INTEGRATED BIOREMEDIATION AND BENEFICIATION OF BIO-BASED WASTE STREAMS

Report to the
Water Research Commission

by

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EXECUTIVE SUMMARY

Proof-of-concept experiments were performed to determine the suitability of recombinant *Aspergillus niger* strains for producing useful enzymes on three bio-based waste streams (apple pomace, grape waste and potato waste). The ability of the recombinant strains to produce enzymes under these cultivation conditions was assessed. Analytical tests were performed to determine the properties of the waste samples and how these properties were altered due to the growth of the fungal strains.

Potato waste proved the best bio-based waste stream for growth of the recombinant *A. niger* strains and the heterologous expression of endoglucanase, endomannanase and endoxylanases. In addition, bioethanol production using *S. cerevisiae* was also achieved using potato wastes for the fermentation feedstock. The wastewater, together with the potato waste can also be used for bio-ethanol production through fermentation and the addition of amylase enzymes.

Trametes pubescens was capable of growing, and producing laccase, on apple and potato wastes without the addition of other nutrients or inducers. The laccase obtained was similar to a purified *T. pubescens* laccase that was produced under optimized media, environment and inducer conditions. Due to the diverse potential applications of laccases, the ease of growth of *T. pubescens* and the lack of additional supplementation, this could be a very valuable value-added product for the biorefinery and warrants further investigation.

Overall, the results showed that the strains identified for high-value enzyme production could successfully produce these enzymes using bio-based waste streams in the cultivation medium.

Biochemical characterization of the enzymes was performed to further assess their fitness for use in waste stream reclamation and lignocellulosic conversion. The three enzymes, endo-β-1,4-glucanase, endo-β-1,4-mannanase and endo-β-1,4-xylanase, showed great potential for being used as key enzymes for lignocellulose degradation. The enzymes are stable at 50°C for at least 24 hours and maintain strong activity at pH 5; conditions often maintained and utilised in bio-refineries. The multi-functionality of the enzymes for different substrates such as carboxymethyl cellulose, locust bean gum and Beechwood xylan indicate that they will play important roles in synergistically degrading the cellulosic and hemicellulosic components of lignocellulosic biomass.

The biochemical characteristics of the three enzymes indicated that they are very suitable for application in the biorefinery sectors of the bioeconomy.

This project, therefore, demonstrated the bioremediation of lignocellulosic wastewater by recombinant *A. niger D15* strains by reduction of organic content, and the concomitant cheap and high production of commercially valuable enzymes on inexpensive and abundant bio-based waste streams.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

PUBLICATIONS AND CONFERENCE PROCEEDINGS

Publications

1. Khan N, Le Roes-Hill M, Welz PJ, Grandin KA, Kudanga T, Van Dyk JS, Ohlhoff C, Van Zyl WH, Pletschke BI (2015). Fruit waste streams in South Africa and their potential role in developing a bio-economy. South African Journal of Science 111:1-11 (Impact factor: 1.031).
2. Le Roes-Hill, M. and Welz, P.J. (2015). Waste to wealth: the hidden potential of waste from fruit. The Conversation, 19 June 2015 (https://theconversation.com/waste-to-wealth-the-hidden-potential-of-waste-from-fruit-43126).
3. Rose SH, Warburg L, Le Roes-Hill M, Khan N, Pletschke B, van Zyl WH. Integrated bioremediation and beneficiation of bio-based waste streams. Submitted as a Chapter for the DST Book: Opportunities for waste biomass and organic waste valorization – seeking alternative solutions to disposal.
4. Another 2-3 manuscripts are currently being prepared for submission.

Conferences

1. Morake, S. T., Rose, S.H., Van Zyl, W.H. and Pletschke, B.I. Effects of synergism between *A. niger* derived enzymes on sugarcane bagasse and corn cob hydrolysis – the role of product inhibition. (Best poster prize). 26th Annual Conference Of The Catalysis Society Of South Africa (CATSA) 2015. Arabella Hotel and Spa, Kleinmond, 15-18 November 2015:
2. Khan, N., Le Roes-Hill, M. and Pletschke, B. The potential of apple processing solid wastes for the generation of value-added products. Poster, 1st Prize. CPUT Research Day, CPUT, 27th November 2014:

LIST OF ABBREVIATIONS

BSA	Bovine Serum Albumin
BWX	Beechwood Xylan
CBP	Consolidated Bioprocessing Process
CMC	Carboxymethylcellulose
COD	Chemical Oxygen Demand
DAFF	Department of Agriculture, Forestry and Fisheries
DNS	3,5-dinitro-salicylic acid solution
DPPH	2,2-diphenyl-1-picrylhydrazyl
EgII	Endo-β-1,4-glucanase
GAE	Gallic acid equivalents
HPLC	High Performance Liquid Chromatography
KD	Kouebelt Detector
LBG	Locust bean gum
LD	Langebelt Detector
ManI	Endo-β-1,4-mannanase
Mt	Million tonnes
OBR-HEC	Ostazin Brilliant Red hydroxyethyl cellulose
OBR-LBG	Ostazin Brilliant Red locust bean gum
PGM	Pressed white grape medium
p-NPG	*p*-nitrophenyl-β-D-glucopyranoside
p-NPM	*p*-nitrophenyl-β-D-mannopyranoside
p-NPX	*p*-nitrophenyl-β-D-xylopyranoside
PPM	Pressed pomace medium
PWM	Potato waste medium
RBB-Xylan	Remazol Brilliant Blue birchwood xylan
RGW	Red grape waste
Rpm	Revolutions per minute
SC	Synthetic Complete
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SK	Skins and Starch
SL	Slivers
SSF	Simultaneous saccharification and fermentation
TDM	*Trametes* defined medium
TEMED	N,N,N',N'-tetramethylethylenediamine
TLC	Thin Layer Chromatography
UPM	Unpressed pomace medium
W-RGW	Washed red grape waste
Xyn2	Endo-β-1,4-xylanase

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CHAPTER 1: **INTRODUCTION AND CONCEPTUAL MODEL (PROOF OF CONCEPT) – BIOCONVERSION OF BIO-RESIDUES TO VALUE-ADDED PRODUCTS**

1.1 Introduction

According to the Department of Agriculture, Forestry and Fisheries, the total fruit production in South Africa is approximately 5.4 million tonnes (Mt) per annum, of which 2.4 Mt are processed (DAFF, 2013). Processing of fruit ranges from canning, juicing, the making of preservatives to drying, to mention a few. During processing, a substantial amount of agri-industrial wastewaters, high in organics, and therefore high chemical oxygen demands (CODs), are generated. According to Burton et al. (2006), the citrus fruit industry alone generates more than 10 000 L of wastewater per tonne of citrus fruit processed, with a COD of 10 000 mg O_2/L. After citrus (which includes oranges, lemons, limes, grapefruit and naartjies), grapes and apples are the dominant fruit crops in South Africa, with more than 1.5 Mt of grapes processed in 2011/2012 and more than 0.24 Mt of apples processed in 2011/2012 (DAFF, 2013). The olive market is also an emerging one in South Africa with an annual production of 3 000 tonnes of table olives and 700 000 L/annum olive oil, most of which is currently being produced in the Western Cape region [http://olivesgowild.co.za/]. For a current review on the role of fruit waste streams in South Africa and their potential role in developing a bioeconomy please refer to Khan et al. (2015).

From previous studies performed by researchers in South Africa and internationally, it was found that the major challenges of fruit and olive mill wastes are the following:

1) Agri-industrial wastewaters typically have low, variable concentrations of solutes and particulate matter, and are not suited to fermentation to generate bio-ethanol or biogas, unless very efficient lignocellulosic carbon conversion is achieved or by the addition of solid wastes (Burton et al., 2006). An alternative approach is bioconversion of the residues to release sufficient carbon nutrients to facilitate uptake through biomass growth, and the subsequent reclamation of the water by separation (Pletschke et al., 2014).
2) Fruit waste streams from deciduous fruits, such as apples and pears, are viscous liquids containing tiny fibrous particles and swollen materials, which hardly settle and make centrifugation or filtration nearly impossible (Perdih et al., 1991).
3) The residues associated with agri-industrial wastewaters are often lignocellulosic, and to provide simple metabolisable carbohydrates, lignocellulosic materials must be depolymerised. The problematic complexity of achieving this efficiently is well documented (Novalin and Zweckmair, 2009).

It is important to understand the composition of fruit wastewaters before suitable disposal and treatment can be implemented. Lignocellulose is one of the major carbohydrates found in fruit processing wastewater and there has and continues to be extensive research carried out on the topic of lignocellulose degradation. Sugars and phenolic compounds are also commonly found in fruit processing water. For example, Friedrich et al. (1986) states that apple slop (containing 3.4% dry matter) from the distillery industry consists of 20.4% cellulose, 14.7% lignin, 14.1% hemicellulose, 1% pectin, 12% glucose and small amounts of proteins and lipids. The processing of fruits in the canning, juicing, winery and distillery industries also produces a certain amount of solid residue, known as pomace, which has a high moisture and sugar content and can be added to

the wastewater streams. In the production of citric juice, the solid residues (up to 50%) can be pressed to generate press cake and a sugar-rich leachate. When the press cake is dried to 10% moisture, it is composed of sugars (30-40%), pectin (15-25%), cellulose (8-10%) and hemicellulose (5-7%) (Widmer et al., 2010). The sugar-rich leachate contains about 10% sugars, which can be concentrated by membrane technology to 25-30% before fermented to ethanol (Garcia-Castello et al., 2006). However, limonene needs to be removed by steam stripping since it can act as inhibitor of yeasts (Pourbafrani et al., 2010) when citrus waste is used. Apple pomace, in particular, represents about 25% of the original fruit after the juice has been extracted (Zheng and Shetty, 1998) and contains about 44% cellulose, 24% hemicellulose and 20% lignin (Nawirska and Kwasniewska, 2005).

Aspergillus spp., notably *A. niger* and *A. oryzae*, have been used for more than 2000 years in the Orient for the production of fermented food and products such as citric acid and soya (Hesseltine, 1983). These fungi produce copious amounts of enzymes that can hydrolyse starch, pectin and cellulosics (de Vries and Visser, 2001; Jin et al., 1998). *Aspergillus* spp. can also degrade and utilize a wide range of phenolic compounds (Garcia Garcia et al., 1997), including compounds present in olive mill wastewaters (Garcia Garcia et al., 2000; Hamdi et al., 1991; Vassilev et al., 1997). The ability of *Aspergillus* spp. to produce extracellular enzymes in large quantities and to utilize recalcitrant phenolic compounds, make them ideal for cleaning up more complex organic matters in waste streams.

Aspergillus niger has long been used for industrial enzyme production, in particular by Novozymes and DSM (http://dsm.com) and remains the preferred organism for industrial enzyme production. In this project, we have access to various *A. niger* strains that overproduce cellulases, xylanases and mannanases (Rose and van Zyl, 2002; 2008) and a laccase (Bohlin et al., 2006). Enzyme production in grams per litre was demonstrated for a mannanase (Van Zyl et al., 2009). In collaboration with the laboratory of Prof. Leif Josson at Umea University, Sweden, Van Zyl and co-workers demonstrated that the *A. niger* strains can be grown on the waste streams of lignocellulosics after the hexose sugars in sugarcane bagasse and northern spruce hydrolysates were fermented to ethanol with industrial yeast strains. At the same time, the *A. niger* strains produced a cellulase and xylanase (Alriksson et al., 2009; Cavka et al., 2011). In principle, it should be possible to grow *A. niger* strains on the spent waste streams after ethanolic fermentation, olive mill waste streams, or fruit processing waste streams with the simultaneous production of high-value enzymes.

1.1.1 Project aims and objectives

The overall project aims and objectives are as follow:

1. The development of an improved understanding of fruit bio-based waste streams in South Africa: Composition and potential for application in bioenergy production and the production of value-added products (Review);
2. The development of a conceptual model for the bioconversion of bio-residues in bio-based waste streams: the use of recombinant fungal strains for the production of enzymes;
3. Identification of successful strains and enzymes for the bioconversion of bio-residues in waste streams. Development of more evidence to fit the conceptual model for this type of waste; and

4. Characterization of the enzymes and their applications in waste stream reclamation and lignocellulosic conversion.

In this chapter, proof-of-concept experiments were performed to determine the ability of recombinant *A. niger* strains to grow on a bio-based waste stream (apple pomace). The ability of the recombinant strains to produce enzymes under these cultivation conditions was also determined (Project Aim/Objective 2). Analytical tests were performed to determine the properties of the apple pomace samples and how these properties were altered due to the growth of the fungal strains.

1.2 Materials and Methods

1.2.1 Apple pomace

Apple pomace for laboratory studies was prepared by homogenizing 'Golden Delicious' apples which were purchased from local supermarkets. Efficient homogenization was only achieved through the addition of water. For every 3 kg of apples processed, 500 mL water was added. The pulp (pomace) was placed in a sieve with muslin cloth and was pressed to remove excess liquid (sample designation: pressed pomace). A second set of pomace was prepared where the excess liquid was retained (sample designation: unpressed pomace). The pomace was stored at -20°C.

1.2.2 Fungal strains

Aspergillus niger strains had previously been constructed in-house (Rose and Van Zyl 2002; 2008) and are known to produce high levels of extracellular enzyme activity. Details regarding the strains are provided in Table 1.1.

Table 1.1: *Aspergillus niger* strains used in the study

Strain	Foreign gene	Activity	Substrate*	References
A. niger D15[pGT1]	None			Rose and Van Zyl (2002; 2008)
A. niger D15[*eg2*]	*Trichoderma reesei egII*	endoglucanase	Lichenan	Rose and Van Zyl (2002; 2008)
A. niger D15[*man1*]	*Aspergillus aculeatus man1*	endomannanase	Locust Bean Gum	Rose and Van Zyl (2008) Van Zyl et al. (2009)
A. niger D15[*xyn2*]	*Trichoderma reesei xyn2*	endoxylanase	Beechwood xylan	Rose and Van Zyl (2002; 2008)

* Lichenan (Sigma); Locust bean gum (Sigma), Birchwood xylan has been discontinued and therefore beechwood xylan (Fluka) had to be used as substitute.

1.2.3 Spore solution preparation

Spores from fresh *A. niger* plate cultures were collected with a toothpick and streaked out onto spore plates in a grid form (Figure 1.1). Spore plates contained 18 g/L agar, 2 g/L neopeptone, 1 g/L yeast extract, 0.4 g/L $MgSO_4.7H_2O$, 10 g/L glucose and 2 g/L casamino acids. Twenty mL Asp+

(300 g/L $NaNO_3$, 76 g/L KH_2PO_4 and 26 g/L KCl) solution and 1 mL of trace elements were added after autoclaving. Plates were incubated at 30°C for 4 to 7 days and subsequently flooded with ±7 mL physiological salt (0.9% NaCl). Spores were loosened with cotton sticks and when a thick spore suspension had formed on the plate, the suspension was transferred into a sterile 15 mL Falcon tube. The tube was vigorously shaken to loosen the spore clumps and the spore dilutions were counted. Spore suspensions were kept at 4°C before being used to inoculate pomace media. Spore solutions could be stored in 50% (v/v) glycerol as cryo-cultures.

1.2.4 Cultivation medium

Fungal strains were cultivated in pressed pomace medium (PPM) and unpressed pomace medium (UPM) (Table 1.2). Traditional medium contained 10 g/L yeast extract, 0.8 g/L $MgSO_4.7H_2O$ and 4 g/L casamino acids. The apple pomace and traditional medium were autoclaved, after which the Asp+ solution and trace elements were added.

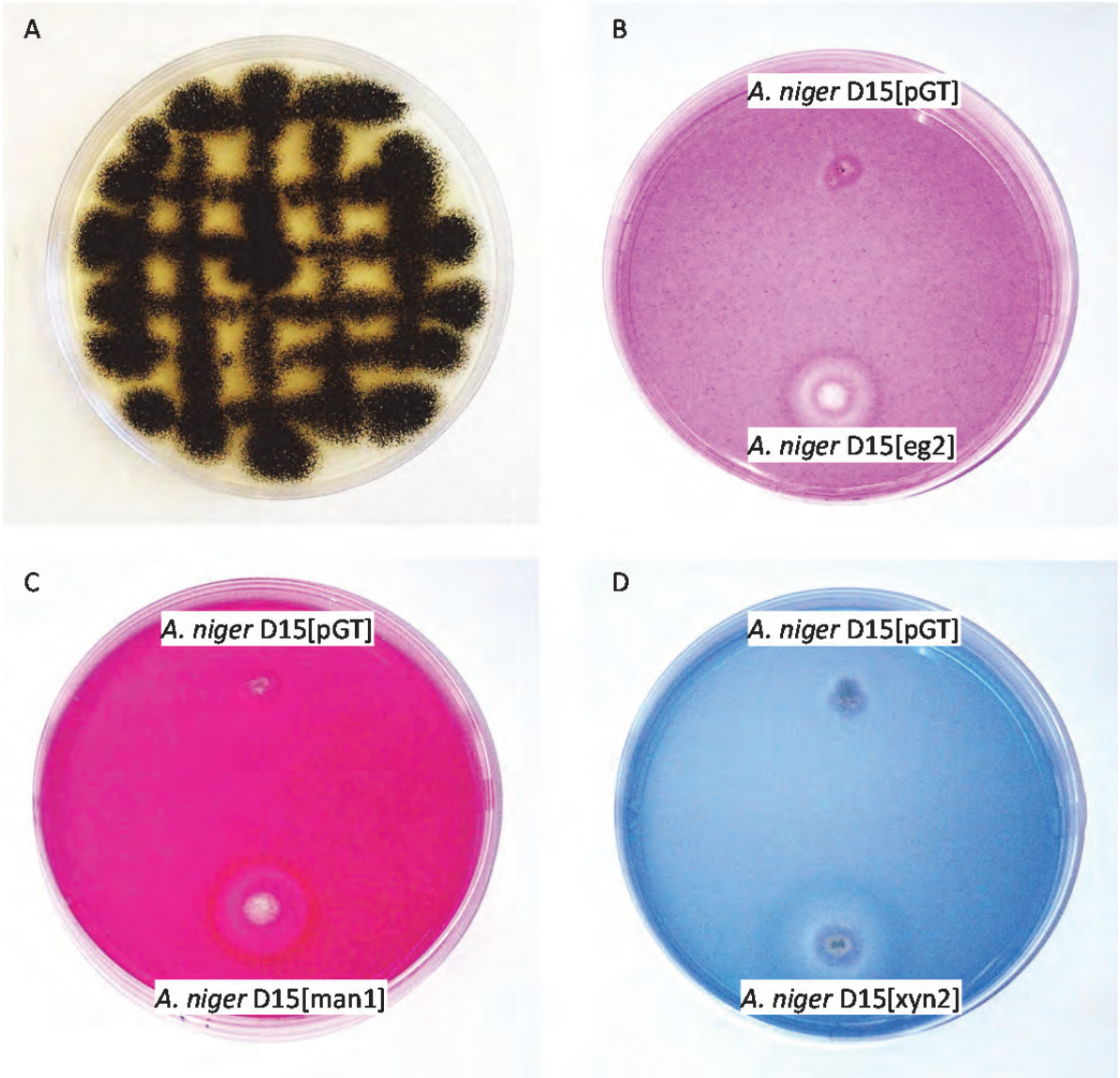


Figure 1.1: Fungal strains are (A) routinely streaked out in grid form to enable a quick colonization of the entire plate. The *A. niger* D15 transformants were tested for (B) endoglucanase, (C) endomannanase and (D) endoxylanase activity. Extracellular enzyme activities are visualized by the presence of clearing zones around the *A. niger* strains.

Table 1.2: The composition of pressed pomace medium and unpressed pomace medium

Pressed pomace medium (PPM)			
Components	**10%**	**20%**	**50%**
Pressed pomace	2 g per flask	4 g per flask	10 g per flask
Traditional medium*	18 mL	16 mL	10 mL
Asp+	800 µL	800 µL	800 µL
Trace elements	40 µL	40 µL	40 µL
Unpressed pomace medium (UPM)			
Components	**10%**	**20%**	**50%**
Unpressed pomace	4 g per flask	8 g per flask	20 g per flask
Traditional medium	16 mL	12 mL	None (no space)
Asp+	800 µL	800 µL	800 µL
Trace elements	40 µL	40 µL	40 µL
Pressed pomace medium (PPM2)			
Components	**10%**	**20%**	**50%**
Pressed pomace	2 g per flask	4 g per flask	10 g per flask
Traditional medium[#]	18 mL	16 mL	10 mL
Asp+	800 µL	800 µL	800 µL
Trace elements	40 µL	40 µL	40 µL
Streptomycin 30 mg/mL	20 µL	20 µL	20 µL
Ampicillin 100 mg/mL	20 µL	20 µL	20 µL

* The amount of traditional medium added to the flasks differed to compensate for the difference in volume of the pomace.

[#] The amount of water in the traditional medium had been amended for the three different concentrations to compensate for the difference in volumes used. Therefore PPM2-10%, PPM2-20% and PPM2-50% are identical in composition accept for the apple pomace. Final volume in the flasks was approximately 21 mL.

1.2.5 Plate assays

Laboratory *A. niger* strains were evaluated and compared with the reference strain. Heterologous enzyme activities were confirmed with plate assays containing Ostazin Brilliant Red hydroxyethyl cellulose (OBR-HEC), Ostazin Brilliant Red locust bean gum (OBR-LBG) and Remazol Brilliant Blue birchwood xylan (RBB-xylan). The OBR-LBG, OBR-HEC and RBB-xylan were prepared according to Biely et al. (1985). Synthetic complete (SC) agar plates (6.7 g/L yeast nitrogen base without amino acids [Difco Laboratories], 100 g/L glucose) were prepared containing 0.1% OBR-HEC, OBR-LBG or RBB-xylan, respectively. Plates contained 10% (w/v) glucose to suppress the native cellulases, mannanases and xylanases.

1.2.6 Enzymatic assays in liquid medium

Strains were inoculated in the pomace media to a spore count of $5x10^5$ spores per mL. Cultivation took place at 30°C at an agitation speed of 200 rpm. The extracellular endoglucanase, endomannanase and endoxylanase activity was determined using the reducing sugar method according to Bailey et al. (1992) and the substrates listed in Table 1.1. All assays were performed at 50°C and pH 5. The supernatant was appropriately diluted prior to the 5 minute incubation with

the substrates. The reactions were terminated by the addition of the 3,5-dinitro-salicylic acid solution (DNS) (Miller, 1959). The samples were boiled for 15 minutes at 100°C to assist in colour development. Glucose, mannose and xylose were used as standards for the endoglucanase, endomannanase and endoxylanase activities (Appendix A). The colorimetric changes were measured spectrophotometrically at 540 nm with an X-MARK™ microtitre plate reader (Biorad, Hercules, CA, USA). The activities were expressed in nkat/mL, where 1 katal equals 1 mole of glucose, mannose or xylose released per second.

1.2.7 Analytical tests

1.2.7.1 Chemical oxygen demand (COD)

The CODs of the different samples was measured using the COD Cell Test kits (photometric method; Merck; equivalent to method 5220D described in the *Standard Methods for the Examination of Water and Wastewater*). Samples (3 mL) were added to COD cells. The tubes were heated in a pre-heated heating block at 148°C for 2 hours. The concentration of COD in mg/L was determined automatically using a Merck (Darmstadt, Germany) Spectroquant Pharo instrument. Measurements were only made of the 10% (w/v) and 20% (w/v) pomace solutions; the 50% (w/v) solution was too viscous to obtain sufficient sample to perform the test.

1.2.7.2 Quantification of total phenolics (Folin-Ciocalteau micro method)

Gallic acid standards of 0 mg/L, 50 mg/L, 100 mg/L, 150 mg/L, 250 mg/L and 500 mg/L were used to compile standard graphs each time the test was performed. Aliquots of 20 µL of each of the standard dilutions (Table 1.3) were added to 1.58 mL distilled water in cuvettes. For the blank, 1.6 mL distilled water was added to a cuvette. Aliquots of 20 µL of the effluent samples were added to 1.58 mL distilled water in cuvettes. An aliquot of 100 µL Folin-Ciocalteau reagent was added to all cuvettes, mixed well and allowed to stand for 5 minutes. Then, 300 µL sodium carbonate solution was added to all cuvettes, mixed well and allowed to stand for 2 hours in the dark. The spectrophotometer was blanked and the absorbance of the standards and test samples were read at 765 nm. A standard graph of absorbance against concentration was plotted and the straight line equation was used to calculate the test concentrations. Results were reported in mg/L gallic acid equivalents (GAE) (Appendix B).

Table 1.3: Standard dilutions for Folin-Ciocalteau standard curve

Final concentration (mg/L)	Stock solution (mL)	Distilled water (mL)
0	0	10.0
50	0.1	9.9
100	0.2	9.8
150	0.3	9.7
250	0.5	9.5
500	1.0	9.0

1.2.7.3 Quantification of dominant sugars

The concentration of the major sugars, sucrose, fructose and glucose, was determined by HPLC using a Phenomenex® Rezex RHM-monosaccharide H+ (8% cross-linkage) column according to the method described by La'Zaro et al. (1989). The concentrations of organic molecules that were

detected were subsequently quantified using relevant standard graphs prepared from HPLC chromatograms. The mobile phase used was 1% (v/v) H_2SO_4.

1.2.7.4 Total protein content (Bradford's assay)

A 1 mg/mL stock solution of bovine serum albumin (BSA) was made by dissolving 10 mg crystalline BSA in 10 mL 0.15 M NaCl. Working standards for the two standard curves were prepared from 1 mg/mL BSA and 100 µg/mL BSA as described in Table 1.4.

Table 1.4: Preparation of standards used in Bradford's Protein Assay

Standard	Vol. 100 µg/mL BSA (µL) or 1 mg/mL BSA	Vol. 0.15 M NaCl (µL)	BSA concentration (µg/mL)
Blank	0	100	0
1	10	90	10 or 100
2	20	80	20 or 200
3	40	60	40 or 400
4	60	40	60 or 600
5	80	20	80 or 800
6	100	0	100 or 1000

In a 96-well microtitre plate, 10 µL of each standard/sample was aliquotted per well (in duplicate) and 90 µL of Bradford's Reagent was added. The reaction was left to stand for 2 minutes and the absorbance was measured at 595 nm using an Anthos Xenyth 1100 microtitre plate reader. The standard curves were constructed by plotting absorbance of standards vs. their respective concentrations. The concentration of each sample was estimated by using the regression line equations from the standard curves (Appendix C).

1.3 Results and discussion

1.3.1 Growth of fungal strains and enzyme activity

The *A. niger* strains were revived from the in-house freeze culture stock collection and spores generated for inoculation. The individual strains were tested on OBR-LBG, OBR-HEC and RBB-xylan plates to confirm the presence of the recombinant enzymes (Figure 1.1). Clearing zones around the colonies are an indication of the extracellular enzyme activity. Although the negative control strain *A. niger* D15[pGT] also produce endoglucanase, endomannanase and endoxylanase activities, no activity was detected on the plates due to the presence of the 10% (w/v) glucose resulting in catabolite repression of the native genes. Constitutive expression of *eg2, man1* and *xyn2* was obtained through the use of the glyceraldehyde 3-phosphate gene (*GPD*) promoter.

The extracellular enzyme activities of all the strains were determined on day 3 in the unpressed pomace medium (UPM) and pressed pomace media (PPM). The unpressed pomace resulted in more enzyme activity detected in the supernatant with all three *Aspergillus* strains on day 3. The pressed and unpressed pomace resulted in reasonably similar levels of activity (Figure 1.2). Increasing the unpressed pomace concentration above 4 g/flask and the pressed pomace above 4 g/flask (i.e. 20%), did not seem to increase the enzyme activity. The flasks containing 10 g pressed pomace (50%, w/v) contained only 10 mL of traditional medium whereas the flasks with 20 g unpressed pomace contained none. This might affect the fungal growth and attribute to the lower yield in enzyme activity.

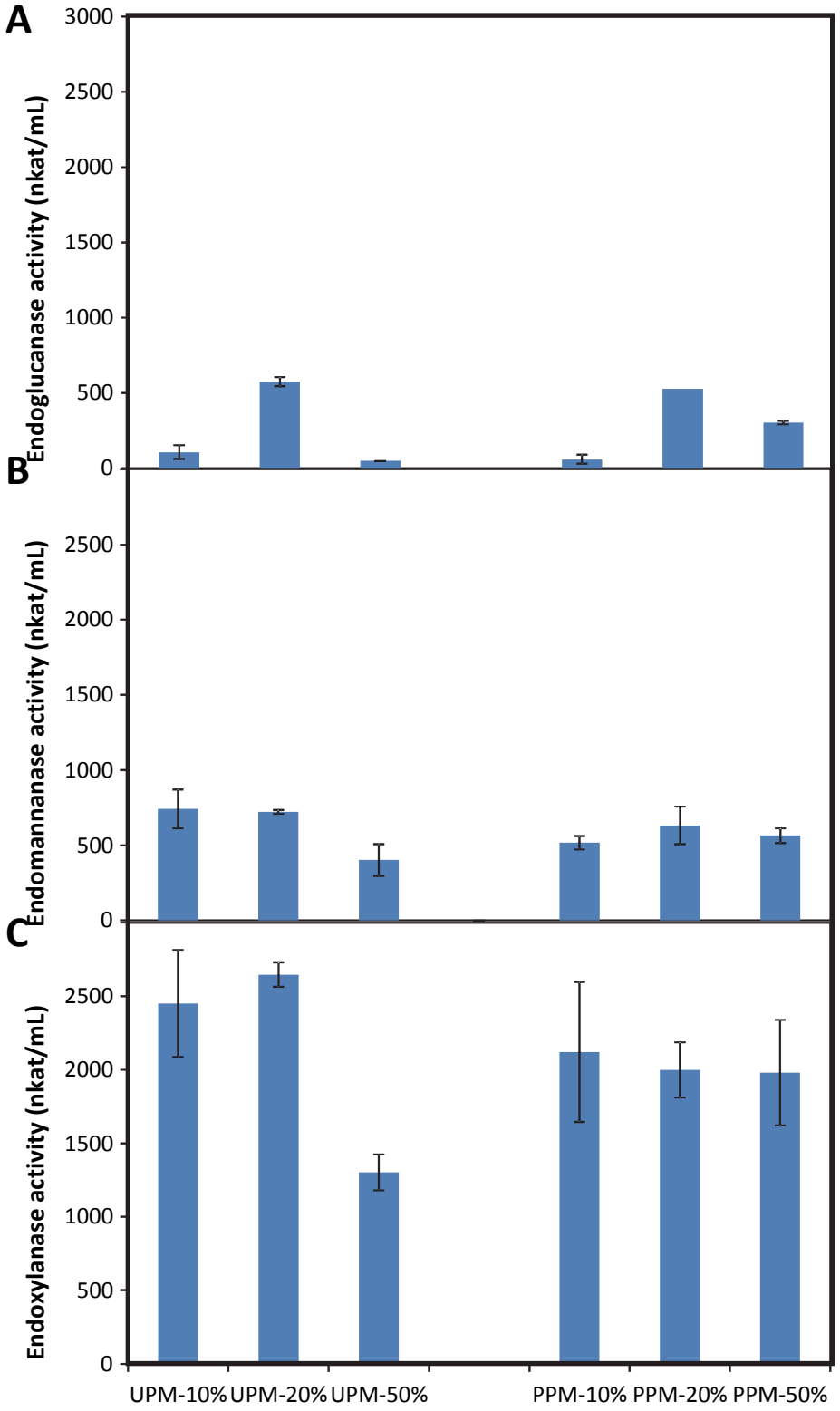


Figure 1.2: The extracellular endoglucanase (A), endomannanase (B) and endoxylanase (C) enzyme activities in nkat/mL detected for *A. niger* D15[egII], D15[*man1*] and D15[xyn2], respectively, after 3 days of cultivation at 30°C in UPM and PPM. Data represents the mean values with errors bars indicating the standard deviation (n=3).

The next experiment focused on the use of the pressed pomace (PPM2) which contained the same amount of traditional medium. Antibiotics were also added to inhibit bacterial growth. The extracellular enzyme activities were measured on day 3 and day 6 (Figure 1.3) and compared with

that of the negative control strain. The highest levels of activity were obtained on PPM2-50% (50% pomace, w/v) medium, probably due to the increase in the native enzyme activities that is evident from the increase in activities of the negative control strain.

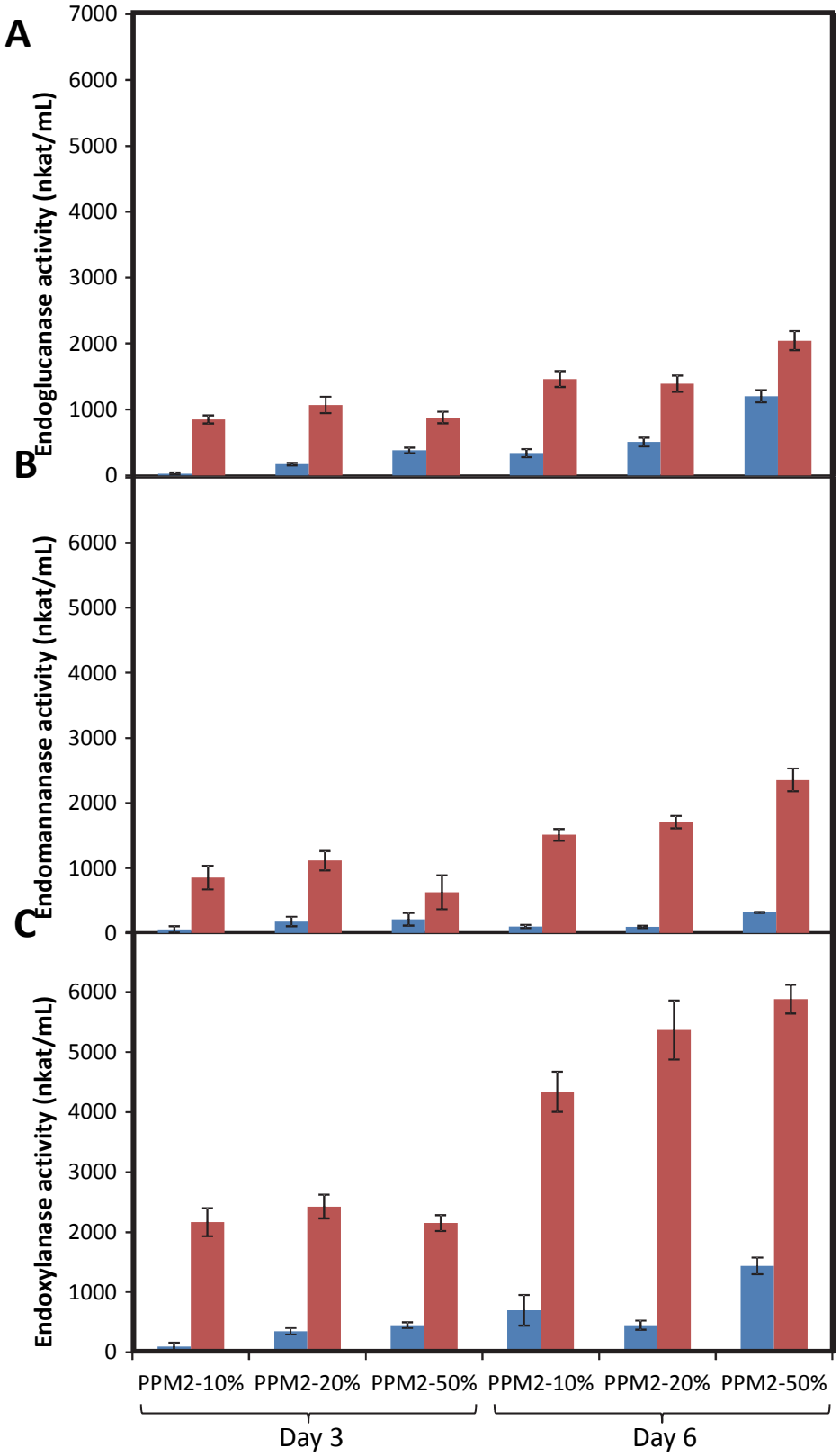


Figure 1.3: The extracellular endoglucanase (A), endomannanase (B) and endoxylanase (C) enzyme activities in nkat/mL detected for the *A. niger* D15[pGT] (blue columns), *A. niger* D15[egII], D15[*man1*] and D15[*xyn2*] (red columns) after 3 and 6 days of cultivation at 30°C in PPM2 medium. Data represents the mean values with errors bars indicating the standard deviation (n=3).

1.3.2 Visual observations

The pomace is a fibrous mass, making it impossible to determine the actual biomass production of the strains by standard filtration and microscopy means. The initial trials had been conducted using only diluted pomace in various concentrations with no addition of a nitrogen source or trace elements. The trials had been based on a similar study (Rose and Van Zyl, 2002) that had been successfully conducted using diluted sugar cane molasses (the compositions differ from apple pomace). No visible growth was observed in any of the pomace dilutions after 7 days of cultivation. No enzyme activity were detected for all three strains. Therefore, the growth medium compositions had been amended (Table 1.1). PPM-10% (10% pomace, w/v) and 20% (20% pomace, w/v), as well as UPM-10% (10% pomace, w/v) and 20% (20% pomace, w/v) is a viscous liquid medium (Appendix C). Agitation allows for mixing, but not necessarily proper oxygen transfer. Oxygen is required for fungal biomass production. Insufficient biomass production directly influences enzyme production. PPM-50%, UPM-50% and UPM2-50% (all three containing 50% pomace, w/v) can be classified as solid state fermentations with agitation resulting in no mixing of the PPM-50% medium and little mixing (slight vibration) of the UPM-50% and UPM2-50% media. The high pomace concentrations were included as solid state fermentations are known to produce high levels of enzyme production (Dhillon et al., 2013).

1.3.3 Chemical analyses of the starting material and spent waste

For the purpose of the chemical analyses, tests were limited to spent waste from fungal strains cultivated in PPM2 (medium in which the highest enzyme production occurred; Figure 1.3). An analysis of the starting materials (unpressed pomace and pressed pomace), revealed that at the lower pomace concentration (10%, w/v), the COD levels of the pomace samples and the spent waste were similar (Figure 1.4). This is probably due to the lower degree of growth at the low concentration, indicating a decreased utilisation of the sugars present and low enzyme production and decreased release of phenolic components in the apple pomace. At the higher concentration of apple pomace (20%, w/v), COD levels in the spent are almost half of that of the pomace samples (Figure 1.4).

The COD concentrations are influenced by both the presence of phenolics as well as any sugars. Components in the traditional medium contributed towards the total phenolics readings, but this was taken into account when the total phenolics for the unpressed pomace and pressed pomace samples were determined. Total phenolics increased in the spent waste for the *A. niger* D15[*egll*] (EG) and D15[*xyn2*] (EX) fungal strains, probably due to the activity of the enzymes on the material present in the pomace, resulting in the release of phenolic components (Figure 1.5).

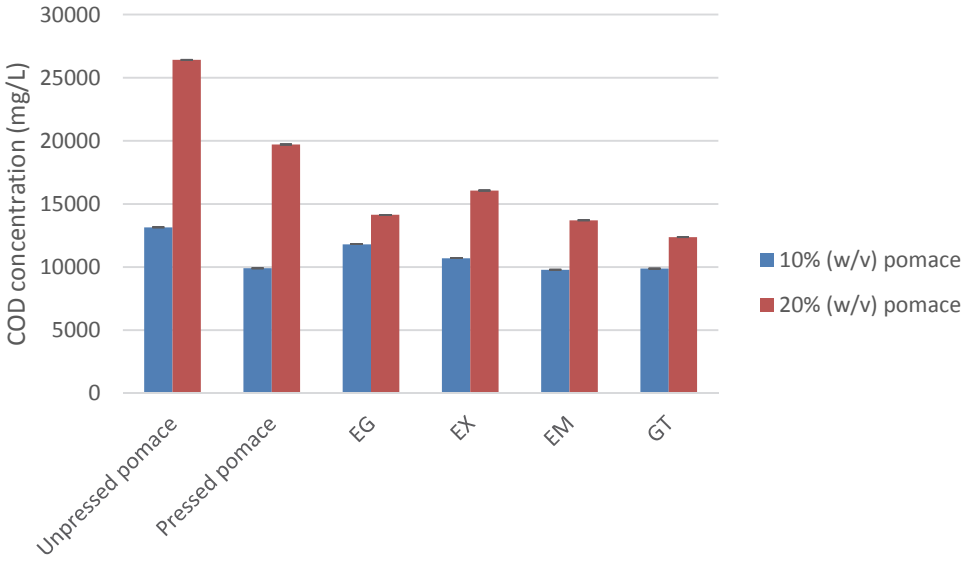


Figure 1.4: The COD levels of starting materials (pressed and unpressed pomace) as well as the spent from fungal strains cultured in PPM2. *A. niger* D15[*egII*] (EG), D15[*man1*] (EM), D15[*xyn2*] (EX) and D15[pGT] (GT). Data represents the mean values with errors bars indicating the standard deviation (n=2).

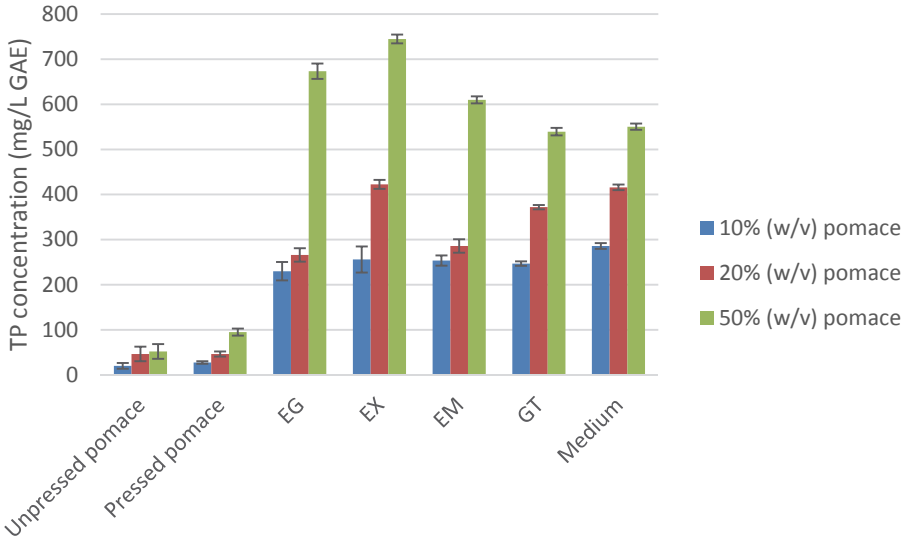


Figure 1.5: Total phenolics (as expressed in gallic acid equivalents (GAE)) as determined for the starting materials as well as the spent from fungal strains cultured in PPM2. *A. niger* D15[*egII*] (EG), D15[*man1*] (EM), D15[*xyn2*] (EX) and D15[pGT] (GT). Data represents the mean values with errors bars indicating the standard deviation (n=2).

Even though the total phenolics increased, the sugar content decreased (Figure 1.6). With the growth of the fungal strains, it was expected to see the decrease in total sugar content. This could also explain the decrease in COD levels observed for the spent waste from fungal strains cultivated in 20% (w/v) PPM, even if the total phenolics had increased. It is clear that all the fungal strains rapidly utilized the fructose (dominant sugar present in the starting material), followed by the glucose and then sucrose, with the exception of the D15[pGT] strain, which completely utilized the sucrose prior to utilisation of the glucose (Figure 1.6).

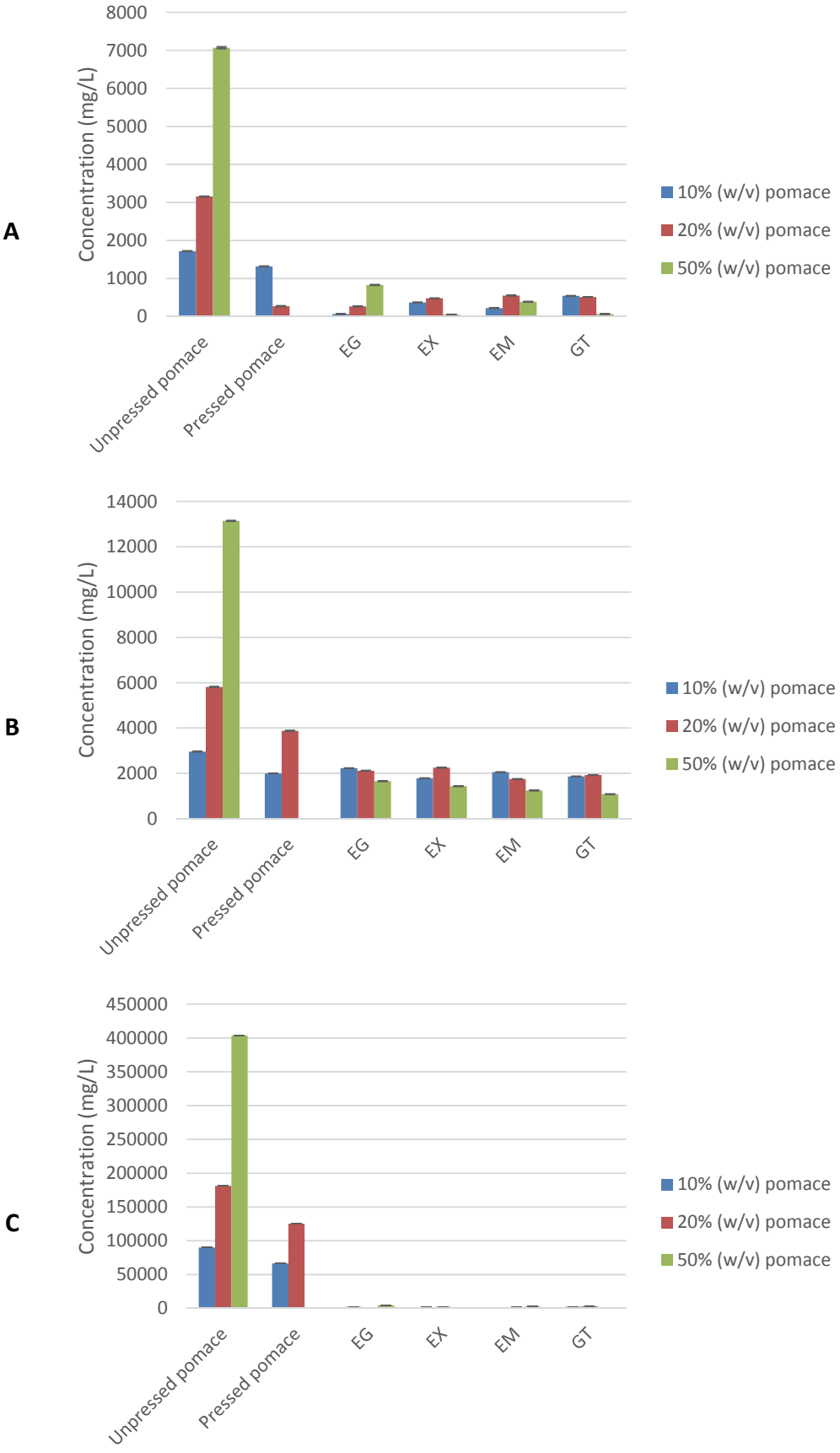


Figure 1.6: Concentrations (mg/L) of the dominant sugars, glucose (A), sucrose (B) and fructose (C) in the starting materials as well as the spent from fungal strains cultured in PPM2. *A. niger* D15[egII] (EG), D15[*man1*] (EM), D15[*xyn2*] (EX) and D15[pGT] (GT). Data represents the mean values with errors bars indicating the standard deviation (n=2).

In conjunction with the utilisation of the sugars in the pomace starting material, an increase in the total protein content in the spent waste was observed (Figure 1.7). This also corresponded with the increase in enzyme activity detected in the spent waste samples (Figure 1.3).

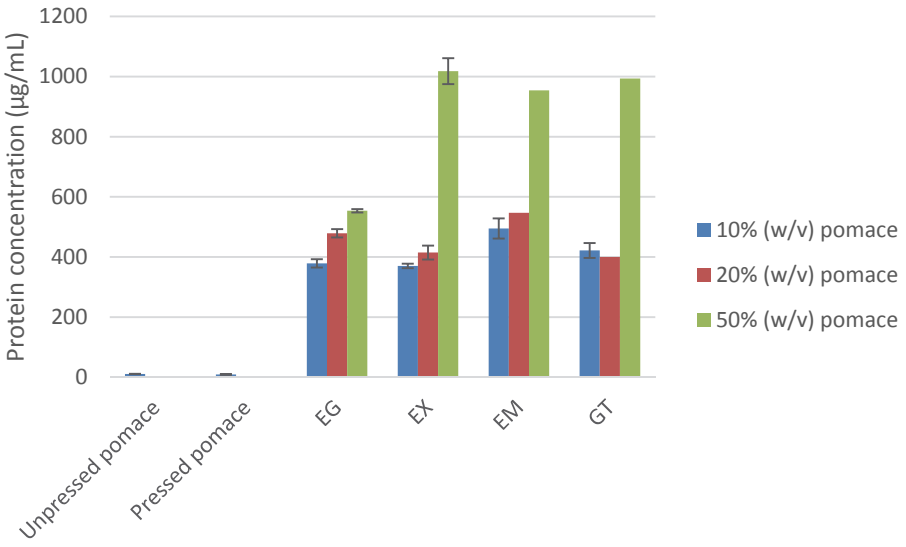


Figure 1.7: Total protein concentration (µg/mL) for the starting materials as well as the spent from fungal strains cultured in PPM2. *A. niger* D15[*egII*] (EG), D15[*man1*] (EM), D15[*xyn2*] (EX) and D15[pGT] (GT). 10% = 10%, w/v; 20% = 20% w/v; 50% = 50% w/v. Data represents the mean values with errors bars indicating the standard deviation (n=2).

1.4 Recommendations for the next phase of the project

Multiple magnetic stirrers will be required to obtain proper mixing and aeration of the medium. Cultivation medium still needs to be optimized in terms of the amount and concentration of pomace that can be used in order to produce an industrially (economically) feasible medium. The next chapter will report on the identification of the best recombinant fungal strains and their enzymes in 3-5 L bioreactors, and developing more evidence to fit the proposed conceptual model.

CHAPTER 2: IDENTIFICATION OF TARGET ENZYMES AND STRAINS

2.1 Introduction

Lignocellulose is one of the major carbohydrates found in fruit processing wastewater and there has, and continues to be, extensive research carried out on the subject of lignocellulose degradation. Sugars and phenolic compounds are also commonly found in fruit processing water. For example, Friedrich et al. (1986) states that apple slop (containing 3.4% dry matter) from the distillery industry consists of 20.4% cellulose, 14.7% lignin, 14.1% hemicellulose, 1% pectin, 12% glucose and small amounts of proteins and lipids. The processing of fruits in the canning, juicing, winery and distillery industries also produces a certain amount of solid residue, known as pomace, which has a high moisture and sugar content and can be added to the wastewater streams. In the production of citric juice, the solid residues (up to 50%) can be pressed to generate press cake and a sugar-rich leachate. When the press cake is dried to 10% moisture, it is composed of sugars (30-40%), pectin (15-25%), cellulose (8-10%) and hemicellulose (5-7%) (Widmer et al., 2010). The sugar-rich leachate contains about 10% sugars, which can be concentrated by membrane technology to 25-30% before fermented to ethanol (Garcia-Castello et al., 2006). However, limonene needs to be removed by steam stripping since it can act as inhibitor of yeasts (Pourbafrani et al., 2010) when citrus waste is used. Apple pomace, in particular, represents about 25% of the original fruit after the juice has been extracted (Zheng and Shetty, 1998) and contains about 44% cellulose, 24% hemicellulose and 20% lignin (Nawirska and Kwasniewska, 2005).

Potatoes are a common root vegetable that are used for a variety of purposes. While we often think of potatoes as a vegetable cooked from fresh, it is estimated that less than 50% of potatoes grown globally are consumed fresh (http://cipotato.org/potato/processing-uses/). The majority of the potato crop is processed into food products and ingredients, used as an additive in animal feed, processed into starch and re-used as seed tubers for the next season's crop. In South Africa, the potato processing industry has grown at a rapid pace over the last decade and currently, 19% of the total potato crop is processed (http://www.potatoes.co.za/processing-industry.aspx). Globally, consumption of potato as food is shifting from fresh potatoes to processed potato products including frozen potatoes. Furthermore, the growth in the potato processing industry in South Africa can be attributed to: the rapid expansion of the fast food industry (increasing demand for frozen French fries and similar products); increase in the average income of the population, enlargement of processing facilities and the rapid rate of urbanization (http://www.potatoes.co.za/processing-industry.aspx). Currently, South Africa processes about 380 000 tons of fresh potatoes, the majority of which is used to make French fries, frozen and chilled products and crisps (http://www.potatoes.co.za/processing-industry.aspx). A small part of South Africa's potato processing industry also produces other products such as canned and frozen mixed vegetables, baby food, reconstructed potato products and potato starch (http://www.potatoes.co.za/processing-industry.aspx). The following picture shows the size of SA's potato crop in 2011 (Table 2.1).

Table 2.1: Potato production in sixteen regions in South Africa in 2011 (http://www.potatoes.co.za/processing-industry.aspx).

Potato production in 16 regions - 2011 crop year

Region	Hectares	% of hectares	10 kg bags	% of total 10 kg bags
Eastern Free State	9 833	19%	29 012 039	13%
Limpopo	9 315	18%	42 552 940	19%
Sandveld	6 818	13%	30 694 945	14%
Western Free State	6 421	12%	30 433 300	14%
KwaZulu-Natal	4 603	9%	19 575 700	9%
Mpumalanga	2 830	5%	12 348 118	6%
North West	2 553	5%	11 887 578	5%
Northern Cape	2 138	4%	8 059 806	4%
South Western Free State	1 600	3%	7 216 350	3%
North Eastern Cape	1 544	3%	7 023 100	3%
Eastern Cape	1 436	3%	6 296 000	3%
Loskop Valley	1 292	2%	4 738 300	2%
Gauteng	1 005	2%	3 996 925	2%
Ceres	885	2%	4 567 772	2%
Southern Cape	183	0.3%	779 869	0.4%
South Western Cape	107	0.2%	478 500	0.2%
	52 563		219 661 242	

Potato processing produces solid wastes that consist of peels and whole or cut potatoes that are discarded due to size, blemishes or in some way failure to meet quality standards for processing. The waste and discarded potatoes can be collected at different stages during the process, constituting different waste streams (e.g. peels/skins separate from potato slivers as they are collected at different points in the process). These high starch-containing wastes may be useful in the implementation of a biorefinery concept.

The application of enzymes for industrial processes is important for economic development and is a pivotal part of South Africa's Bioeconomy Strategy. Laccases (benzenediol/oxygen oxidoreductases; EC 1.10.3.2) are well-known blue multi-copper oxidases. Laccases are industrially significant enzymes as they have a number of diverse applications, including various biocatalytic purposes such as delignification of lignocellulosics, cross-linking of polysaccharides and various bioremediation applications among others. The white-rot fungus, *Trametes pubescens*, is recognised as an efficient laccase enzyme producer. The use of industrial and agricultural wastes for laccase production by white-rot fungi is an effective way to reduce the cost of production (Rodriguez, 2008) and has the added potential to improve the nutritional value of agri-industrial by-products for animal feed or soil fertilizer (Gonzalez et al., 2013). The application of fungal laccases for industrial application is only feasible if high levels of the enzyme are available. While many reports mention convenient, reliable production systems (which include inexpensive, optimized media for large-scale production), growth of laccase-producing fungi using agricultural residues presents a promising alternative. These residues are abundant and readily available and fall into the global bioeconomy aim of utilisation and value-addition of waste products.

Additionally, they provide the nutrients required for fungal growth, while the presence of lignin acts as a natural inducer for the ligninolytic enzymes, including laccase (Couto and Sanromán, 2005).

Aspergillus spp., notably *A. niger* and *A. oryzae*, have been used for more than 2000 years in the Orient for the production of fermented food and products such as citric acid and soya (Hesseltine, 1983). These fungi produce copious amounts of enzymes that can hydrolyse starch, pectin and cellulosics (de Vries and Visser, 2001; Jin et al., 1998). *Aspergillus* spp. can also degrade and utilize a wide range of phenolic compounds (Garcia Garcia et al., 1997), including compounds present in olive mill wastewaters (Garcia Garcia et al., 2000; Hamdi et al., 1991; Vassilev et al., 1997). The ability of *Aspergillus* spp. to produce extracellular enzymes in large quantities and to utilize recalcitrant phenolic compounds, make them ideal for cleaning up more complex organic matters in waste streams.

Aspergillus niger has long been used for industrial enzyme production, in particular by Novozymes and DSM and remains the preferred organism for industrial enzyme production. In this project, we have access to various *A. niger* strains that overproduce cellulases, xylanases and mannanases (Rose and van Zyl, 2002; Rose and van Zyl, 2008) and a laccase (Bohlin et al., 2006). Enzyme production in grams per litre was demonstrated for a mannanase (Van Zyl et al., 2009). In collaboration with the laboratory of Prof. Leif Jonsson at Umea University, Sweden, they demonstrated that the *A. niger* strains can be grown on the waste streams of lignocellulosics after the hexose sugars in sugarcane bagasse and northern spruce hydrolysates were fermented to ethanol with industrial yeast strains. At the same time, the *A. niger* strains produced a cellulase and xylanase (Alriksson et al., 2009; Cavka et al., 2011). In principle, it should be possible to grow *A. niger* strains on the spent waste streams after ethanolic fermentation, olive mill waste streams, or fruit processing waste streams with the simultaneous production of high-valued enzymes.

In this section of the work, proof-of-concept experiments were performed to determine the suitability of recombinant *A. niger* strains to produce useful enzymes on three bio-based waste streams (apple pomace, grape waste and potato waste). The abilities of the recombinant strains to produce enzymes under these cultivation conditions were determined. Analytical tests were performed to determine the properties of the waste samples and how these properties were altered due to the growth of the fungal strains. This section also reports on small-scale production of laccase from *T. pubescens* on apple pomace and potato wastes and larger scale (3 L airlift reactor) laccase production on potato waste in order to determine the suitability of this strain for industrially useful enzyme (laccase) production using bio-based waste streams.

2.2 Materials and Methods

2.2.1 Biomass (carbohydrate source)

2.2.1.1 Apple pomace

Apple pomace for laboratory studies was prepared by extracting the juice from 'Golden Delicious' apples purchased from local supermarkets. The pulp (pomace) remaining after juicing was homogenized in a blender. For 'pressed pomace' (PP), the homogenised pomace was placed in a sieve with cheesecloth and washed with tap water to remove residual sugars. For 'unpressed

pomace' (UP), the pomace was not washed and excess liquid in the homogenate after homogenisation was retained. The pomace was stored at -20°C until use.

2.2.1.2 Grape pomace

The pressed white and red grape waste, were supplied by a local winery located in Stellenbosch and stored at -20°C until use. The waste was obtained through the assistance of Mr Jacques Roussouw, Environmental Manager, Distell.

2.2.1.3 Potato waste

Lamberts Bay Foods supplied four different potato wastes (kouebelt detector [KD], langebelt detector [LD], slivers [SL], skins and starch [SK]). The KD waste consisted of chips with defects collected after the steam peeler and oil fryer treatment. The LD waste consisted of chips with defects collected after the steam peeler treatment. The SL waste consisted of small potato off-cuts. SL and SK were collected after the steam peeler treatment. The potato waste was stored at -20°C until use. The wastes were thawed and roughly chopped before use.

2.2.2 *Aspergillus* strains and media

Strains had previously been constructed in-house (Rose and Van Zyl 2002; 2008) and are known to produce high levels of extracellular enzyme activity.

2.2.2.1 Spore solution preparation

Spores were collected with a toothpick and streaked out onto spore plates in a grid form. Spore plates contained (g/L) 18 agar, 2 neopeptone, 1 yeast extract, 0.4 $MgSO_4.7H_2O$, 10 glucose and 2 casamino acids. Twenty mL Asp+ (g/L, 300 $NaNO_3$, 76 KH_2PO_4 and 26 KCl) and 1 mL of trace elements, which were added after autoclaving. Plates were incubated at 30°C for 4 to 7 days. The spore plates were flooded with ±7 mL physiological salt (0.9%, w/v, NaCl) and the spores loosened with cotton sticks. When a thick spore suspension formed on the plate, the suspension was pipetted into a sterile 15 mL Falcon tube. The tube was vortexed to loosen the spore clumps and the spores were counted. The spore suspension was stored at 4°C or in 50% glycerol as cryocultures at -80°C.

2.2.2.2 Cultivation medium

Fungal strains were cultivated in pressed pomace medium (PPM3), pressed white grape medium (PGM) and potato waste medium (PWM) (Table 2.2)

Table 2.2: The composition of the pressed apple pomace medium, pressed grape medium, and potato waste medium

A. Pressed apple pomace medium (PPM)		
Components	PPM-10%	PPM-20%
Pressed pomace	2 g	4 g
Traditional medium[1]	18 mL	16 mL
Asp+	800 µL	800 µL
Trace elements	40 µL	40 µL
Streptomycin 30 mg/mL	20 µL	20 µL
Ampicillin 100 mg/mL	20 µL	20 µL

[1] The amount of traditional medium added to the flasks differ to compensate for the difference in volume of the biomass. [2] KD – Koue belt, chips defects, [3] LD – Lang belt, chips defects, [4] Slivers – small off cuts

Table 2.2 B. Pressed grape medium (PGM)					
Components	PGM-10%		PGM-20%	PGM-50%	
Pressed white grape	2 g		4 g	10 g	
Traditional medium[1]	18 mL		16 mL	10 mL	
Asp+	800 µL		800 µL	800 µL	
Trace elements	40 µL		40 µL	40 µL	
Streptomycin 30 mg/mL	20 µL		20 µL	20 µL	
Ampicillin 100 mg/mL	20 µL		20 µL	20 µL	
C. Potato waste medium (PWM-10%)					
Components	KD[2]	LD[3]	Slivers[4]	Skins	Starch
Biomass (grated)	2 g	2 g	2 g	2 g	2 g
Traditional medium[1]	18 mL	18 mL	18 mL	18 mL	18 mL
Asp+	800 µL	800 µL	800 µL	800 µL	800 µL
Trace elements	40 µL	40 µL	40 µL	40 µL	40 µL
Streptomycin 30 mg/mL	20 µL	20 µL	20 µL	20 µL	20 µL
Ampicillin 100 mg/mL	20 µL	20 µL	20 µL	20 µL	20 µL
D. Potato waste medium (PWM-20%)					
Components	KD[2]		Slivers[4]	Starch	
Biomass (warring blender)	4 g		4 g	4 g	
Traditional medium[1]	18 mL		18 mL	18 mL	
Asp+	800 µL		800 µL	800 µL	
Trace elements	40 µL		40 µL	40 µL	
Streptomycin 30 mg/mL	20 µL		20 µL	20 µL	
Ampicillin 100 mg/mL	20 µL		20 µL	20 µL	

[1] The amount of traditional medium added to the flasks differ to compensate for the difference in volume of the biomass. [2] KD – Koue belt, chips defects, [3] LD – Lang belt, chips defects, [4] Slivers – small off cuts

2.2.2.3 Cultivation

Strains were inoculated in the different media to a spore count of 5×10^5 spores per mL. Cultivation took place at 30°C at an agitation speed of 200 rpm.

2.2.2.4 Enzyme preparation

All *A. niger* strains were cultivated in 500 mL 2×MM for three days (Rose and van Zyl, 2002, 2008).

The supernatant (approximately 300 mL) was harvested through myracloth, frozen at -80°C overnight and lyophilized in a Vitis freeze dryer. PPM3 has the same media composition as PPM2 (previous chapter), but the traditional medium and apple pomace was used without autoclaving. Traditional medium contains (g/L) 10 yeast extract, 0.8 $MgSO_4.7H_2O$ and 4 casamino acids. The biomass (grape waste and potato waste) and traditional medium were autoclaved, after which the Asp+ and trace elements were added. The amount of water in the traditional medium had been amended for the different media to compensate for the difference in volumes used. Therefore, all media were identical in composition except for the biomass component. Final volume in the flasks was approximately 21 mL.

2.2.3 *Trametes pubescens* culture preparation

Trametes pubescens cultures available at the BTB research group (CPUT) were maintained on *Trametes* Defined Media (TDM) agar plates, g/L: 10 glucose, 5.25 peptone, 2 KH_2PO_4, 0.5 $MgSO_4$, 0.1 $CaCl_2$, 0.3 NaCl, 12 agar and 10 mL trace elements solution consisting of, g/L: 0.6 $FeSO_4$, 0.03

$CuSO_4$, 0.07 $ZnCl_2$, 0.34 $MnSO_4$, 0.19 $CoCl_2$, 0.002 $NiCl_2$, 0.62 $(NH_4)_2MoO_4$. Cultures were incubated at 28°C for 5-7 days and plates were stored at 4°C.

2.2.3.1 Inoculum

A homogenate of *T. pubescens* was used to inoculate experimental cultures. One TDM agar plate covered in *T. pubescens* (5-7 days' growth) was homogenised per 100 mL sterile dH_2O in a Waring blender. 0.5 mL homogenate per 10 mL culture volume was used for inoculation. 300 mL homogenate was used to inoculate 3 L (final culture volume) airlift reactors.

2.2.4 Assays and analytical tests

2.2.4.1 Endoglucanase, endomannanase and endoxylanase

The extracellular endoglucanase, endomannanase and endoxylanase activity was determined using the reducing sugar method according to Bailey et al. (1992) and the substrates specific for each enzyme (Table 1.1). All assays were performed at 50°C and pH 5. The supernatant was appropriately diluted prior to the 5 minute incubation with the substrates. The reactions were terminated by the addition of the 3,5-dinitro-salicylic (DNS) acid solution. The samples were boiled for 15 minutes at 100°C to assist in colour development. Glucose, mannose and xylose were used as standards for the endoglucanase, endomannanase and endoxylanase activities. The colorimetric changes were measured spectrophotometrically at 540 nm with an X-MARK™ microtitre plate reader (Biorad, Hercules, CA, USA). The activities were expressed in nkat/mL, where 1 katal equals 1 mole of glucose, mannose or xylose released per second.

2.2.4.2 Laccase

Laccase from *Trametes versicolor* (TvL) was purchased from Sigma-Aldrich and resuspended in 50 mM sodium tartrate buffer (pH 4.5) for use. Dilutions of TvL were used as positive controls for laccase assays. Laccase activity in experimental cultures was routinely measured in microtitre plate format (Anthos Xenyth 1100 instrument) using the ABTS assay [0.5 mM ABTS; 420 nm; ε = 36 000 /M/cm; sodium acetate buffer (pH 5.0)].

2.2.4.3 Chemical Oxygen Demand (COD)

Chemical oxygen demand (COD) of the different samples was measured using the COD Cell Test kits (photometric method; Merck; equivalent to method 5220D described in the *Standard Methods for the Examination of Water and Wastewater*). Samples (3 mL) were added to COD cells. The tubes were heated in a pre-heated heating block at 148°C for 2 hours. The concentration of COD in mg/L was determined automatically using a Merck (Dramstradt, Germany) Spectroquant Pharo instrument.

2.2.4.4 Total Phenolics (Folin-Ciocalteau micro method)

Gallic acid standards of 0 mg/L, 50 mg/L, 100 mg/L, 150 mg/L, 250 mg/L and 500 mg/L were used to compile standard graphs each time the test was performed. 20 µL of each of the standard dilutions was added to 1.58 mL distilled water in cuvettes. For the blank, 1.6 mL distilled water was added to a cuvette. 20 µL of the effluent samples were each added to 1.58 mL distilled water in cuvettes. 100 µL Folin-Ciocalteau reagent was added to all cuvettes, mixed well and allowed to stand for 5 minutes. 300 µL sodium carbonate solution was added to all cuvettes, mixed well and allowed to stand for 2 hours in the dark. The spectrophotometer was blanked and the absorbance of the standards and test samples were read at 765 nm. A standard graph of absorbance against

concentration was plotted and the straight line equation was used to calculate the test concentrations. Results were reported in mg/L gallic acid equivalents (GAE).

2.2.4.5 Quantification of dominant sugars

The concentrations of the major sugars (sucrose, fructose and glucose) were determined by HPLC using a Phenomenex® Rezex RHM-monosaccharide H+ (8% cross-linkage) column according to the method described by La'Zaro et al. (1989). The concentrations of organic molecules that were detected were quantified using relevant standard graphs prepared from HPLC chromatograms. The mobile phase used was 1% H_2SO_4.

2.2.5 Yeast fermentation on potato waste

2.2.5.1 Biomass (carbohydrate source)

Lamberts Bay Foods supplied different potato wastes from different points in the processing operation. The potato waste was stored at -20°C prior to use.

2.2.5.2 Yeast strain and pre-culture preparation

The commercially available yeast strain, Ethanol Red, was used for the fermentation of the potato waste. Pre-cultures were prepared in YPD medium (g/L: 10 yeast extract, 20 peptone and 20 glucose). Cultures were grown overnight to a cell density of 3×10^8 cells per mL. A 10% inoculum was used for the fermentations. YPWM-10% and YPWM-20% was used as fermentation media (Table 2.3). The YPWM-10% was used without sterilization. The YPWM-20% medium (50 mL) was heated in a microwave oven to 60°C (approximately 2 minutes; remove most of the contaminating organisms), followed immediately by the addition of the remaining medium (at room temperature).

Fermentation bottles were inoculated to a concentration of 1×10^7 cells per mL (Appendix D). Ampicillin (100 µg/mL) and streptomycin (15 µg/mL) were added to inhibit bacterial contamination. Agitation and incubation were performed on a magnetic multi-stirrer at 37°C, with regular sampling through a syringe needle pierced through the rubber stopper.

Table 2.3: Composition of the fermentation media

Yeast Potato waste medium (YPWM-10%) – No heat treatment			
Components	KD[2]	Slivers[4]	Starch
Biomass	10 g	10 g	10 g
YP medium[1]	90 mL	90 mL	90 mL
Streptomycin 30 mg/mL	100 µL	100 µL	100 µL
Ampicillin 100 mg/mL	100 µL	100 µL	100 µL
Stargen 002[4]	100 µL	100 µL	100 µL
Yeast Potato waste medium 2 (YPWM-20%) – heated to 60°C in microwave			
Components	KD[2]	Slivers[4]	Starch
Biomass	20 g	20 g	20 g
YP medium[1]	90 mL	90 mL	90 mL
Streptomycin 30 mg/mL	100 µL	100 µL	100 µL
Ampicillin 100 mg/mL	100 µL	100 µL	100 µL
Stargen 002[4]	100 µL	100 µL	100 µL

[1]YP medium (10 g/L yeast extract, 20 g/L peptone). [2] KD – Koue belt, chips defects, [3] LD – Lang belt, chips defects, [4] Slivers – small off cuts, [4] Control fermentations did not contain Stargen 002

2.2.5.3 Ethanol analysis

Ethanol concentrations (in supernatant) were quantified with high performance liquid chromatography (HPLC), using a Surveyor Plus liquid chromatograph (Thermo Scientific) consisting of a LC pump, auto sampler and Refractive Index Detector. The compounds were separated on a Rezex RHM Monosaccharide 7.8 × 300 mm column (00H0132-K0, Phenomenex) at 60°C with 5 mM H_2SO_4 as mobile phase at a flow rate of 0.6 mL/min.

2.2.5.4 Calculations

The HPLC data (ethanol concentration in the supernatant) was converted to percentage ethanol (of the maximum theoretical yield) taking into account the dry weight of the potato waste. Examples of the calculations are presented in Table 2.4.

Table 2.4: Conversion of ethanol (g/L) to % ethanol of theoretical yield

	Starch 100 g/L = 100 g DW/L		Slivers 100 g/L = 30 g DW/L	
Time in hours	Ethanol in supernatant	% ethanol (of a theoretical yield of 51 g/L)	Ethanol in supernatant (HPLC)	% ethanol (of a theoretical yield of 15.3 g/L)
0	0.55	1.08	0.45	2.95
44	2.82	5.53	3.81	24.91
92	5.39	10.57	4.41	28.81
140	7.40	14.52	4.85	31.72
188	11.58	22.71	4.63	30.24

2.2.6 Laccase production by *T. pubescens*

2.2.6.1 Apple pomace (10 mL culture volumes)

Aliquots of 0.5 mL 5-day *T. pubescens* pre-cultures (in TDM) was used to inoculate 10%, 20%, 30% and 50% pressed (PP) and unpressed (UP) apple pomace suspended in dH_2O in 10 mL final culture

volumes. Flasks were incubated at 28°C, shaking at 200 rpm. Samples were taken after 3, 5, 7 and 9 days of incubation and assayed for laccase activity using the ABTS assay.

2.2.6.2 Potato wastes (20 mL culture volumes)

Aliquots of 1 mL *T. pubescens* homogenate was used to inoculate flasks with each of the four potato waste types (KD, LD, SL and SK) at 10% and 20% potato waste suspended in dH_2O in 20 mL final culture volumes. Flasks were incubated at 28°C, shaking at 200 rpm. Samples were taken after 5 and 7 days of incubation and assayed for laccase activity using the ABTS assay.

2.2.6.3 Potato wastes (airlift reactor, 3 L)

Subsamples of 300 mL *T. pubescens* homogenate was used to inoculate an airlift reactor containing 10% roughly chopped potato chips (McCains) suspended in dH_2O. McCains frozen chips were used as a substitute for potato waste as there was insufficient authentic potato waste sample for the reactor requirements. The frozen chips were thawed and roughly chopped before use. Antifoam 204 (Sigma-Aldrich) was added to the reactor (at 1 mL/L) upon inoculation to prevent foaming and approximately 500 mL sterile dH_2O was added to the reactor daily to maintain the volume. The reactor was incubated at 28°C for 5 days and filtered air was supplied at a rate of 8 L/min.

2.2.7 Biocatalysis using T. pubescens laccase

Laccase produced by *T. pubescens* during small-scale (10 mL) production using KD potato waste and larger-scale production (3 L) using substitute potato waste was assessed for the ability to couple ferulic acid monomers (Adelakun et al., 2012). Biocatalysis reactions were setup in 10 mL volume. Each reaction contained 2.5 U of *T. pubescens* laccase or 1 mL supernatant from the potato waste reactor or small scale tests, 10 mM ferulic acid and 80% (v/v) ethyl acetate. The ferulic acid was prepared in 100 mM sodium acetate buffer, pH 5. Reaction mixtures were incubated at 30°C for 24 hrs, shaking at 160 rpm. After 24 hrs, the ethyl acetate layer was removed and allowed to dry down in a fume hood. The dried extract was resuspended in 1 mL ethyl acetate and analysed by thin layer chromatography (TLC). 1 µL of each sample was applied to a silica F_{254} plate and the samples separated using the following solvent system: chloroform – ethyl acetate – formic acid (5:4:1, v/v/v). Products were visualised under UV, 254 nm. The antioxidant activity of the products formed was determined by dabbing the TLC plate with a 2,2-diphenyl-1-picrylhydrazyl (DPPH) solution (0.025 mg/mL DPPH in methanol). Reduction of the purple pigment in DPPH to a clear solution, serves as an indication that antioxidant activity is present; for a TLC plate, the plate would stain pink/purple, while areas with antioxidants would remain white.

2.3 Results and discussion

2.3.1 Enzyme production by *Aspergillus* strains

2.3.1.1 Apple pomace

The extracellular enzyme activities of the *A. niger* D15[pGT1], [*eg2*], [*man1*] and [*xyn2*] strains were determined on day 3 and day 6 in PPM3-10% and 20% medium (Figure 2.1). No additional (to the control strain) endoglucanase activity was detected, whereas reasonable levels of endomannanase and endoxylanase activity was detected after three days of cultivation, similar to what had previously been obtained on the autoclaved PPM2-10% and PPM2-20% medium. The endomannanase activity and endoxylanase activity dropped significantly on day 6 whereas in

PPM2-10% and PPM2-20%, the activity increased about 1.5 to 2-fold. The strains used in the study were designed to work best under high sugar concentrations (Rose and van Zyl 2002, 2008; van Zyl et al., 2009). The lack of autoclaving (no pre-treatment) resulted in less sugar being released. The available sugars are metabolized quickly. After 6 days of cultivation, little sugar remained resulting in less enzyme being secreted.

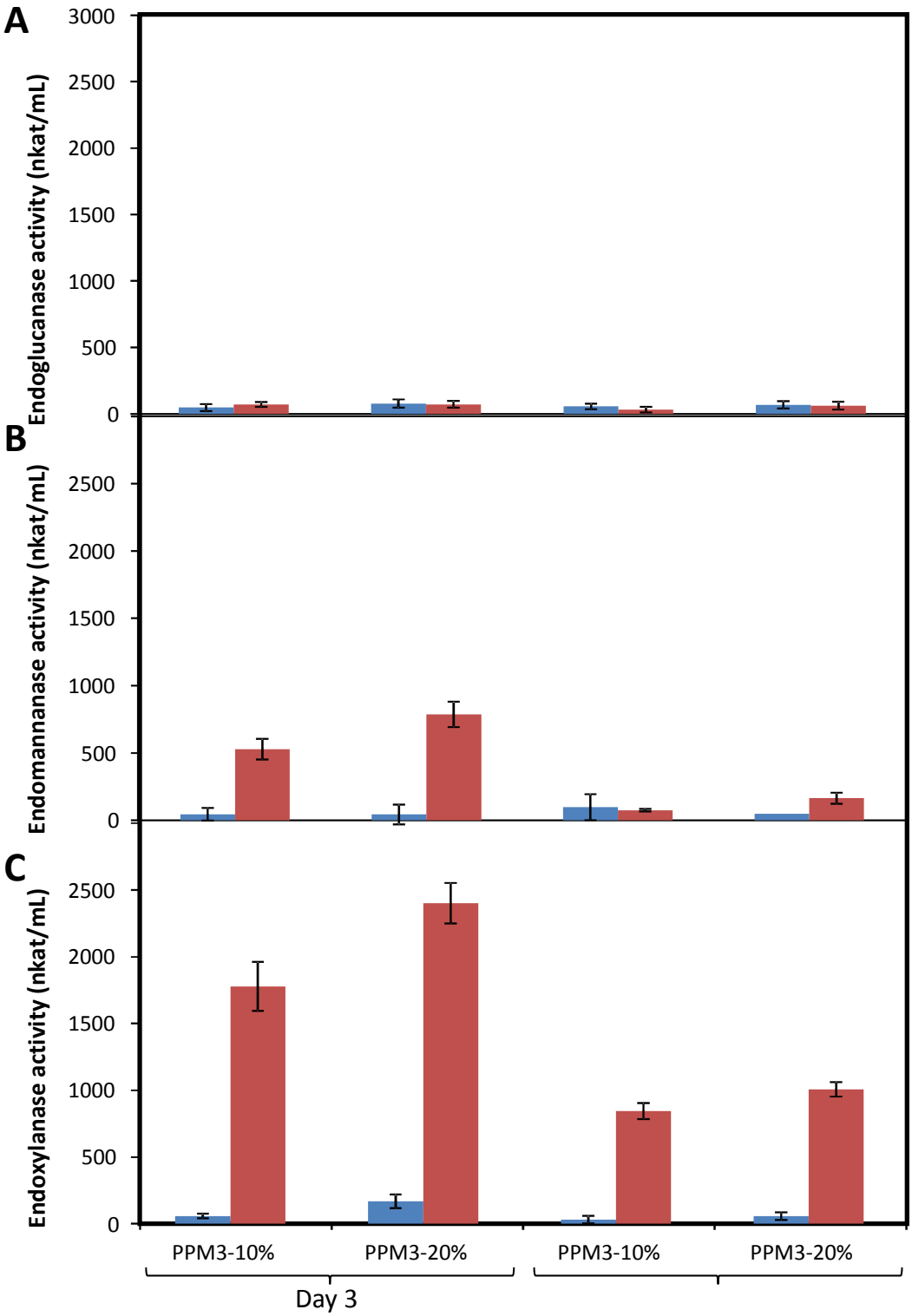


Figure 2.1: The extracellular (A) endoglucanase, (B) endomannanase and (C) endoxylanase enzyme activities detected for the *A. niger* D15[*eg2*], D15[*man1*] and D15[*xyn2*] after 3 and 6 days of cultivation at 30°C in PPM3 (unautoclaved apple pomace). Data represents the mean values with errors bars indicating the standard deviation (n=3).

The COD concentrations in both PPM3-10% and PPM3-20% preparations decreased with fungal treatment (Figure 2.2), indicating that the fungal strains most probably utilized the components in the PPM3 preparations, such as the sugars and phenolics, during growth. Total phenolic concentrations in both PPM3-10% and PPM3-20% preparations also decreased with fungal treatment (Figure 2.3), confirming the result obtained for the decreased COD readings.

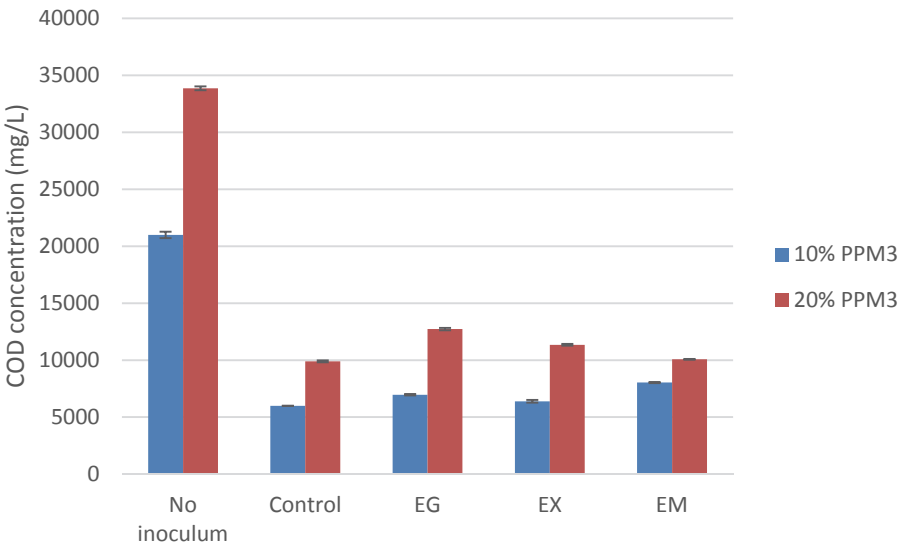


Figure 2.2: COD of the *A. niger* D15[*eg2*], D15[*man1*] and D15[*xyn2*] supernatants after 6 days of cultivation at 30°C in PPM3 (unautoclaved apple pomace). *A. niger* D15[*egII*] (EG), D15[*man1*] (EM), D15[*xyn2*] (EX) and D15[pGT] (control). Data represents the mean values with errors bars indicating the standard deviation (n=2).

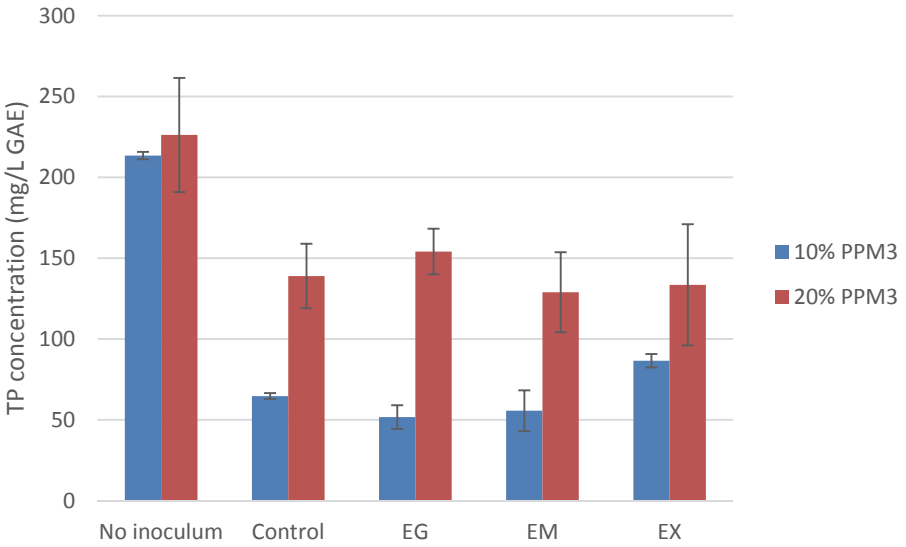


Figure 2.3: Total phenolics expressed as mg/L gallic acid equivalents (GAE) of the *A. niger* D15[*eg2*], D15[*man1*] and D15[*xyn2*] supernatants after 6 days of cultivation at 30°C in PPM3 (unautoclaved apple pomace). *A. niger* D15[*egII*] (EG), D15[*man1*] (EM), D15[*xyn2*] (EX) and D15[pGT] (control). Data represents the mean values with errors bars indicating the standard deviation (n=3).

2.3.1.2 Grape wastes

There was very little variation in the pH of the untreated grape waste suspensions, but overall, it was in the acidic range (<pH 4.0) (Table 2.5).

Table 2.5: pH of winery wastewater and grape solid wastes suspended in winery wastewater and dH_2O

		10%	20%	50%
White grape	solid + winery wastewater	pH 3.79	pH 3.8	pH 3.88
White grape	solid + distilled water	pH 3.75	pH 3.77	pH 3.82
Red grape	solids + winery wastewater	pH 3.63	pH 3.6	pH 3.56
Red grape	solids + distilled water	pH 3.79	pH 3.6	pH 3.57
Authentic winery wastewater		pH 3.97		

As expected, COD concentrations increased with increasing concentrations of untreated grape solids in different suspensions (
Table **2.**6).

Table 2.6: COD (mg/L) of winery wastewater and grape solid wastes suspended in winery wastewater and dH_2O

	10%	20%	50%
White grapes + winery wastewater	6370	10100	27360
White grapes + distilled water	6744	12250	31920
Red grapes + winery wastewater	3850	6380	19410
Red grapes + distilled water	4848	9990	16200
Authentic winery wastewater	3210		

White grape wastes contained less phenolics than red grape wastes (Figure 2.4), which was expected (it is well known that red grapes contain more phenolic compounds than white grapes; Kennedy, 2008).

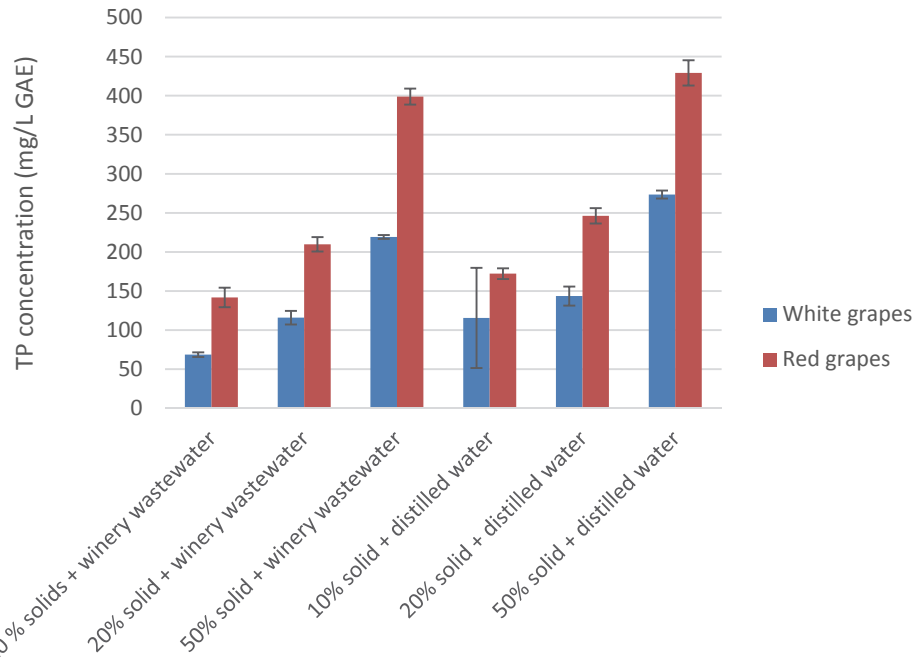


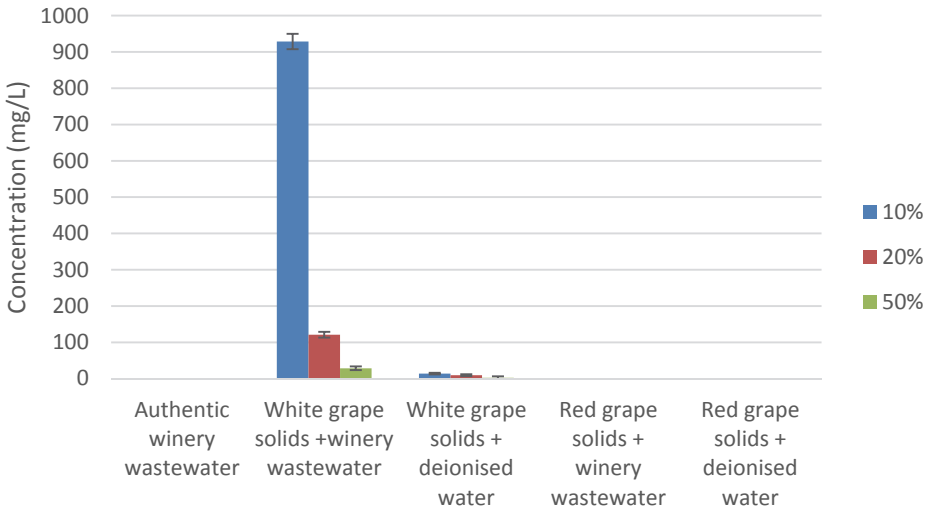
Figure 2.4: Total phenolic (TP) concentration expressed as mg/L gallic acid equivalents (GAE) present in white grape and red grape winery wastes.

The results for the three different sugars tested in untreated winery wastes were interesting (Figure 2.5). No sucrose could be detected in the authentic winery wastewater (Figure 2.5a) or in the red grape solutions. There also seems to be an inhibitory effect on the total sucrose concentration as the concentration of white grape solid waste added to the winery wastewater was increased (Figure 2.5a). The white grape waste contained higher sugar (fructose, glucose and sucrose) concentrations than red grape as was expected, since the white grape waste was obtained from the processing of special late harvest grapes. Initial experiments showed that the *A. niger* strains used in this study did not grow well in the presence of the red grape waste due to the low sugar concentrations, and the study concentrated on using white grape waste in the medium (PGM; Table 1.2).

The extracellular enzyme activities of all the *A. niger* D15[pGT1], [*eg2*], [*man1*] and [*xyn2*] strains were determined on day 3 and day 6 in PGM-10% and PGM-20% (Figure 2.6). Increasing the grape biomass had little effect on the activity produced by the strains. In general, the activity of all the strains was significantly lower than that obtained using apple pomace. Similar to the abovementioned study (with PPM3), this can be attributed to the lack of easily metabolisable sugars in the grape biomass.

The COD concentrations decreased considerably from >30000 mg/L (PGM-10%) and >55000 mg/L (PGM-20%) to <10000 mg/L (PGM-10%) and <20000 mg/L (PGM-20%) (Figure 2.7).

A



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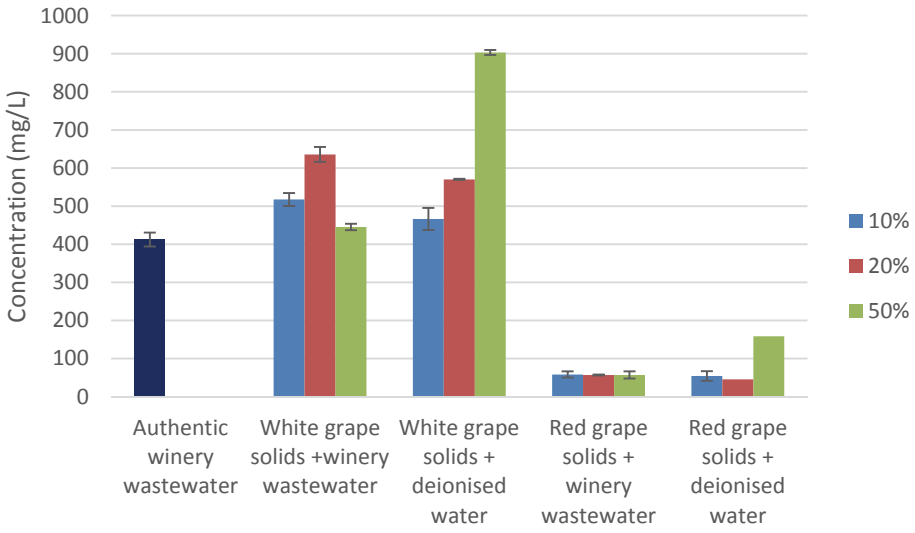


C

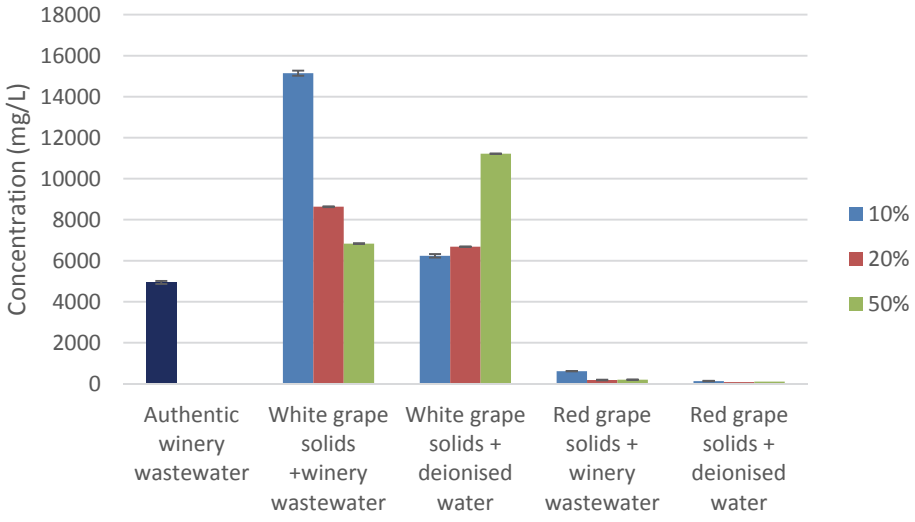


Figure 2.5: Sugar concentrations in winery wastewater, white grape solid wastes, and red grape winery wastes. A: Sucrose; B: Glucose; and C: Fructose.

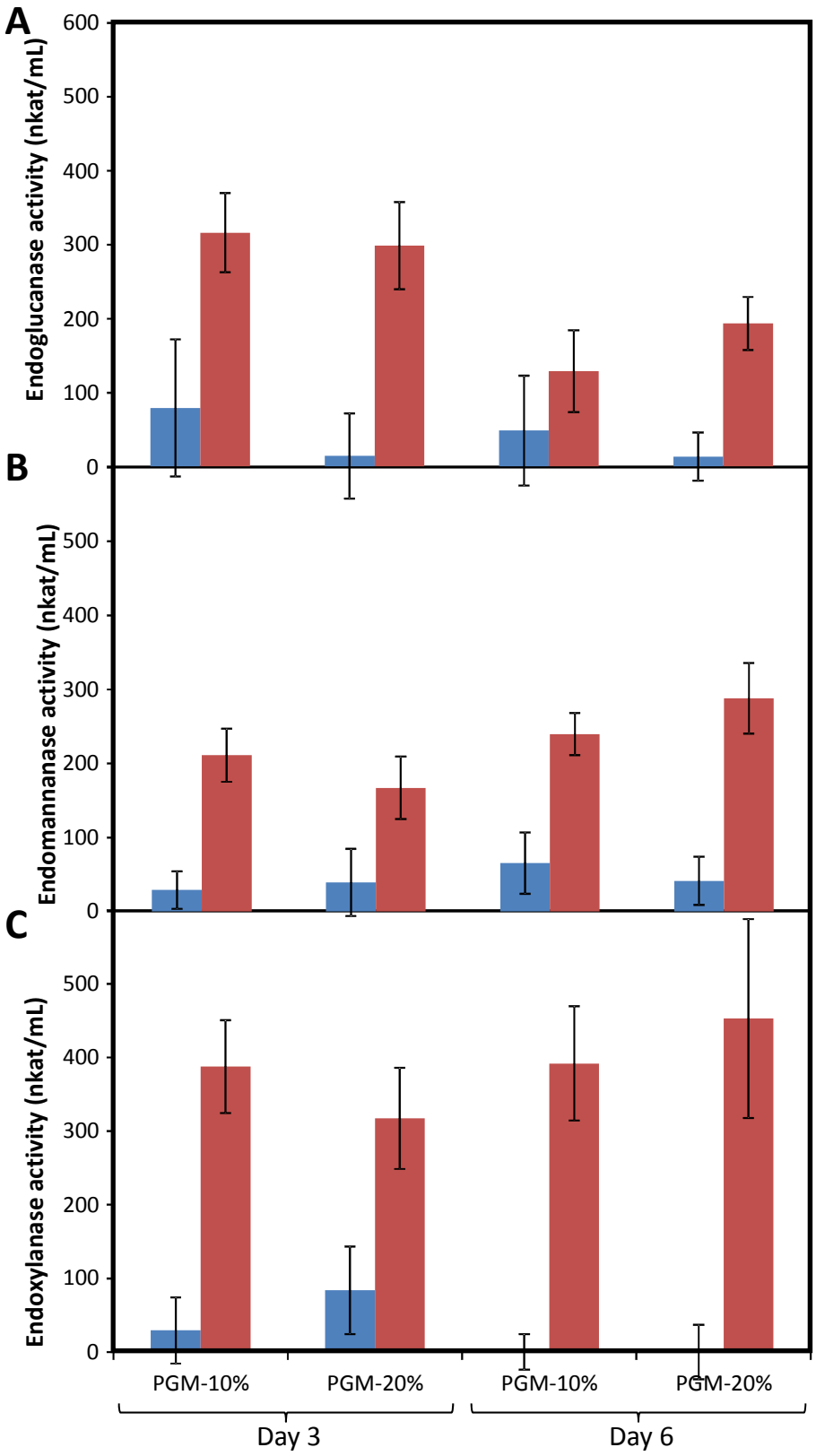


Figure 2.6: The extracellular (A) endoglucanase, (B) endomannanase and (C) endoxylanase enzyme activities detected for the *A. niger* D15[*eg2*], D15[*man1*] and D15[*xyn2*] after 3 and 6 days of cultivation at 30°C in PGM (10% and 20% white grape solids). Data represents the mean values with errors bars indicating the standard deviation (n=3).

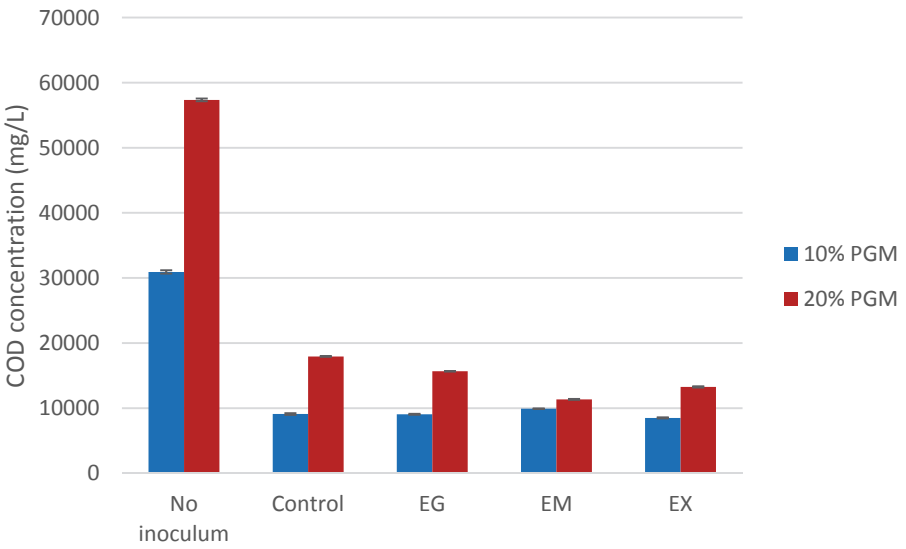


Figure 2.7: The COD concentrations detected for the *A. niger* D15[pGT1] (control), D15[*eg2*] (EG), D15[*man1*] (EM), and D15[*xyn2*] (EX) supernatants after 6 days of cultivation at 30°C in PGM (10% and 20% white grape solids). Data represents the mean values with errors bars indicating the standard deviation (n=2).

Analysis of sugar concentration showed that all the glucose present was consumed during growth and enzyme production (Figure 2.8).

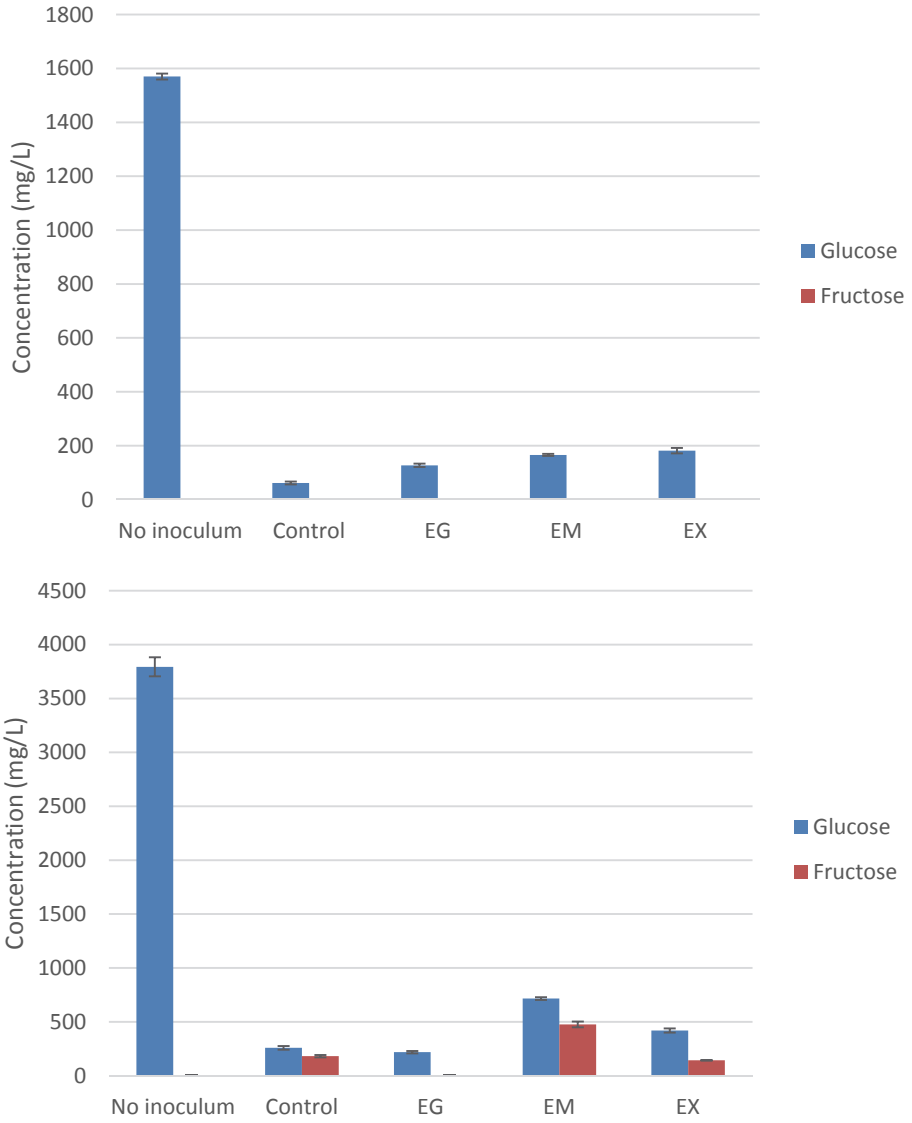


Figure 2.8: The sugar concentrations detected for the *A. niger* D15[pGT1], D15[*eg2*], D15[*man1*] and D15[*xyn2*] supernatants after 6 days of cultivation at 30°C in PGM (top) 10% and (bottom) 20% white grape solids. Data represents the mean values with errors bars indicating the standard deviation (n=2).

Total phenolic concentration in the media also decreased (Figure 2.9). However, while the starting concentration of phenolic compounds was lower (as expected) with lower grape waste concentrations (PGM-10%), the final concentrations after cultivation of *A. niger* strains remained higher than that of PGM-20% (Figure 2.9).

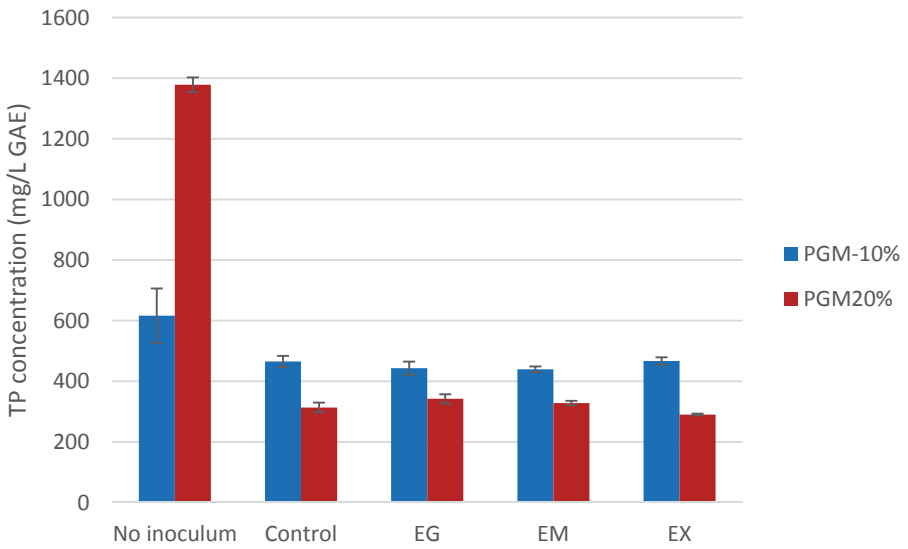


Figure 2.9: Total phenolics concentration detected for the *A. niger* D15[pGT1] (control), D15[*eg2*] (EG), D15[*man1*] (EM), and D15[*xyn2*] (EX) supernatants after 6 days of cultivation at 30°C in PGM (10% and 20% white grape solids). Data represents the mean values with errors bars indicating the standard deviation (n=2).

2.3.1.3 Potato wastes

The extracellular enzyme activities of all the *A. niger* D15[pGT1], [*eg2*], [*man1*] and [*xyn2*] strains were determined on day 3 and day 6 in PWM-10% (Figure 2.10). Potato waste was grated prior to autoclaving. In general, the PWM-10% resulted in similar levels of activities for KD, slivers and starch as biomass carbohydrate source. In most cases, an increase in activity was observed on day 6 (compared to day 3), but the increase in activity does not justify an additional 3 days of cultivation. The levels of activity compares well with the data obtained on apple pomace on day 3. For all three strains, the activity observed using skins as biomass source, was much lower that with the other carbohydrate sources. The skins are removed from the potatoes after a heat treatment. This results in the skins peeling off and containing little starch material. The skins contain primarily cellulose, which is more difficult for the strains to utilize.

The KD, slivers and starch were used to prepare PWM-20%. The potato waste was homogenised in a warring blender prior to autoclaving. Similar levels of activity were observed on day 3 and day 6 for the endoglucanase and endoxylanase producing strains (Figure 2.11). The increase in potato waste content only benefitted the endomannanase production.

Untreated, 10% potato wastes suspended in dH_2O ranged in COD contents from <1000 to approximately 200 mg/L (Figure 2.12a). Upon preparation of the medium (PWM-10%), the COD increases to 5000-10000 mg/L (Figure 2.12b). Cultivation of the *A. niger* strains in PWM-10% results in small decreases in COD content in most cases. This could be due to the production of enzymes and other organic compounds that affect COD content.

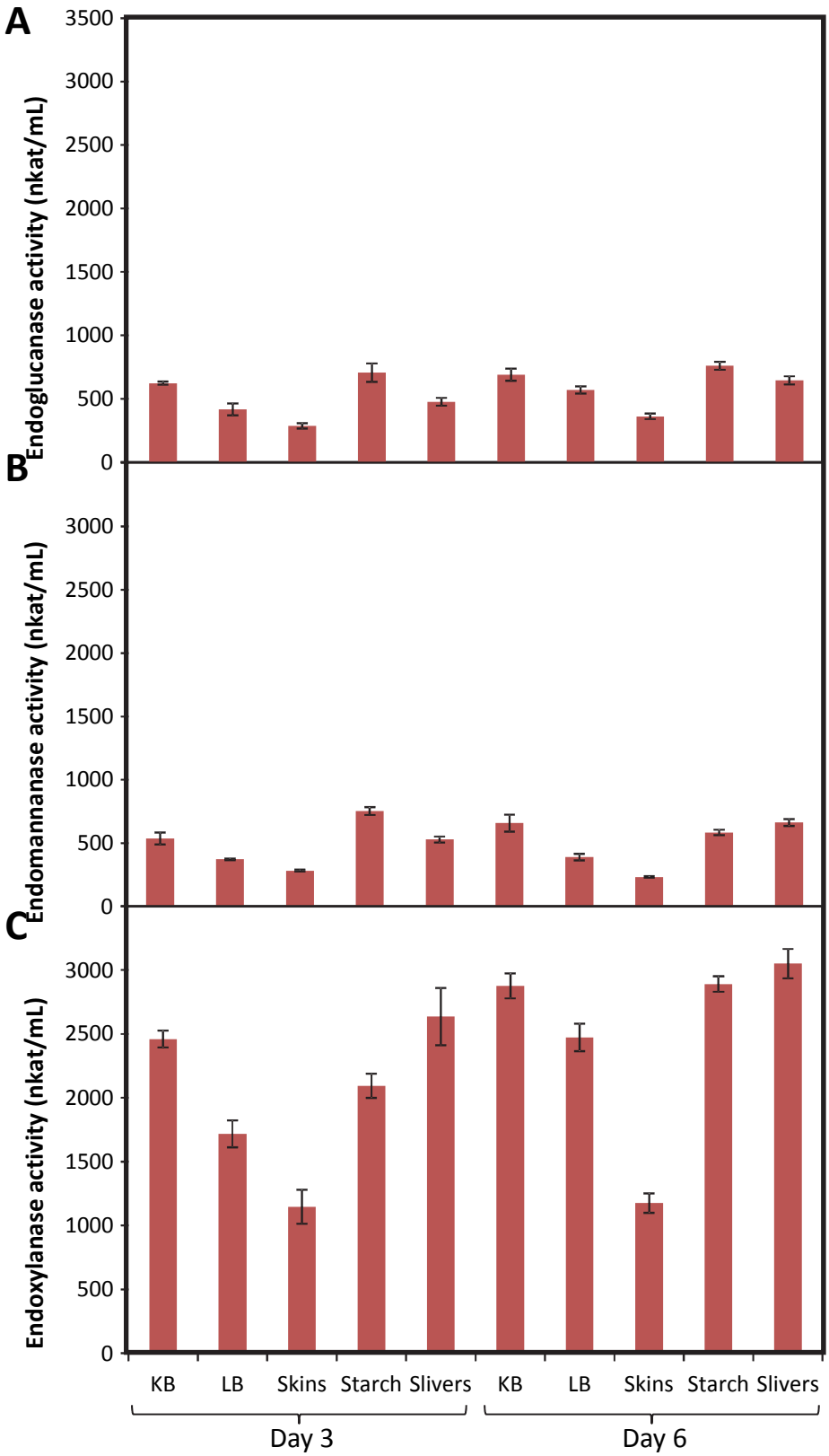


Figure 2.10: The extracellular (A) endoglucanase, (B) endomannanase and (C) endoxylanase enzyme activities detected for the *A. niger* D15[*eg2*], D15[*man1*] and D15[*xyn2*] after 3 and 6 days of cultivation at 30°C in PWM-10%. The activity levels had been normalised (data obtained for the *A. niger* D15[pGT] had been deducted from the values obtained for *A. niger* D15[*eg2*], D15[*man1*] and D15[*xyn2*]. The highest level of endoglucanase and xylanase activity for *A. niger* D15[pGT] was 50 nkat/mL, while 100 nkat/mL was obtained for endomannanase activity. Data represents the mean values with errors bars indicating the standard deviation (n=3).

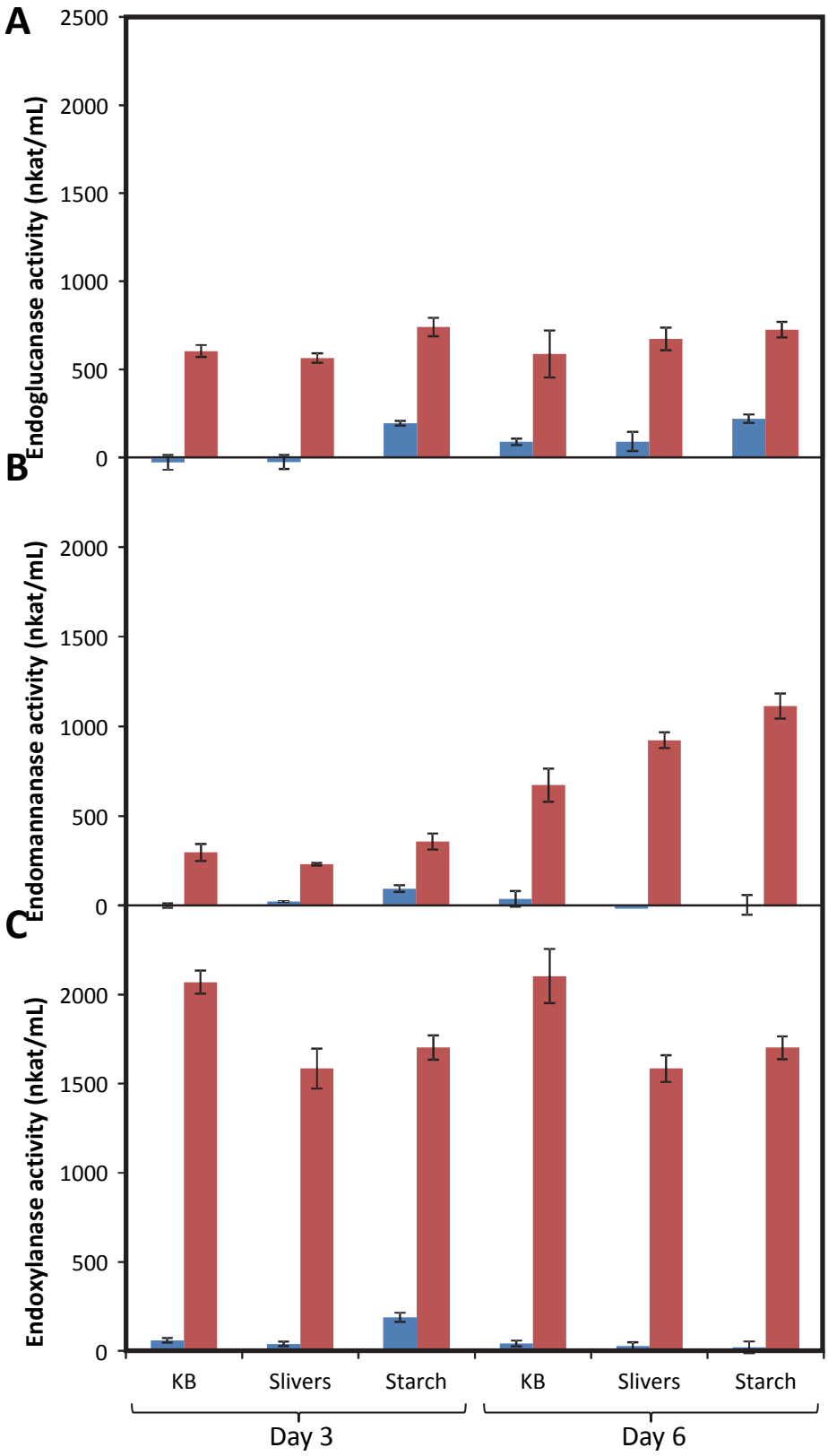


Figure 2.11: The extracellular (A) endoglucanase, (B) endomannanase and (C) endoxylanase enzyme activities detected for the *A. niger* D15[*eg2*], D15[*man1*] and D15[*xyn2*] after 3 and 6 days of cultivation at 30°C in PWM-20%. Data represents the mean values with errors bars indicating the standard deviation (n=3).

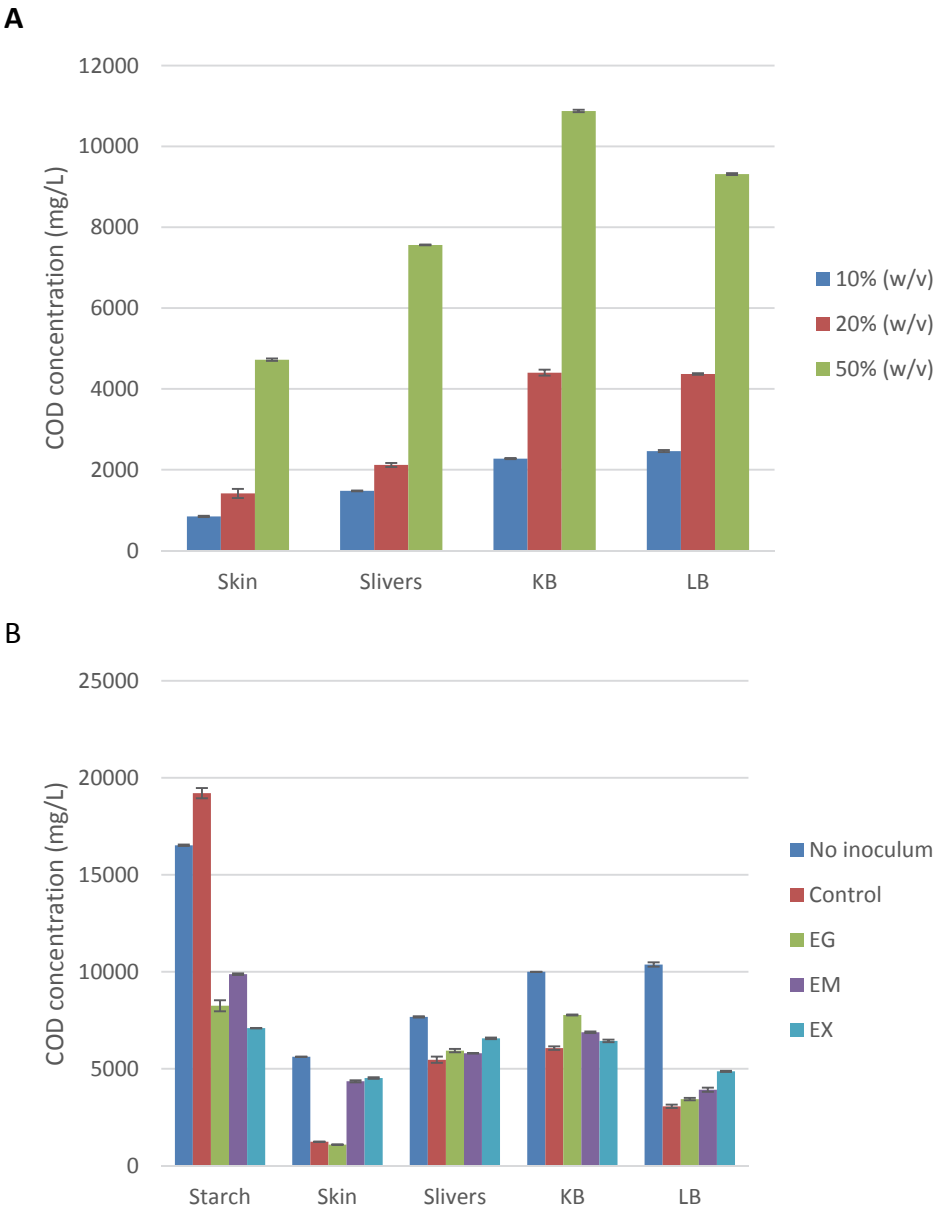
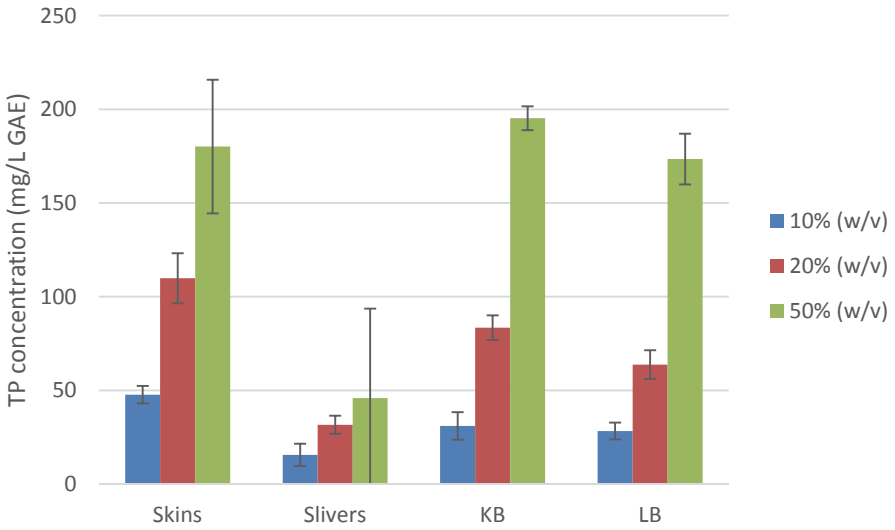


Figure 2.12: COD concentrations in potato waste supernatants (a) before and (b) after growth of *A. niger* D15[*egII*] (EG), D15[*man1*] (EM), and D15[*xyn2*] (EX) in PWM-10%. Data represents the mean values with errors bars indicating the standard deviation (n=2).

Untreated, 10% potato wastes suspended in dH_2O showed very low levels of phenolic content (Figure 2.13a). Even at 50% concentration, phenolic content remained low (Figure 2.13). As with COD, preparation of the medium for cultivation (PWM-10%) resulted in an increase in phenolics concentration (Figure 2.13b). Cultivation of the *A. niger* strains in PWM-10% resulted in only minor decreases in phenolic content in the strains MAN 1 on starch and EG2 on skins and LD (Figure 2.13b). For the other wastes, there was an increase in total phenolics after cultivation. Of the potato wastes tested, KD contained the highest glucose concentrations, while LD contained similar levels of fructose and glucose (Figure 2.14a).

A



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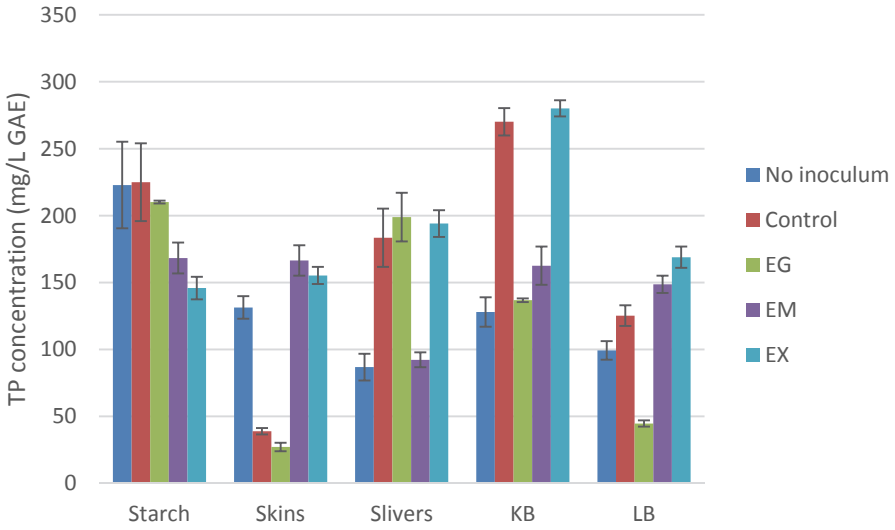


Figure 2.13: Total phenolic concentrations in potato waste supernatants (A) before and (B) after growth of *A. niger* D15[*egII*] (EG), D15[*man1*] (EM), and D15[*xyn2*] (EX) in PWM-10%. Data represents the mean values with errors bars indicating the standard deviation (n=3).

Visual observations

The grape winery biomass waste is a fibrous mass, making it impossible to determine the actual biomass production of the strains. The biomass contained mainly stalks, pips and grape skins. The pips and stalks (>50% of the biomass) was difficult to utilize within 6 days of cultivation by the *A. niger* strains. This implies that the utilizable carbohydrate concentration was in fact much lower than the amount of biomass used. The PGM-50% medium was difficult to work with. The grape waste soaked up all the liquid (solid state fermentation) making it impossible to harvest supernatant for the assays. PGM-10% and PGM-20% are viscous liquid media, similar to the apple pomace medium. Agitation allows for mixing, but not necessarily proper oxygen transfer. The grape waste proved difficult to work with and resulted in low levels of activity. Therefore, it does not currently merit further investigation.

The PWM-10% and PWM-20% (both containing starch) has a high level of viscosity. The gel-like consistency makes mixing and aeration difficult. The high levels of activity obtained with this waste could potentially improve if better aeration can be established in a bioreactor setup.

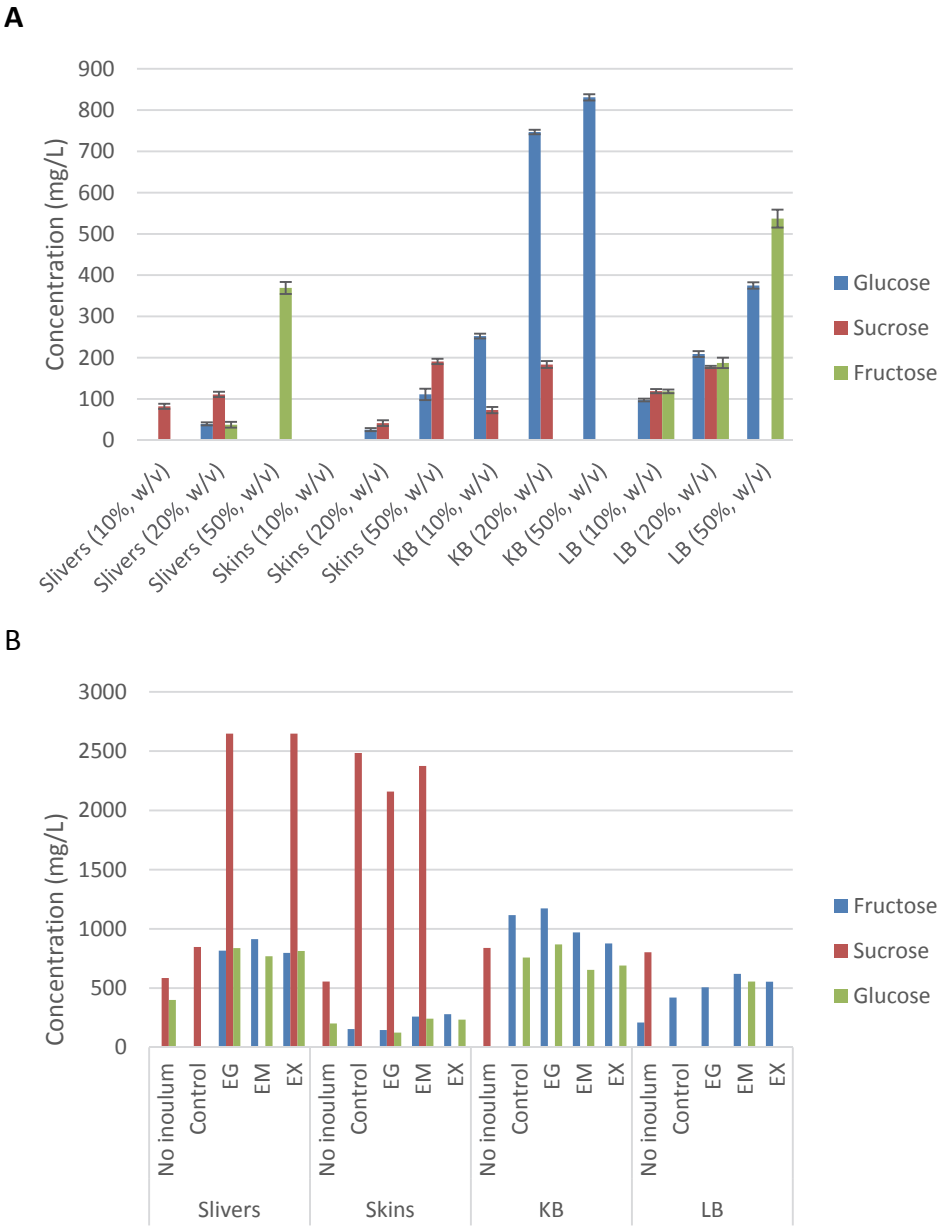


Figure 2.14: Sugar concentrations in potato waste supernatants (A) before and (B) after growth of *A. niger* D15[*egII*] (EG), D15[*man1*] (EM), and D15[*xyn2*] (EX) in PWM-10%. Data represents the mean values with errors bars indicating the standard deviation (n=2).

2.3.2 Yeast fermentation on potato waste

The possible use of potato waste for bio-ethanol production was investigated. The Ethanol Red strain was selected for use in the fermentation study as it is currently used globally for starch conversion to ethanol due to its robust nature. Ethanol Red was incubated with the potato waste supplemented with commercial amylase enzymes (Stargen 002) known to hydrolyze raw starch (no heating required). The fermentations were monitored over time (Figure 2.15). More ethanol

was produced from the corn starch, but the starch conversion (ethanol obtained as a percentage of the theoretical yield) of the KD was higher.

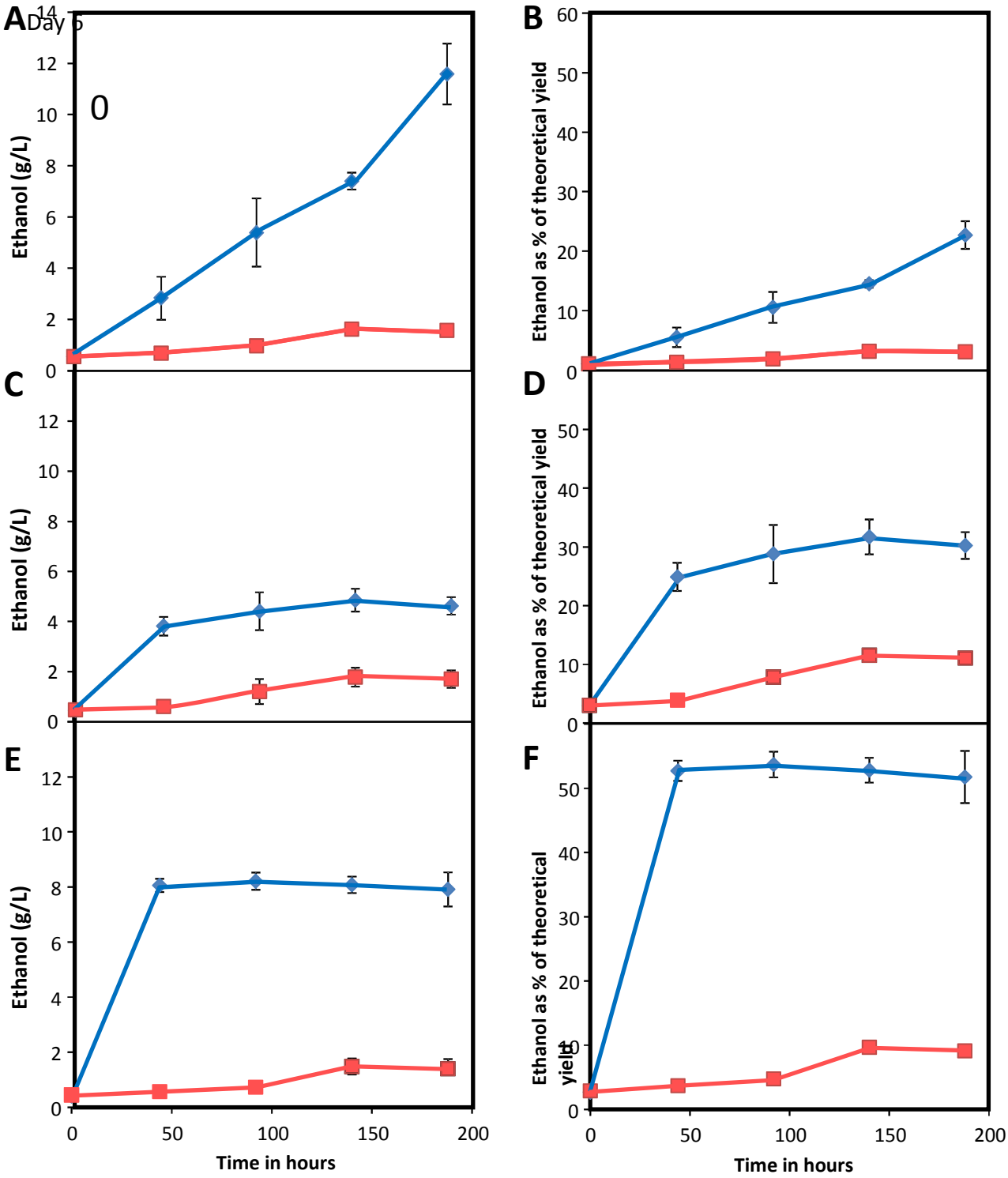


Figure 2.15: Ethanol concentrations were monitored over time for the Ethanol Red strain with (♦) and without (■) Stargen cultivated in YPWM-10% containing (A) starch, (C) slivers and (E) KD. Data were converted to % ethanol per theoretical yield (B-starch, D-slivers, F-KD), taking into account the amount of DW (100 g/L slivers and KD = approximately 30 g (DW)/L = maximum of 15.3 g/L ethanol). Data represents the mean values with errors bars indicating the standard deviation (n=3). Error bars are not visible on all data points.

contaminating organisms. However, after 24 hours bacterial contamination was evident despite the addition of the antibiotics. The bacterial contamination also channelled some of the available carbon away from the ethanol production (to biomass), resulting in the lower than expected starch conversion (% ethanol per theoretical yield).

Fermentations were repeated with YPWM-20% (Figure 2.16). The 2 minute heat treatment (refer to methods section) made a significant difference in the ethanol yield obtained from the potato waste, probably due to the decrease in bacterial contamination and the heat pre-treatment. Similar results were obtained for the KD and Slivers, reaching levels of about 70% ethanol (% ethanol per theoretical yield) after 48 h of fermentation. The data compared well with that obtained with the corn starch. The potato waste can therefore be used for heterologous enzyme production by *A. niger* as well as for bio-ethanol production by *S. cerevisiae*.

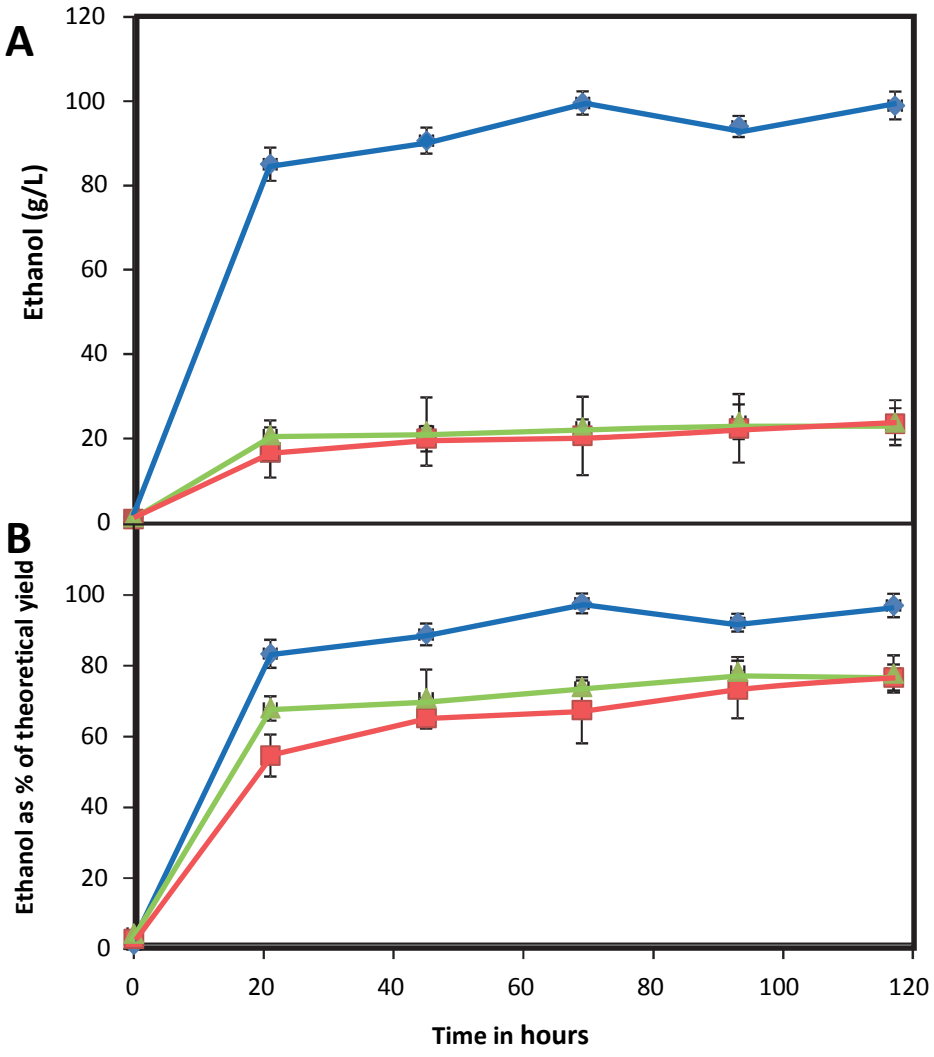


Figure 2.16: (A) Ethanol concentrations were monitored over time for the Ethanol Red strain with Stargen in YPWM-20% using ♦ starch, ■ slivers and ▲ KD as carbon source. Data were converted to % ethanol per theoretical yield (B), taking into account the amount of DW (200 g/L slivers and KD = approximately 60 g (DW)/L = maximum of 30.6 g/L ethanol). Data represents the mean values with errors bars indicating the standard deviation (n=3). Error bars are not visible on all data points.

2.3.3 Laccase production by *T. pubescens*

2.3.3.1 Apple pomace (10 mL culture volumes)

Trametes pubescens was inoculated onto pressed (PP) and unpressed (UP) apple pomace prepared in dH_2O at concentrations ranging from 10% to 50% pomace wet wt/vol. Laccase activity was monitored (Figure 2.17). At 30% and 50% pomace, the preparations were highly viscous, which made agitation (hence aeration) and sampling very difficult. While laccase activity appears optimal at 30% and 50% apple pomace concentrations, these concentrations are not practical for larger scale production due to the high viscosity and were not considered further. In general, laccase production increased with increasing pomace concentrations and was higher using PP than UP. Production in 10 and 20% PP remained similar (0.7-0.8 U/mL and 0.7-1 U/mL, respectively) from day 5 of cultivation.

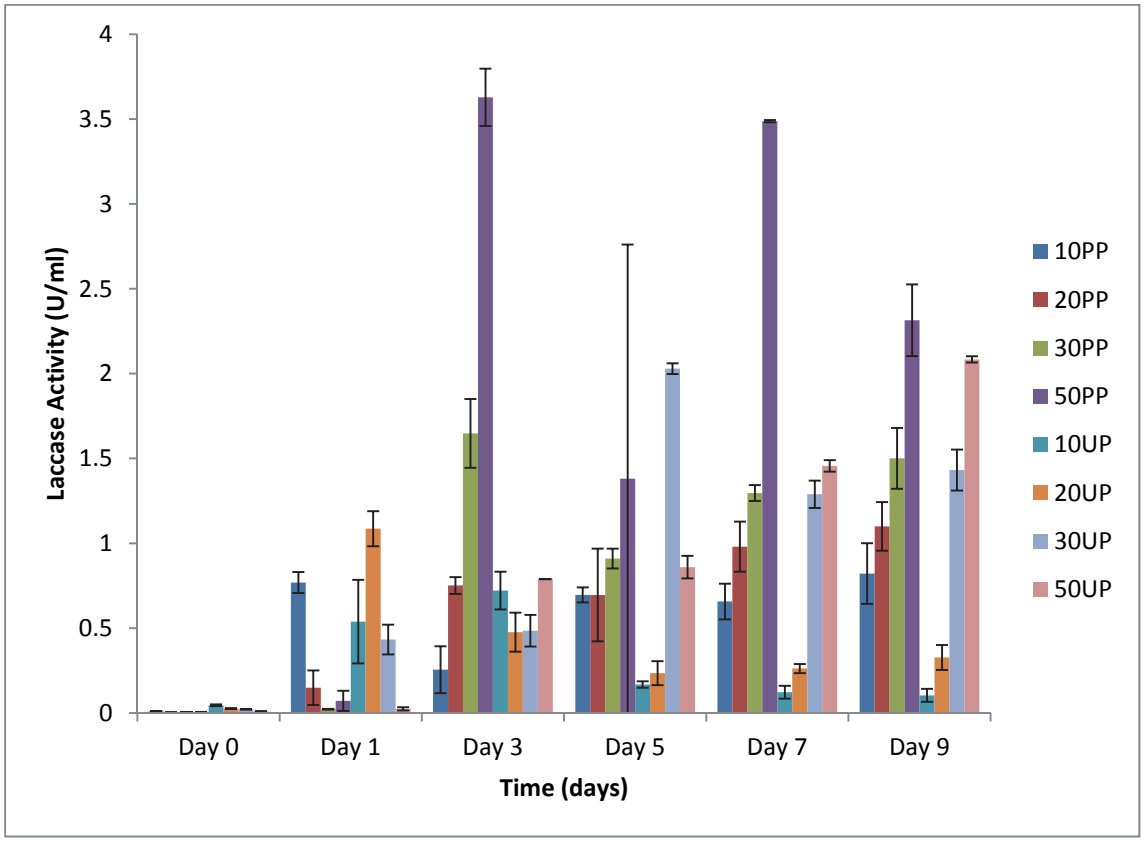


Figure 2.17: Laccase production by *T. pubescens* cultured in pressed (PP) and unpressed (UP) apple pomace. Data represents the mean values with errors bars indicating the standard deviation (n=3).

2.3.3.2 Potato wastes (20 mL culture volumes)

Initial experiments using the potato wastes (suspended in dH_2O only) showed that the medium became highly viscous with increasing potato waste concentrations. At concentrations of 20% and more, all the liquid was taken up by the waste (solid state fermentation). Furthermore, standard autoclave times of 20 min were insufficient and cultures were contaminated (especially LD) with other microorganisms. Autoclave times were extended to 30 min, which resolved the challenge of contamination.

The wastes were then used at 10% and 20% concentration only (Figure 2.18). These flasks were inoculated using a homogenate of *T. pubescens*, without first growing a pre-culture. Visual observations showed that the lack of pre-culture did not inhibit growth and *T. pubescens* showed excellent growth in all waste types.

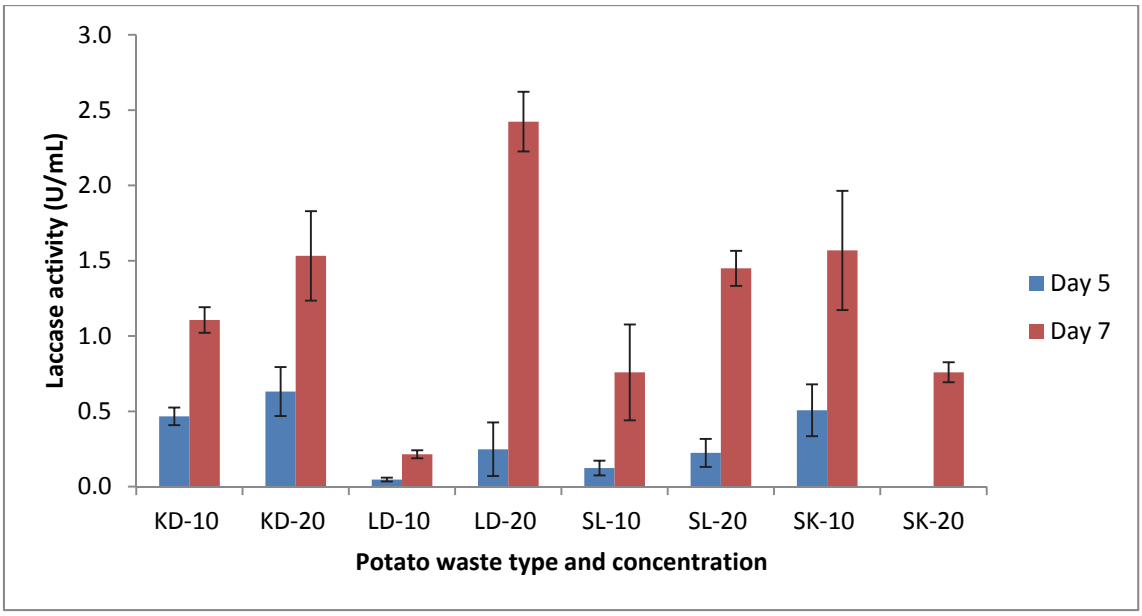


Figure 2.18: Laccase production by *T. pubescens* cultured in potato wastes (KD, LD, SL and SK refer to the waste type while 10 and 20 refer to the percent waste suspended in dH_2O). Data represents the mean values with errors bars indicating the standard deviation (n=3).

SK-20 – which consisted of skins, was effectively a solid state fermentation as all the liquid was absorbed by the waste after autoclaving. Therefore, a sample was not taken on Day 5. *T. pubescens* grew very well on the surface (thick white mycelial growth) and after 7 days, 20 mL 0.1 mM sodium-acetate buffer (pH 5) was added to the culture and incubated shaking at 200 rpm for 2 hrs before a sample was taken for analysis.

In all cases (except SK), 20% potato waste resulted in much higher laccase production than 10% waste after 7 days. LD wastes showed the most dramatic difference, with a greater than 10 times increase in laccase production in LD-20 after 7 days compared to LD-10 (Figure 2.18).

2.3.3.3 Potato wastes (airlift reactor, 3 L)

McCains frozen rustic chips were used as a substitute potato waste during the airlift reactor experiment (at 10% wet wt/vol) as we had an insufficient amount of authentic potato waste for the reactor requirements. A homogenate of *T. pubescens* was used to inoculate the reactor as previous experiments showed excellent growth and laccase production without the use of a pre-culture. Figure 2.19 shows the airlift reactor with substitute potato waste (10% wet wt/vol) used in this study.

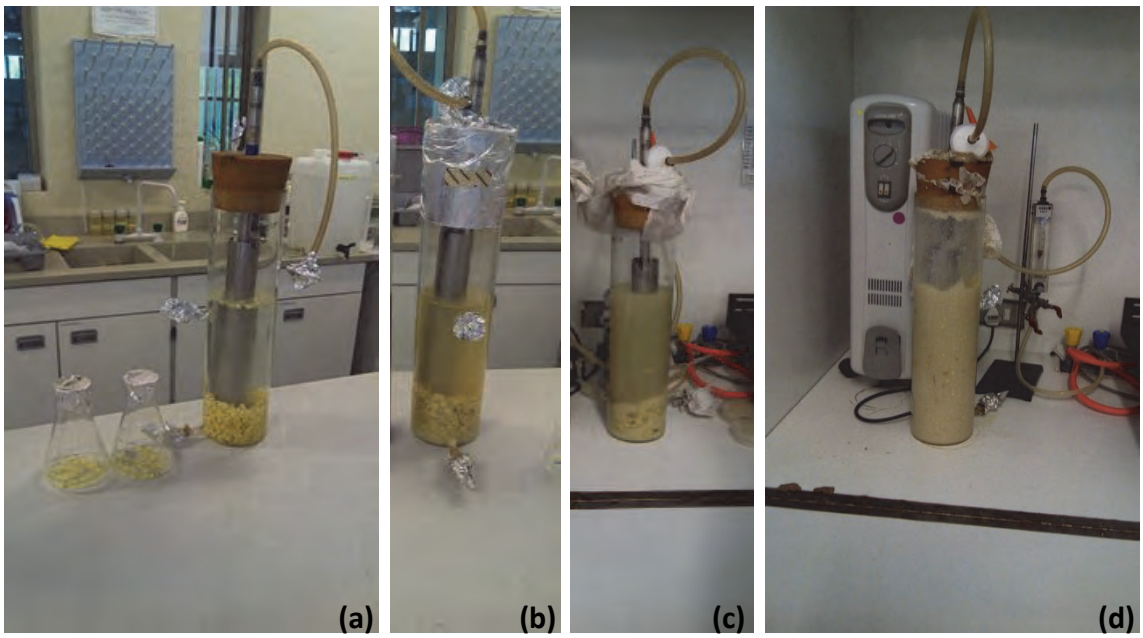


Figure 2.19: 3 l (final volume) airlift reactors used for the cultivation of *T. pubescens* on 10% substitute potato waste: (a) before autoclaving; (b) after autoclaving; (c) after addition of the *T. pubescens* homogenate and (d) after 5 days.

It must be noted that the air supplied to the reactor is dried and results in high levels of evaporation. To counter the decreasing volume over time, 500 mL sterile dH_2O was added to the reactor daily. Initially, there were some concern regarding mixing and aeration in the reactor as the thick potato wastes settled at the bottom of the reactor. However, within 24-48 hrs, this layer had dissipated and mixing and aeration appeared more efficient. After 5 days, the reactor contents were harvested. Laccase production in the airlift reactor (0.5 U/mL after 5 days) was similar to laccase production in 20 mL culture volumes using KD (discarded frozen chips) after 5 days (Figure 2.18). In future, longer cultivation times will be monitored to determine if production improves with time.

2.3.4 Biocatalysis using *T. pubescens* laccase

The laccase produced using 10% and 20% KD waste in small-scale (20 mL) culture volumes and using the substitute potato waste (10% in the airlift reactor) were used in biocatalysis reactions. Earlier studies (Adelakun et al., 2012) have shown that fungal laccases can be used to couple phenolic monomers to improve the antioxidant capacity of those monomers. In this study, ferulic acid was used to determine if the laccase produced using potato wastes could accomplish similar results. After 24 hrs of incubation of culture supernatants and ferulic acid, product formation was evident from the presence of a dark, yellow-orange product (Figure 2.20).

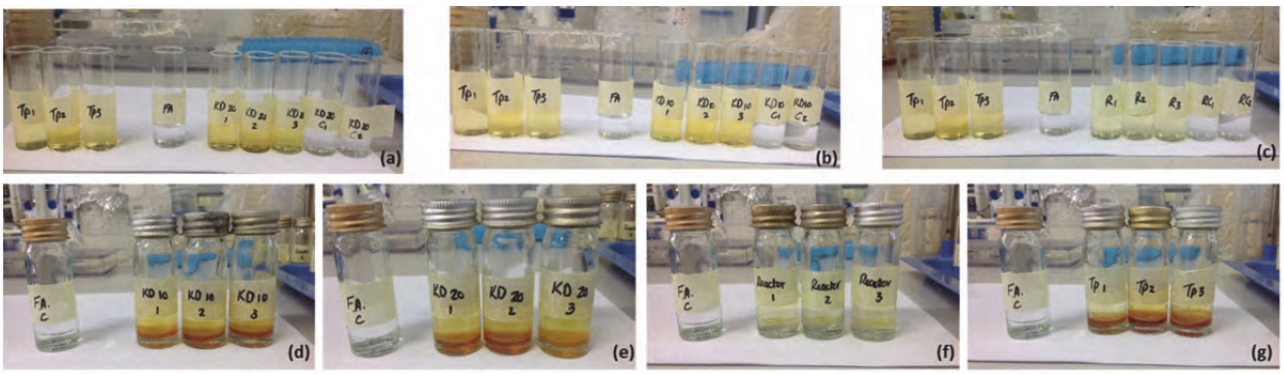


Figure 2.20: Comparison of colour between purified *T. pubescens* laccase (TP 1-3), ferulic acid (FA) and (a) supernatants from *T. pubescens* cultured on 20% KD potato waste (KD20 1-3) and uninoculated experimental controls (KD20 C1-C2); (b) supernatants from *T. pubescens* cultured on 10% KD potato waste (KD10 1-3) and uninoculated experimental controls (KD10 C1-C2) and (c) supernatants from *T. pubescens* cultured on 10% substitute potato waste in an airlift reactor (R 1-3) and uninoculated experimental controls (R C1-C2). Product formation after 24 hrs reaction with ferulic acid (FA) was evident as a yellow-orange colour change: (d) 10% KD supernatant, (e) 20% KD supernatants, (f) airlift reactor supernatants and (g) purified *T. pubescens* laccase.

Analysis by TLC revealed that similar products were obtained, irrespective of whether the purified *T. pubescens* laccase was used or the supernatant fluid from the "proof of concept" flasks or reactor (Figure 2.21).

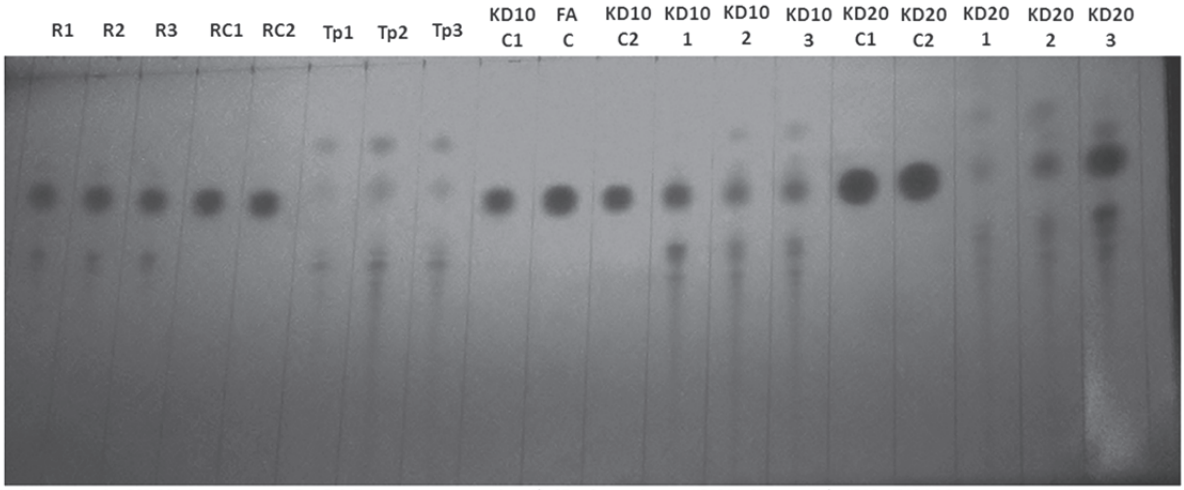


Figure 2.21: TLC analysis of products from coupling reactions with FA and: reactor samples (R1, R2 and R3); reactor controls (RC1 and RC2); purified *T. pubescens* laccase (Tp1, Tp2 and Tp3); 10% KD controls (KD10 C1 and C2); 10% KD supernatants (KD10 1, 2 and 3); 20% KD controls (KD20 C1 and C2); 20% KD supernatants (KD20 1, 2 and 3). The FA control was spotted in the middle lane for comparison.

The TLC plates were also stained with DPPH and, similar to using purified *T. pubescens* laccase, clear spots indicating antioxidant activity were observed (Figure 2.22).

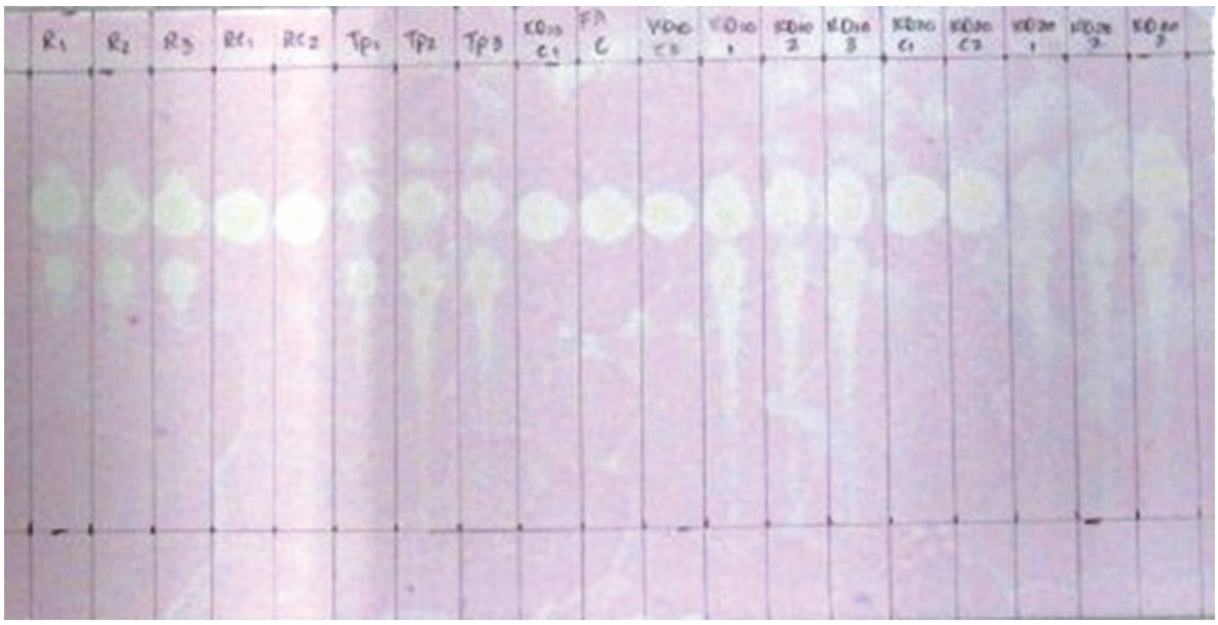


Figure 2.22: TLC plate showing antioxidant activity (clear/white spots) with ferulic acid and biocatalysis reaction products: reactor samples (R1, R2 and R3); reactor controls (RC1 and RC2); purified *T. pubescens* laccase (Tp1, Tp2 and Tp3); 10% KD controls (KD10 C1 and C2); 10% KD supernatants (KD10 1, 2 and 3); 20% KD controls (KD20 C1 and C2); 20% KD supernatants (KD20 1, 2 and 3). The FA control was spotted in the middle lane for comparison.

2.4 Conclusion

Potato waste proved the best bio-based waste stream for growth of the recombinant *A. niger* strains and heterologous expression of endoglucanase, endomannanase and endoxylanases. Furthermore, bioethanol production using *S. cerevisiae* was also achieved using potato wastes for the fermentation feedstock. Enzyme and ethanol production by these strains using bio-based waste streams show potential and should be further investigated.

Trametes pubescens is capable of growing, and producing laccase, on apple and potato wastes without the addition of other nutrients or inducers. The laccase obtained was similar to a purified *T. pubescens* laccase that was obtained under optimized media, environment and inducer conditions when used directly in biocatalysis reactions, without any inhibition observed. Due to the diverse potential applications of laccases, the ease of growth of *T. pubescens* and the lack of additional supplementation, this could be a very valuable value-added product under the biorefinery concept and merits further investigation.

Overall, the results presented in this chapter show that the strains identified for high-value enzyme production could successfully produce these enzymes using bio-based waste streams in the cultivation medium.

It must be noted that attempts were made to produce laccase from *T. pubescens* on winery waste solids (grape skins, pips, etc.) in an airlift reactor (3 L). However, initial experimentation showed that the airlift reactor format did not cope well with the grape solids, which tended to sink to the bottom and clump, making it difficult to disperse in the reactor. In future, amendments to the airlift reactor will be investigated to attempt to improve mixing and aeration with this waste.

CHAPTER 3: CHARACTERISATION OF ENZYMES AND APPLICATIONS 1

3.1 Introduction

This section of the report addresses the characterization of the enzymes and their applications in waste stream reclamation and lignocellulosic conversion. This WRC project aims to demonstrate bioremediation of lignocellulosic wastewater by recombinant *Aspergillus niger* D15 strains by reduction of organic content and cheap and high production of commercially valuable lignocellulose hydrolysing enzymes on inexpensive and abundant bio-based waste streams.

Removal of organic matter from bio-based waste stream allows for reuse or more environmentally friendly disposal of the wastewater. Apple processing waste (apple pomace) is used as a model waste stream in this study. Of the approximately 5.4 million tonnes of fruit produced annually in South Africa, 2.4 million tonnes are processed (Anon, 2013). A lot of waste is produced in the process. Apples are the third most produced and processed fruit in South Africa after grapes and oranges. About 0.766 million tonnes are produced each year, and of that, 0.214 million tonnes are processed (Anon, 2012a). In apple juice production, 25-30% of the original apple remains as apple pomace, which is a solid waste product (Dhillon et al., 2013). Solid fruit wastes are where most fruit waste polysaccharides are found, liquid wastes generally have a low and variable sugar content (Burton et al., 2006). Apple pomace contains 7.2-43.6% (% w/w) cellulose, 4.26-24.40% (% w/w) hemicellulose and 15.3-23.5% (% w/w) lignin (Dhillon et al., 2013). It can be added to agri-industrial wastewater streams to increase the polysaccharide content for fermentation of waste streams to produce bioalcohols (Burton et al., 2006). It has already been demonstrated in the WRC project that the recombinant *A. niger* strains being used in the WRC project can grow on apple pomace and produce, at laboratory scale, the recombinant lignocellulolytic enzymes. The recombinant endoxylanase 2 (E.C.3.2.1.8; GH family 5) that was characterized for this study is one of those enzymes. The enzyme characterized in this study was, however, obtained from *Aspergillus niger* D15 [*xyn2*] grown on double strength minimal media, for ease of growth of the fungi and production and extraction of the enzymes. This strain has high levels of expression of endoxylanase 2 (Rose and van Zyl, 2002; Rose and Zyl, 2008).

3.2 Enzyme characterization

Enzymes need to be biochemically characterized before their industrial use can be assessed. When using enzymes industrially, it is important to know what molecules inhibit the enzymes as this affects enzyme activity. Inhibition of lignocellulolytic enzymes may occur due to inhibitory compounds formed or released during pretreatment and hydrolysis. Enzyme activity may also be affected by products formed by the enzyme itself (feedback inhibition) or by the products of other lignocellulose hydrolysing enzymes. Product inhibition is important to consider in enzyme synergy studies. Inhibition may also occur due to fermentation products such as ethanol (van Dyk and Pletschke, 2012). Inhibition may also occur due to metal ions found in feed stocks, process water or in reagents used in a biorefinery. Metal ions may also come from dissolving or corrosion of reactor vessels or pipe lines (Tejirian and Xu, 2010). This study aims to characterize endoxylanase 2 from recombinant *A. niger* for use in various industrial applications. The efficient use of these enzymes for lignocellulose hydrolysis requires enzyme characterization to determine the optimal selection and ratio of enzymes for synergistic hydrolysis.

3.3 Characterization of *Trichoderma reesei* endoxylanase 2

Biochemical characterization of endoxylanase 2 produced by recombinant *Aspergillus niger* D15 [*xyn2*], has been performed (protein size, activity, pH optimum, and temperature optimum, thermal stability) (Rose and van Zyl, 2002). In depth biochemical characterization, including substrate specificity on a wide variety of substrates, the effect of inhibitors that could affect enzyme activity such as phenolics, metal ions and fermentation products such as ethanol, has not been performed on these recombinant enzymes. The properties of recombinant heterologous enzymes can differ from the properties of the native enzyme (Setati et al., 2001). This may occur, for example, due to glycosylation of the recombinant enzyme (Setati et al., 2001). *T. reesei* enzymes expressed in *A. niger* have lower activity than when expressed in *T. reesei* (Cavka et al., 2014). This may be due to the different glycosylation patterns of the two fungi (Takashima et al., 1998). Glycosylation may decrease activity by blocking enzyme active sites (Rose and van Zyl, 2002). The medium in which the fungi grow can also affect glycosylation patterns (Alriksson et al., 2009). Glycosylation can also improve enzyme activity by increasing temperature stability of enzymes (Samanta et al., 2012). Qin et al. (2008) found that the endoglucanase 2 of *T. reesei*, when expressed in *Saccharomyces cerevisiae*, was more glycosylated than the native enzyme. They also found that an increase in glycosylation increased the pH activity range and thermal stability of the enzyme. The focus of endoxylanase 2 characterization was on the effect of phenolic, organic acid and fermentation product inhibition, as this has not been done for this enzyme, expressed in any host, to the best of our knowledge. The only endoxylanase 2 inhibition characterization found was done by Dekker (1983) where endoxylanase 2 was found to be competitively inhibited by xylose. Characterization of lignocellulolytic enzyme inhibition by various substances that would be found in a bioreactor has been identified as an important gap in the literature which would be useful to produce more efficient enzyme cocktails for lignocellulose hydrolysis (Van Dyk and Pletschke, 2012).

In this chapter, biochemical characterization in terms of substrate specificity, temperature optimum and pH optimum was compared between crude and purified endoxylanase 2 (Xyn2). The Xyn2 was purified by ultrafiltration devices. Further characterization was performed on the crude enzyme including: temperature stability and the inhibitory effect of organic acids, phenolics, metal ions and fermentation products. Lignocellulosic enzymes used for biomass hydrolysis may come into contact with fermentation products in a simultaneous saccharification and fermentation (SSF) process or consolidated bioprocessing process (CBP). SSF is where hydrolysis and fermentation occur in the same reaction vessel and CBP is where the fermentation organism produces the lignocellulolytic enzymes used for hydrolysis (Van Dyk and Pletschke, 2012). The aim of these experiments was to better characterize the enzymes for better determination of their feasibility for application in industry.

3.4 Materials and methods

3.4.1 Materials

Production of the recombinant Xyn2 used in this study was performed by Dr SH Rose from Stellenbosch University. The recombinant *Trichoderma reesei* Xyn2 was expressed in *Aspergillus niger* D15 grown in double strength minimal media with 10% (w/v) glucose and the extracellular

growth medium, containing the secreted recombinant Xyn2, was freeze-dried and sent to Rhodes University.

3.4.2 Purification

Freeze dried enzyme was dissolved in 0.05 M pH 5 citric acid buffer (100 mg freeze dried enzyme/mL). Aliquots of the crude enzyme solution were concentrated and purified using 30 K Amicon® Ultra-2 centrifugal filtration devices as described in the Amicon® Ultra-2 centrifugal filtration device User Guide (Anon, 2012b). The devices were pre-rinsed with buffer and multiple devices were used to increase the volume of obtained purified protein. The centrifugal devices were centrifuged at 4000 rpm for 30 minutes at 4°C in a swinging bucket rotor (Heraeus Megafuge 1.0 R). The concentrated and purified endoxylanase was recovered by inserting the concentrate collection tube into the filter device then inverting the filter device and removing the filtrate collection tube. The concentrate was then collected into the concentrate collection tube by centrifuging the filter device with the inserted concentrate collection tube at 1000 rpm for 2 minutes at 4°C. The membrane of each filter device was washed with one 200 µl volume of 0.05 M pH 5 citrate buffer to recover enzyme which may have bound to the membrane. The 30 K device filtrate was then concentrated and purified using 10 K Amicon® Ultra-2 centrifugal filtration devices as was done with the 30 K Amicon® Ultra-2 centrifugal filtration devices. Protein concentration, enzyme activity on beechwood xylan and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis was performed on the crude enzyme, 30 K device and 10 K device to access purification. SDS-PAGE was performed on 10% and 14% gels (Laemmli, 1970) using a Biorad Mini-protean ® Tetra Cell and the method outlined in the Biorad Mini-protean ® Tetra Cell Instruction Manual. Staining was done as described in (Bollag et al., 1996). The gel was imaged with a Chemidoc™ XRS+ system using Image lab™ software.

3.4.3 Protein determination

Protein concentration was determined using a modified Bradford method (Bradford, 1976) and bovine serum albumin as a standard. Bradford reagent (230 µl) was added to 25 µl of protein sample and mixed on a plate shaker for 12 minutes at room temperature prior to absorbance measurement at 595 nm with a Powerwave$_x$ Spectroquant spectrophotometer and Kc Junior software (v1.41.8).

3.4.4 Enzyme assays

Enzyme activity was measured by the release of reducing sugars formed in a modified DNS (dinitrosalicyclic acid) method (Miller, 1959) using xylose as the standard. The DNS reagent was composed of 2 g sodium hydroxide, 2 g 3,5- dinitrosalicylic acid, 40 g potassium sodium tartrate, 0.4 g phenol and 0.1 g sodium bisulphite in 200 mL H_2O. The enzyme assay reaction mixture and assay conditions varied for the different experiments. For all the enzyme assays, excluding the *p*-nitrophenol substrate assays, the DNS assay was performed the same. The samples were centrifuged at 13 000 *x g* for 2 minutes after the enzyme substrate hydrolysis reaction. A 150 µl aliquot of the supernatant was then added to 300 µl DNS reagent, heated at 100°C for 10 minutes then cooled on ice for 5 minutes. Absorbance was measured at 540 nm using a Powerwave$_x$ micro plate reader (Bio-Tek instruments Inc. with KC Junior software V1.41.8). The reducing sugars released were determined as xylose equivalents using a xylose standard curve. Activity (U) was defined as µmol xylose equivalents released per mL of substrate per min under the assay

conditions specified. For all the enzyme assays, the buffer used was 0.05 M pH 5.5 citric acid buffer and the substrate used for was beechwood xylan, which was at a concentration of 1% (w/v) in the reaction medium unless otherwise stated. Appropriate substrate, enzyme and assay controls were also done for each assay. For the enzyme assays for purification determination, pure enzyme substrate specificity, pH optimum for the crude and pure enzyme, the enzyme assays were conducted as follows. The reaction mixture consisted of 350 µl of 1.14% (w/v) beechwood xylan in 0.05 M pH 5 citrate buffer and 50 µl of diluted enzyme (1.6 µg/ mL) in 0.05 M pH citrate buffer. For the purification determination assays, 350 µl of 0.57% (w/v) beechwood xylan in 0.05 M pH 5 citrate buffer. For the *p*-nitrophenol substrate assays for the pure enzyme, the reaction volumes were as follows: 318 µl buffer, 32 µl 25 mM substrate and 50 µl enzyme. The assay was conducted for 15 minutes and stopped by the addition of 2M $CaCO_3$, the absorbance was then measured at 405 nm.

For the enzyme assays for the crude enzyme, the enzyme assays were conducted as follows. The reaction mixture consisted of 200 µl of 0.05 M pH 5 citrate buffer, 100 µl of 4% (w/v) beechwood xylan and 50 µl of diluted enzyme (0.5 µg/ mL) in 0.05 M pH citrate buffer. For the p-nitrophenol substrate assays for the crude enzyme, the assay was conducted the same as with the pure enzyme except the protein concentration of the 50 µl volume added to the reaction mixture was 0.5 µg/ mL.

3.4.5 Substrate specificity

Assays were conducted as described under the enzyme assays section for both crude and pure enzymes. The substrates used were: beechwood xylan, oatspelt xylan, locust bean gum, Arabinogalactan, Avicel ®, carboxymethylcellulose and 4-nitrophenyl-monosaccharide substrates.

3.4.6 pH optima

For the pH optima assays, the reaction mixture consisted of 200 µl of 0.267 M boric, acetic and phosphoric (BAP) buffer (Britton and Robinson, 1931), 100 µl of 4% (w/v) beechwood xylan and 50 µl of diluted enzyme (1.6 µg/mL). The pH range assessed was pH 3.37 to pH 8.5 with 0.5 pH unit intervals.

3.4.7 Temperature optima

For the temperature optima assays, the reaction mixture consisted of 200 µl of pH 5 (determined pH optima) 0.267 M BAP buffer (Britton and Robinson, 1931), 100 µl of 4% (w/v) beechwood xylan and 50 µl of diluted enzyme (0.9 µg/mL). The temperatures assessed were: 30°C, 35°C, 40°C, 45°C, 50°C, 55°C, 60°C, 65°C, 70°C and 83°C.

3.4.8 Temperature stability

This was done for the crude enzyme at 37°C and 50°C. The diluted enzyme solution was incubated for 0h, 0.5h, 1h and 3h and then assayed as described in the enzyme assay section.

3.4.9 Inhibition

For the inhibition assays, which were only done with the crude enzyme, a portion of the buffer was replaced with inhibitors at various concentrations and the assay was conducted as with the non-inhibition assays. The lignin, phenolics and organic acids used were dissolved or suspended in

water, stock solutions of 20 g/L were made and added to the reaction mixture. The metal ions (and the associated anion) and EDTA were dissolved in 0.05 M pH 5 citrate buffer, stock solutions of 50 mM were made and added to the reaction mixture. The fermentation inhibitors used were added directly to the reaction mixture. All inhibition assays were conducted in a 50°C incubator on a shaking platform (Labcon platform shaker).

3.4.10 Laccase production by *Trametes pubescens*

3.4.10.1 Trametes pubescens culture preparation

Trametes pubescens cultures available at the BTB research group (CPUT) were maintained on *Trametes* Defined Media (TDM) agar plates, g/L: 10 glucose, 5.25 peptone, 2 KH_2PO_4, 0.5 $MgSO_4$, 0.1 $CaCl_2$, 0.3 NaCl, 12 agar and 10 mL trace elements solution consisting of, g/L: 0.6 $FeSO_4$, 0.03 $CuSO_4$, 0.07 $ZnCl_2$, 0.34 $MnSO_4$, 0.19 $CoCl_2$, 0.002 $NiCl_2$, 0.62 $(NH_4)_2MoO_4$. Cultures were incubated at 28°C for 5-7 days and plates were stored at 4°C. A homogenate of *T. pubescens* was used to inoculate experimental cultures. One TDM agar plate covered in *T. pubescens* (5-7 days' growth) was homogenised per 100 mL sterile dH_2O in a Waring blender. Homogenate (1 mL) per 20 mL culture volume was used for inoculation.

3.4.10.2 Laccase assay

Laccase from *Trametes versicolor* (TvL) was purchased from Sigma-Aldrich and resuspended in 50 mM sodium tartrate buffer (pH 4.5) for use. Dilutions of TvL were used as positive controls for laccase assays. Laccase activity in experimental cultures was routinely measured in microtitre plate format (Anthos Xenyth 1100 instrument) using the ABTS assay [0.5 mM ABTS; 420 nm; ε = 36 000 M/cm; sodium acetate buffer (100 mM, pH 5.0)].

3.4.10.3 Laccase production using red grape waste

The red grape waste (RGW; collected from a Stellenbosch winery and stored at -20°C until use) was used directly, without further crushing or similar processing. As anti-fungal agents may be used in the vineyards, washed red grape waste (W-RGW) was also tested. For W-RGW, the RGW was washed with running tap water, while constantly mixing, in a sieve lined with muslin for a minimum of 10 minutes.

A volume of 1 mL *T. pubescens* homogenate was used to inoculate flasks containing 10, 20, 30 and 50% RGW and W-RGW (20 mL culture volume). Flasks were incubated at 28°C. 10 and 20% RGW and W-RGW cultures were incubated shaking at 200 rpm, while 30 and 50% RGW and W-RGW cultures were static cultures (solid-state fermentation, SSF) as there was no visible liquid remaining after sterilisation (autoclave). Samples were taken after 3, 5 and 14 days of incubation for shaking cultures. 5 mL sodium acetate buffer (100 mM, pH 5.0) was added to each SSF culture and set to shake at 200 rpm for 1 hr. After centrifugation at 10 000 $\times g$, sample supernatants were assayed for laccase activity using the ABTS assay.

3.4.10.4 Laccase production using potato waste with red grape waste

Potato waste collected from two points in the processing of potatoes at Lamberts Bay Foods, Lamberts Bay, was used: Wastes generated after the removal of off-cuts (from cutting into fries) and defects (cutting and defects, CD) and the solid waste that is separated from the mixed solid-liquid waste washed out of the factory (final solids, FS). After collection, the waste samples were homogenised in a food processor and stored at -20°C until use.

The CD and FS wastes were used at 20% w/v, with RGW added at 1, 3, 5 and 10% w/v. mL. *T. pubescens* homogenate was used to inoculate flasks containing the mixed wastes (20 mL culture volume). Flasks were incubated at 28°C, shaking at 200 rpm. Samples were taken after 3, 5, 7 and 10 days of incubation and assayed for laccase activity using the ABTS assay.

3.4.11 Enzyme production by *A. niger*

3.4.11.1 Biomass preparation

Apple pomace was prepared by homogenizing 'Golden Delicious' apples purchased from local supermarkets. No water was added during homogenisation and therefore pressing was unnecessary. Lamberts Bay Foods supplied different potato wastes. A mixture of potato waste solids were prepared (homogenising) in a food processor (Morphy Richards) and stored at -20°C. Pressed apple pomace differed from the batch used in the previous reports.

3.4.11.2 Fungal strains

Aspergillus niger strains had previously been constructed in-house (Rose and Van Zyl 2002; 2008) and is known to produce high levels of extracellular activity. Details regarding the strains are provided in Table 1.1.

3.4.11.3 Spore solution preparation

Fungal spores were collected with the toothpick and streaked out onto spore plates in a grid form. Spore plates contained 18 g/L agar, 2 g/L neopeptone, 1 g/L yeast extract, 0.4 g/L $MgSO_4.7H_2O$, 10 g/L glucose and 2 g/L casamino acids. Twenty mL Asp+ (300 g/L $NaNO_3$, 76 g/L KH_2PO_4 and 26 g/L KCl) and 1 mL of trace elements were added after autoclaving. The plates were incubated at 30°C for 4 to 7 days. The spore plates were flooded with ±7 mL physiological salt (0.9% NaCl) and the spores loosened with cotton sticks. Once a thick spore suspension had formed on the plate, it was pipetted into a sterile 15 mL Falcon tube. The tube was vortexed to loosen the spore clumps and count the spores. The spore suspension was stored at 4°C. Spore solutions could be stored in 50% glycerol as cryocultures.

3.4.11.4 Cultivation medium

Fungal strains were cultivated in potato waste medium (PWM) (Table 3.1). The traditional medium contained 10 g/L yeast extract, 0.8 g/L $MgSO_4.7H_2O$ and 4 g/L casamino acids. The biomass (potato waste) and traditional medium were autoclaved, after which the Asp+ and trace elements were added. The amount of water in the traditional medium had been amended for the different media to compensate for the difference in volumes used. Therefore all media were identical in composition accept for the biomass component. The final volume in the flasks was approximately 21 mL.

Table 3.1: The composition of PPM2 (pressed apple pomace medium 2) and PWM

Pressed apple pomace medium (PPM2)			
	PPM-10%	**PPM-20%**	**PPM-50%**
Pressed pomace	2 g	4 g	10 g
traditional medium[1]	18 mL	16 mL	10 mL
Asp+	800 µL	800 µL	800 µL
trace elements	40 µL	40 µL	40 µL
Streptomycin 30 mg/mL	20 µL	20 µL	20 µL

Table 3.1 Continued Potato waste medium (PWM-20%)	
Components	**Solids Mix**
Biomass	4 g
traditional medium[1]	18 mL
Asp+	800 µL
trace elements	40 µL
Streptomycin 30 mg/mL	20 µL
Ampicillin 100 mg/mL	20 µL

[1] The amount of traditional medium added to the flasks differ to compensate for the difference in volume of the biomass. The amount of water in the traditional medium had been amended for the 3 different concentrations to compensate for the difference in volumes used. [2] Mixture of potato waste solids

3.4.11.5 Enzymatic assays in liquid medium

Aspergillus strains were inoculated in the different media to a spore count of 5×10^5 spores per mL. Cultivation took place at 30°C at an agitation speed of 200 rpm. The extracellular endoglucanase, endomannanase and endoxylanase activities were determined using the reducing sugar method according to Bailey et al. (1992) and the substrates listed in Table 1.1. All assays were performed at 50°C and pH 5. The supernatant was appropriately diluted prior to the 5 minute incubation with the substrates. The reactions were terminated by the addition of the DNS acid solution. The samples were boiled for 15 minutes at 100°C to assist in colour development. Glucose, mannose and xylose were used as standards for the endoglucanase, endomannanase and endoxylanase activities. The colorimetric changes were measured spectrophotometrically at 540 nm with an X-MARK™ microtitre plate reader (Biorad, Hercules, CA, USA). The activities were expressed in nkat/mL, where 1 katal equals 1 mole of glucose, mannose or xylose released per second.

3.4.11.6 Sample preparation

The supernatants of all the fungal samples were obtained at the end of the cultivation. The supernatants were filtered under vacuum through myracloth and stored at -20°C for further analysis.

3.5 Results

3.5.1 Purification

The recombinant Xyn2 was the dominant secreted protein in the freeze-dried extracellular solution of the *Aspergillus niger* D15 [*xyn2*] strain as could be seen by the single dominant band in Figure 3.1. The size of the Xyn2 was about 20 kDa which is similar to the reported relative molecular mass (21 kDa) of the Xyn2 expressed by the same *A. niger* D15 strain used in this study as well as the native Xyn2 expressed in *Trichoderma reesei* (Rose and van Zyl, 2002; Lappalainen et al., 2000). The crude secreted endoxylanase enzyme had proteins other than the dominant recombinant Xyn2 as could be seen by the faint bands in Lane 2 and 3 of Figure 3.1. An increase in the protein loaded into the SDS-PAGE gel of the crude secreted proteins (Lane 1 and 2 of Figure 3.1) showed an increase in the amount of visible protein bands (Lane 1: 2.4 µg protein loaded, Lane 2: 2.1 µg protein loaded. The proteins other than the recombinant Xyn2 seen in the gel would have been native proteins secreted by the *A. niger* D15 and possibly proteins from the yeast extract which was added to the culture medium. Native cellulase and xylanase production was suppressed by addition of glucose to the culture medium. The Xyn2 was constitutively expressed, which is why the production was high relative to the other proteins. The low molecular weight proteins that were less than 18.4 kDa, as seen in the crude secreted proteins (Figure 3.1; lane 2

and 3), did not show in the 30 K filter device concentrate (Figure 3.1; Lane 4) as they were not retained by the 30 K filter device membrane. The 30 K filter devices have a nominal molecular weight cut off of 30 KDa but proteins with a molecular weight around 30 K may be partially retained as movement through the membrane depends not only on molecular weight but also on shape (Anon, 2012b). This is probably why Xyn2, which has a molecular mass of around 20 kDa, was retained on the 30 K membrane (Figure 3.1; Lane 4). The Xyn2 may have also been retained due to binding to the Amicon® Ultra-2 30 k centrifugation device membrane which is made up of Ultracel® low binding regenerated cellulose. The 10 K filter device concentrate of the 30 K filter devices filtrate showed a single band of about 20 kDa (Figure 3.1; Lane 7-8) that was identified as Xyn2 based on its molecular weight as well as its specific endoxylanase activity (Table 3.1). This indicates purification to electrophoretic homogeneity.

The endoxylanase from the secreted extracellular proteins of *Aspergillus niger* D15 was concentrated and purified with Amicon® Ultra-2 centrifugation devices in a two-step ultrafiltration. The first ultrafiltration with the 30 K membrane showed that Xyn2 was retained on the membrane (Figure 3.1; Lane 4) and the specific activity on Beechwood xylan increased 1.44 fold compared to the crude secreted enzyme solution (Table 3.2). The protein concentration also increased 2.6 fold (Table 3.2). The activity yield (29%) was however lower than the protein recovery (52.6%) which possibly indicates that the enzyme was denatured given that the endoxylanase was the dominant protein (Figure 3.1; Lane 4). The enzymes were centrifuged at 4°C and kept on ice during centrifugation and the crude enzyme was stable at 4°C for at least 20 days, the purified enzyme was found to be stable (retained > 80% activity) for at least 3 days (data not shown).

The centricon filtration process only takes about an hour and a half and was done at 4°C and is not a process which should cause enzyme denaturation so the Xyn2 should have retained most of its activity. The specific activity increased 1.36 fold indicating that the xylanase was purified, although to a small extent. Retention of Xyn2 on the 30 K and 10 k membranes (Figure 3.1) is one reason for the low activity yield of the purified 10 K concentrate. A large fold purification was not expected, as the Xyn2 was clearly the dominant secreted protein (Figure 3.1).

The combined protein recovery of the 30 K concentrate and 10 K concentrate of the 30 K filtrate was 84.8%. The rest of the proteins would have been bound to the filtration membrane or were small enough to pass through the 30 K and 10 K membranes. The combined activity yield was 54%, the rest of the Xyn2 may have bound to the 10 K and/or 30 K membrane or possibly passed through the membrane although it should have been mostly retained on the 10 K membrane.

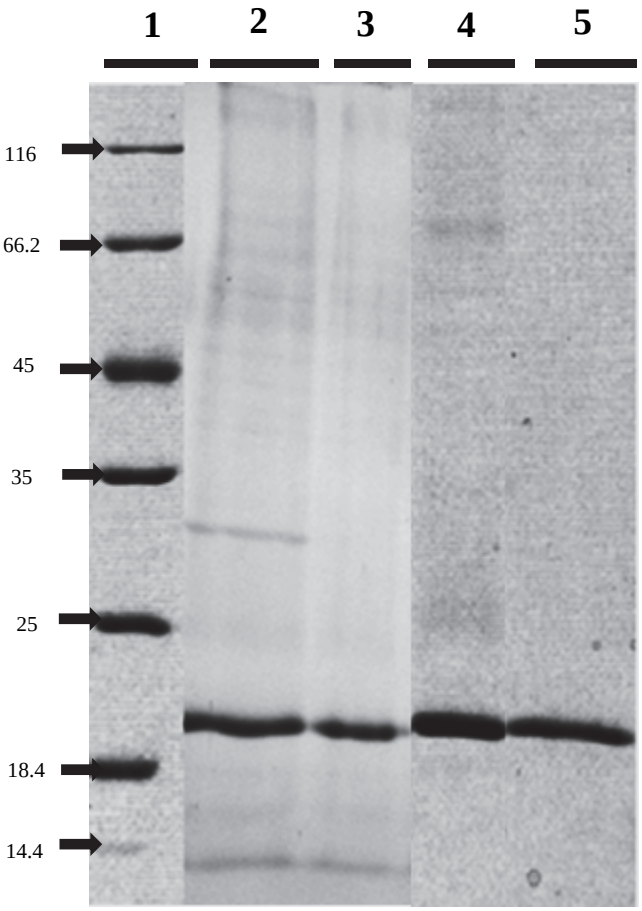


Figure 3.1: Separation of the crude and the concentrated and purified total extracellular protein fractions (using Amicon ® Ultra-2 centrifugation devices) of *A. niger* D15 [*xyn2*] on 10% and 14% SDS-PAGE. Lane 1: Molecular weight markers (unstained, Thermo Scientific) with sizes, in kDa, indicated on the left. Lane 2 and 3: crude enzyme separated on 10% SDS-PAGE. Lane 4: 30 K filter device concentrate separated on 14% SDS-PAGE. Lane 5: 10 K filter device concentrate of 30 K filter device filtrate separated on 14% SDS-PAGE. The amount of protein loaded into wells was as follows: lane 2: 2.4 µg, lane 3: 2.1 µg, lane 4: 2.1 µg, lane 5: 3.7 µg.

Table 3.2: Purification of Xyn2 from secreted proteins of *A. niger* D15

Purification step[a]	Vol. (mL)	Protein concentration (mg/mL)	Total protein (mg)	Protein recovery (%)	Activity [b] (U/mL)	Total activity (U)	Specific Activity (U/mg protein)	Purification fold	Activity yield (%)
Crude stock (100 mg/mL FDS [a])	3.00	0.15	0.46	100	37.19	111.57	1865	1	100
30 K concentrate	0.61	0.39	0.24	52.6	53.85	32.76	2688	1.44	29.4
10 K concentrate of 30 K filtrate	0.55	0.27	0.15	32.2	50.66	27.73	2533	1.36	24.9

[a] FDS = freeze dried solution

[b] U = µmol reducing sugars released/mL.minute

3.5.2 Substrate specificity

The crude and purified Xyn2 had high substrate specificity for beechwood xylan and oat spelt xylan, which were the substrates high in xylan content (Table 3.3; Figure 3.2). Activity on the other holocellulosic substrates tested was absent although the crude enzyme did display a small amount of α-L-arabinofuranisidase activity (1.23% relative activity). About 15% of the residues of oatspelt xylan are glucose which is found in the backbone and about 10% of the residues are arabinose residues which branch off the backbone, xylose makes up 75% of the residues (Wagschal et al., 2009). Beechwood xylan from Sigma, which was used in this study, consists of greater than 90% xylan residues, and about 5% (w/v) arabinose (Wagschal et al., 2009). The endoxylanase activity was highest on beechwood xylan for both the crude and the pure enzyme (Table 3.3, Figure 3.2). The activity was higher on beechwood xylan than oatspelt xylan probably because of less arabinose branches. There was no significant difference between the specific activity of the crude and purified endoxylanase on beechwood xylan (Figure 3.2). This was not expected as the crude and pure endoxylanase did show a significant difference in specific activity after Xyn2 purification (Table 3.3). Enzyme activity on oatspelt xylan was higher for the crude enzyme than for the pure enzyme (Figure 3.2). This was possibly due to the α-L-arabinofuranosidase activity of the crude enzyme.

Table 3.3: Activities of Xyn2 on different holocellulosic substrates. Values are represented as mean values ± standard deviation (n = 3).

	Activity measured	**Specific activity (U/mg)**		**Relative activity (%)**	
		Crude enzyme	**Pure enzyme**	**Crude enzyme**	**Pure enzyme**
Beechwood xylan	Endoxylanase and arabinofuranosidase	2479 ± 64.3	2533 ± 77.2	100 ± 2.59	100 ± 3.05
Oat spelt xylan	Endoxylanase, arabinofuranosidase and endoglucanase	1834 ±26.8	1369 ± 97.5	74 ± 1.08	54 ± 3.85
4-nitrophenyl- α -L-arabinofuranoside	Exo-α -L-arabinofuranosidase	30.4 ± 13.4	0	1.23 ± 0.54	0
Carboxymethyl cellulose	Endoglucanase	0	0	0	0
locust bean gum	endomannanase	0	0	0	0
Avicel ®	endoglucanase	0	0	0	0
Arabinogalactan	Arabinase, galactosidase	0	0	0	0
4-nitrophenyl-β-D-xylopyranoside	Exo-β-D-xylopyranosidase	0	0	0	0
4-nitrophenyl-β-D-glucopyranoside	Exo-β-D-glucopyranosidase	0	n.d.	0	n.d.
4-nitrophenyl-β-D-mannopyranoside	Exo-β-D-mannopyranosidase	0	n.d.	0	n.d.
4-nitrophenyl-β-D-galactopyranoside	Exo-β-D-galactopyranosidase	0	n.d.	0	n.d.
4-nitrophenyl-α-D-galactopyranoside	Exo-α -D-galactopyranosidase	0	n.d.	0	n.d.

n.d. = not determined

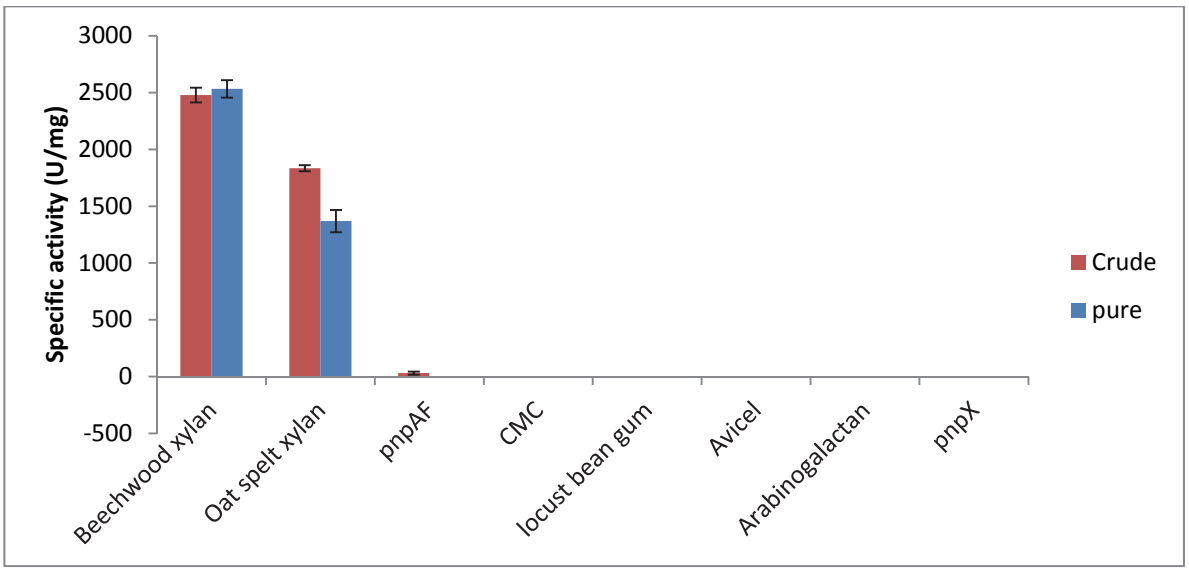


Figure 3.2: Crude and pure Xyn2 activity on different substrates. Values are represented as mean values ± SD (n=3).

3.5.3 pH optima

The pH profile for crude and purified Xyn2 as seen in Figure 3.3 indicated that the crude enzyme had an optimal pH between 5.5 and 6.5. The pure enzyme had a definite peak at pH 6.5. The pH activity range seemed to shift towards more basic activity from the crude to the pure enzyme. The crude Xyn2 retained >80% activity between pH 5.5 and pH 7. The pure Xyn2 retained > 80% activity between pH 6 and pH 7.

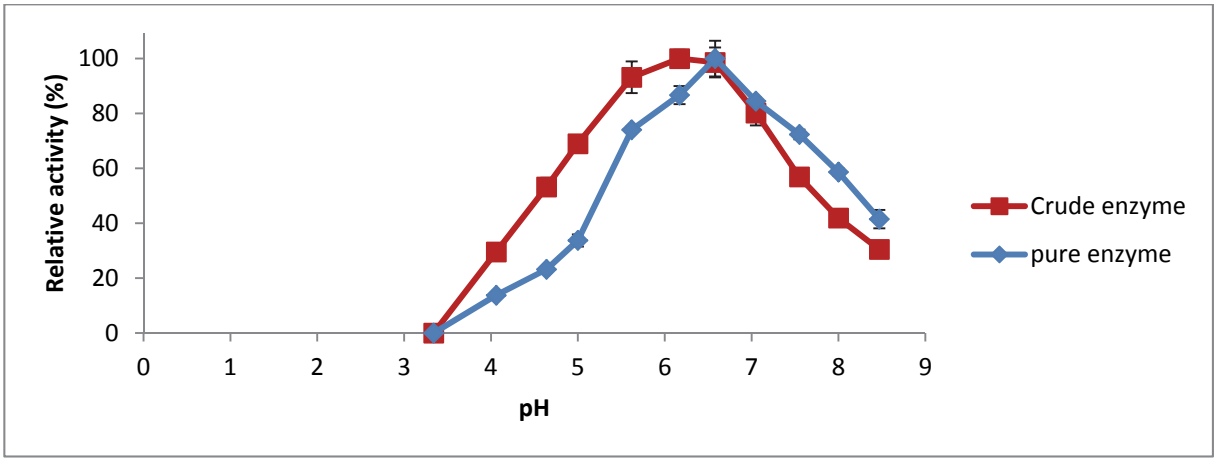


Figure 3.3: pH optima of crude and pure Xyn2 activity. Activity was measured using 0.5% (w/v) beechwood xylan as a substrate and 0.1 M BAP buffer from pH 3.37 to 8.5. The enzyme assay was 30 minutes and the protein load was 0.2 µg/mL. Values are represented as mean values ± SD (n=3) and in some cases errors bars are too small to be seen.

3.5.4 Temperature optima

The temperature optima of the crude and pure Xyn2 can be seen in Figure 3.4. The temperature optimum profiles were very similar for the crude and pure enzyme. The only apparent differences were at the extremes of the graph. At 30°C and 40°C the crude enzyme had a higher activity.

Between 55°C to 70°C the crude enzyme formed a second peak, perhaps indicating a second active enzyme. The temperature optima for the crude and pure enzymes were 50°C under the reaction conditions used.

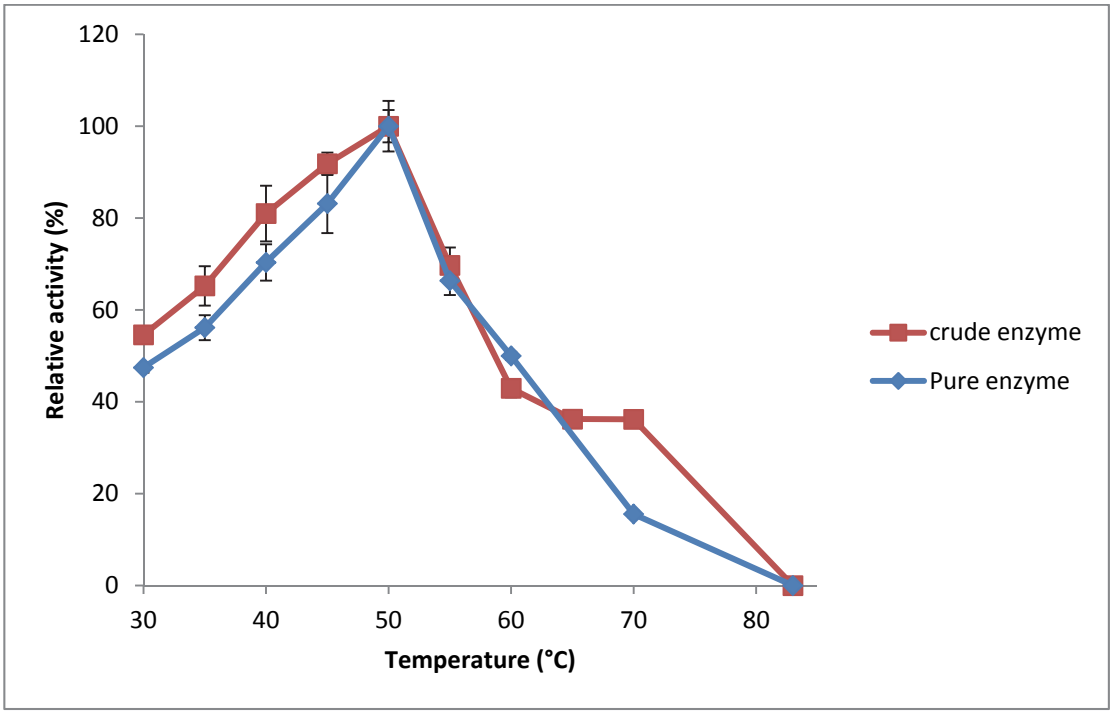
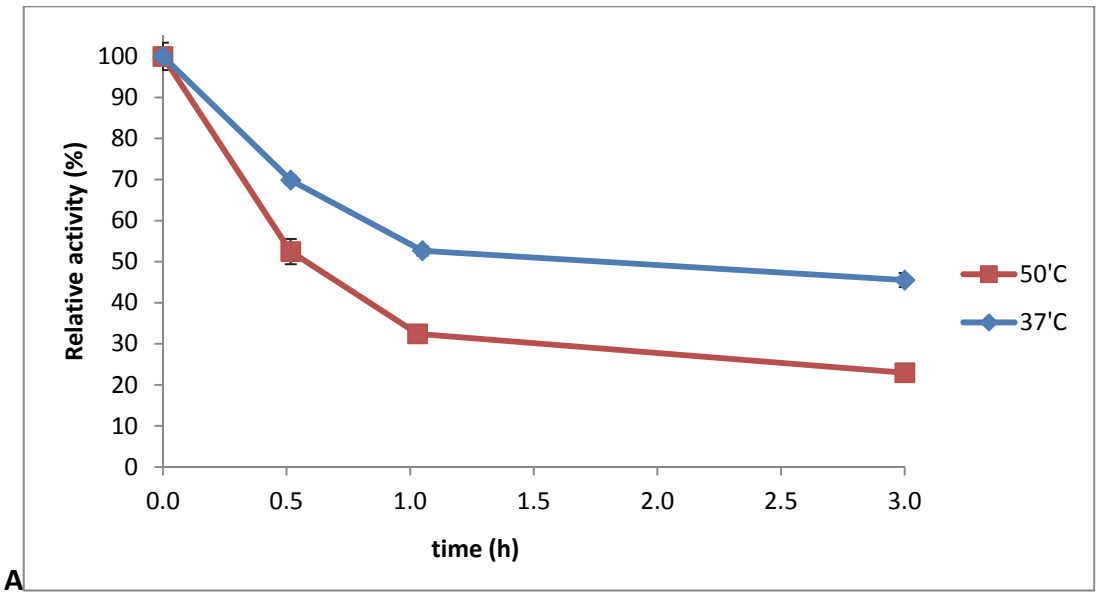


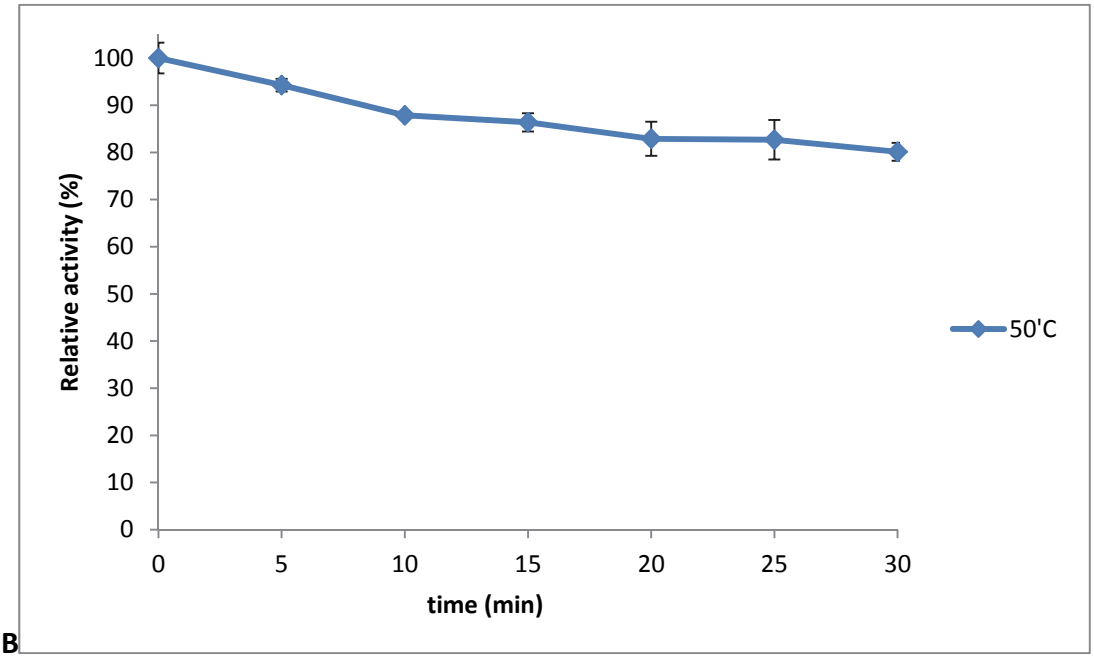
Figure 3.4: Temperature optima of crude and pure Xyn2 activity. Activity was measured using 0.5% (w/v) beechwood xylan as a substrate and 0.1 M BAP buffer from pH 3.37 to 8.5. The enzyme assay was 30 minutes and the protein load was 0.1 µg/mL. Values are represented as mean values ± SD (n=3) and in some cases errors bars are too small to be seen.

3.5.5 Temperature stability

The temperature stability of crude Xyn2 was measured over a 3 hour period at 37 °C and 50 °C (Figure 3.5A). The half-life at 37 °C was just over 1 hour. The half-life at 50 °C was about 30 minutes. Given that the standard assay used in this study is at 50 °C and for 30 minutes, and that the half-life at 50 °C was 30 minutes an additional stability assay was performed at 50 °C to see if the enzyme stability would be increased if the assay reaction time decreased. Figure 3.5B shows the Xyn2 stability at 50°C with a reduced assay time of 5 minutes. Above 80% of the Xyn2 activity was retained with this modified assay.



A



B

Figure 3.5: Temperature stability of the crude Xyn2. A: Crude enzyme activity was measured using 1% (w/v) beechwood xylan as a substrate and 0.05 M pH 5 citrate-NaOH buffer. The enzyme assay was 30 minutes and the protein load was 0.5 µg/mL. B: Purified enzyme activity was measured using 1% (w/v) beechwood xylan as a substrate and 0.05 M pH 5 citrate-NaOH buffer. The enzyme assay was 5 minutes and the protein load was 0.5 µg/mL. Values are represented as mean values ± SD (n=3).

3.5.6 Inhibition

The effect of soluble lignin on Xyn2 activity was investigated at lignin concentrations ranging from 0 to 2 mg/mL (Figure 3.6). The investigated concentration range seemed to have an insignificant effect on Xyn2 activity.

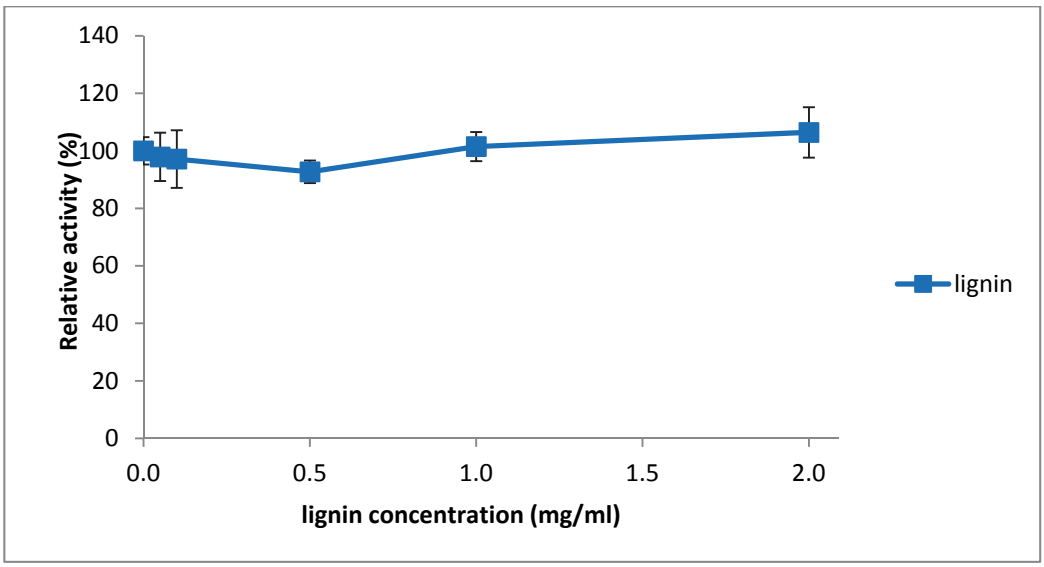


Figure 3.6: The effect of lignin on Xyn2 activity was determined using beechwood xylan as a substrate in the presence of soluble lignin concentrations from 0 to 2 mg/mL. Values are presented as mean values ± SD (n=3); SD error bars are sometimes too small to be seen. Values are represented as mean values ± SD (n=3) and in some cases errors bars are too small to be seen.

The effect of selected monophenolics on Xyn2 activity is shown in Figure 3.7. Vanillin, *p*-coumaric acid and vanillic acid had no significant inhibitory effect on the Xyn2 at the concentrations used. Gallic acid was significantly inhibitory (16% inhibition) at 0.2% (w/v).

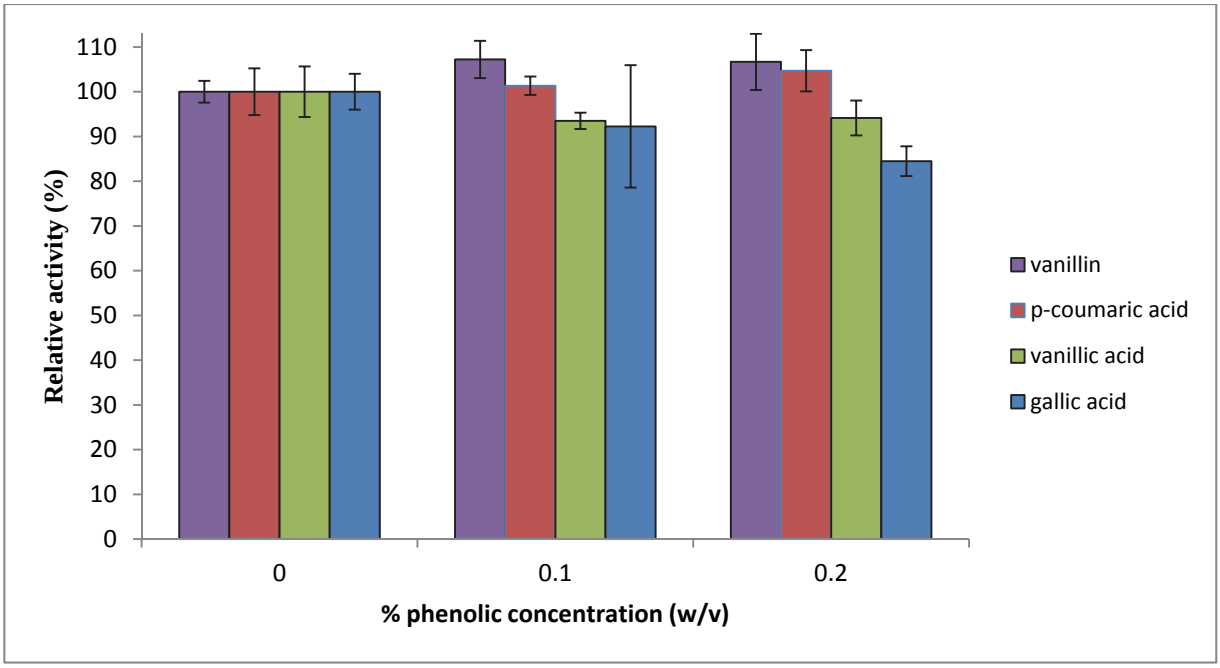


Figure 3.7: The effect of selected monophenolics on Xyn2 activity was determined using beechwood xylan as a substrate in the presence of 0.1% (w/v) or 0.2% (w/v) monophenolics. Values are presented as mean values ± SD (n=3); SD error bars are sometimes too small to be seen.

The effect of furfural, acetic acid and formic acid on Xyn2 activity is shown in Figure 3.8. Furfural and acetic acid did not cause a significant change in Xyn2 activity. Formic acid caused a 24%

decrease in enzyme activity. Formic acid is a stronger acid than acetic acid (Jönsson et al., 2013). A change in acidity of about 0.5 pH units was noted upon addition of the formic acid and acetic acid to the reaction mix using pH paper strips.

The effect of divalent cations and EDTA on Xyn2 activity on beechwood xylan was determined (Figure 3.9). At the concentrations tested Ca^{2+}, Fe^{2+}, Zn^{2+}, Fe^{3+} and Cu^{2+} did not cause any significant inhibition of Xyn2. Mg^{2+} caused inhibition at both 2 mM and 10 mM with an increase in inhibition between 2 mM and 10 mM. The same pattern was observed for EDTA and Mn^{2+}, the amount of inhibition was however larger. Mn^{2+} was the strongest inhibitor. The amount of inhibition caused at 10 mM inhibitor concentration was: Mg^{2+} – 14%; EDTA – 23%; Mn^{2+} – 47%.

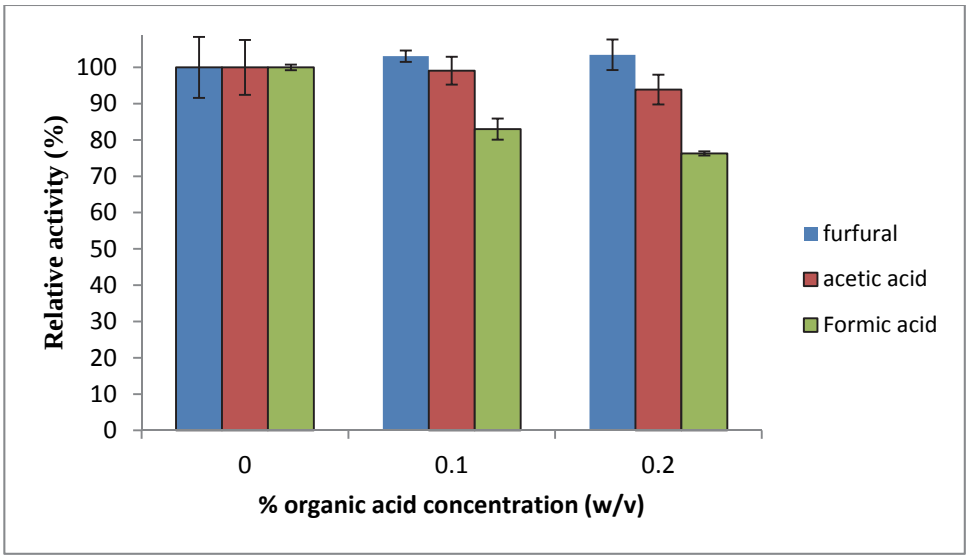


Figure 3.8: The effect of acetic acid, formic acid and furfural on Xyn2 activity was determined using beechwood xylan as a substrate in the presence of 0.1% (w/v) or 0.2% (w/v) organic acids. Values are presented as mean values ± SD (n=3).

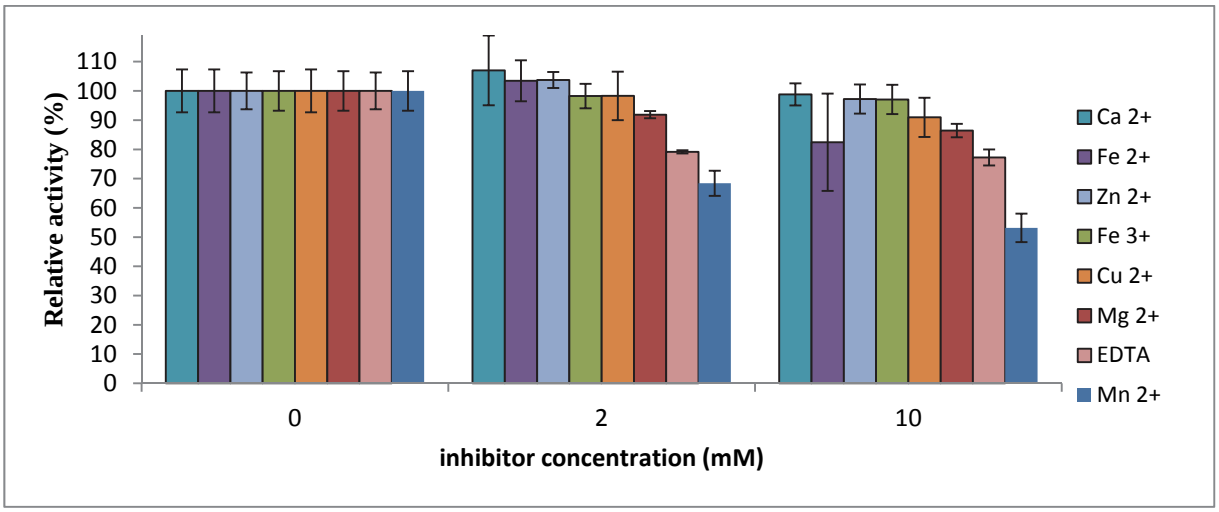


Figure 3.9: Effect of metal cations: Ca^{2+}, Fe^{2+}, Zn^{2+}, Fe^{3+}, Cu^{2+}, Mg^{2+}; and EDTA on Xyn2 activity on beechwood xylan. Values are represented as mean values ±SD (n=3).

The inhibitory effect of common fermentation products on Xyn2 activity was determined (Figure 3.10). It was found that 2-propanol, 1-butanol and ethanol caused a similar degree of inhibition. Inhibition effects only started to occur for acetone at 30 g/L, ethanol at 40 g/L and 2-propanol at 50 g/L. 1-butanol was the most inhibitory; at 60 g/L there was a decrease in activity of about 70%.

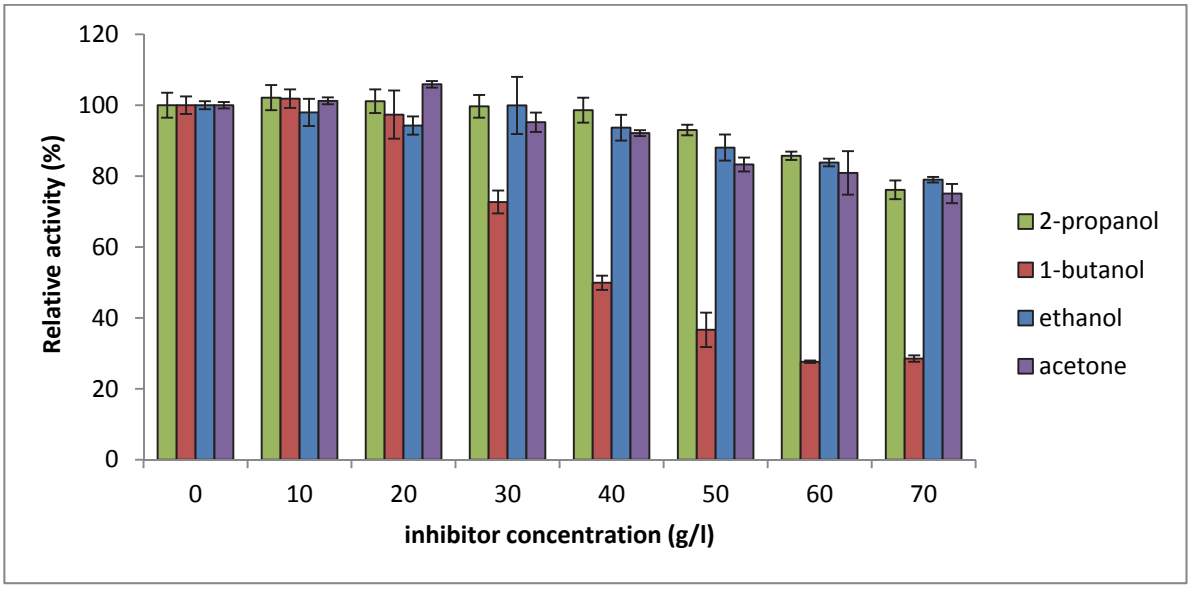


Figure 3.10: Effect of the fermentation products 2-propanol, 1-butanol, ethanol and acetone (10-70 g/L) on Xyn2 activity on beechwood xylan. Values are represented as mean values ±SD (n=3).

3.5.7 Laccase production using red grape waste

Figure 3.11 shows laccase production by *T. pubescens* cultured in RGW and W-RGW. Only shaking cultures (10 and 20% grape waste) were sampled and assayed for laccase production on Days 3 and 5. All cultures were assayed on Day 14.

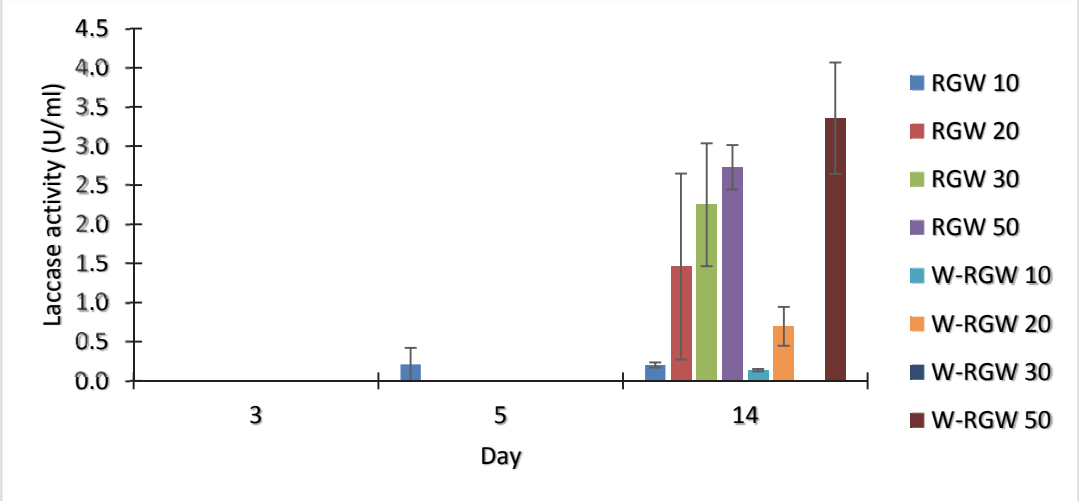


Figure 3.11: Laccase production by *T. pubescens* cultured on red grape waste (RGW) and washed red grape waste (W-RGW) at 10, 20, 30 and 50% (w/v). Values are represented as mean values ± SD (n=3) and in some cases errors bars are too small to be seen.

There was little to no laccase production after 5 days of incubation, which corresponded to little or no visible fungal growth in the medium. After 14 days, fungal growth in the shaking cultures was observed stuck to the sides of the flask. Good growth within the medium was difficult to determine due to the non-homogenous nature and dark colour of the grape waste. Growth of the SSF cultures was excellent, forming a thick white mycelial mat over the top and within the grape waste material, with the exception of 30% W-RGW (W-RGW 30). Laccase production was higher in the SSF cultures (2.3-3.3 U/mL) than shaking cultures (0.2-1.5 U/mL), with the exception of 30% W-RGW (W-RGW 30). There was little to no growth in flasks containing 30% W-RGW, which resulted in no laccase production. Laccase production using potato waste with red grape waste.

Previous work showed that *T. pubescens* produced up to 2.5 U/mL laccase within 7 days using 20% w/v potato wastes. In this study, 20% w/v potato wastes were supplemented with RGW to determine if laccase production could be increased (Figure 3.12).

The addition of low concentrations of RGW (up to 5%) enhances laccase production by *T. pubescens* in both types of potato waste (> 4 U/mL with RGW compared to approximately 2 U/mL when no RGW was added). Furthermore, while maximum laccase production occurred on day 7 in potato waste alone, production when RGW was added was not only greater, but also occurred earlier (Day 3-5). Laccase activity was detected in cultures supplemented with 10% RGW only after 10 days incubation (Figure 3.12).

3.5.8 Heterologous gene expression in *A. niger*

The *A. niger* strains were cultivated on apple pomace medium (PPM2) and the enzyme activities determined after 3 and 6 days of cultivation (Figure 3.13). The extracellular levels of enzyme activities were much lower than that obtained previously indicating that different results can be expected when different methods are used for homogenizing the substrate or when different cultivars are used. The total phenolics in the media was determined after 6 days of cultivation (Figure 3.14). In general, the growth of the strains does not seem to affect the total phenolics present in the medium (when compared to the uninoculated medium). The cultivation of the strains results in a decrease in COD.

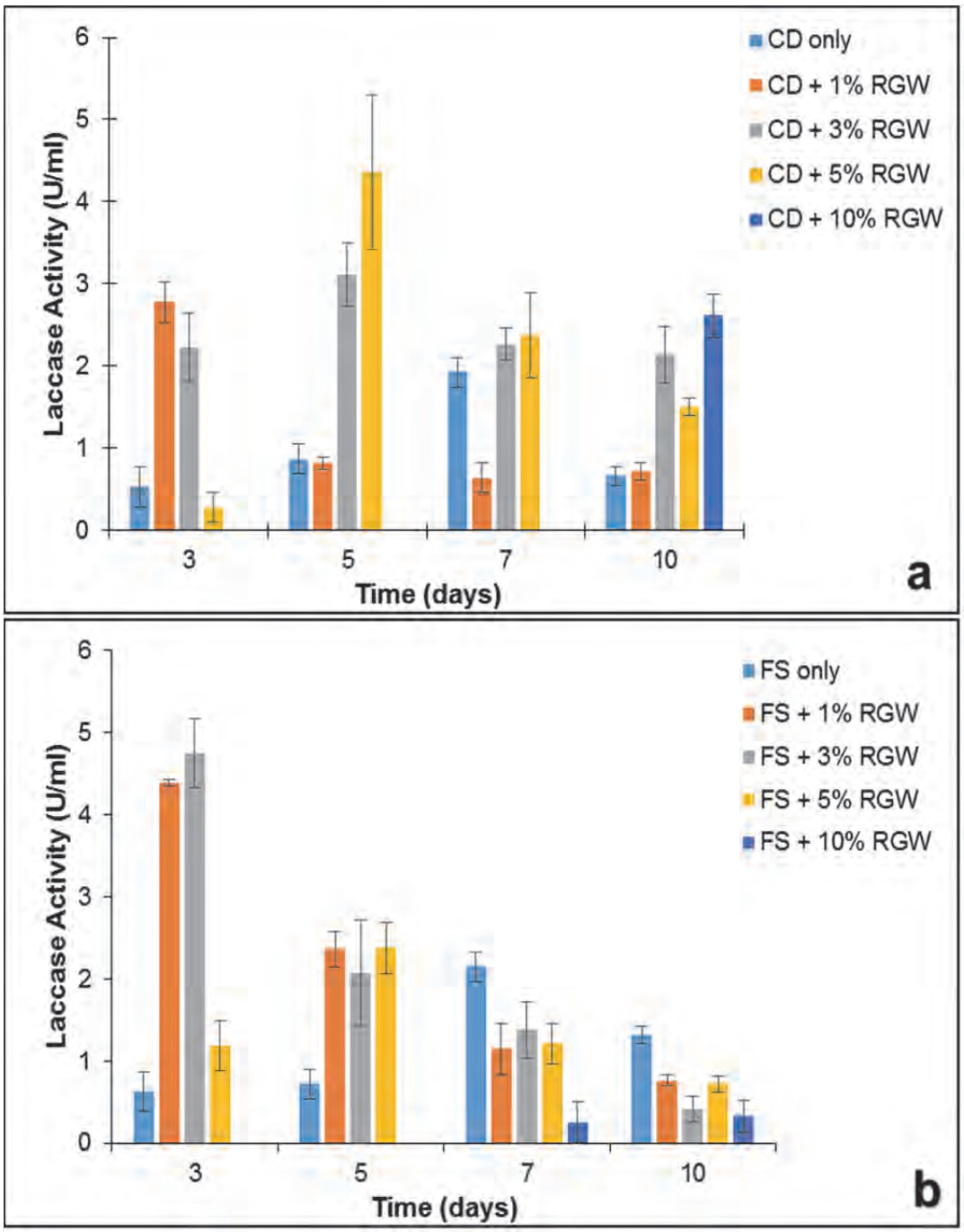


Figure 3.12: Laccase production by *T. pubescens* in 20% w/v (a) Cutting and defects (CD) potato waste and (b) final solids (FS) potato waste supplemented with 1, 3, 5 and 10% w/v RGW. Values are represented as mean values ± SD (n=3).

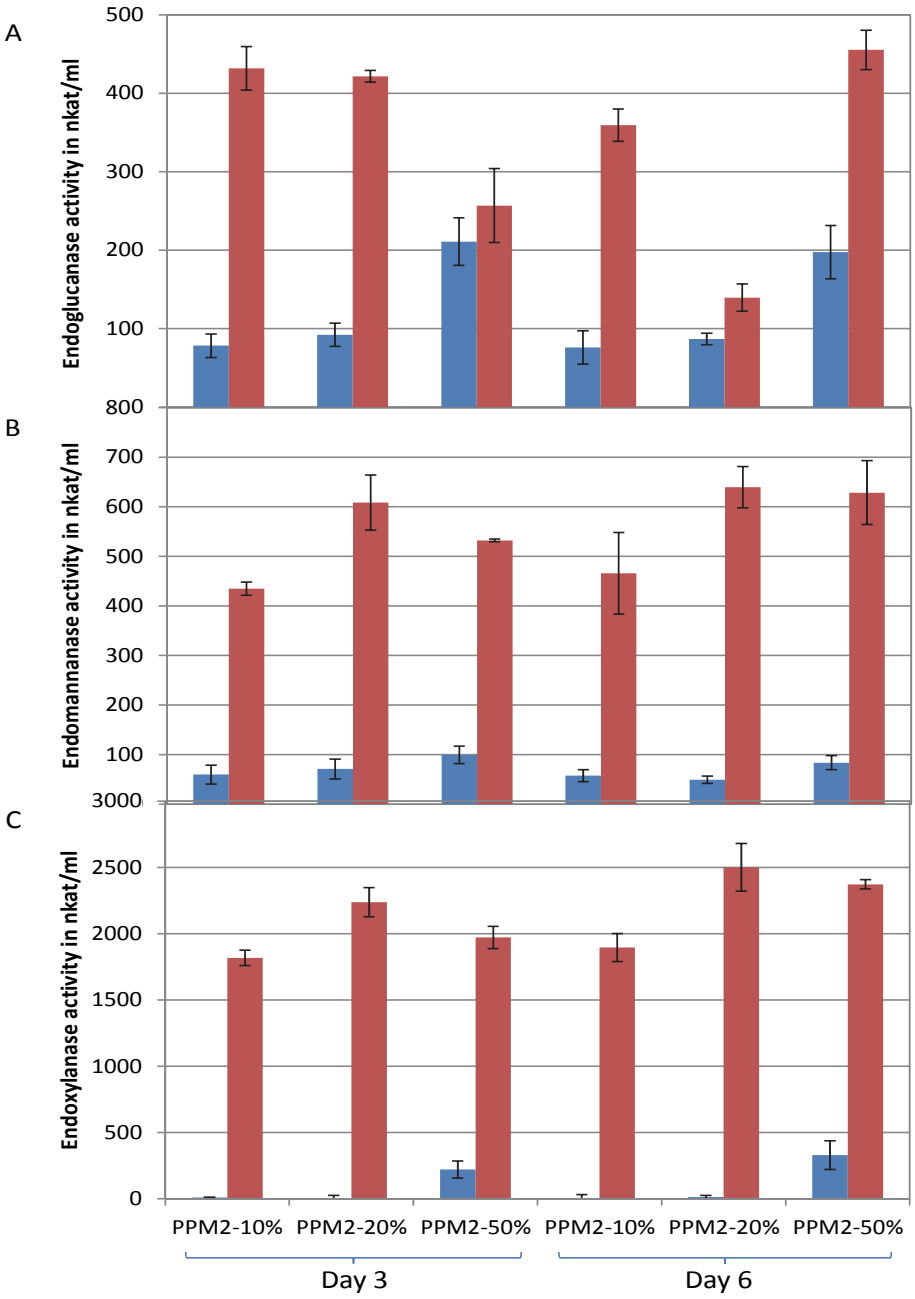


Figure 3.13: The extracellular (A) endoglucanase, (B) endomannanase and (C) endoxylanase enzyme activities detected for the *A. niger* D15[*eg2*], D15[*man1*] and D15[*xyn2*] after 3 and 6 days of cultivation at 30°C in PPM2 (apple pomace medium). Values are represented as mean values ± SD (n=3).

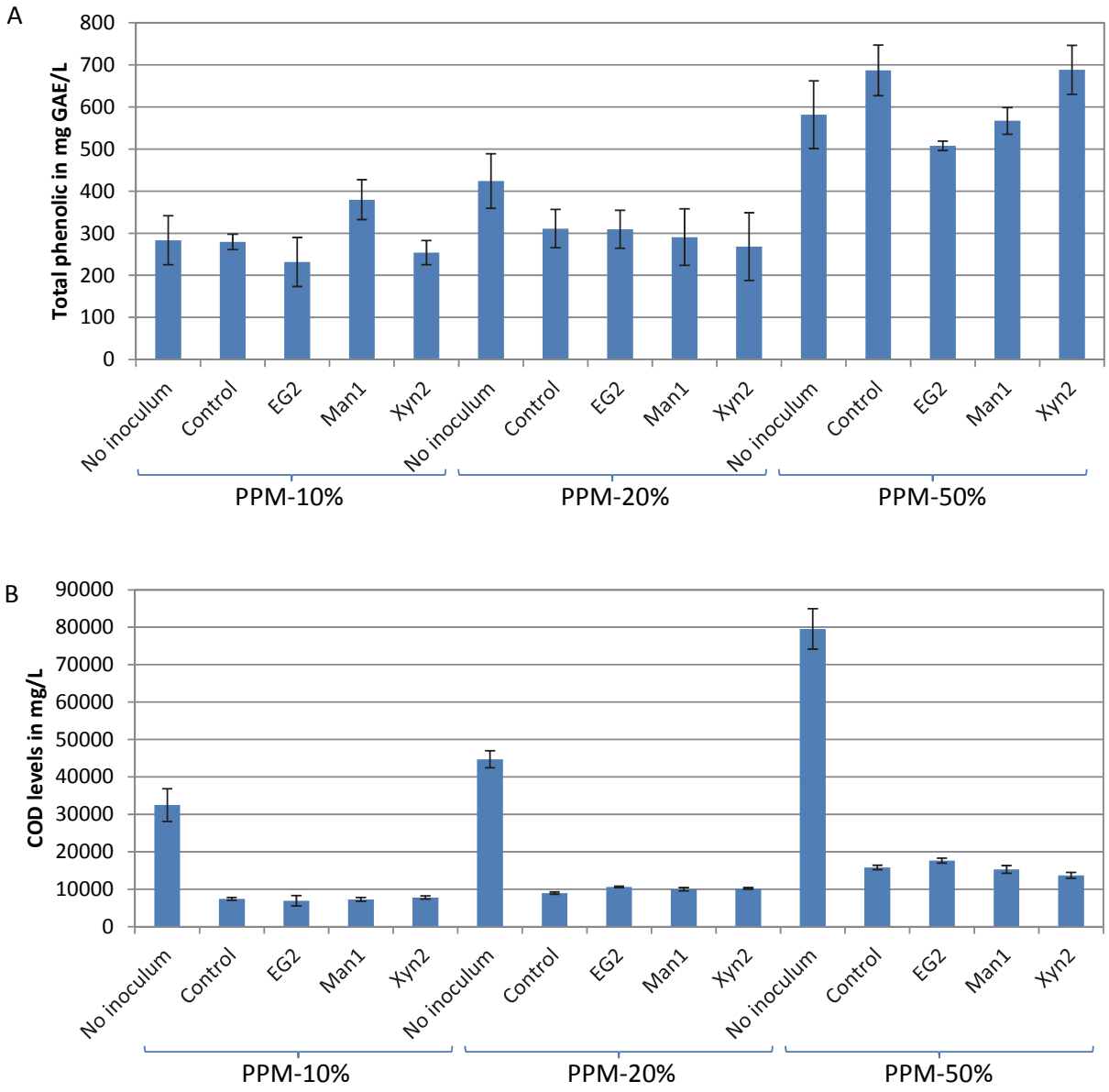


Figure 3.14: The (A) total phenolics and (B) COD levels were determined for the *A. niger* D15[*eg2*], D15[*man1*] and D15[*xyn2*] supernatants after 6 days of cultivation at 30°C in PPM2 (apple pomace). Values are represented as mean values ± SD (n=3).

The previous chapter focused on the use of the individual waste streams of the potato waste industry to determine which waste stream was most suited for maximum levels of extracellular activities produced by recombinant *A. niger* strains. For this report the potato solids were prepared (homogenized) using a mixture of all the different solids as it would be obtained from the potato processing industry. The solids were used to prepare PWM-20% (Table 3.1). The traditional medium was prepared with either distilled water (to compare with the previous results) or with wastewater obtained from the potato processing industry. Enzyme activities were determined after 3 and 6 days of cultivation (Figure 3.15).

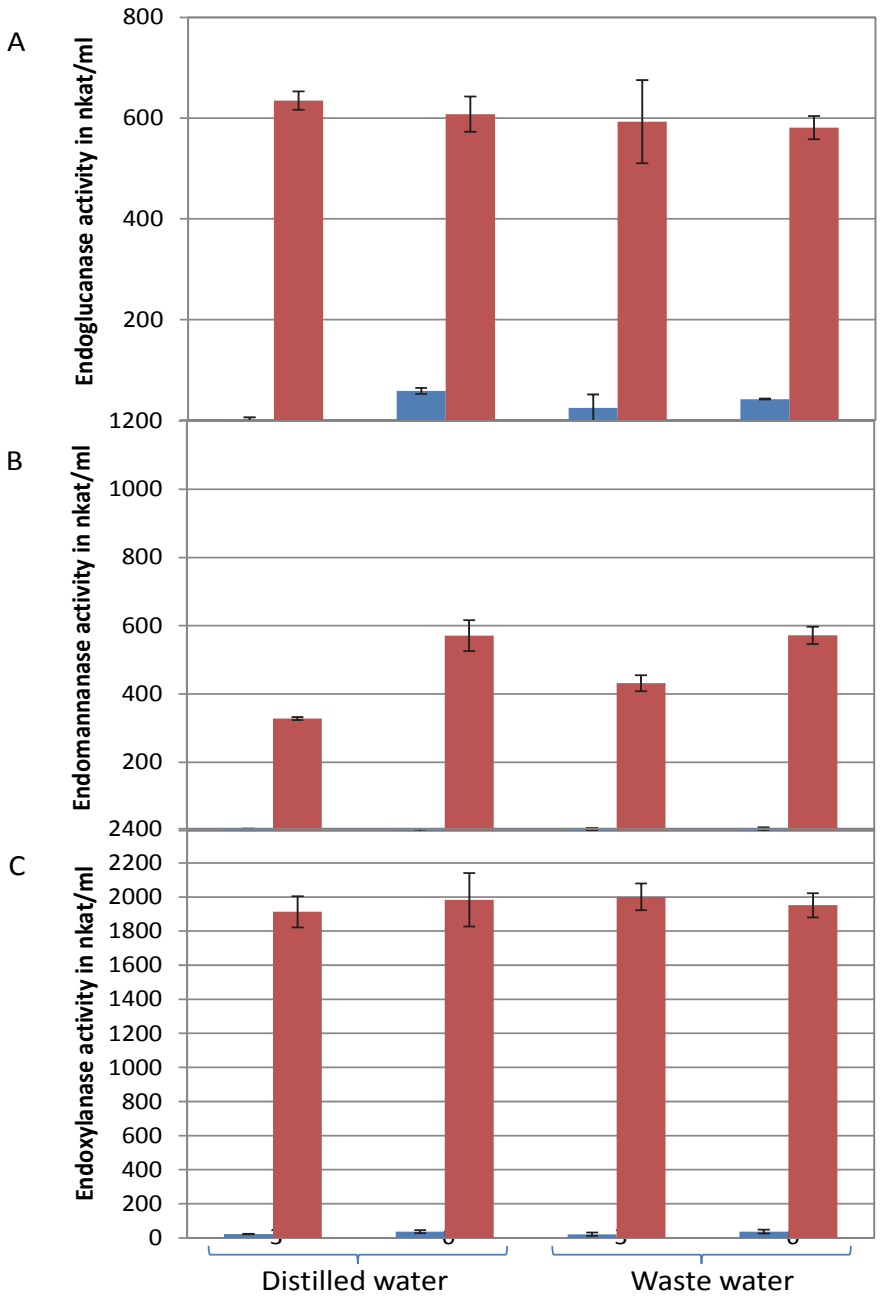


Figure 3.15: The extracellular (A) endoglucanase, (B) endomannanase and (C) endoxylanase enzyme activities detected for the *A. niger* D15[*eg2*], D15[*man1*] and D15[*xyn2*] respectively, after 3 and 6 days of cultivation at 30°C in PWM-20% (containing a 20% mixture of potato solids). Traditional medium was either prepared with distilled water or wastewater obtained from the potato industry. The *A. niger* D15[GT] reference strain is in blue. Values are represented as mean values ± SD (n=3).

Extracellular enzyme activities were determined after 3 and 6 days of cultivation (Figure 3.15). The endoglucanase and endoxylanase levels of activities for the mixture of solids were similar to the values obtained for the individual potato wastes, whereas the mannanase activity was similar to that previously obtained with KB, but much lower than that obtained with slivers and starch (Figure 3.15). The addition of wastewater had little effect on the levels of extracellular enzyme production, indicating that the wastewater can be used for the cultivation of *A. niger* strains for enzyme production.

The residual sugar concentration were determined at the end of the fermentations (data not shown) through HPLC analysis. As expected no glucose, fructose or sucrose were detected. The dry weight composition of the different potato wastes varied: 10 g of wet weight equals approximately 3 g KB, 3.14 g LB, 1.71 g skins, 3 g slivers, 2 g solids mixture and 9 g starch. The dry weight determined for the solids mixture was used to determine the theoretical enzyme yield obtained from PWM-20% (Table 3.4).

The total phenolics and COD levels in the media was determined after 6 days of cultivation (Figure 3.16). The use of wastewater did not affect the COD or levels of total phenolics (with the exception of the Xyn2, probably experimental error). In general, the growth of the strains did not seem to affect the COD levels in the medium (when compared to the uninoculated medium). The addition of the traditional medium, however, significantly increases the levels of the total phenolics and the COD.

Table 3.4: Data conversion of activities obtained by the recombinant strains on PWM-20% (solids)

		Activity in nkat/mL		**Activity in nkat/g DW[1]**		**Activity in U/g DW**		**Enzyme in g/L[2]**
Endoglucanase	Time	[pGT1]	[Eg2]	[pGT1]	[Eg2]	[pGT1]	[Eg2]	Eg2
Distilled water	day 3	0.00	634.89	0.00	15872.22	0.00	952.14	0.17
	day 6	58.22	607.78	1455.56	15194.44	87.32	911.48	0.17
Wastewater[3]	day 3	24.22	592.67	605.56	14816.67	36.33	888.82	0.16
	day 6	41.78	581.11	1044.44	14527.78	62.65	871.49	0.16
Mannanase	Time	[pGT1]	[Man1]	[pGT1]	[Man1]	[pGT1]	[Man1]	Man1
Distilled water	day 3	5.26	328.07	131.58	8201.75	7.89	492.01	0.07
	day 6	0.97	570.76	24.37	14269.01	1.46	855.97	0.13
Wastewater[3]	day 3	4.68	431.58	116.96	10789.47	7.02	647.24	0.10
	day 6	3.70	571.93	92.59	14298.25	5.55	857.72	0.13
Endoxylanase	Time	[pGT1]	[Xyn2]	[pGT1]	[Xyn2]	[pGT1]	[Xyn2]	Xyn2
Distilled water	day 3	22.79	1912.99	569.68	47824.86	34.17	2868.92	0.27
	day 6	36.16	1983.62	903.95	49590.40	54.23	2974.83	0.28
Wastewater[3]	day 3	21.09	2001.32	527.31	50032.96	31.63	3001.38	0.29
	day 6	36.53	1951.41	913.37	48785.31	54.79	2926.53	0.28

[1] DW refers to the dry weight of the substrate; [2] calculated from the specific activities obtained from Samanta et al. (2012) for the EG2 (3676 nkat/mg), Setati et al. (2001) for Man1 (4400 nkat/mg) and Tenkanen et al. (1992) for Xyn2 (7000 nkat/mg); [3] wastewater was used to prepare the traditional medium for fungal cultivation

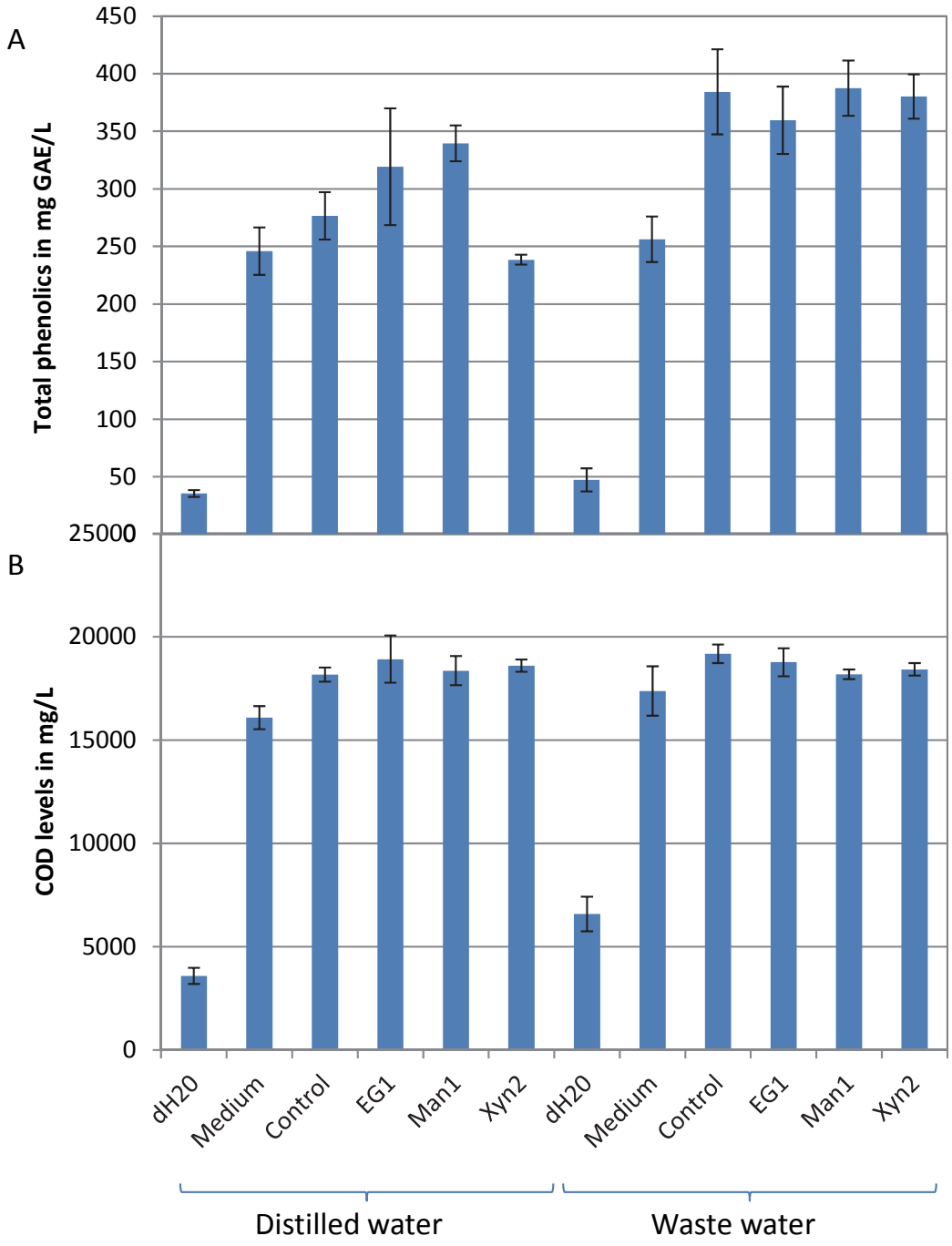


Figure 3.16: The (A) total phenolics and (B) COD levels were determined for the *A. niger* D15[*eg2*], D15[*man1*] and D15[*xyn2*] supernatants after 6 days of cultivation at 30°C in PWM-20% (containing a 20% mixture of potato solids). Values are represented as mean values ± SD (n=3).

3.6 Discussion

3.6.1 Purification

The size of Xyn2 (20 kDa, Figure 3.1) was as expected. The Xyn2 produced by *A. niger* D15 [*xyn2*] was found to be 21 kDa by Rose and van Zyl (2002). When expressed by its native host, *Pichia pastoris* and *Pichia stipitus*, the size has been found to be between 19 kDa and 21 kDa (Lappalainen et al., 2000; Tenkanen et al., 1992). These differences in size are not significantly

different as they were determined by SDS-PAGE which has an error in molecular weight calculation of 10% (Walker, 2010). The molecular weight when expressed in *Saccharomyces cerevisiae* was found to be 27 kDa, which is significantly larger than the Xyn2 expressed in other hosts (La Grange et al., 1996) was probably due to glycosylation. Glycosylation depends on the fungal strain (even within the same species), cultivation medium and pH, and secretion of carbohydrate modifying molecules (Seiboth et al., 2011). Xyn2 is N-glycosylated and has 15 possible N-glycosylation sites (La Grange et al., 2001) which may have been glycosylated in *S. cerevisiae* to a larger extent than in the other hosts. *S. cerevisiae* is known to hyperglycosylate secreted proteins (He et al., 2009).

The purification procedure using sequential filtrations with 30 K and then 10 K Amicon® Ultra-2 centrifugation devices purified the Xyn2 to electrophoretic homogeneity (Figure 3.1). These devices were chosen for purification as they potentially provided a cheap, simple method for purification, which could potentially be up scaled to industrial scale. The specific activity obtained was 2533 U/mg protein, which is a higher specific activity than what has been found in the literature. The specific activity for purified Xyn2 of *Trichoderma reesei* expressed in *Escherichia coli* has been found to be 1757 U/mg (Turunen et al., 2001). The enzyme assay method used by (Turunen et al., 2001) was one of the standard DNS methods used for xylanase activity (Bailey et al., 1992) and UV spectroscopy was used to determine the protein concentration (Turunen et al., 2001). Tenkanen et al. (1992) found the specific activity for purified Xyn2 from *T. reesei* to be 810 U/mg. The Lowry assay was done for protein determination. The enzyme assays used in these studies were different to the assay method used in this study, which could account for the difference in determined specific activity. The substrates were different or from different suppliers and the protein determination methods were different as well as the assay times. The commonly used method for determining xylanases activity of Bailey et al. (1992) was not used in this study as the reaction volume is large (2 mL) and is not suitable for enzymology-based research, which requires many enzyme assays. The protocol used in this study was the standard holocellulase protocol used in our laboratory which has an enzyme activity assay reaction volume of 0.4 mL.

The activity yield of the purification of Xyn2 was low (24.9%; Table 3.2). This was due in part to retention on the 30 K membrane and possibly also due to adsorption to the filter membrane. The possible cellulose binding convex face of β-sheet A of Xyn2 may be the cause for the binding. (Törrönen and Rouvinen, 1997). Samanta et al. (2012) purified heterologously produced and secreted endoglucanase 2 from *Trichoderma reesei* and achieved 45.35% activity recovery. Activity yield may have been improved by using different Amicon® Ultra-2 centrifugation device filters. A 50 kDa filter may be used instead of a 30 kDa filter. This would possibly minimize retention on the 30 kDa membrane, unless the binding affinity of the Xyn2 to the regenerated cellulose membrane is high. This may, however, have caused a decrease in purification as some of the proteins with a molecular mass larger than Xyn2 and lower than 50 kDa may not separate from Xyn2. The low fold purification of the electrophoretically homogeneous Xyn2 and the relatively high amount of Xyn2 in the crude extract compared to other extracellular proteins is an indication that purification is probably not necessary as it is already quite pure. Characterization of pure enzymes is important for the design of enzyme cocktails where the types and ratio of enzymes can be optimized to maximize monomeric sugar yield and minimize protein loading for lignocellulose hydrolysis for specific substrates which could save costs (Van Dyk and Pletschke, 2012). The problem with

purification is that it is often expensive and may not be necessary. Expression of *T. reesei* Xyn2 in *Saccharomyces cerevisiae* produced an almost pure protein (95%) (He et al., 2009), this may seem to indicate it as a better expression host than *A. niger* for secreted Xyn2. However, enzyme production from *S. cerevisiae* is low compared to *A. niger* (van Zyl et al., 2009), *A. niger* can also utilize phenolics and organic acids as nutrients which are toxic to yeast (Alriksson et al., 2009). This is one of the reasons why *A. niger* is being used in bioremediation of lignocellulosic wastes as well as in the production of valuable enzymes from the waste (Joshi and Attri, 2006)

3.6.2 Substrate specificity

The crude and pure Xyn2 had a high specific activity for the xylan polymers tested (Table 3.3; Figure 3.2). The activity was higher on beechwood xylan than on oat spelt xylan probably because beechwood xylan has less arabinose branching than oat spelt xylan (Wagschal et al., 2009). Hydrolysis of the xylan backbone is less sterically hindered by arabinose side chains in beechwood xylan than in oat spelt xylan (Wagschal et al., 2009). The higher oatspelt xylan activity of the crude enzyme compared to the pure enzyme (Figure 3.2) could be explained by the α-L-arabinofuranosidase activity of the crude enzyme, no α -L-arabinofuranosidase activity was detected in the pure enzyme. Removal of arabinose branches in the beechwood xylan backbone would have given the Xyn2 better access and allowed for higher hydrolysis rates by Xyn2. The enzymes seemed to display synergy. Synergy has been observed between endoxylanases and α-L-arabinofuranosidases on oatspelt xylan (Van Dyk and Pletschke, 2012). The arabinofuranosidases of *Aspergillus niger* that have been characterized are all greater than 34 kDa in size (de Vries and Visser, 2001). Therefore, they probably would have not passed through the 30 KDa Amicon® filtration device membrane so they would not have been present in the purified enzyme solution. The low yield, low fold purification and lowered activity on oatspelt xylan indicate that purification is not beneficial.

The high xylanase activity and undetectable cellulase activity potentially make this Xyn2 ideal for use in pre-bleaching in the paper and pulp industry, which could reduce chlorine use by up to 30%. Chlorine forms toxic organochlorines in pulp bleaching when chlorine reacts with lignin degradation products (Polizeli et al., 2005).

3.6.3 pH optima

The pH optima for the crude Xyn2 (pH 5.5-6.5) (Figure 3.3) was about the same as what was found for the Xyn2 expressed by the same *Aspergillus niger* D15 strain (pH 5-6; Rose and van Zyl, 2002). The pH optimum for the pure enzyme was 6.5 which was slightly higher than what was found by Rose and van Zyl (2002). The pH activity range of the pure enzyme was also slightly narrower and shifted about 0.5 pH units towards more basic conditions. The pH activity profile found by Rose and van Zyl (2002) was broader than what was found in this study, however, Rose and van Zyl (2002) measured pH optima between pH 4 and 7 with 1 pH unit intervals so their pH optima curve was less detailed than the pH optima curve constructed in this study. The enzyme assay protocol used by Rose and van Zyl (2002) was the commonly used method of Bailey et al. (1992). The enzyme assay was therefore 5 min long as opposed to 30 min long in this study; they also used a different buffer with different ionic strength (0.05 M citrate phosphate buffer). The protein concentration they used in the assay is not given, but it could have played a role in stability, as proteins become less stable as their concentrations decrease (Deutscher, 2009). The ideal protein

concentration to use should be based on enzyme loading determination studies. This was not done in this study. The decrease in stability may have been due to a decrease in ionic strength caused by ultra-centrifugation, which could have caused a decrease in enzyme stability. The pH stability range of Xyn2 has been extended by expression in *Pichia pistoris* (He et al., 2009). The Xyn2 retained 60% relative activity between pH 3 and 8 (He et al., 2009) whereas the endoxylanase in this study retained 0% activity at pH 3.37 and 42-58% activity at pH 8 (Figure 3.3).

3.6.4 Temperature optima

The temperature optimum was found to be 50°C for the crude and purified enzyme (Figure 3.4). This is within the range of Xyn2 produced by the same recombinant *Aspergillus* niger D15 strain and in *Pichia pastoris* (50°C-60°C); (Rose and van Zyl, 2002; He et al., 2009). The temperature activity range was, however, different. The Xyn2 from this study had the highest relative activity at temperatures below 50°C but its activity at higher temperatures was much lower. The Xyn2 expressed in *Pichia pastoris* retained relative activity more at high temperatures above the pH optima (50-60°C) than at low temperatures below the pH optima. This increased thermal stability may have been due to glycosylation or amino acid mutation (He et al., 2009). The apparent higher thermostability at high temperatures compared with this study may be due to the difference in assay conditions used. Rose and van Zyl (2002) conducted their enzyme assay for 5 minutes, while He et al. (2009) conducted their assay for 10 minutes. Differences in protein concentrations between the assays may have also played a role.

For the reaction conditions used in this study, the specific activity of the enzymes was highest at the optimal temperature and pH. This does not necessarily mean that this is the optimal temperature and pH of the enzymes to use in a bioreactor for example, as temperature and pH stability play a role. If the assay was conducted for a longer period of time, a different pH and temperature optima may be found. The optimal temperature was 50°C (Figure 3.4) but the enzyme was not very stable at 50°C (Figure 3.5). The temperature optimum would probably decrease with increased reaction time. In a bioreactor, the problem with low reaction temperatures would be low reaction rate. There would need to be a balance between reaction rate and stability. The temperature and pH used would also need to take into account what type of process will be used for hydrolysis and fermentation. Simultaneous saccharification and fermentation (SSF) or consolidated bioprocessing (CBP) would need to take into account the optimal conditions of not only the enzymes but also the fermenting organisms for example fermenting organisms often have an optimal growing temperature between 30 and 32°C (Van Dyk and Pletschke, 2012). However, genetic engineering of *Saccharomyces cerevisiae*, the current most suitable industrial fermentation organism, is improving the thermotolerance of the yeast that would minimize contamination and increase hydrolysis rate in SSF or CBP. Industrially used strains exhibit optimal growth between 28 and 30°C, Liu et al. (2014) increased the optimal growth temperature range to between 28°C and 35°C by introduction of heat shock genes from thermophiles.

3.6.5 Temperature stability

The half-life at 37 °C was just over 1 hour and the half-life at 50 °C was about 30 minutes of the Xyn2 (Figure 3.5 A). Turunen et al. (2001) found that, at 55 °C, native Xyn2 had a half-life of about 18 min, when expressed in *E. coli* it was 5-7 min. They also produced an Xyn2 with a disulphide

bridge and weakly stabilizing mutations that increased the half-life at 55°C to 112 minutes. Xyn2 expressed in *Saccharomyces cerevisiae* retained greater than 90% of its activity after 60 minutes at 50°C (La Grange et al., 1996). When Xyn2 was expressed in *Pichia pastoris,* it retained about 94% of its activity after incubation at 50°C for 30 minutes (He et al., 2009). Direct comparison between the results obtained in this study and results that have been reported is not easy as the enzyme assays are generally conducted using the protocol of Bailey et al. (1992). This protocol has a shorter assay time (5 minutes) than the assay time used in this study (30 minutes) so the stability measurements performed with the assays in this study may be lower than the same activity measurements performed using the standard protocol of Bailey et al. (1992). The stability of the Xyn2 with 5 minute activity assays (80% activity retained; Figure 3.5 B) was similar to the activity retention of the native enzyme when expressed in *P. pastoris* (94% activity retention; He et al., 2009). Xiong et al. (2004) created a mutant Xyn2 with disulphide bridges and amino acid alterations that had a half-life of 56h at 65°C. The half-life at 75°C was 25 minutes. This is an indication that mutations such as these are potentially very useful for improving the industrial use of enzymes.

3.6.6 Inhibition

Soluble lignin had a negligible effect of Xyn2 activity at the concentrations that were tested (Figure 3.6). Lignin inhibits cellulases by non-specific absorption to cellulases or to substrates which inhibits enzyme activity (Pan, 2008). Pan (2008) pre-incubated lignin with the substrate (Avicel®) overnight before enzyme assays. There was only a 5 minute pre-incubation of the substrate with inhibitor in the heating blocks prior to the enzyme assays. An increased incubation time may decrease enzyme activity in the presence of lignin by increased association of inhibitors with the substrate. This would need to be tested. Morrison et al. (2011) found that XynA of *Clostridium cellulovorans* was inhibited by 25% low concentrations of lignin (0.075 mg/mL) but further increases in lignin concentration up to 1 mg/mL did not increase inhibition; inhibition fluctuated between 80% and 90%.

Tejirian and Xu (2011) found that of polymeric lignin, oligomeric phenolics and monophenolics; oligomeric phenolics were the most inhibitory followed by monophenolics. Of the monophenolics tested for their inhibition effect on Xyn2, only gallic acid showed significant inhibition (Figure 3.7). The phenolic inhibitors were dissolved in water but none of them were completely soluble in water and this is why the inhibition reactions were done on a shaker to ensure interaction of the monophenolics with the enzyme. No published study on the inhibitory effect of monophenolics on the activity of *Trichoderma reesei* Xyn2 could be found. Morrison et al. (2011) determined the effect of p-coumaric acid and gallic acid on the endoxylanase XynA of *Clostridium cellulovorans* and found that 0.2% (w/v) p-coumaric acid caused 37% inhibition and 0.2% gallic acid caused 48% inhibition. The higher inhibition found for XynA by Morrison et al. (2011), compared to the 0% inhibition by p-coumaric acid and 16% by gallic acid found in this study, may be due to fact that, in the Morrison et al. (2011) study, the inhibitors were dissolved in methanol, which may have increased interactions of the monophenolics with the Xyn2, therefore increasing inhibition. In a bioreactor, phenolics could affect enzyme hydrolysis in an aqueous lignocellulose hydrolysate. In a simultaneous saccharification and fermentation process or a consolidated bioprocessing process, ethanol, acetone or other fermentation products could increase the solubility of phenolics, which could interact with the holocellulolytic enzymes.

Formic acid had a greater inhibitory effect on Xyn2 activity that acetic acid and furfural (Figure 3.8). The strong inhibitory effect of formic acid may be because it changed the pH of the reaction medium from between 4.5-5 to 4-4.5. This could account for about 15% to 39% decrease in activity based on the pH optimum curve (Figure 3.3). This could be the reason for the 24% decrease in activity caused by formic acid (Figure 3.3). Jing et al. (2009) found that formic acid was a competitive inhibitor of the commercial cellulase mix Spezyme CP (Genencor International, Rochester, NY, U.S.A). They also found that furfural was a stronger inhibitor of spezyme than organic acids. Formic acid was a stronger inhibitor than furfural in this study (Figure 3.8).

Aspergillus niger can grow on waste media with a high phenolic and organic acid content, such as spent hydrolysates, and utilize phenolics and acetic acid, furfural and hydroxymethyl furfural that inhibit enzyme activity and microbial growth. This could minimize inhibition caused by these compounds as *A. niger* utilizes them allowing higher growth. This is beneficial for remediating the spent waste and for potentially producing enzymes on site in a biorefinery (Alriksson et al., 2009).

Inhibition of Xyn2 by EDTA indicated the requirement of a divalent cation cofactor as EDTA chelates metal cations (Seiboth et al., 2011; Tejirian and Xu, 2010). Carmona et al. (1998) found that 10 mM EDTA has been found to cause 51% inhibition of a purified endoxylanase from *Aspergillus versicolor*, Xyn2 in this study was inhibited 23% (Figure 3.9). To determine which metal ions may be important for activity, metal ions could be added after EDTA addition to see which metal ions restore activity to determine those that are important for activity.

A selection of metal ions was tested for their inhibition of Xyn2 activity. Only Mn^{2+} and Mg^{2+} showed significant inhibition at the concentration of metal ions used (Figure 3.9). Metal ions have been known to have inhibitory effects on cellulases as well as endoxylanases (Tejirian and Xu, 2010; Lee et al., 2008; Carmona et al., 1998). Tejirian and Xu (2010) propose that their inhibitory effect may be due to various reasons. They may bind to histidine residues and cause conformational changes, replace metal ion cofactors, bind to the substrate and cause steric hindrance to enzymatic hydrolysis or may oxidize the reducing ends of cellulose making the reducing ends less susceptible to hydrolysis by lower reactivity or decreased cellulase accessibility. A study by Tejirian and Xu (2010) suggested that Cu^{2+} and Fe^{2+} inhibit cellulases by a redox mechanism. There was no correlation between redox potential of the inhibitors and the extent of inhibition. There was also no correlation between metal ion reduction potential and inhibitory effect although it was not determined whether the metal ions used were stable to reduction or oxidation under the conditions used. There was also no correlation between the ionic radius or charge of the ion and the extent of inhibition. The inhibition was different to what has been found for other endoxylanases. Mn^{2+} and Mg^{2+} were the only inhibitory metal ions. In two previous studies, Mn^{2+} and Mg^{2+} had no effect or activated the enzyme and Cu^{2+} inhibited the enzyme by more than 70 % whereas in this study Cu^{2+} had no inhibitory effect (Figure 3.9). The assays were however all conducted under different conditions.

Inhibition of Xyn2 by 2-propanol, 1-butanol and ethanol (Figure 3.10) was similar to inhibition by these fermentation products of XynA of *Clostridium cellulovorans* (Morrison et al., 2011). 1-butanol was a potent inhibitor with around 70% inhibition at a concentration of 70 g/L and ethanol and 2-propanol displayed a similar inhibition pattern. Ethanol has been produced at titres

up to 65 g/L (Cavka et al., 2014) but butanol is produced at much lower titres, the maximum reported being 30 g/L with continuous butanol removal (Shen et al., 2011). The mechanism of inhibition was not determined in this study; Morrison et al. (2011) determined that ethanol is a mixed inhibitor of XynA from *C. cellulovorans.* (Holtzapple et al., 1990) found that butanol is a non-competitive inhibitor of crude commercial cellulase mix. Understanding the effect of fermentation product inhibitors is important in simultaneous saccharification and fermentation and consolidated bioprocessing processes.

3.6.7 Laccase production using red grape waste

At concentrations > 20% w/v RGW, SSF had to be used as there was little to no liquid available after autoclaving. RGW without supplementation inhibited fungal growth during the first week of incubation. This could be due to the lack of readily available carbon in the form of simple sugar. After two weeks, growth in SSF had improved, with the exception of 30% w/v W-RGW (W-RGW 30). Washing the RGW before use did not improve the substrate for production of laccase by *T. pubescens*. It was noted that growth within duplicate W-RGW flasks was inconsistent. In future, RGW without washing will be used. In the unshed RGW, laccase production up to 2.7 U/mL was achieved using 50% w/v RGW under SSF. At lower RGW concentrations, laccase production decreased, with greater variability between duplicate flasks. High variability can be expected due to the non-homogenous nature of the substrate, which consists of an unequal mix of grape skins, seeds and stalks.

3.6.7.1 Laccase production using potato waste with red grape waste

FS potato waste contained more potato skins, while CD potato waste contained more potato flesh. Previous work in this study reported on the suitability of potato waste as a substrate for laccase production. However, under airlift reactor conditions, <1 U/mL activity was achieved. Phenolic compounds are known to induce laccase production in *T. pubescens* (Strong, 2011); therefore, RGW was used to induce laccase production in the potato medium. Optimal RGW supplementation to 20% w/v potato waste appears to be between 1-5% w/v RGW with CD potato waste and 1-3% w/v with FS potato waste. However, higher concentrations (10% w/v RGW) appeared to delay the onset of laccase production. Laccase activity was only detected in cultures with 10% RGW after 10 days incubation (Figure 3.12).

3.6.8 Heterologous enzyme production *A. niger*

Apple and potato waste can be used for the successful cultivation of recombinant strains of *A. niger*. However, the method used for the processing of the biomass need to be optimised if consistent results are to be obtained (Figure 3.13). The use of wastewater from the potato industry was evaluated and used as substitute for the distilled water (traditional medium composition). No significant difference was observed in terms of enzyme activity, COD or levels of total phenolics indicating that potato wastewater can be used for the production of economically important enzymes.

3.7 Conclusions

The Xyn2, which was the dominant extracellular secreted protein, was purified to electrophoretic homogeneity using 10 K and 30 K Amicon® Ultra-2 centrifugal filtration devices. The purification fold (1.36) and activity yield (24.9%) was however quite low. This may have been due to binding to

the regenerated cellulose membrane of the filter. The crude Xyn2 had endoxylanase activity and some (1.23% ± 0.54%) exo-α-L-arabinofuranosidase activity when tested on various holocellulosic substrates. The amount of contaminating activity was low so the crude enzyme was quite pure and further purification is probably unnecessary. The exo-α-L-arabinofuranosidase activity was beneficial in terms of oatspelt xylan hydrolysis. The pH optimum for the crude Xyn2 was between 5.5 and 6.5 and pure enzymes pH optimum was about 6.5. The crude enzymes pH optimum was within the range reported in the literature, the pure enzymes pH optima was slightly higher than what has been reported. There was a shift towards more basic pH for the pure enzyme although the reason for this was not determined.

The temperature optima for the crude and pure enzyme was 50°C for both the crude and pure enzyme which is within the range that has been reported for Xyn2 expressed in *A. niger.* Crude endoxylanase was less thermostable than expected at 37°C and 50°C. This may have been due to the different protocols used as the assay protocol used in our lab has a longer reaction time than the standard method of Bailey et al. (1992) that is generally used. This standard method does however have a short reaction time, which probably does not reflect the long hydrolysis time that would occur in a bioreactor. In terms of inhibition, lignin had no significant effect on activity; 2 g/L gallic acid caused 16% inhibition. At a concentration of 2 g/L formic acid caused a 24% loss in activity that was probably caused by a decrease in pH. EDTA caused 23% inhibition of Xyn2 at a concentration of 10 mM suggesting that a metal cation is required for activity. Among the metal ions tested, only Mg^{2+} and Mn^{2+} caused significant inhibition, 14% and 47% respectively, at the highest concentration of inhibitor used (10 mM). These ions were not expected to be the most inhibitory based on the literature.

All the fermentation products tested for inhibition had an inhibitory effect on Xyn2, which increased with increased inhibitor concentration. The extent inhibition caused by 2-propanol, ethanol and acetone were all similar, the decrease in activity was about 22% at 70 g/L. The most inhibitory was 1-butanol: at 70 g/L, it caused a decrease in activity of about 70%. The highest 1-butanol achieved through fermentation is 30 g/L, at this concentration, the inhibition (decrease in activity) is about the same as the inhibition of the other inhibitors at 70 g/L. Inhibition of Xyn2 should be a consideration for fermentation where 1-butanol is produced. Future experiments should use a more standardized assay and longer assay times on complex substrates that would be used industrially, to better characterize the enzyme under the conditions that it would be used. Synergy with other holocellullases is an important aspect of characterization that has not been addressed well in the literature for Xyn2. Synergy studies should be done to determine what combinations of enzymes to use for maximal hydrolysis and this would be done on complex substrates, which would be used for bioethanol production.

Previous studies have shown increased laccase production by *T. pubescens* in winery wastewaters with high phenolic concentrations (Strong and Burgess, 2008; Strong, 2011). Furthermore, grape seeds were found to be an excellent substrate for laccase production in another *Trametes* species (*T. hirsuta*, Moldes et al., 2003). Combining the excellent potential of potato waste for growth of *T. pubescens* with the induction potential of RGW could generate feasible amounts of laccase and will be further optimised and scaled up to 3L in future.

The potato waste and the potato wastewater can be used for the cultivation of *A. niger* strains and the production of recombinant enzymes. The wastewater does not contribute much to the total phenols or COD levels and since it does not seem to affect the growth or the enzyme production by *A. niger* strains, it is worth investigating its possible use for the cultivation of *S. cerevisiae.* The wastewater, together with the potato waste can be used for bio-ethanol production through fermentation and the addition of amylase enzymes.

CHAPTER 4: CHARACTERISATION OF ENZYMES AND APPLICATIONS 2

4.1 Introduction

This project previously demonstrated the bioremediation of lignocellulosic wastewater by recombinant *Aspergillus niger* D15 strains by reduction of organic content and cheap and high production of commercially valuable lignocellulose hydrolysing enzymes on inexpensive and abundant bio-based waste streams. This section addresses the follow-on characterization of the enzymes and their applications in waste stream reclamation and lignocellulosic conversion. This study focuses primarily on characterising three glycoside hydrolases: endo-β-1,4-glucanase, endo β-1,4-mannanase and endo-β-1,4-xylanase in order to understand how these three enzymes function synergistically on model substrates. This study aims to enrich the understanding of enzymes as they function in synergy and establish their potential for applications in biorefineries, and the greater bioeconomy.

4.1.1 Project Aims and Objectives

The overall aim of this section of this study is to characterise three *Aspergillus niger* derived enzymes: endoglucanase (EgII), endomannanase (ManI) and endoxylanase (Xyn2) using biochemical techniques. Furthermore, synergy studies will be conducted to determine their cooperative ability on model substrates.

The specific objectives of this study were as follow:

a) To partially purify the three enzymes using centrifugal filtration;
b) To conduct *in-silico* characterisation studies on the known protein sequences using ProtParam and UniProt;
c) To determine the enzymes' molecular weight using SDS-PAGE;
d) To determine the pH optima of the enzymes;
e) To determine the temperature optima and thermal stability of the enzymes;
f) To obtain the kinetic parameters of the enzymes; and
g) To conduct simultaneous binary synergy studies on the three enzymes on defined substrates: carboxymethyl cellulose, locust bean gum and beechwood xylan.

4.2 Methods and Materials

4.2.1 Enzymes

The three enzymes used in this study, endoglucanase, endomannanase and endoxylanase, were produced through recombinant technology as specified in the previous study.

Endo β 1,4 glucanase was expressed through the *Trichoderma reesei egII* gene in *A. niger* D15[*eg2*], while endo-β-1,4-mannanase was produced through the *Aspergillus aculeatus man1* gene in *A. niger* D15[*man1*] and the endo-β-1,4-xylanase enzyme was produced by the incorporation of the *Trichoderma reesei xyn2* gene in *A. niger* D15[*xyn2*]. The fungal strains were grown on minimal media plates in the presence of 10% glucose to repress the catabolite expression of the endogenous intracellular enzymes. The secreted (extracellular) enzymes were then isolated, freeze-dried and sent to Rhodes University.

4.2.2 Centrifugal Filtration

Centrifugal filtration was conducted to partially purify the freeze-dried enzymes by removing the excess glucose. The filtration was performed by using the 10 K Amicon® Ultra-2 Centrifugal Filter Devices with a Nominal Molecular Weight Limit of 10 000 Da. Prior to filtration, the membranes were soaked in 50 mM sodium citrate buffer, pH 5.0, at 4 °C for one hour. Freeze-dried enzyme solutions (100 mg/mL) were filtered through the 10 K devices in a swinging bucket rotor at 4000 rpm (Heraeus Megafuge 1.0 R) for 30 minutes at 4 °C. The membrane-retained content was recovered by inverting the filtration devices and spinning the concentrate down at 4000 rpm for 20 minutes. To ensure maximal sample recovery, the membrane was washed with 250 µl 50 mM sodium phosphate buffer (pH 5.0), and centrifuged further into the inverted collection tube for 10 minutes at 4000 rpm. The concentrates were quantified using the Bradford protein assay and diluted accordingly.

4.2.3 Protein Concentration Determination

The Bradford assay was used to determine the protein content of the purified enzymes (Bradford, 1976). The standard curve was developed using Bovine Serum Albumin (BSA) as the standard with concentrations ranging from 0-10 µg/mL. With the appropriately diluted protein samples, 100 µl of protein and 100 µl Bradford's reagent were added to a 96-well plate and incubated at room temperature on a rocker platform for 15 min before absorbance readings were measured at 595 nm with a PowerWave Spectroquant and analysed using the KC Junior software.

4.2.4 *In silico* characterisation

The sequences for the enzymes – EgII, ManI and Xyn2 – were retrieved from the National Centre for Biotechnology Information (NCBI) protein database. The sequences were analysed using the ProtParam tool as well as Uniprot to predict the enzymes' various biochemical and physico-chemical properties.

4.2.5 Molecular Weight Analysis: SDS-PAGE

For the molecular weight analysis, discontinuous sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) was used with a 10% resolving gel and 5% stacking gel. The 10% resolving gel was composed of: 4.1 mL Milli-Q water, 3.3 mL of 30% (w/v) acrylamide/bis-methylene; 2.6 mL 1.5 M Tris-HCl buffer (pH 8.8) and 100 µl of 10% (w/v) SDS. The gel was polymerised using 100 µl 10% (w/v) ammonium persulphate and 20 µl of pure N,N,N',N'-tetramethylethylenediamine (TEMED). For the 5% stacking gel, the following was used: 2.975 mL Milli-Q water; 0.670 mL 30% (w/v) acrylamide/bis-methylene, 1.25 mL 0.5 M Tris-HCl buffer (pH 6.8) and 50 µl SDS. 50 µl of 10% (w/v) ammonium persulphate and 10 µl TEMED were used to catalyse the polymerisation of the gel.

The protein fractions were mixed in a 4:1 ratio with SDS loading buffer that contained: 50 mM Tris HCl (pH 6.8); 50% (v/v) glycerol; 3% (w/v) SDS, and 0.2 % (w/v) bromophenol blue). 50 µl of fresh β-mercaptoethanol was added to 950 µl sample buffer. The sample volumes were made up to 20 µl in a sterile Eppendorf™ tube and heated at 100°C for 5 min in a Labnet AccuBlock™ digital dry bath. The electrophoresis was conducted using Mini-Protein® 3 cell tank with the SDS running buffer containing: 25 mM Tris, base; 192 mM glycine; 1% (w/v) SDS. The gels were run at a constant 120 V for approximately 80 minutes using a Bio-Rad PowerPac™ Basic.

The gels were stained using the Coomassie staining solution (0.1% (w/v) Coomassie Brilliant Blue G250; 30% (v/v) methanol; 10% (v/v) glacial acetic acid; 60% Milli-Q water) for 1 hour. The gels were further destained for 6 hours to overnight (until the blue background has disappeared) using the destain solution (50% (v/v) methanol; 10% (v/v) glacial acetic acid and 40% Milli-Q water). The gels were then imaged using the ChemiDoc™ XRS gel imaging system to confirm the presence of the separated proteins.

4.2.6 Enzyme Activity Assays

The enzymes activities were determined through their respective assays and quantified using the DNS method for reducing sugars (Bailey et al., 1992). The DNS reagent was made up using 1% (w/v) NaOH, 1% (w/v) 3,5-dinitrosalicylic acid, 20 % (w/v) sodium potassium tartrate, 0.2% (w/v) phenol, and 0.05% (w/v) sodium metabisulphite (Miller, 1959). Standard curves generated for this part of the study is provided in Appendix E.

4.2.6.1 Cellulase Activity Assay

Using a 1.5 mL Eppendorf™ tube, 100 µl enzyme solution was added to 400 µl 1.25% (w/v) CMC to make up a final 1% substrate concentration. The reaction proceeded at 50 °C for 45 minutes in a Labnet AccuBlock™ digital dry bath. The reaction was then stopped by the addition of 500 µl DNS reagent. The samples were then centrifuged down at 13 000 *x g* for 5 minutes using a desktop Heraeus pico centrifuge to pellet out any insoluble substrate. The samples were then heated at 100°C for 5 minutes to accelerate the colour development process followed by cooling on ice for a further 10 minutes before 250 µl of the solution was added to a 96-well plate for absorbance measurements at 540 nm using a Powerwave spectrophotometer with the KC Junior software. The assays were conducted in triplicate.

The cellulase activity was determined by using D-(+)-glucose standards (0-1 mg/mL). Units of enzyme activity were defined as µmol glucose equivalents released per minute.

4.2.6.2 Mannanase Activity Assay

The mannanase activity was determined using 0.5% (w/v) locust bean gum (LBG) dissolved in 50 mM sodium phosphate buffer (pH 6.0) as the substrate as described by Puchart et al. (2004). 400 µl 0.67 % LBG was mixed with 100 µl enzyme solution in a sterile Eppendorf™ tube. The reaction was conducted at 50°C for 45 minutes. 500 µl DNS reagent was added to stop the reaction, this was followed by centrifugation at 13 000 *x g* for 5 minutes. The samples were then heated at 100°C for 5 minutes and cooled for 10 minutes further. 250 µl was used for the spectrophotometric analysis at 540 nm using D(+)-mannose (0-1 mg/mL) as the standard. All measurements were conducted in triplicate. Units of endomannanase activity were defined as µmol of mannose equivalents released per minute.

4.2.6.3 Xylanase Activity Assay

The xylanase activity of the endoxylanase enzyme was determined by measuring the reducing sugars liberated by 1% (w/v) beechwood xylan in 50 mM sodium phosphate buffer (pH 7.0) as the substrate using the DNS method. 400 µl of 1.25% beechwood xylan was mixed with 100 µl of enzyme solution in a sterile Eppendorf™ tube. The reaction was carried out at 50°C in a Labnet AccuBlock™ digital dry bath for 45 minutes followed by the addition of the DNS reagent to stop the reaction. The samples were then heated at 100°C for 5 minutes before cooling them on ice for

another 10 minutes. D(+)-xylose (0-1 mg/mL) was used as the standard for the spectrophotometric analysis. Triplicate samples (250 µl each) were measured at 540 nm. Units of enzyme activity were defined as µmol of xylose equivalents released per minute.

4.2.7 pH Optima

The pH optima studies were conducted from pH 4-8 (with 0.5 pH unit increments) for the EgII, ManI and Xyn2 enzymes using their respective substrates as mentioned in Chapter 3. A 50 mM sodium citrate buffer was used from pH 4-6 while a 50 mM sodium phosphate buffer was used from pH 6-8. The assays were conducted in triplicate at 50°C for 45 minutes using the protocols described in Chapter 3.

4.2.8 Temperature optima and stability determination

The temperature optima studies were performed from 20-80°C using a Labnet AccuBlock™ digital dry bath at pH 5.0 (50 mM sodium citrate buffer) for 45 minutes. The enzyme activities were determined using the DNS method in Chapter 3 in triplicate. The enzymes' thermal stability were measured over 72 hours in triplicate at temperatures 50, 55, 60 and 65°C using a 50 mM sodium citrate buffer (pH 5.0). The residual activities were determined using the DNS method after incubation at a set temperature for a specific period.

4.2.9 Determination of Kinetic parameters

The kinetic parameters V_{max} and K_M were determined using KaleidaGraph® Synergy Software using multiple concentrations of carboxymethyl cellulose (0-30 mg/mL), locust bean gum (0-20 mg/mL) and beechwood xylan (0-30 mg/mL).

4.2.10 Substrate Specificity determination

Specific activity studies were conducted to determine if the three enzymes had additional functions with other substrates.

4.2.10.1 Avicelase activity

The cellobiohydrolase activity was measured against Avicel (hence called Avicelase activity) by combining 400 µl 2.25% Avicel (2% final concentration) and 100 µl of appropriately diluted enzymes in 50 mM sodium citrate buffer (pH 5.0). The reactions were carried out at 50°C for 24 hours after which the samples were centrifuged for 5 minutes (13 000 × g). The Avicelase activity was determined using the DNS method in triplicate and the reducing sugars released were measured against a glucose standard curve at 540 nm. One enzyme unit was defined as the amount of enzyme required to liberate 1 µmol of glucose equivalent per minute.

4.2.10.2 Glucosidase activity

The β-glucosidase activity of the enzymes was measured using *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG) as the substrate. *p*NPG (450 µl) (2.25 mM final concentration) was combined with 50 µl appropriately diluted enzyme at pH 5.0 in 50 mM sodium citrate buffer. The reactions were carried out at 50°C for 30 minutes and stopped by the addition of 500 µl 2 M sodium carbonate. 250 µl triplicate samples were added to a 96-well microtitre plate, the β-glucosidase activity was determined by the release of *p*-nitrophenyl product, and this was measured at 405 nm. The activity was measured against a *p*-nitrophenol standard curve with one unit of enzyme activity

defined as the amount of enzyme required to liberate 1 µmol *p*-nitrophenol per minute under the specified assay conditions.

4.2.10.3 Mannosidase activity

The specific β-mannosidase activities of the enzymes were measured by their ability to hydrolyse *p*-nitrophenyl-β-D-mannopyranoside (*p*NPM). The assay reaction mixture contained 450 µl of 2.25 mM *p*NPM and 50 µl of appropriately diluted enzyme in 50 mM sodium citrate buffer (pH 5.0). The reaction was conducted at 50°C for 30 minutes and terminated by the addition of 500 µl 2 M sodium carbonate. The released *p*-nitrophenyl product was measured at 405 nm with a PowerWave$_x$ Spectroquant on K$_c$ Junior software. *p*-Nitrophenol was used as suitable standard and one enzyme unit was defined as the amount of enzyme that released 1 µmol of *p*-nitrophenol per minute under the specific assay conditions.

4.2.10.4 Xylosidase activity

The xylosidase activity of the enzymes was determined using *p*-nitrophenyl-β-D-xylopyranoside (*p*NPX). The assay mixture was composed of 450 µl of 2.25 mM *p*NPX and 50 µl enzyme in 50 mM sodium citrate buffer (pH 5.0). The reaction was allowed to proceed for 30 minutes and was terminated by adding 500 µl 2 M sodium carbonate. The released *o*-nitrophenyl product was measured at 410 nm using *o*-nitrophenol as the suitable standard with one enzyme unit defined as the amount of enzyme required that released 1 µmol of *o*-nitrophenol per minute under the specific assay conditions.

4.2.11 Synergy Studies

The enzyme synergy studies were conducted on the EgII, ManI and Xyn2 enzymes with defined model substrates: carboxymethyl cellulose (CMC), locust bean gum (LBG) and beechwood xylan (BWX) in various binary combinations (Appendix F). Within the varying combinations, the total protein loading was kept constant at 11 µg/mL (0.025 mg/g of CMC or BWX and 0.0125 mg/g LBG). The experiments were carried out in triplicate at a total volume of 500 µl at 50°C with 50 mM sodium citrate buffer (pH 5.0) for 60 minutes. The released reducing sugars were determined using the DNS reaction. Substrate and enzyme controls were included in the analysis.

4.3 Results and Discussion

4.3.1 *In silico* Characterisation

For the *in silico* characterisation, the physico-chemical parameters of the enzymes were obtained using the ProtParam tool in the ExPASy Bioinformatics Resource Portal (Gasteiger et al., 2005) as well as the UniProt (Universal Protein Resource) catalogue. Table 4.1 displays the ProtParam and UniProt results obtained from the sequence analysis of each of the enzymes.

The EgII from *T. reesei* has a sequence length of 418 amino acids, which translates to a molecular mass of 44.2 kDa and a pI of 4.97. The *A. aculeatus* ManI has a sequence length of 377 amino acids, 41.1 kDa and 4.88 is the molecular weight and pI respectively. The Xyn2 from *T. reesei* has a molecular weight of 24.1 kDa from a sequence length of 223 and a pI value of 7.87 (Table 4.1).

Table 4.1. Physico-chemical properties of EgII, ManI and Xyn2 enzymes predicted by UniProt and ProtParam

Protein Name	**Endoglucanase II (EgII)**	**Endomannanase I (ManI)**	**Endoxylanase B (Xyn2)**
UniProt ID	P43317	Q00012	Q99015, P36217
GenBank Accession Number	ABA64553.1	AAA67426.1	AAB29346.1
Organism	*Trichoderma reesei (Hypocrea jecorina)*	*Aspergillus aculeatus*	*Trichoderma reesei (Hypocrea jecorina)*
Sequence Length	418	377	223
Molecular Weight (kDa)	44.2	41.1	24.1
pI	4.97	4.88	7.87
Binding Sites	-	-	-
Active Sites	- 27 (Nucleophile) - 134 (Proton Donor)	- 198 (Proton Donor) - 306 (Nucleophile)	- 118 (Nucleophile) - 209 (Proton Donor)
Glycosylation Site(s)	182	105, 255, 326, 357	70, 90, 129

The results show that all three enzymes contain at least one glycosylation site, which is relevant to the study because the enzymes are being expressed in *Aspergillus niger* – a eukaryotic, fungal organism – and it is likely that the enzyme might undergo glycosylation as a post-translational modification.

4.3.2 Molecular weight determination

An SDS-PAGE analysis of the individual enzymes was conducted to determine the level of purity (after centrifugal filtration) and to confirm the molecular weight of the respective enzymes. The SDS-PAGE image in Figure 4.1 shows single bands in each loaded lane, thus confirming their purity.

The molecular weights of the enzymes as estimated by SDS-PAGE were: 49.6 kDa for the *T. reesei* EgII, 46.4 kDa for *A. aculeatus* ManI and 21.6 kDa for the Xyn2 from *T. reesei.* Based on the comparison between the SDS-PAGE analysis (Figure 4.1) and the theoretical molecular weights given in Table 4.1, there are slight differences in molecular weights. For both the EgII and ManI, the observed molecular weights are higher by 5.4 and 5.3 kDa respectively and the observed Xyn2 molecular weight is lower by 2.5 kDa. However, these differences are not surprising, as similar trends have been found in literature.

In a study by Setati et al. (2001), the *A. aculeatus* endo-β-1,4-mannanase, which was expressed in *Saccharomyces cerevisiae,* was found to be 50 kDa in size. The authors attribute this increase in observed molecular weight to be due to the glycosylation and upon de-glycosylation, the enzyme had a molecular weight of 41 kDa, which correlates with the theoretical molecular weight in Table 4.1. Furthermore, a study by Van Zyl et al. (2009) found that the *A. aculeatus* endo-β-1,4-mannanase expressed in *A. niger* had a molecular weight of approximately 46 kDa – their findings are in line with the SDS-PAGE analysis in Figure 4.1. These two papers thus show that the fungal

system used for expression plays a critical role in determining the correct level of glycosylation for secreted enzymes.

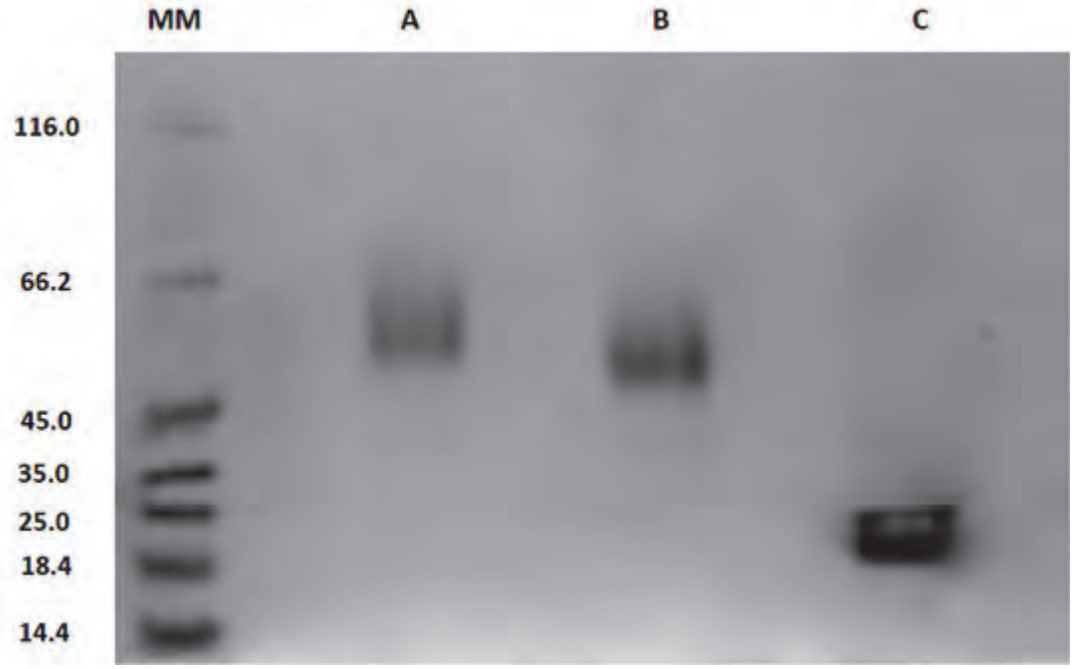


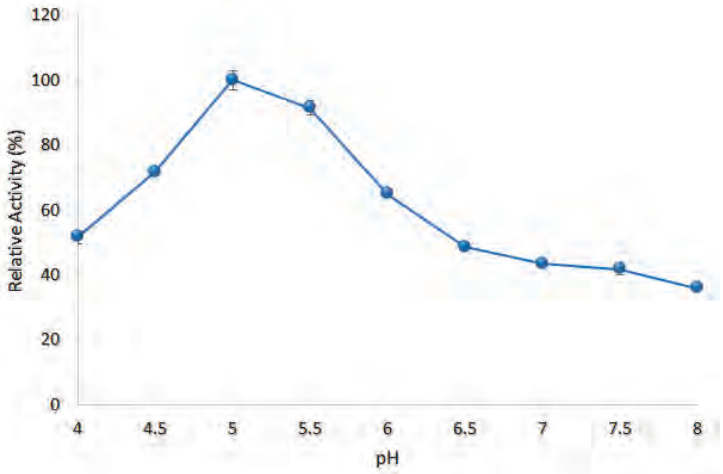
Figure 4.1. Molecular weight analysis of the enzymes using SDS-PAGE purified through centrifugal filtration. Lane MM: Unstained protein molecular weight marker 26612 (Thermo Scientific®); Lane A: Endo-β-1,4-glucanase (EgII); Lane B: Endo-β-1,4-mannanase (ManI); Lane C: Endo-β-1,4-xylanase (Xyn2). The SDS-PAGE separation occurred using a 10% resolving gel with a 5% stacking gel and electrophoresed at 120 V for 80 minutes.

Rose and Van Zyl (2002) reported on the constitutive expression of *T. reesei* endo-β-1,4-xylanase and endo-β-1,4-glucanase in *A. niger.* Their study found that the molecular weight of the endoxylanase was about 21 kDa and this is in line with the findings of this study.

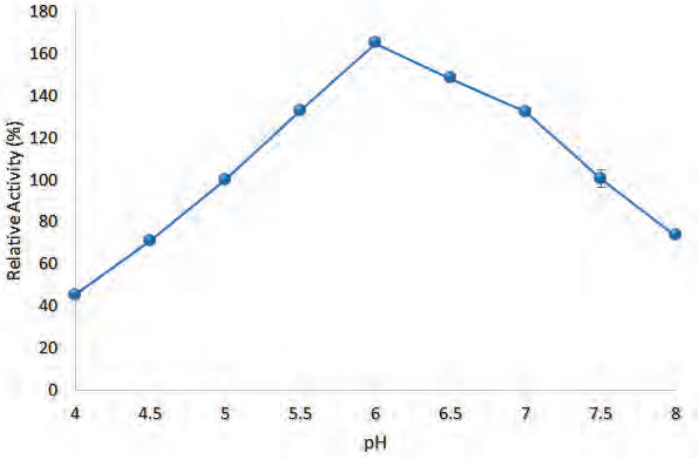
4.3.3 pH Optima Determination

The pH optimum profiles of the three enzymes (Figure 4.2) were determined from a pH range of 4.0-8.0 using 5.0 mM sodium citrate (pH 4.0-6.0) and 50 mM sodium phosphate (pH 6.0-8.0) at 50°C for 45 minutes. The three enzymes exhibited their optimal pH between pH 5.0 and 6.0. This is a common range as reported in literature for many glycoside hydrolases. The optimal pH for the EgII and Xyn2 was at 5 and 5.5 respectively. However, they both experienced a sharp drop in activity at pH 6.0. The ManI, on the other hand, displayed a sharp, distinct peak at pH 6 (165.04 ± 1.55%), but gradually lost activity at greater pH levels.

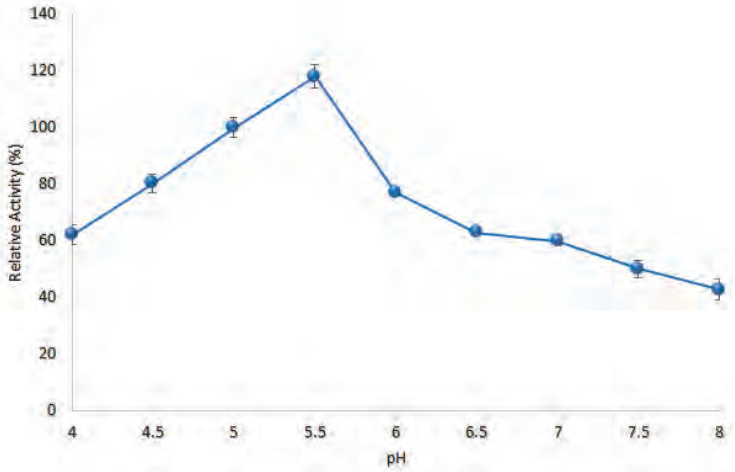
The results of this study are in agreement with previous studies in literature. Rose and van Zyl (2002) found that the endoxylanase had an optimum pH range between 5 and 6 and the endoglucanase was most active at 5. Christgau et al. (1994) found the optimum to be between 5.5 and 6.0 for the *A. aculeatus* ManI.



(A)



(B)

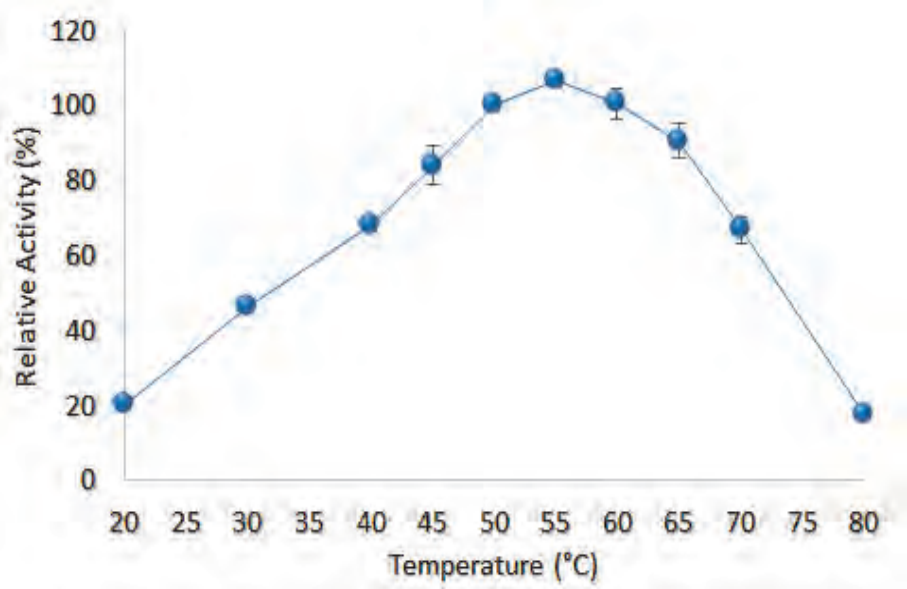


(C)

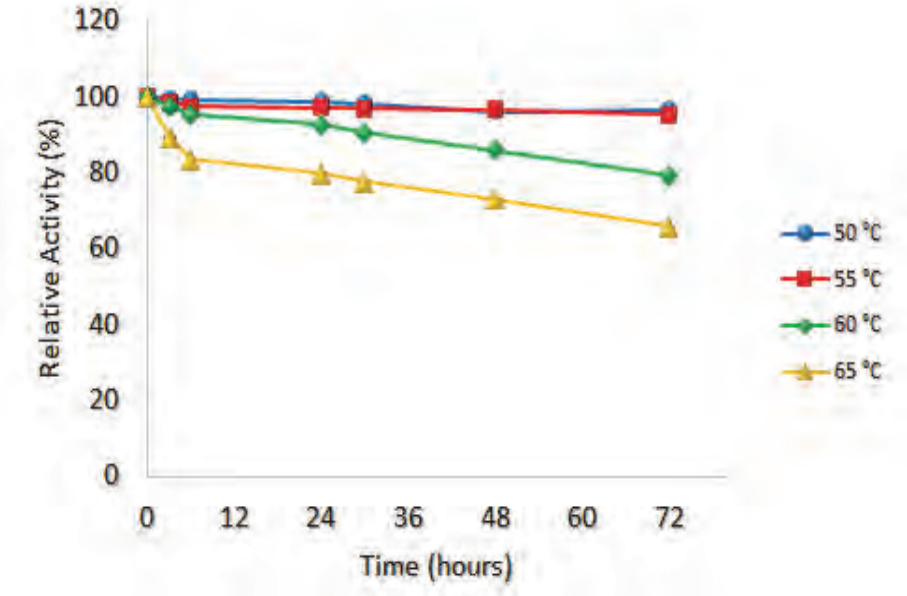
Figure 4.2: The pH optima profiles of the glycoside hydrolase enzymes; (A) EgII, (B) ManI and (C) Xyn2 on their defined model substrates ranging from pH 4.0 to 8.0 using 50 mM sodium citrate (pH 4.0-6.0) and 50 mM sodium phosphate (pH 6.0-8.0) buffers. Values are represented as mean values ± SD (n=3) and in some cases errors bars are too small to be seen.

4.3.4 Temperature Optima and Stability Determination

The temperature optima and thermal stability for each of the glycoside hydrolase enzymes were investigated using 50 mM sodium citrate buffer (pH 5.0). Figures 4.3 to 4.5 illustrate the thermal properties of the EgII, ManI and Xyn2 enzymes, respectively.



(A)



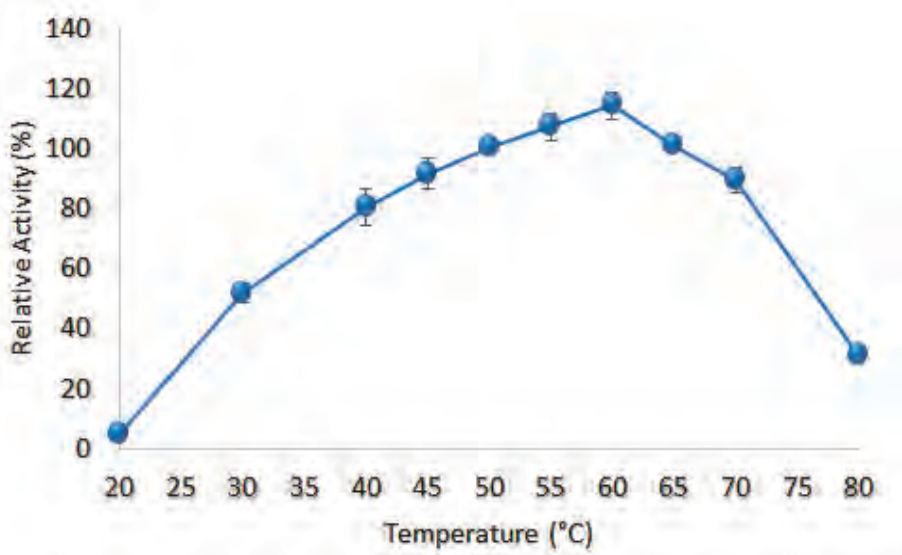
(B)

Figure 4.3: The temperature profile of the endo-β-1,4-glucanase (EgII); (A) temperature optimum from 20-80°C and (B) temperature stability at 50, 55, 60 and 65°C over 72 hours. Values are represented as mean values ± SD (n=3) and in some cases errors bars are too small to be seen.

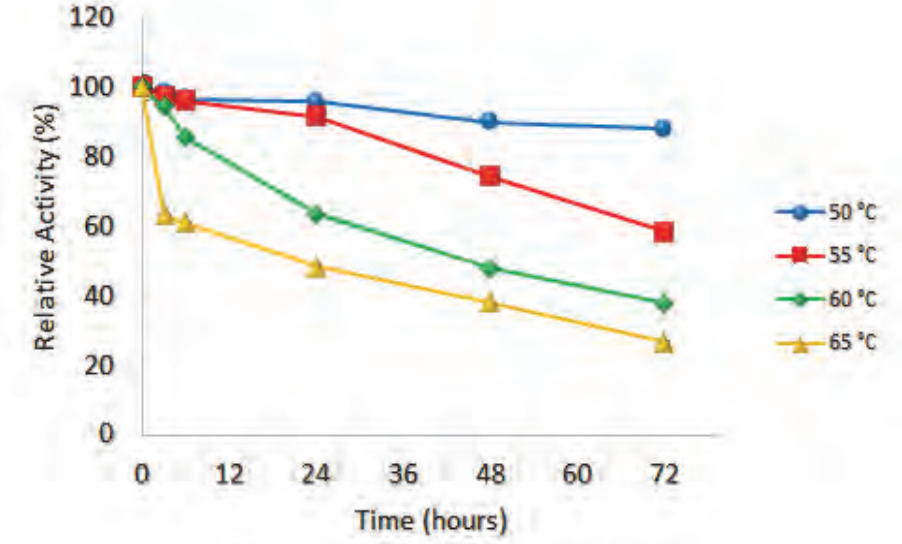
The temperature profile for EgII showed that the enzyme had a temperature optimum at 55°C, but still maintains high levels of activity at 50 and 60°C (Figure 4.3.A). The enzyme stability graph (Figure 4.3.B) showed that EgII is a thermostable enzyme over 72 hours; the enzyme exhibited comparable residual activities at 50 and 55°C over the 72 hours, while a moderate decline occurred at 60°C and a much more noticeable decline at 65°C for the same time period.

The results in Figure 4.3.A are in agreement with studies that previously determined that the *T. reesei* EgII to be optimally active between 50 and 55°C (Qin et al., 2008; Samanta et al., 2012). The thermostability profile of the EgII (Figure 4.3.B) is not in entirely in agreement with reported data by Samanta et al. (2012). The study found that the EgII rapidly lost activity after 40 minutes at 60°C. However, the study by Rose and van Zyl (2002) corroborate the results seen in Figure 4.3.B. The study found that the endoglucanase was stable at 50 and 60°C for the entire length of their study (180 minutes), losing between 10-15% of residual activity at 60°C in the process. These studies highlight the importance of selecting the most suited fungal strain for the production of a common enzyme and further studies need to look at how the fungal system used in the expression can affect enzyme functionality.

The optimum temperature for the ManI occurred at 60°C and a decline in activity was observed at temperatures greater than 60°C (Figure 4.4.A). The ManI displayed a relatively stable residual activity over 72 hours at 50°C, but the decline in stability increased with increasing temperature (Figure 4.4.B).



(A)

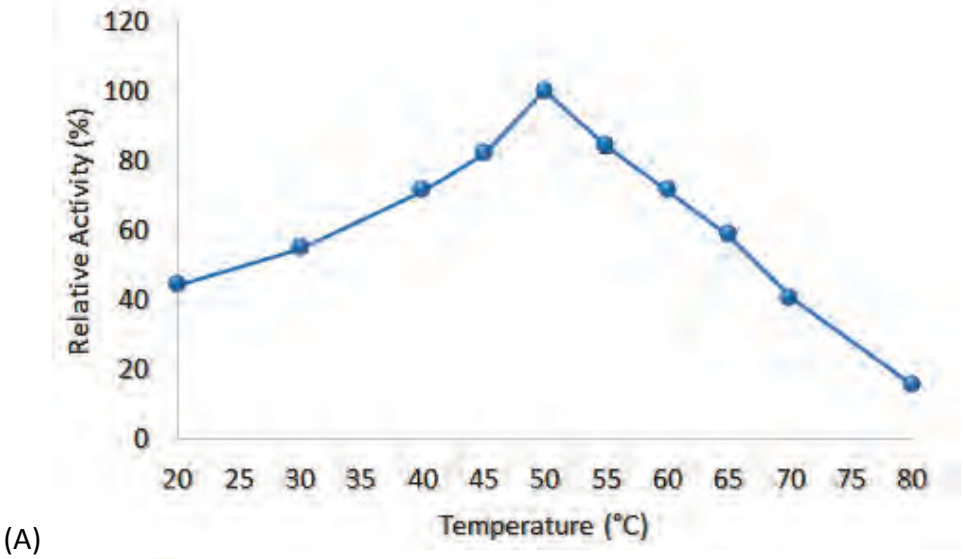


(B)

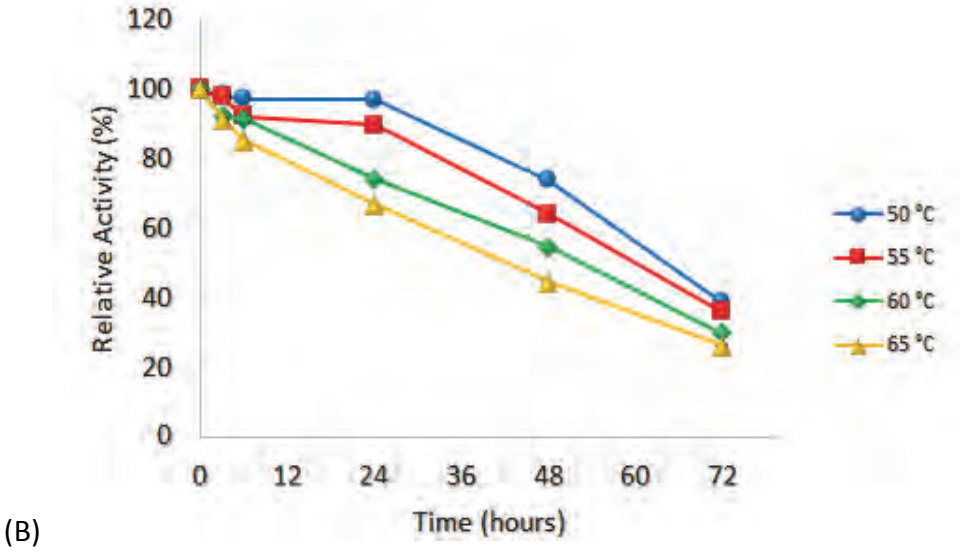
Figure 4.4: The temperature profile of the endo-β-1,4-mannanase (ManI); (A) temperature optimum from 20-80°C and (B) temperature stability at 50, 55, 60 and 65°C over 72 hours. Values are represented as mean values ± SD (n=3) and in some cases errors bars are too small to be seen.

A similar trend is observed in literature with regards to the temperature optima of the ManI (Figure 4.4.A). Christgau et al. (1994) and Setati et al. (2001) both found that the ManI is optimally at 60°C. ManI thermostability studies in literature are inconsistent to accurately compare the profile in Figure 4.4.B. With that said however, a study by Puchart et al. *(*2004) on *A. fumigatus* endo-β-1,4-mannanase found that even though the enzyme was optimally active at 65°C, the stability at this temperature was severely impacted within an hour of incubation, with a reported loss of over 90%. Furthermore, the study found that the enzyme retained almost 100% of activity at 55°C after 6 hours of incubation.

The study by Puchart et al. (2004) agreed with the observations seen in Figure 4.4.B, where the enzyme's optimal temperature did not always correlate with the stability over time. Therefore, this study can be used to demonstrate the value of conducting both temperature optima and stability studies (over an extended time period) and both measurements need to be taken into account.



(A)



(B)

Figure 4.5: The temperature profile of the endo-β-1,4-xylanase (Xyn2); (A) temperature optimum from 20-80°C and (B) temperature stability at 50, 55, 60 and 65°C over 72 hours. Values are represented as mean values ± SD (n=3) and in some cases errors bars are too small to be seen.

The Xyn2 displayed a clear temperature optimum at 50°C (Figure 4.5A). However, the Xyn2 expressed lower thermal stabilities over 72 hours. The enzyme retained most of its activity by 24 hours at 50 and 55°C, but a sharp decline is observed afterwards at these temperatures. At 60 and 65°C, the enzyme has lost over 20% of its activity, and further declines by 50% at 72 hours.

The Xyn2 temperature optimum observed in Figure 4.5.A of 50°C corresponds to the study conducted by Rose and Van Zyl (2002), where a *T. reesei* Xyn2 was used. Similarly, another study by Fialho and Carmona (2004) on two *A. giganteus* xylanases (XynI and XynII) showed similar results. This indicates that the functionality of these xylanases in terms of optima are the same; however, the thermostability profiles of these xylanases seem to differ. The Xyn2 in study by Rose and van Zyl (2002) exhibits thermostability from 40-60°C for 180 minutes (losing less than 20% residual activity overall), while the XynI and XynII from the Fialho and Carmona (2004) study lost significantly high levels of activity at 50 and 60°C – approximately 35 and 70%, respectively, in the same time period.

The profiles in this study are one of the few that detail the EgII, ManI and Xyn2 thermal stabilities over 72 hours in this temperature range. The thermal behaviours of the enzymes are an important component in the application of enzymes for use in lignocellulosic degradation and it is advantageous that the enzymes are highly active and stable at 50-60°C, which indicates their potential for use in biorefineries.

4.3.5 Specific Activity Determination

The specific activities of the enzymes were evaluated on various model substrates at 50°C using a 50 mM sodium citrate buffer at pH 5.0 (Table 4.2). Protein content was determined using the Bradford method.

Table 4.2: Specific activities of the EgII, ManI and Xyn2 enzymes on defined substrates.

Substrate	**Activity**	**Enzyme Specific Activity (U/mg)**		
		EgII	ManI	Xyn2
Avicel	*Avicelase*	0.49	0.19	0.14
Carboxymethyl cellulose	*Cellulase*	64	3.8	28
Locust Bean Gum	*Mannanase*	6.2	72	35
Beechwood xylan	*Xylanase*	8.3	6.3	193
4-Nitrophenyl α-D-glucopyranoside (*p*NPG)	*β-glucosidase*	0.42	0.48	4.1
4-Nitrophenyl α-D-mannopyranoside (*p*NPM)	*β-mannosidase*	n.d.*	n.d.*	n.d.*
4-Nitrophenyl β-D-xylopyranoside (*p*NPX)	*β-xylosidase*	0.08	0.09	1.6

1 U is the amount of enzyme releasing 1 µmol of product per minute

** Not detected*

The substrate specificity analysis of the EgII (Table 4.2) indicates that the enzyme can moderately hydrolyse locust bean gum and beechwood xylan with specific activities at 6.2 and 8.3 U/mg, respectively. These observations are strongly corroborated by a similar study conducted by Sachslehner et al. (1998). The authors found that a *Sclerotium rolfsii* endoglucanase exhibited noticeable activities towards beechwood xylan (2.91 U/mL) and locust bean galactomannan (3.33 U/mL). This study therefore shows that the enzyme has the ability to degrade not only cellulose-based substrates (as seen with the CMC specific activity of 64 U/mg in this study), but can also degrade mannan- and xylan-based substrates to some extent.

A similar trend can be observed with the ManI substrate specificity analysis: the enzyme had a clear preference for its natural substrate, locust bean gum (72 U/mg), as expected. Moreover, the enzyme shows some xylan and cellulose hydrolysing activities with specific activities at 6.3 and 3.8 U/mg, respectively. Once again, the study by Sachslehner et al. (1998) revealed the same with activities at 2.90 and 3.69 U/mL.

Interesting trends can be observed when it comes to the Xyn2 substrate specificity analysis. As expected, the enzyme displayed a strong preference towards its substrate – beechwood xylan – with a specific activity at 193 U/mg. The enzyme showed strong activities towards CMC and LBG with specific activities of 28 and 35 U/mg respectively. Sachslehner et al. (1998) somewhat further

confirmed these findings with their study, although the xylanase had lower activities compared to the Xyn2 towards these substrates.

The highlight of these results was the fact that the Xyn2 exhibited moderate β-glucosidase and β-xylosidase activities (4.1 and 1.6 U/mg, respectively). This is an interesting discovery as it points to the possibility of endoxylanase being a multifunctional enzyme with different active sites. These observations are in contradiction to literature and thus would need to be further investigated using additional, non-synthetic substrates.

The three enzymes did not display any β-mannosidase and strong cellobiohydrolase activity with Avicel. However, the fact the enzymes are able to hydrolyse additional substrates show promising signs towards the application in lignocellulose hydrolysis. As a result, the synergy studies will play an important role in further understanding how the additional functionalities affect the enzymes and their ability to degrade model substrates.

4.3.6 Kinetic Parameter Determination

The kinetic parameters for the glycoside hydrolases were analysed using KaleidaGraph® to determine the V_{max}, K_M, k_{cat} and k_{cat}/K_M values. CMC (0-30 mg/mL), LBG (0-20 mg/mL) and BWX (0-30 mg/mL) were used as the respective model substrates for the individual enzymes.

From Table 4.3, the kinetics of EgII showed that the EgII has a high affinity towards CMC with a K_M of 2.19 mg/mL and a V_{max} of 90.42. The K_M value was in agreement with 2.1 mg/mL reported by Samanta et al. (2012); however, the V_{max} differed quite substantially with the authors reporting 220.57 µmol/min. The differences in kinetic parameters seem to be attributable largely to the fungal system in which the enzymes were produced. Karnchanatat et al. (2007) used a *Daldinia eschscholzii* endoglucanase – a K_M and V_{max} of 1.74 mg/mL and 63 µmol/min, respectively, was observed. In contrast, the reported kinetic parameters for a *Fomitopsis pinicola* were found to be 11.6 mg/mL and 1250 µmol/min, respectively (Yoon et al., 2008).

Table 4.3: Kinetic parameters estimated for EgII, ManI and Xyn2 enzymes

Enzyme	Substrate	V_{max} (µmol/min)	K_M (mg/mL)	k_{cat} (min^{-1})	k_{cat}/K_M ($min^{-1}mg^{-1}$ mL)
EgII	*CMC*	90.42	2.19	7931	3621
ManI	*LBG*	62.01	0.098	4733	48295
Xyn2	*BWX*	160.75	2.57	14225	5535

The estimated values for V_{max} and K_M for *A. aculeatus* ManI were 62.01 µmol/min and 0.09 mg/mL, respectively, with locust bean gum. These parameters indicated that the ManI has a very strong affinity towards the substrate; however, it has a lower turnover number (4733 min^{-1}) when compared to the other two enzymes. The K_M of the enzyme is in agreement with the 0.11 mg/mL reported by Naganagouda et al. (2009) with *A. niger* ManI; once again, the V_{max} values differ from each other and could thus be attributed to the fungal source.

The estimated kinetic parameters of the *T. reesei* Xyn2 were 160.75 µmol/min and 2.57 mg/mL for the V_{max} and K_M with beechwood xylan. The results suggest that the Xyn2 has a lower affinity to

the beechwood xylan (relative to the other enzymes on their respective substrates), but has the highest turnover number of 14 225 units/min. These results are in close agreement with the data found by Sandrim et al. (2005) on an *A. caespitosus* XynII. The authors found the V_{max} and K_M to be 113 µmol/min and 3.1 mg/mL with Birchwood xylan.

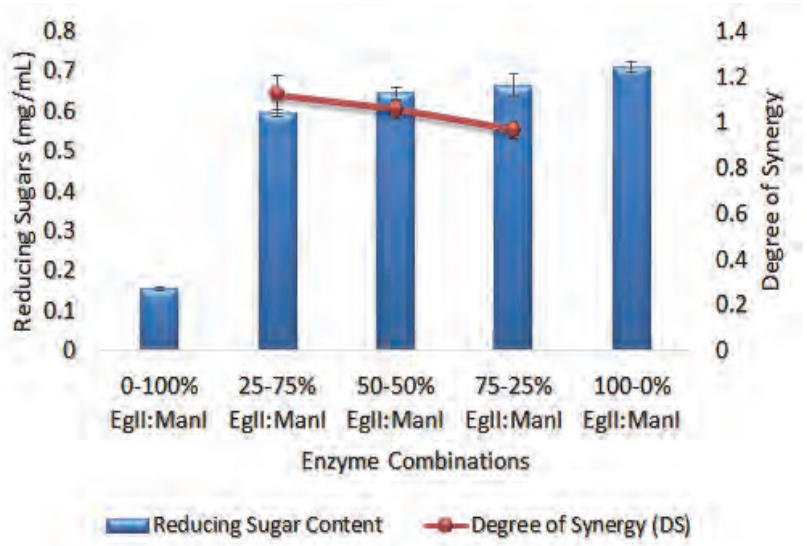
The kinetic data show that when the K_M is too low, the turnover number is lowered as a result. This suggests that the binding interactions between the enzyme and substrate are too strong and that they hinder the efficiency of the enzyme in the degradation of the product. On the other hand, considering that these three enzymes exhibit mild activities towards other substrates, it is possible that the hydrolysis might be enhanced when the enzymes function simultaneously.

4.3.7 Synergy Studies

The synergistic associations between the EgII, ManI and Xyn2 were determined through the quantification of the reducing sugars released during the degradation of the model substrates: carboxymethyl cellulose, locust bean gum and beechwood xylan. Being the first study of its kind, the purpose of the synergy experiments in this format was not only to determine which enzymes produce the most reducing sugars but rather (more importantly) to understand how the enzymes can potentially cooperate when applied to lignocellulose biomass. Therefore, the key objective for this study was to determine whether or not synergy occurs between certain enzymes.

4.3.7.1 Bi-synergy on Carboxymethyl cellulose

Figure 4.6 demonstrates the synergistic relationship found between the three enzymes on carboxymethyl cellulose over 60 minutes at 50°C using 50 mM sodium citrate buffer (pH 5.0).



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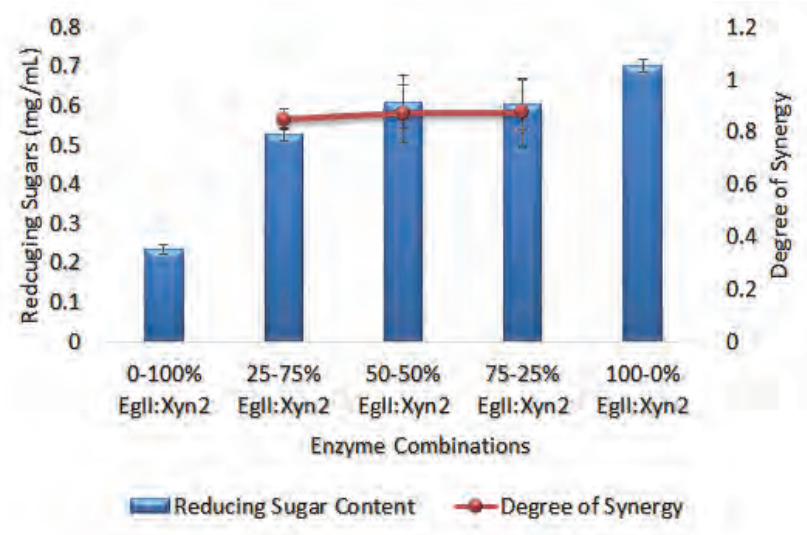

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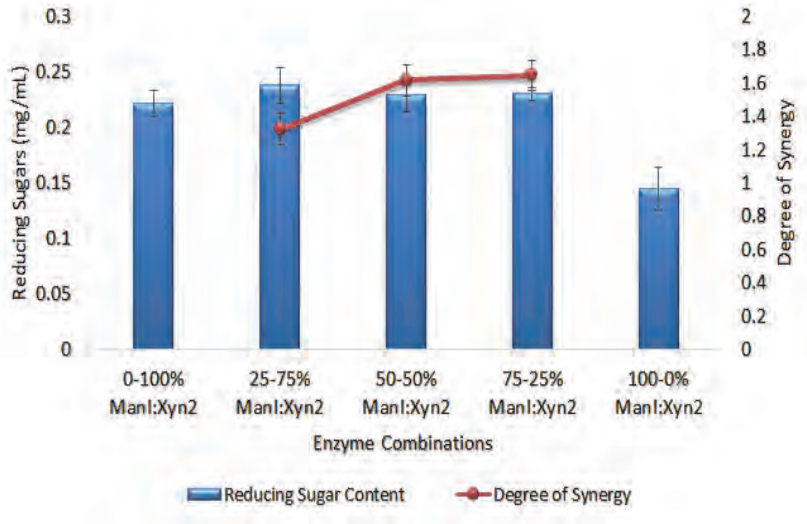


(C)

Figure 4.6. The synergistic interactions obtained on carboxymethyl cellulose and the reducing sugars released by pre-determined enzyme combinations. (A) EgII and ManI, (B) EgII and Xyn2 and (C) ManI and Xyn2. Hydrolysis reactions were carried out for 60 minutes at 50°C. Bar graphs represent the released reducing sugars and the line graph is the degree of synergy between each combination. Data represent the mean values and error bars indicate the deviation from the mean (n =3).

With carboxymethyl cellulose, the maximum reducing sugars (0.71 mg/mL) were produced by the EgII at 100% protein loading. This was expected as the CMC is the natural substrate for the EgII. Using the degree of synergy, the specific mechanisms of the combinations can be understood further. Looking at the ManI:Xyn2 synergy (Figure 4.6.C), it is clear that these two enzymes cooperated the best with each other leading to the highest degree of synergy of 1.6. With this said, there is a possibility that these enzymes potentially occupy different sites on the CMC, especially since they produce more reducing sugars when in synergy (with the highest content seen at 0.24 mg/mL).

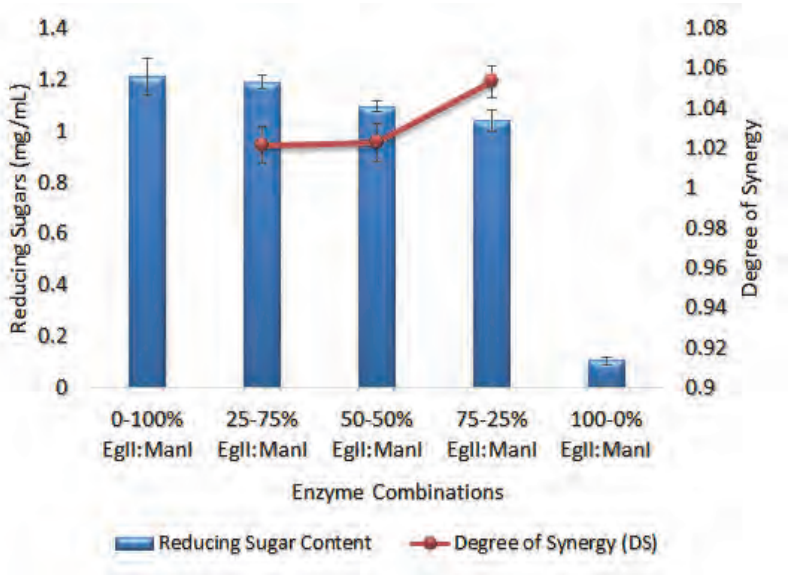
Interestingly, the interactions between the EgII:Xyn2 have a different outcome (Figure 4.6.B). The combination of the two enzymes yielded the lowest degree of synergy with 0.84 being the highest observed. This suggests that Xyn2 could potentially be in competition with EgII for the substrate, CMC, which is not surprising seeing as the Xyn2 exhibited 44% CMC activity when compared to the EgII (Table 4.2). This observation is also evident when the EgII:ManI combinations (Figure 4.6.A) are compared to the EgII:Xyn2 combinations (Figure 4.6.B). By comparing the reducing sugars between the 25%, 50% and 75% EgII in synergy between ManI and Xyn2, it is clear than the EgII is less effective (in the same concentrations) when combined with Xyn2.

4.3.7.2 Bi-synergy on Locust bean gum

Figure 4.7 demonstrates the synergistic relationship found between the three enzymes on locust bean gum over 60 minutes at 50°C using 50 mM sodium citrate buffer (pH 5.0). The synergy on locust bean gum indicated a different picture; however, some aspects remained the same compared to the synergy on carboxymethyl cellulose (Figure 4.6). The DS on the EgII:ManI combination was 1.05 at the highest. Therefore, the assumption would be that the ManI was virtually the only enzyme active (Figure 4.7A) – especially because at 100% enzyme loadings, the ManI produced 1.2 mg/mL of reducing sugars, while the EgII only managed 0.11 mg/mL (which amounts to less than 10%).

The EgII:Xyn2 combination produced the highest degree of synergy – 1.44 – at 50 – 50% enzyme loading on locust bean gum (Figure 4.7B). In light of the observations made in, the results are expected, as the enzymes are no longer in competition for a common substrate. This then suggests that the enzymes occupy different sites on the locust bean gum.

The combination between the ManI and Xyn2 yielded the lowest degree of synergy for the locust bean gum at 0.82. It is likely that the two enzymes were competing for substrates and thus the assumption would be that they occupy the same sites on the locust bean gum for hydrolysis. These results show that synergy could be effected by the substrates the enzymes are presented with in a particular reaction.



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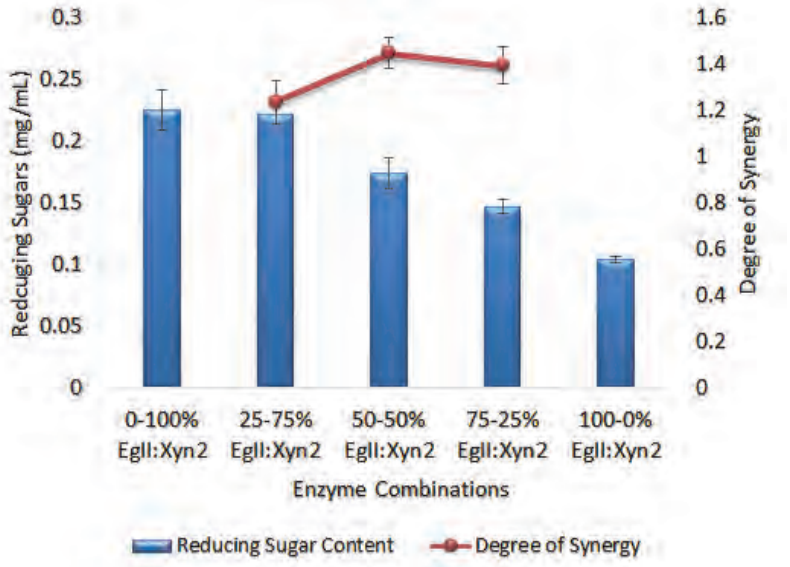

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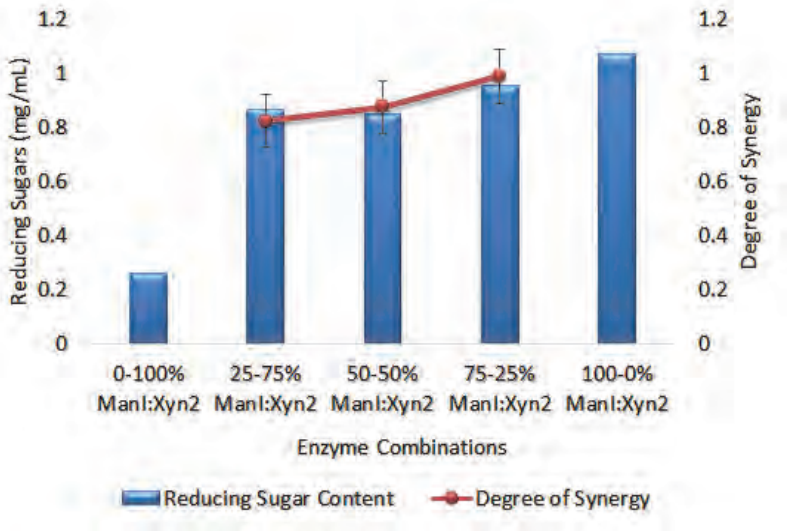


(C)

Figure 4.7. The synergistic interactions obtained on locust bean gum and the reducing sugars released by pre-determined enzyme combinations. (A) EgII and ManI, (B) EgII and Xyn2 and (C) ManI and Xyn2. Hydrolysis reactions were carried out for 60 minutes at 50°C. Bar graphs represent the released reducing sugars and the line graph is the degree of synergy between each combination. Data represent the mean values and error bars indicate the deviation from the mean (n =3).

4.3.7.3 Bi-synergy on Beechwood xylan

Figure 4.8 demonstrates the synergistic relationship detected between the three enzymes on beechwood xylan over 60 minutes at 50°C using 50 mM sodium citrate buffer (pH 5.0). The synergy on beechwood xylan follows the same trends as observed in Figures 4.6 and 4.7 above. One of the key highlights in Figure 4.8 is that any individual (or combination of) enzyme/s that do not have Xyn2, produced low amounts of reducing sugars and this was affirmed by the specific activity analysis (Table 4.2). Thus, the low DS observed between EgII:ManI combinations were expected (Figure 4.8A).

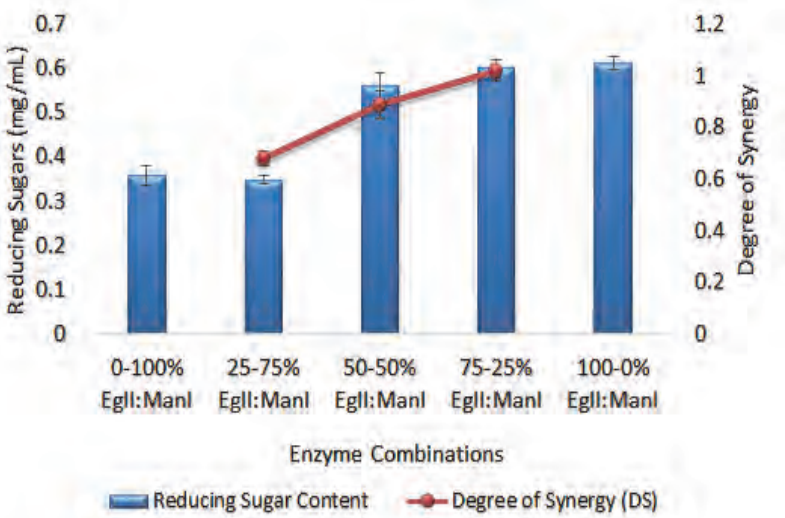
In light of the specific activity analysis (Table 4.2), it was not possible to attribute the relatively low DS values on enzyme competition entirely; it is likely that the enzymes (EgII and ManI) were not efficient in hydrolysing the backbone. Therefore, it is essential that further kinetic studies be conducted on the individual enzymes to confirm these observations.

4.3.7.4 Bi-synergy on combined substrates

Figure 4.9 demonstrates the synergistic relationship found between the three enzymes on a combined substrate mixture between carboxymethyl cellulose, locust bean gum and beechwood xylan (4:3:3) over 60 minutes at 50°C using 50 mM sodium citrate buffer (pH 5.0). The results of the combined substrates show that the interaction between the Xyn2 and ManI yielded the highest degree of synergy with the highest being 2.03 (Figure 4.9C). The enzymes were able to successfully cooperate with each other to obtain a maximum of 1.42 mg/mL reducing sugars (at 50-50% protein loadings) as compared to the 1.34 and 1.38 mg/mL reducing sugars for the ManI and Xyn2 respectively.

The interaction between EgII and ManI yielded higher reducing sugar content than the ManI and Xyn2 combination. The highest released reducing sugar was 1.9 mg/mL at 75% EgII and 25% ManI and this related to a degree of synergy of 0.93. This then suggests that the EgII and ManI functioned independently of each other. The lowest degree of synergy was observed for the EgII:Xyn2 combinations with the highest being 0.27 and the highest reducing sugar content was measured at 1.12 mg/mL with a 50% ratio of EgII and Xyn2. The degree of synergy clearly shows that the two enzymes are not only competing with each other for substrate, but they display anti-synergy. Thus, these two enzymes need to be investigated further and more importantly, determine if this behaviour is consistent with complex lignocellulosic biomass.

The results in Figures 4.6-4.7 showed that the enzymes possess potential for synergistic use. It would be important to compare how synergistic interactions between the three enzymes are affected by different natural lignocellulosic substrates. This aspect is currently under investigation.



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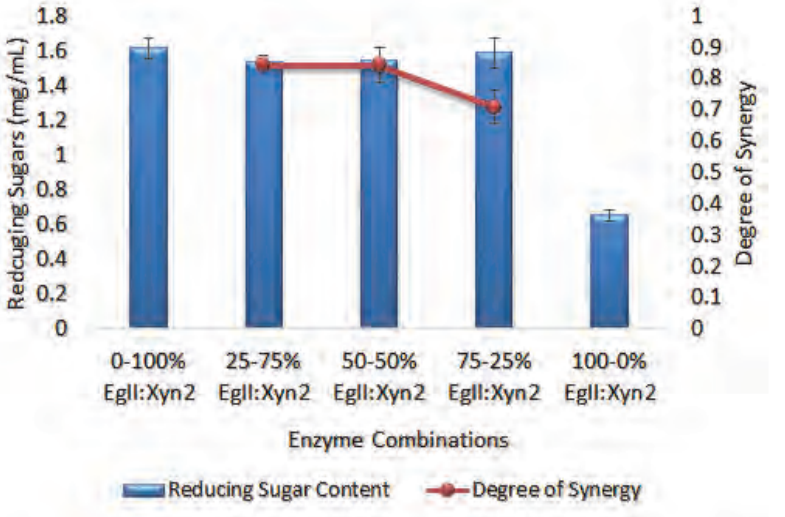

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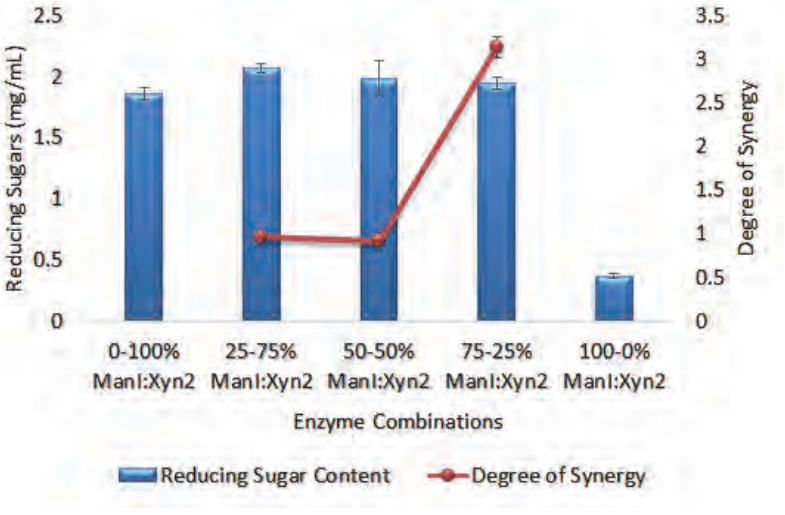
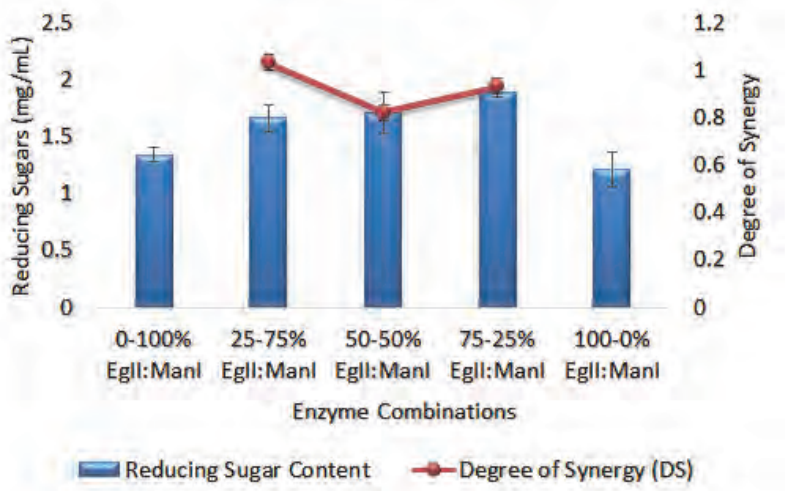


(C)

Figure 4.8. The synergistic interactions obtained on Beechwood xylan and the reducing sugars released by pre-determined enzyme combinations. (A) EgII and ManI, (B) EgII and Xyn2 and (C) Man and Xyn2. Hydrolysis reactions were carried out for 60 minutes at 50°C. Bar graphs represent the released reducing sugars and the line graph is the degree of synergy between each combination. Data represent the mean values and error bars indicate the deviation from the mean (n =3).



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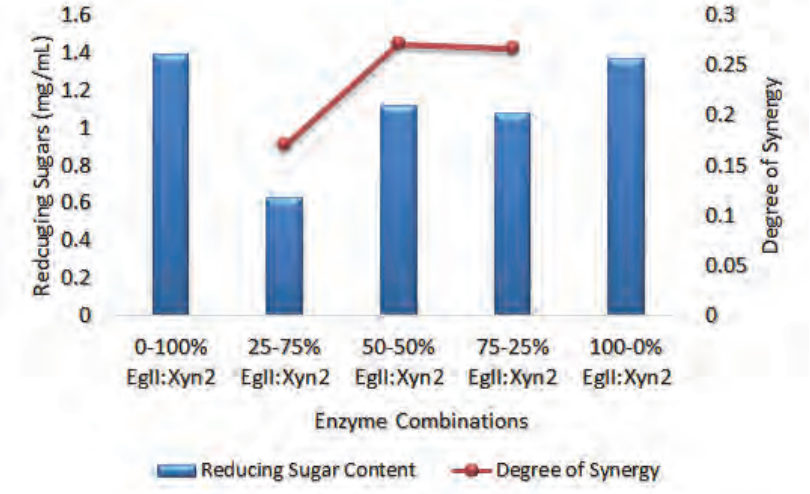

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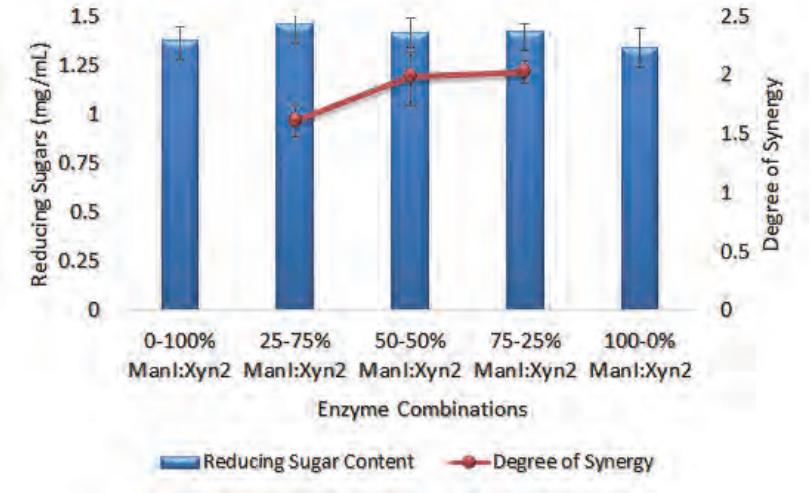

(C)

Figure 4.9. The synergistic interactions obtained on a combined substrate mixture between carboxymethyl cellulose, locust bean gum and beechwood xylan (4:3:3) and the reducing sugars released by pre-determined enzyme combinations. (A) EgII and ManI, (B) EgII and Xyn2 and (C) ManI and Xyn2. Hydrolysis reactions were carried out for 60 minutes at 50°C. Bar graphs represent the released reducing sugars and the line graph is the degree of synergy between each combination. Data represent the mean values and error bars indicate the deviation from the mean (n =3).

CHAPTER 5: CONCLUDING REMARKS

General Discussion and Conclusions

The development of an improved understanding of fruit bio-based waste streams in South Africa is vital for applications in bioethanol production as well as the creation of value added products. In light of this, the overall aim of this project has been to develop a conceptual model for the bioconversion of bio-residues in bio-based waste streams, through the use of fungal strains to produce enzymes, which, in turn, have further potential for applications in waste stream reclamation as well as bioethanol production through lignocellulosic biomass.

The proof-of-concept experiments explored the suitability of recombinant *Aspergillus niger* and *Trametes pubescens* strains to produce industrially-relevant enzymes (cellulase, mannanase, xylanase and laccase) through bio-based waste streams: apple pomace, grape waste and potato waste. The production of extracellular cellulases, mannanases and xylanase through *A. niger* was observed in all three waste streams. This indicates that *A. niger* is evidently effective at degrading a variety of fruit waste stream components, thereby reinforcing the organism's applicability in bio-residue bioconversion. Furthermore, Chapter 1 shows that the expression of the recombinant enzymes was optimised to produce the respective extracellular enzyme, while suppressing the catabolite expression of the *A. niger* endogenous enzymes.

The study showed that potato waste was the more effective bio-based waste stream for improving the growth of the recombinant *Aspergillus niger* strains, which lead to greater heterologous expression of endoglucanase, endomannanase and endoxylanase enzymes. In addition, the production of bioethanol was achieved with *S. cerevisiae* utilising potato waste as the fermentation feedstock. Therefore, this study provided evidence that shows that the use of bio-based waste stream has potential in the production of both enzymes and bioethanol.

Trametes pubescens showed the ability to grow and produce laccases on apple and potato waste without the supplementation of additional nutrients or inducers. Furthermore, the laccase produced from these waste streams was similar to a purified *T. pubescens* laccase obtained under optimised media, environment and inducer conditions. The combination of these experiments showed that bio-based waste streams do have noticeable potential for application in enzyme and bioethanol production.

Characterisation studies showed that the properties of the *A. niger* derived enzymes: endoglucanase (EgII), endomannanase (ManI) and endoxylanase (Xyn2) were largely in agreement with what was reported previously in literature. All three enzymes had temperature optima between 50 and 60 °C and were stable at these temperatures for at least 24 hours, while the pH optima of the three enzymes were between 5 and 6. These properties – pH and temperature – indicate that these enzymes are well suited for applications in bio-refineries due to the fact that the industrial processes occur within these ranges. Xyn2 was shown to have cross-functional activities on carboxymethyl cellulose (CMC) and locust bean gum (LBG); the enzyme further displayed slight glucosidase and xylosidase activities as well. This adds to the versatility of the enzyme's potential application in a number of processes.

Synergy studies between the EgII, ManI and Xyn2 enzymes were conducted on CMC, LBG and Beechwood xylan (BWX), as well as combined mixture. The synergistic interactions between EgII and Xyn2 seemed to have the most promising implications in the production of fermentable monomeric sugars, that are relevant for biofuel production, as well as additional value-added products. The synergy between these two enzymes is significantly higher in cellulose and beechwood xylan mixtures.

This report provides strong evidence that demonstrates that *A. niger* can use a variety of waste streams to produce industrially-relevant enzymes, which can be further applied in a biorefinery setting, through the production of fermentable sugars for biofuel production as well as generating value added products. The synergistic relationships further reveal that these enzymes can be used together to enhance their degradation capabilities to transform waste to value.

Recommendations for Further Research

The interesting data generated in this report leads the way to further investigation that would ultimately inform the establishment of biorefineries in South African and internationally. Future studies could focus on further developing *A. niger* strains that produce a consortium of enzymes that also have further application in waste stream reclamation as well as lignocellulosic biomass degradation. The synergy between EgII, ManI and Xyn2 could be expanded to a variety of fruit waste streams as well as lignocellulosic biomass. Laccase could be added as an additional enzyme to examine the interactions with other enzymes. Furthermore, pilot scales can be conducted using an optimised *A. niger* derived enzyme cocktail to determine and improve large-scale prospects.

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Appendix A: Standard curves used to convert optical density to activity

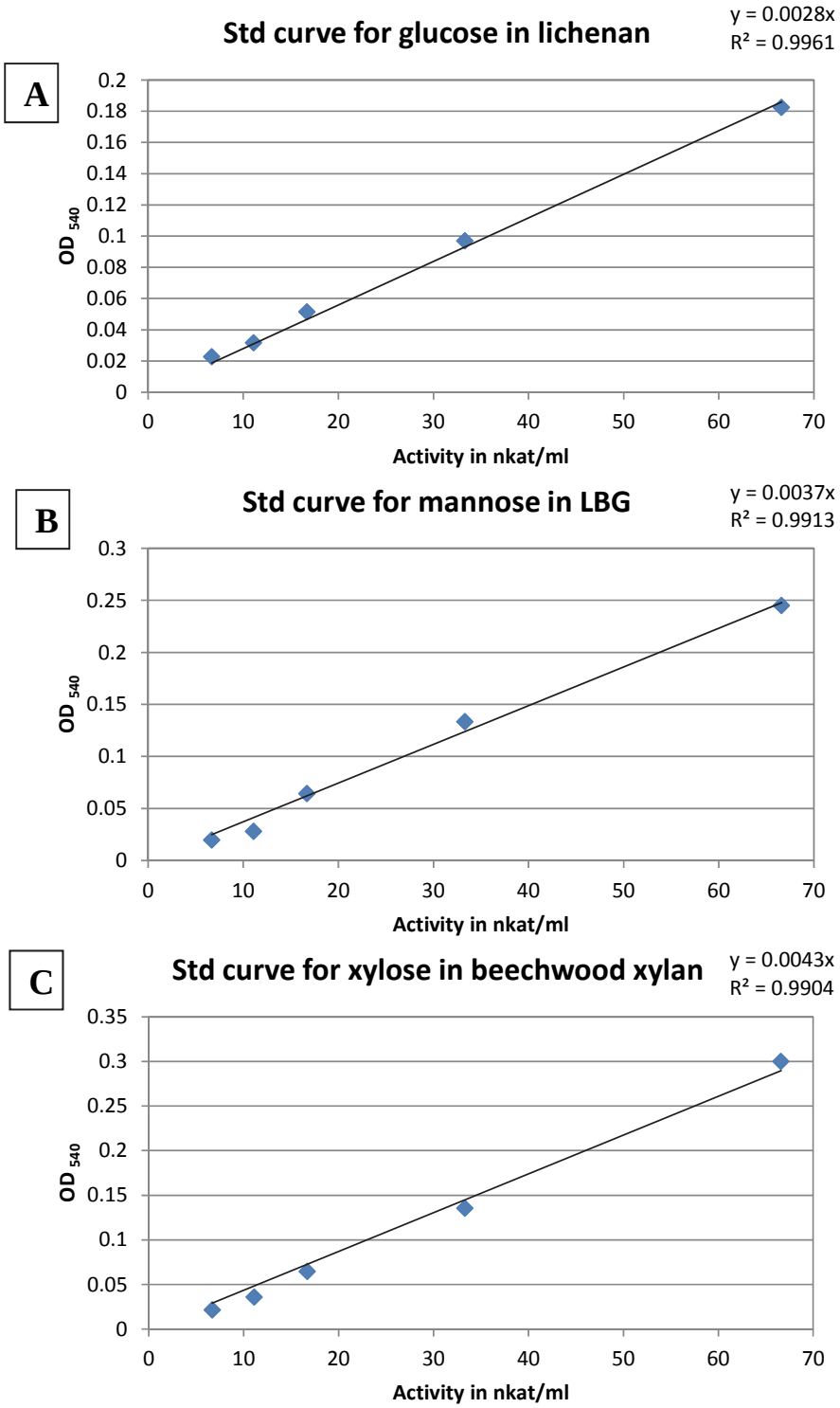


Figure A1.1. Standard curves for (A) Glucose in lichenan (B) Mannose in Locust Bean Gum (LBG) and (C) Xylose in Beechwood xylan against enzyme activity in nkat/mL.

Appendix B: Gallic acid standard curve for the determination of total phenolics

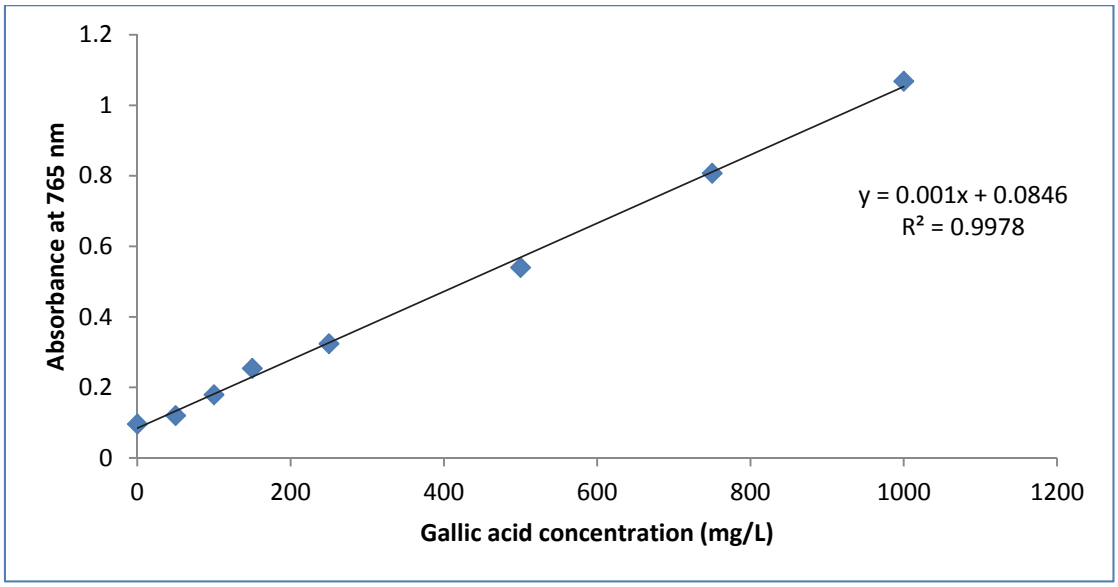
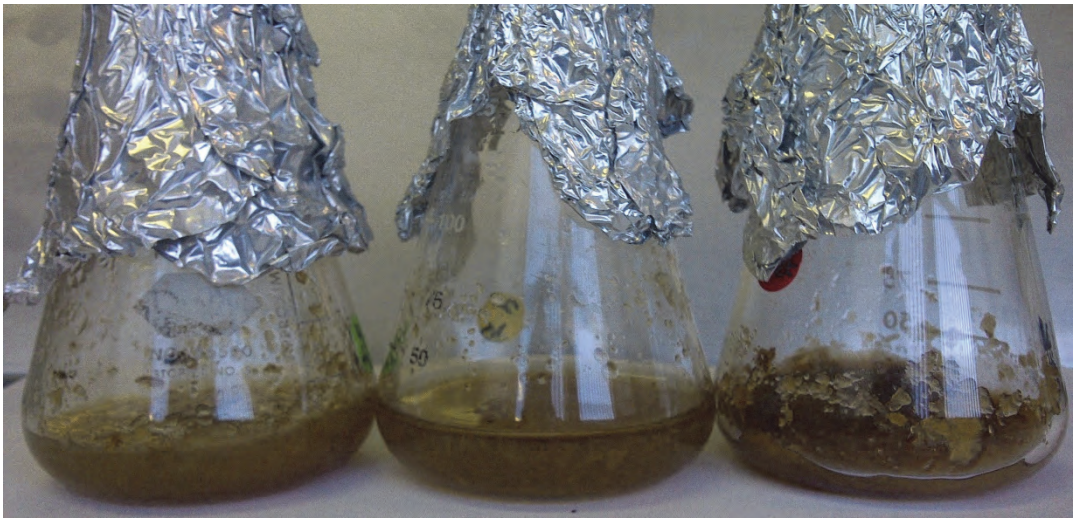


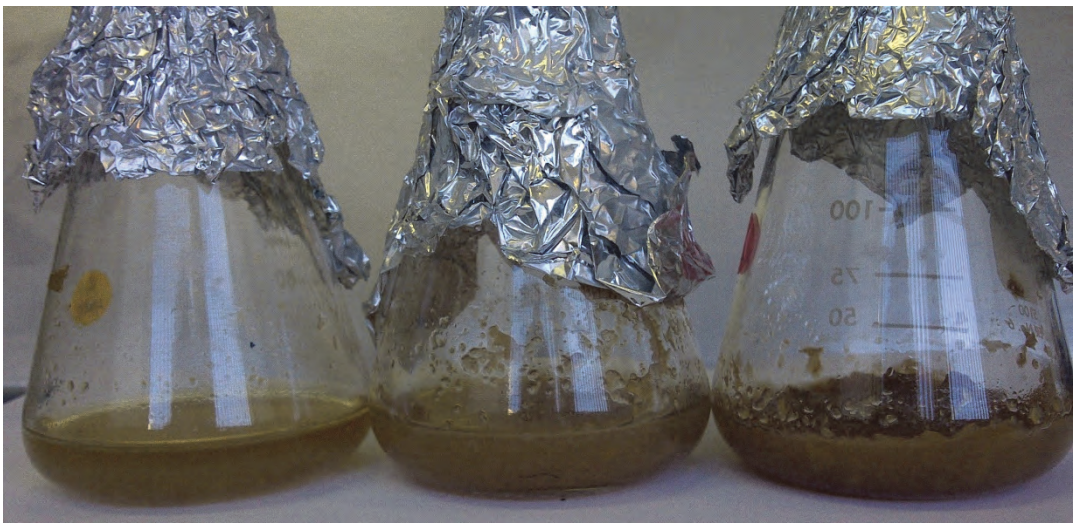
Figure B1.1. Gallic acid standard curve determined the Folin-Ciocalteu Method for total phenolic determination.

Appendix C: Medium before inoculation

PPM2-1, PPM2-2 and PPM2-3 looks similar to PPM-1, PPM-2 and PPM-3, respectively



UPM 10% UPM 20% UPM 50%



PPM 10% PPM 20% PPM 50%

Figure C1.1. Visual inspection of varying percentage composition of Unpressed Pomace Media (UPM) and Pressed Pomace Media (PPM) before inoculation.

Appendix D: Picture of fermentation of potato waste

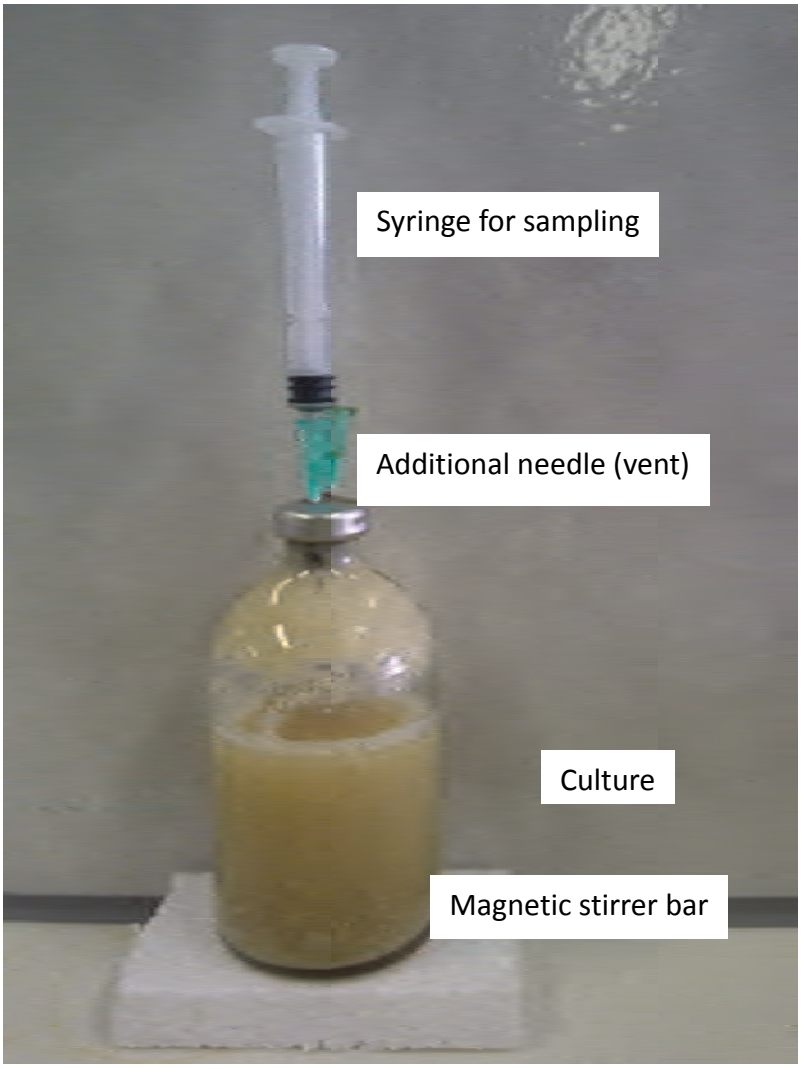


Figure D1.1. Picture of fermentation of potato waste.

Appendix E: Standard Curves for protein and enzyme activity

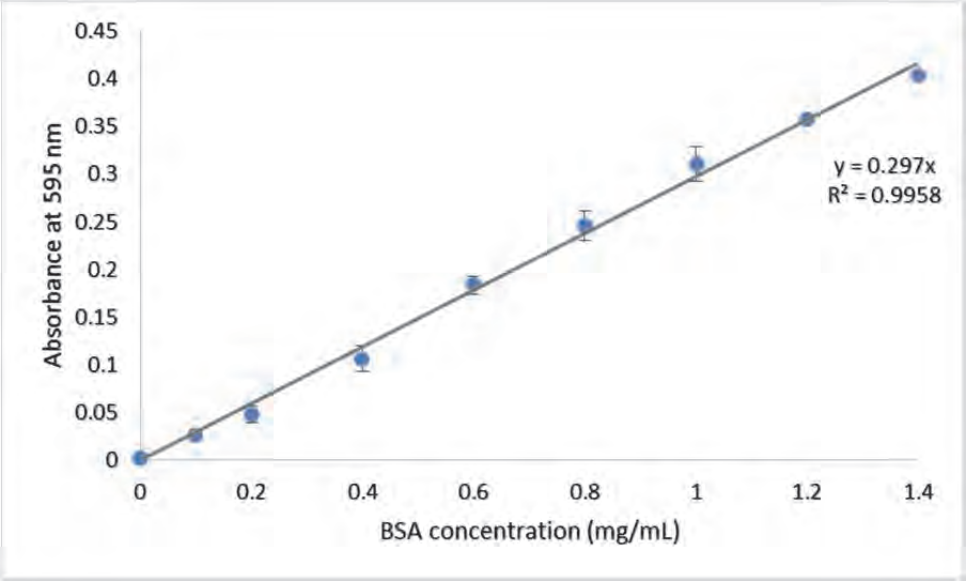


Figure E1.1: Bovine serum albumin standard curve obtained using the Bradford assay for the quantification of protein content. Data represents the mean values with errors bars indicating the standard deviation (n=3).

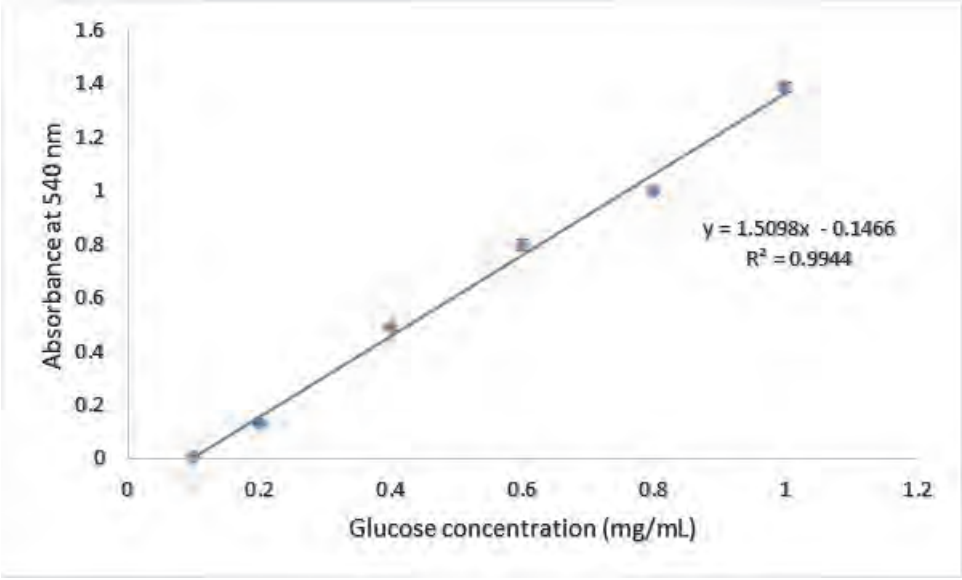


Figure E1.2: Glucose standard curve obtained using the DNS assay for quantifying the reducing sugar content. Data represents the mean values with errors bars indicating the standard deviation (n=3).

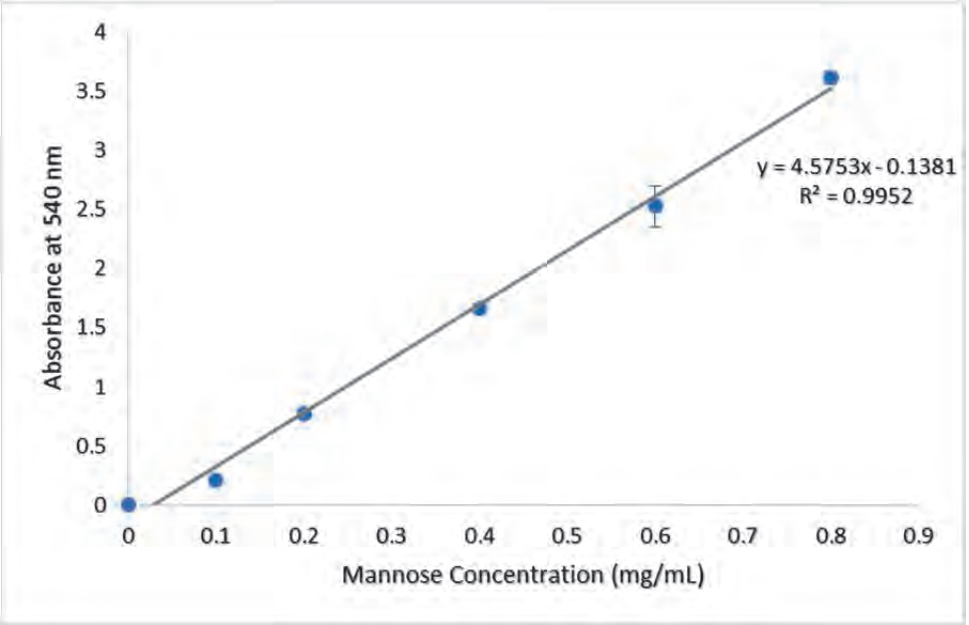


Figure E1.3: Mannose standard curve obtained using the DNS assay for quantifying the reducing sugar content. Data represents the mean values with errors bars indicating the standard deviation (n=3).

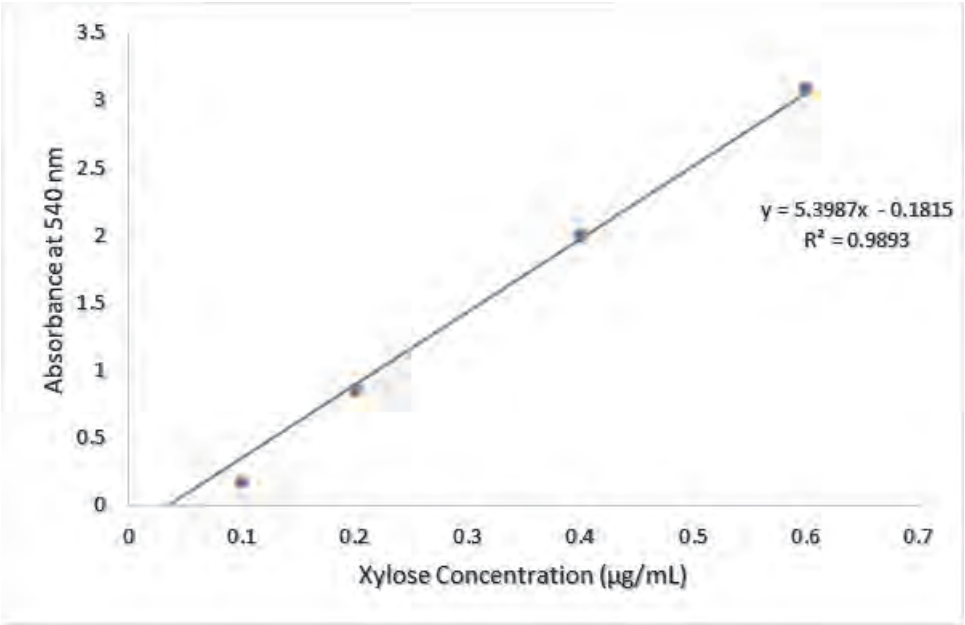


Figure E1.4: Xylose standard curve obtained using the DNS assay for quantifying the reducing sugar content. Data represents the mean values with errors bars indicating the standard deviation (n=3).

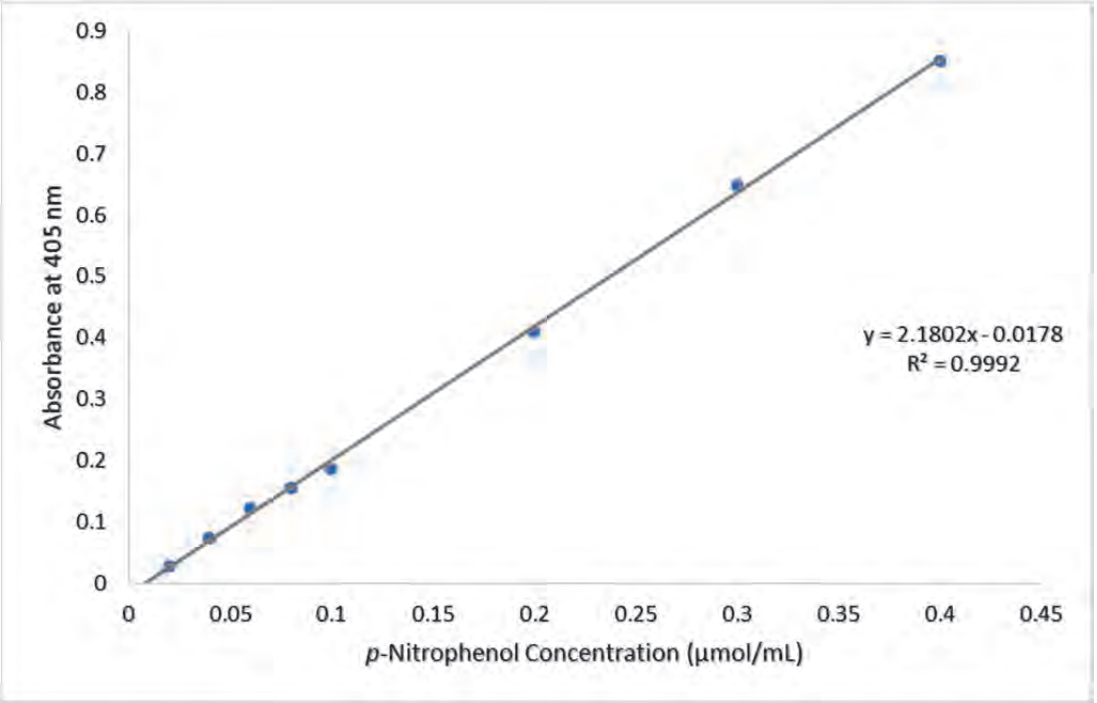


Figure E1.5: *p*-Nitrophenol standard curve obtained for the β-glucosidase and β-mannosidase activity assays. Data represents the mean values with errors bars indicating the standard deviation (n=3).

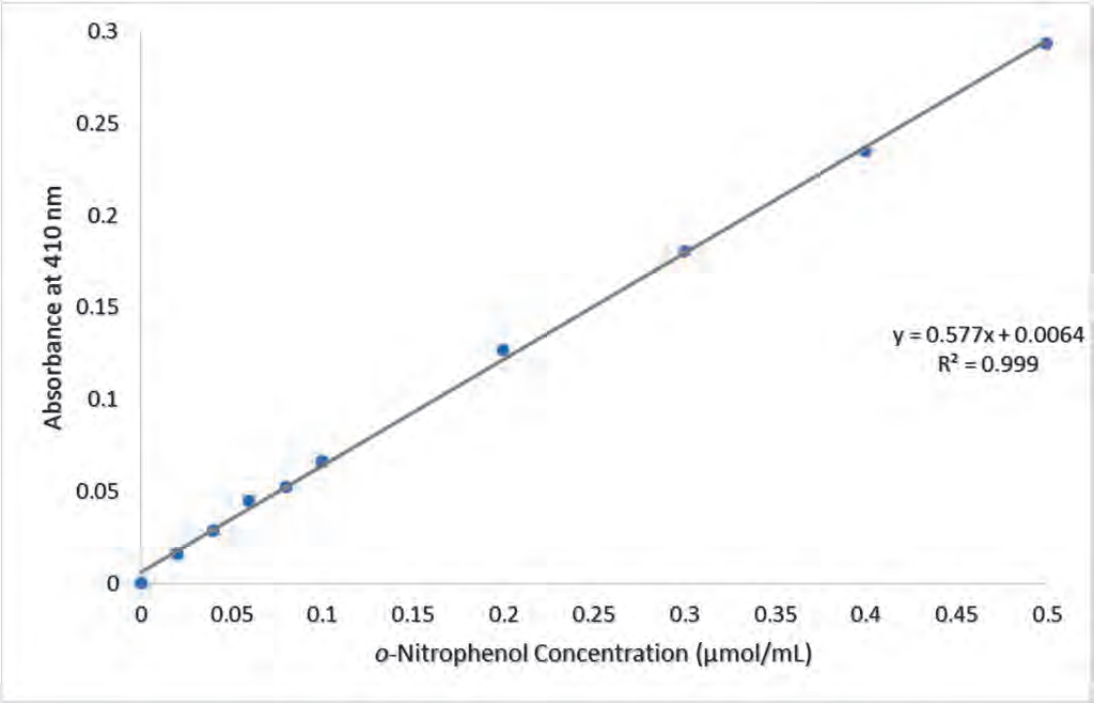


Figure E1.6: *o*-Nitrophenol standard curve obtained for the β-xylosidase activity assays. Data represents the mean values with errors bars indicating the standard deviation (n=3).

Appendix F: Synergy Studies

The reaction mixtures for the binary synergy assays were made up to a final volume of 500 µl using 50 mM sodium citrate buffer (pH 5.0). 400 µl of 1.25% (w/v) carboxymethyl cellulose or beechwood xylan and 0.66% (w/v) locust bean gum was added for each respective reaction; 100 µl of appropriately dilute enzymes were added in different combinations as shown in table A6.1. The total protein concentration was kept constant in all combinations at 11.5 µg/mL. The assays were run in triplicate at 50°C for 60 minutes.

Table F1.1. Enzyme combinations for bi-synergy studies during model substrate hydrolysis

Combination	EgII (µl)	ManI (µl)	Xyn2 (µl)
0-100% EgII:ManI	0	100	0
25-75% EgII:ManI	25	75	0
50-50% EgII:ManI	50	50	0
75-25% EgII:ManI	75	25	0
0-100% EgII:Xyn2	0	0	100
25-75% EgII:Xyn2	25	0	75
50-50% EgII:Xyn2	50	0	50
75-25% EgII:Xyn2	75	0	25
0-100% ManI:Xyn2	0	0	100
25-75% ManI:Xyn2	0	25	75
50-50% ManI:Xyn2	0	50	50
75-25% ManI:Xyn2	0	75	25