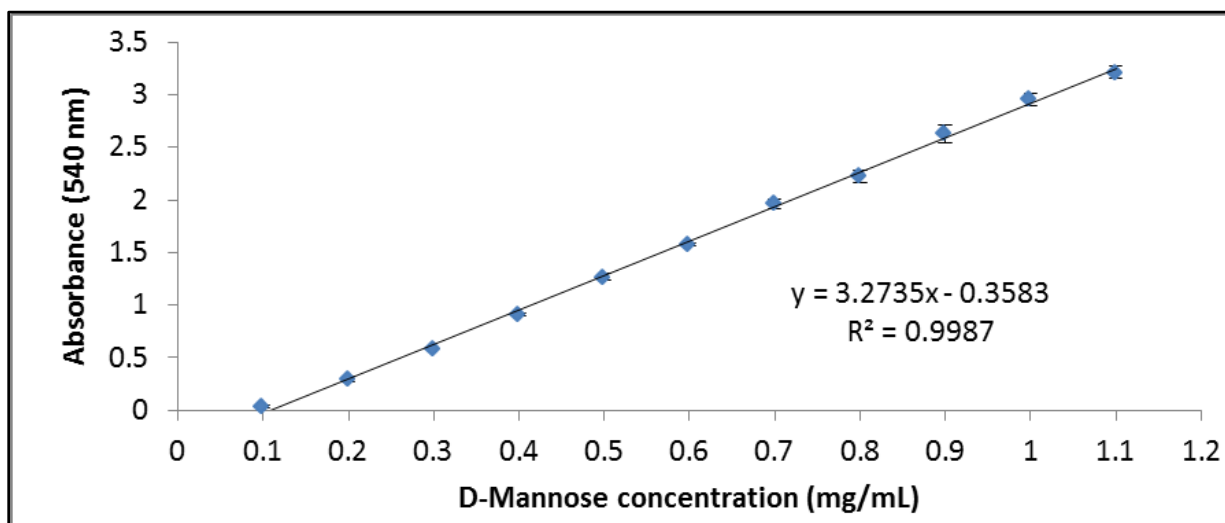
**Figure B2: D-Glucose standard curve for concentrations between 0.1 and 1.0 mg/mL, generated with the DNS assay with absorbance measured at 540 nm. Values represent means,  $\pm$ SD, n = 3.**



**Figure B3: D-Xylose standard curve for concentrations between 0.1 and 1.0 mg/mL, generated with the DNS assay with absorbance measured at 540 nm. Values represent means,  $\pm$ SD, n = 3.**



**Figure B4: D-Galacturonic acid standard curve for concentrations between 0.1 and 1.4 mg/mL, generated with the DNS assay with absorbance measured at 540 nm. Values represent means,  $\pm$ SD, n = 3.**



**Figure B5: D-Mannose standard curve for concentrations between 0.1 and 1.1 mg/mL, generated with the DNS assay with absorbance measured at 540 nm. Values represent means,  $\pm$ SD, n = 3.**

### **Appendix C: Synergy study enzyme loading volume and ratio table**

Enzyme activity assays for synergy studies were prepared according to normal enzyme activity assays with the enzyme fraction loaded as per Table C1, C2 and C3. Reactions conditions were as per standard enzyme activity assays with assay time periods varying. Total volumes of assay solutions were adjusted as needed per experiment with concentrations kept constant.

**Table C1: Enzyme loading volumes and ratios for bi-synergy studies.**

<b>Enzyme A volume (μL)</b>	<b>Enzyme B volume (μL)</b>	<b>Ratio</b>
40	10	A80:B20
30	20	A60:B40
20	30	A40:B60
10	40	A20:B80

**Table C2: Enzyme loading volumes and ratios for tri-synergy studies.**

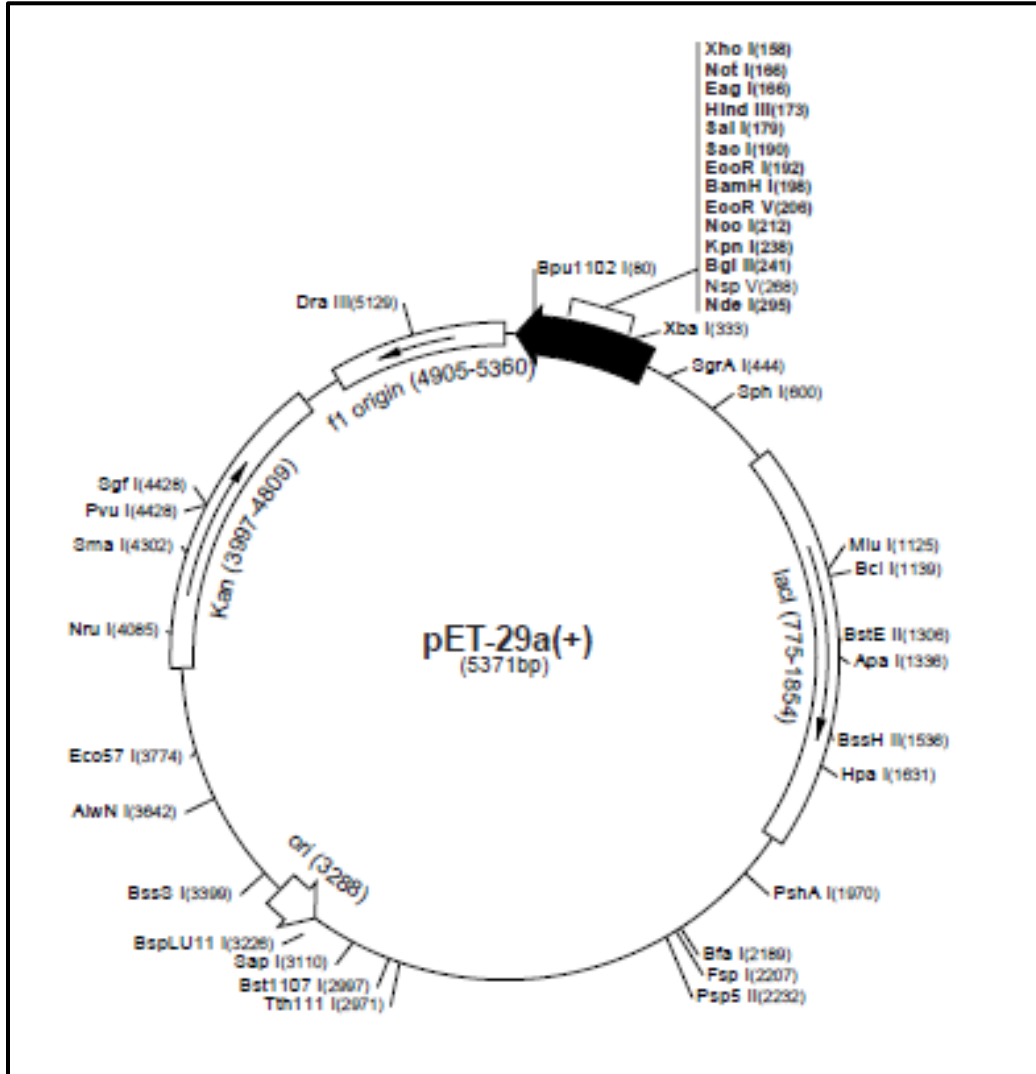
<b>Enzyme A volume (μL)</b>	<b>Enzyme B volume (μL)</b>	<b>Enzyme C volume (μL)</b>	<b>Ratio</b>
30	10	10	A60:B20:C20
20	20	10	A40:B40:C20
20	10	20	A40:B20:C40
10	20	20	A20:B40:C40
10	30	10	A20:B60:C20
10	10	30	A20:B20:C60

**Table C3: Enzyme loading volumes and ratios for quad-synergy studies.**

<b>Enzyme A volume (μL)</b>	<b>Enzyme B volume (μL)</b>	<b>Enzyme C volume (μL)</b>	<b>Enzyme D volume (μL)</b>	<b>Ratio</b>
20	10	10	10	A40:B20:C20:D20
10	20	10	10	A20:B40:C20:D20
10	10	20	10	A20:B20:C40:D20
10	10	10	20	A20:B20:C20:D40

## Appendix D: Map of expression vector

A plasmid map of the pET-29a(+) expression vector system used to express ManA and XynA in *E. coli* BL21 (DE3) cells has been depicted in Figure D1.

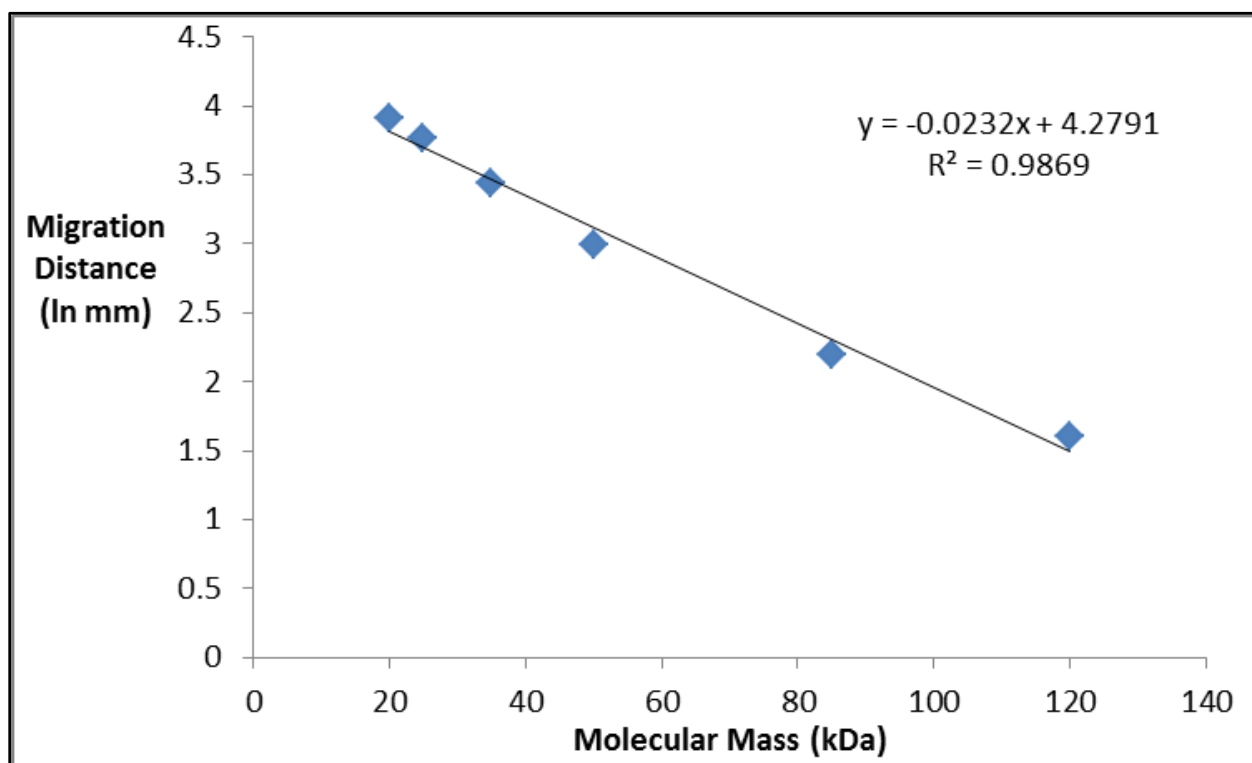


**Figure D1: Plasmid map of the pET-29a(+) Expression vector system used for the expression of ManA and XynA from *C. cellulovorans*. The mannanase and xylanase genes were inserted between the EcoRI and BamHI restriction sites (Novagen, 1998).**



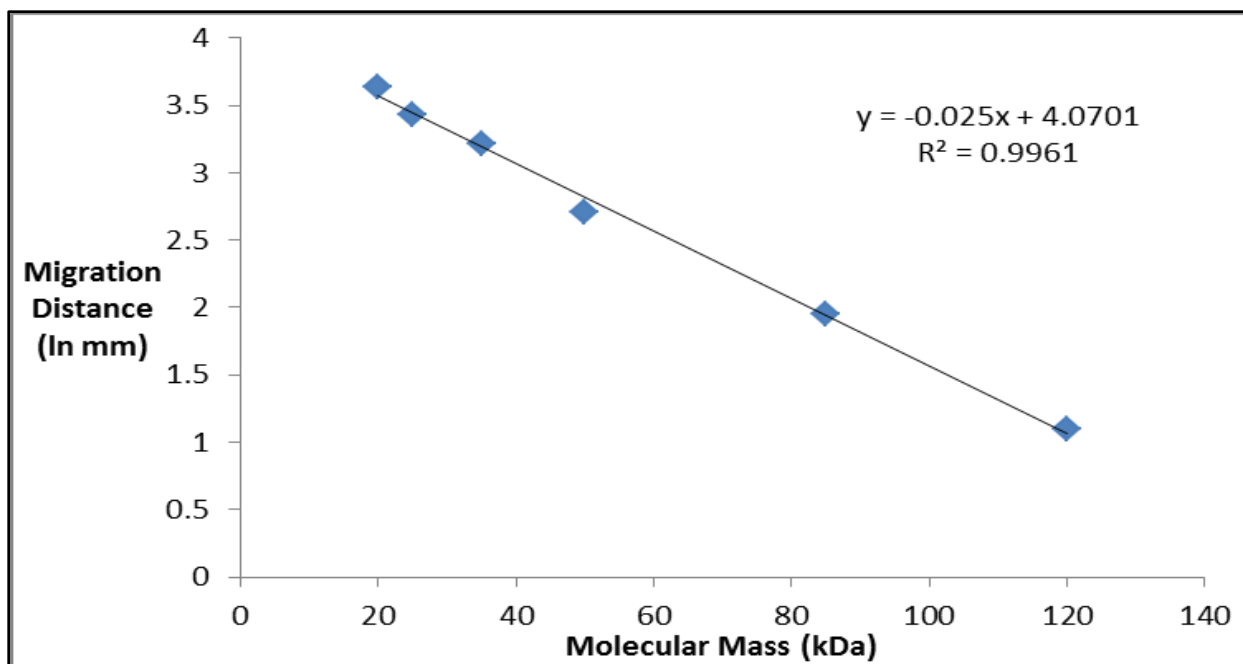
## Appendix E: Standard curves of log regression analysis of molecular weights graphs

To quantify the masses of each protein band within a SDS-PAGE gel, a standard curve relating distance migrated by a protein band of interest and its molecular weight had to be established. This was achieved with the measurement of the distance migrated (mm) by each band within the molecular marker and the conversion with natural log (ln mm) to establish a linear relationship when plotted against their known molecular masses. Then through regression analysis an equation was formed to relate the distance migrated to molecular mass. This was performed for the SDS-PAGE study of *T. reesei* cellulase and *A. niger* pectinase of Figure 4.1 and the resultant standard curve is depicted in Figure E1.

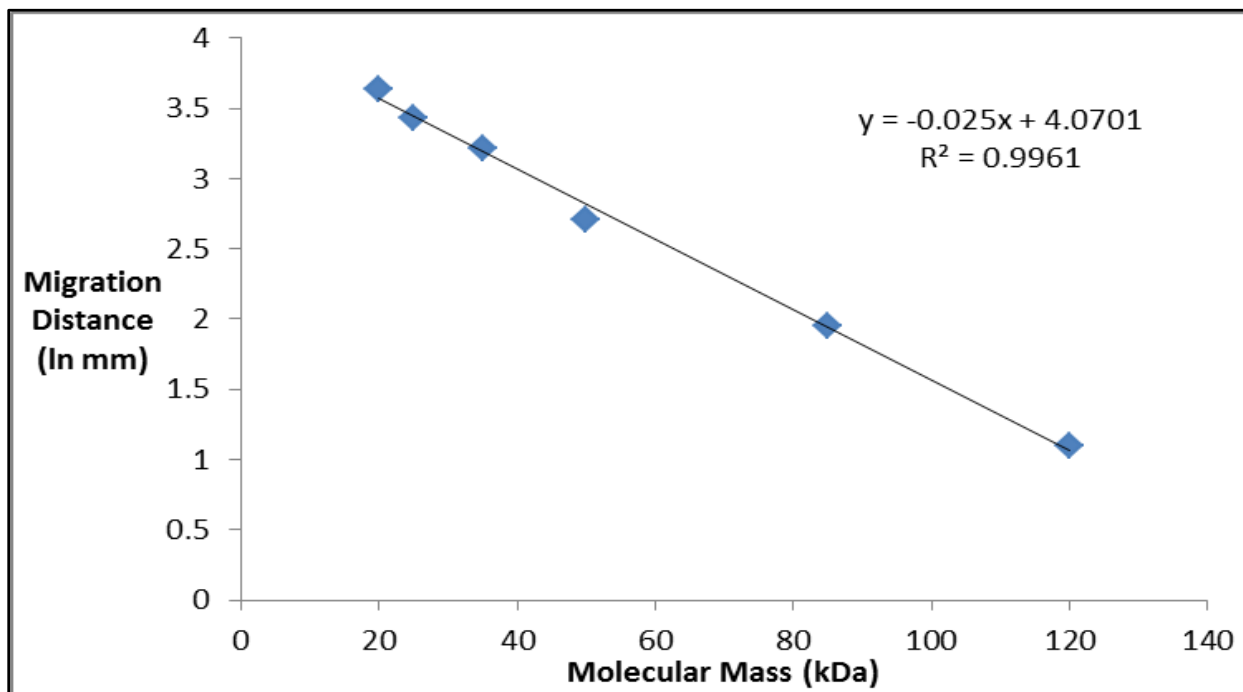


**Figure E1: A standard curve of the masses for the molecular marker in relation to their natural log migration distances for the SDS PAGE of *T. reesei* cellulase and *A. niger* pectinase.**

To obtain the molecular weights of the protein fractions of the discontinuous activity gels of Figures 4.2-3, a standard curve of natural log molecular weights for the protein markers were constructed and have been represented in Figure E2-3.



**Figure E2: A standard curve of the masses for the molecular marker in relation to their natural log migration distances for the 0.1% (w/v) PGA discontinuous activity gel of *A. niger* pectinase and *C. cellulovorans* ManA and XynA.**



**Figure E3: A standard curve of the masses for the molecular marker in relation to their natural log migration distances for the 0.3 % (w/v) pectin discontinuous activity gel of *A. niger* pectinase and *C. cellulovorans* ManA and XynA.**

## **Appendix F: Physical processing of pineapple pomace and microscopy**

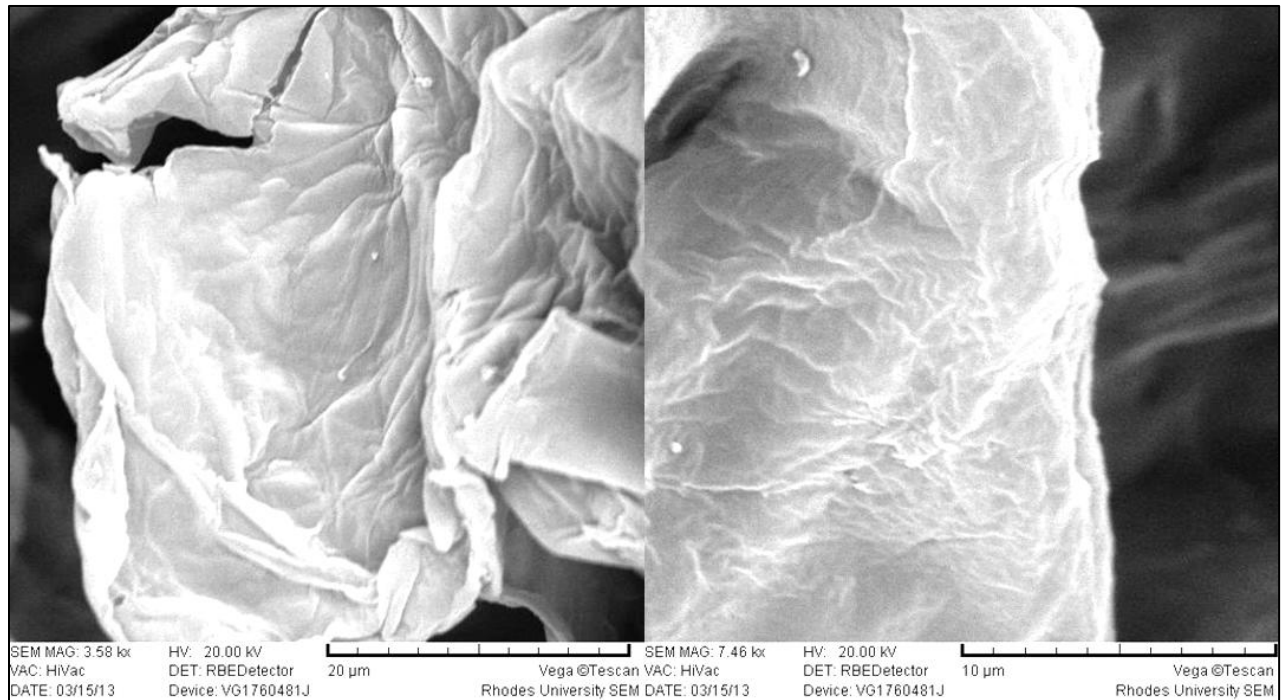
The initial stages of processing pineapple fruit to pomace, before pre-treatment procedures, have been depicted in Figure F1.



**Figure F1: Photographs of the stages of processing smooth *A. comosus* pineapple fruit for pineapple pomace preparation.**

The top picture of Figure F1 depicts, from left to right, a whole pineapple fruit, separated crown, base, skins and skinned fruit. The base and skins were juiced and the resultant products are depicted in the bottom left picture. These are from left to right, measuring cylinder of foam and juice, bag of pomace, and the precipitated slurry from the juice. The bottom right picture is a close up photograph of the pineapple pomace.

Photographs of the pineapple pomace, after pre-treatment, taken with electron microscopy at 3580x and 7460x magnification, are displayed in Figure F2.



**Figure F2: An electron microscopy image of *A. comosus* pineapple pomace powder at magnifications of 358x (left) and 7460x (right).**