A Lignocellulolytic Enzyme System for Fruit Waste Degradation: Commercial Enzyme Mixture Synergy and Bioreactor Design

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Abstract

Studies into sources of alternative liquid transport fuel energy have identified agro-industrial wastes, which are lignocellulosic in nature, as a potential feedstock for biofuel production against the background of depleting nonrenewable fossil fuels. In South Africa, large quantities of apple and other fruit wastes, called pomace, are generated from fruit and juice industries. Apple pomace is a rich source of cellulose, pectin and hemicellulose, making it a potential target for utilisation as a lignocellulosic feedstock for biofuel and biorefinery chemical production. Lignocellulosic biomass is recalcitrant in nature and therefore its degradation requires the synergistic action of a number of enzymes such as cellulases, hemicellulases, pectinases and ligninases. Commercial enzyme cocktails, containing some of these enzymes, are available and can be used for apple pomace degradation. In this study, the degradation of apple pomace using commercial enzyme cocktails was investigated. The main focus was the optimisation of the release of sugar monomers that could potentially be used for biofuel and biorefinery chemical production. There is no or little information reported in literature on the enzymatic degradation of fruit waste using commercial enzyme mixtures.

This study first focused on the characterisation of the substrate (apple pomace) and the commercial enzyme cocktails. Apple pomace was found to contain mainly glucose, galacturonic acid, arabinose, galactose, lignin and low amounts of xylose and fructose. Three commercial enzyme cocktails were initially selected: Biocip Membrane, Viscozyme L (from *Aspergillus aculeatus*) and Celluclast 1.5L (a *Trichoderma reesei* ATCC 26921 cellulase preparation). The selection of the enzymes was based on activities declared by the manufacturers, cost and local availability. The enzymes were screened based on their synergistic cooperation in the degradation of apple pomace and the main enzymes present in each cocktail. Viscozyme L and Celluclast 1.5L, in a 50:50 ratio, resulted in the best degree of synergy (1.6) compared to any other combination. The enzyme ratios were determined on Viscozyme L and Celluclast 1.5L based on the protein ratio. Enzyme activity was determined as glucose equivalents using the dinitrosalicylic acid (DNS) method. Sugar monomers were determined using Megazyme assay kits.

There is limited information available on the enzymes present in the commercial enzyme cocktails. Therefore, the main enzymes present in Viscozyme L and Celluclast 1.5L were identified using different substrates, each targeted for a specific enzyme and activity. Characterisation of the enzyme mixtures revealed a large number of enzymes required for apple pomace degradation and these included cellulases, pectinases, xylanases, arabinases and mannanases in different proportions. Viscozyme L contained mainly pectinases and hemicellulases, while Celluclast 1.5L displayed largely cellulase and xylanase activity, hence the high degree of synergy reported. The temperature optimum was 50°C for both enzyme mixtures and pH optima were observed at pH 5.0 and pH 3.0 for Viscozyme L and Celluclast 1.5L, respectively. At 37°C and pH 5.0, the enzymes retained more that 90% activity after 15 days of incubation, allowing the enzymes to be used together with less energy input. The enzymes were further characterised by determining the effect of various compounds, such as alcohols, sugars, phenolic compounds and metal ions at various concentrations on the activity of the enzymes during apple pomace hydrolysis. Apart from lignin, which had almost no effect on enzyme activity, all the compounds caused inhibition of the enzymes to varying degrees. The most inhibitory compounds were some organic acids and metal ions, as well as cellobiose and xylobiose.

Using the best ratio for Viscozyme L and Celluclast 1.5L (50:50) for the hydrolysis of apple pomace, it was observed that synergy was highest at the initial stages of hydrolysis and decreased over time, though the sugar concentration increased. The type of synergy for optimal apple pomace hydrolysis was found to be simultaneous. There was no synergy observed between Viscozyme L and Celluclast 1.5L with ligninases - laccase, lignin peroxidase and manganese peroxidase. Hydrolysing apple pomace with ligninases prior to addition of Viscozyme L and Celluclast 1.5L did not improve degradation of the substrate.

Immobilisation of the enzyme mixtures on different supports was performed with the aim of increasing stability and enabling reuse of the enzymes. Immobilisation methods were selected based on the chemical properties of the supports, availability, cost and applicability on heterogeneous and insoluble substrate like apple pomace. These methods included cross-linked enzyme aggregates (CLEAs), immobilisation on various supports such as nylon mesh, nylon beads, sodium alginate beads, chitin and silica gel beads. The immobilisation strategies

were unsuccessful, mainly due to the low percentage of immobilisation of the enzyme on the matrix and loss of activity of the immobilised enzyme.

Free enzymes were therefore used for the remainder of the study. Hydrolysis conditions for apple pomace degradation were optimised using different temperatures and buffer systems in 1 L volumes mixed with compressed air. Hydrolysis at room temperature, using an unbuffered system, gave a better performance as compared to a buffered system. Reactors operated in batch mode performed better (4.2 g/L (75% yield) glucose and 16.8 g/L (75%) reducing sugar) than fed-batch reactors (3.2 g/L (66%) glucose and 14.6 g/L (72.7% yield) reducing sugar) over 100 h using Viscozyme L and Celluclast 1.5L. Supplementation of β-glucosidase activity in Viscozyme L and Celluclast 1.5L with Novozyme 188 resulted in a doubling of the amount of glucose released. The main products released from apple pomace hydrolysis were galacturonic acid, glucose and arabinose and low amounts of galactose and xylose. These products are potential raw materials for biofuel and biorefinery chemical production.

An artificial neural network (ANN) model was successfully developed and used for predicting the optimum conditions for apple pomace hydrolysis using Celluclast 1.5L, Viscozyme L and Novozyme 188. Four main conditions that affect apple pomace hydrolysis were selected, namely temperature, initial pH, enzyme loading and substrate loading, which were taken as inputs. The glucose and reducing sugars released as a result of each treatment and their combinations were taken as outputs for 1–100 h. An ANN with 20, 20 and 6 neurons in the first, second and third hidden layers, respectively, was constructed. The performance and predictive ability of the ANN was good, with a R^2 of 0.99 and a small mean square error (MSE). New data was successfully predicted and simulated. Optimal hydrolysis conditions predicted by ANN for apple pomace hydrolysis were at 30% substrate (wet w/v) and an enzyme loading of 0.5 mg/g and 0.2 mg/mL of substrate for glucose and reducing sugar, respectively, giving sugar concentrations of 6.5 mg/mL and 28.9 mg/mL for glucose and reducing sugar, respectively. ANN showed that enzyme and substrate loadings were the most important factors for the hydrolysis of apple pomace.

It can therefore be concluded that a 50:50 combination of Viscozyme L and Celluclast 1.5L, supplemented with Novozyme 188 was able to efficiently degrade apple pomace (75% conversion) using a batch reactor operated at room temperature without buffering the system. The sugar monomers released from apple pomace hydrolysis are potential raw materials for biofuel and biorefinery chemical production. ANN can successfully predict glucose and reducing sugar release from apple pomace. Recommendations for future studies include: scaling-up bioreactors to larger volumes, production of value added products from the released sugars, measuring of amounts of oligosaccharides released and identification and quantification of phenolic compounds.

Declaration

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

Signed: Repson Gama

On this _____12_____of ___December_____2013_____

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Table of Contents

List of Figures

List of Tables

List of Abbreviations

µL	Microliter
APS	Ammonium persulphate
BSA	Bovine serum albumin
$CaCl_2$	Calcium chloride
CBM	Carbohydrate binding module
CMC	Carboxymethylcellulose
E. coli	*Escherichia coli*
KDa	Kilodalton
h	Hours
g	Gram
GH	Glycosyl hydrolase
HCl	Hydrochloric acid
HPLC	High performance liquid chromatography
GC	Gas chromatography
mg	Milligrams
min	Minutes
mL	Millilitre
mM	Millimolar
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NREL	National Renewable Energy Laboratory
SEM	Scanning electron microscope
SDS	Sodium dodecyl sulphate
PAGE	Polyacrylamide gel electrophoresis
SD	Standard deviation
DH_2O	Distilled Millipore water
TEMED	N, N, N', N'-tetramethylethylenediamine
TLC	Thin layer chromatography
TFA	Trifluoroacetic acid
CLEAs	Cross-linked enzyme aggregates
ID	Internal diameter

BG	Beta-glucosidase
EG	Endoglucanase
CBH	Cellobiohydrolase
EDTA	Ethylenediaminetetraacetic acid

Research outputs emanating from this study

Publications in peer-reviewed scientific journals:

1. Van Dyk, J.S., **Gama, R.**, Morrison, D., Swart, S., Pletschke, B.I. (2013). Food processing waste: problems, current management and prospects for utilisation of the lignocellulose component through enzyme synergistic degradation. *Renewable and Sustainable Energy Reviews*, **26:** 521-531.
2. **Gama R**., Van Dyk, J.S., Burton, S.G., Pletschke, B.I. (2014). Evaluation of commercial enzyme mixtures for the hydrolysis of apple pomace and characterisation of these mixtures. *In preparation.*
3. **Gama R**., Van Dyk, J.S., Burton, S.G., Pletschke, B.I. (2014). Optimization of enzymatic apple pomace hydrolysis conditions for an industrial bioconversion process. *In preparation.*
4. **Gama R.**, Van Dyk, J.S., Burton, M., Pletschke, B.I. (2014). Artificial neural network for the prediction of apple pomace hydrolysis using commercial enzyme preparations. *In preparation.*

International conference proceedings:

1. **Gama R**., Van Dyk, J.S., Pletschke, B.I. Commercial enzyme mixtures can be used for efficient hydrolysis of apple pomace, a lignocellulosic substrate: V International conference on Environmental, Industrial and Applied Microbiology – BioMicroWorld2013, Faculty of Medicine-Complutense University, Madrid, Spain, 2-4 October 2013 (poster).

National conference proceedings:

1. Pletschke B.I., Beukes N., **Gama R.**, Van Dyk J.S. Cellulase and Hemicellulose Synergy for Lignocellulose Degradation: 16th South African Society of Microbiology (SASM) conference, Cape Sun Hotel, Cape Town, South Africa, 6-8 November 2011 (Oral).
2. **Gama R**., Van Dyk J.S., Pletschke B.I. Bioconversion of apple pomace using *Aspergillus* and *Trichoderma* commercial enzyme cocktails: 17th South African Society of Microbiology (SASM) conference, Bela-Bela, South Africa, 25-27 November 2013 (poster).

CHAPTER 1: GENERAL INTRODUCTION

1.1. Literature Review

1.1.1. Biofuels

1.1.1.1. Introduction

The world population is estimated to grow to about 10 billion people by 2050 (Dashtban *et al*., 2009). In order to meet the ever-increasing energy demands, the world is therefore exploring alternative sources of energy against the background of rapid depletion of traditionally used non-renewable fossil fuels (coal, oil, and natural gas), currently a major source of transport energy (87%) (Merino and Cherry, 2007). Petroleum currently constitutes about 98% of transport fuel and alternative sources have to be found (Gomez *et al*., 2008). Globally, oil production has been predicted to fall from 25 billion barrels to about 5 billion barrels in 2050 (Dashtban *et al*., 2009; Sun and Cheng, 2002). Besides being non-renewable, fossil fuels have been a source of global conflict as a result of their control and geographical distribution. Fossil fuels are also a major contributor of climate change and global warming due to emission of greenhouse gases (carbon dioxide, carbon monoxide, methane, nitrous oxide, ozone). Unstable price fluctuations has been a major concern for many countries as fuel price increases tend to affect other sectors of the economy as well (Dashtban *et al*., 2009; Garcia-Aparicio *et al*., 2011; Himmel *et al*., 2007; Merino and Cherry, 2007; Solomon *et al*., 2007). Global attention is now being shifted towards finding alternative sources of energy for liquid transportation fuels, e.g. biofuels.

1.1.1.2. Biofuel production

Biofuels are fuels produced from biological sources such as plant materials and include bioethanol, biodiesel, biobutanol, bio-oil, dimethyl ether and dimethyl furan. The major biofuels are bioethanol, which is produced from fermentation of sugars, and biodiesel, which is produced from plant/ animal oil. The advantages of biofuels over fossil fuels is that biofuels are produced from materials that are renewable, abundantly and locally available, while their use cause less emission of greenhouse gases, less impact on the environment and

is sustainable (Balat, 2011; Del Rio *et al*., 2012; Hahn-Hagerdal *et al*., 2006; Merino and Cherry, 2007).

There has been an increase in biofuel production around the world from 2000, e.g. bioethanol production levels increased from 16.9 – 72.0 billion litres from 2000 – 2009 (Sorda *et al*., 2010). In the United States of America (USA), Brazil and some countries in Europe, bioethanol production is already commercially established, and in 2007, 46 billion litres of bioethanol were produced globally. Ethanol can be used as an alternative to transport fuel or mixed with gasoline (24%: 76%, ethanol: gasoline), thus relieving pressure on dwindling fossil fuel reserves (Sun and Cheng, 2002; Wyman, 1994). Bioethanol is currently being produced from different starch crops in different countries, e.g. corn and sweet sorghum in the USA, sugar cane in Brazil, corn and wheat in China, wheat, barley and sugar beet in the EU and corn and wheat in Canada (Balat, 2011; Dashtban *et al*., 2009; Ge *et al*., 2011). The increase in biofuel production in these countries has been promoted mainly by policies of national governments. The use of biofuel is also being supported in South Africa (Garcia-Aparicio *et al*., 2011).

Currently, most bioethanol is being produced from food crops like corn starch, sugar cane, sugar beet, palm oil and oilseeds (first generation biofuels) (Hu *et al*., 2008). The process involves conversion of starch in these crops into simple sugars which are subsequently fermented to ethanol. It has been reported that in 2006, the USA reallocated about 20% of total corn supply to bioethanol production (Sorda *et al*., 2010). The use of food-based crops to produce biofuels poses a direct competition with food supply for humans and animals and thus causes global increases in food prices (Balat, 2011; Ferreira *et al*., 2009; Gomez *et al*., 2008). Balat (2011) reported that, in the USA, the prices of some food commodities like beef, eggs, milk, bread and cereals rose between 10 and 30% due to the use of corn for bioethanol production. Water, land and energy resources, which are supposed to be used to produce food for humans, will be diverted to crops for biofuel production. First generation biofuels also causes some emission of greenhouse gases due to clearing of large pieces of land to grow crops and the use of fertilisers and pesticides (Ge *et al*., 2011). Large-scale production of first generation biofuels is thus unsustainable and is less likely to be used in the near future. Other sources of biofuels are being investigated to replace first generation biofuels.

Second generation biofuels, which target non-food, cheap and widely available cellulosic wastes, e.g. agricultural wastes, forestry residues, paper and pulp waste, food and animal waste and municipality wastes, are a potential feedstock for ethanol production and much attention is now directed towards their utilisation. Some of the lignocellulose wastes that has drawn the attention of many researchers include corn stover, wheat straw, sugarcane bagasse, rice straw, rice hull, corncob, oat hull, corn fibre, saw dust, wood bark, switch grass and municipality wastes (Balat, 2011; Ge *et al*., 2011; Gomez *et al*., 2008; Hu *et al*., 2008; Wyman, 2007). Lignocellulose consists of 75% complex polysaccharides that are naturally degraded in the environment by microbes which utilise them as energy and carbon sources (Dashtban *et al*., 2009; Pedrolli *et al.*, 2009). The microorganisms, such as bacteria and fungi, produce enzymes that work synergistically to degrade lignocellulosic biomass. Degradation of lignocellulose is, however, a complex process due to the heterogeneous and recalcitrant nature of lignocellulose, the presence of lignin and hemicellulose, while the crystallinity of cellulose limits cellulose availability to cellulases. Physico-chemical pre-treatment methods have been employed to remove lignin and make cellulose and hemicellulose accessible for enzyme degradation (Garcia-Aparicio *et al*., 2011). Extensive research on enzymatic conversion of lignocellulose has been an ongoing process (Kovacs *et al*., 2009; Merino and Cherry, 2007; Sun and Cheng, 2002).

There are also some concerns with respect to biofuel production from lignocellulose feedstocks, for example their impact on food security and environment. If large pieces of land are to be used to grow plants for biofuels, it will reduce available land for food crops, affecting food supply and prices. The planting of crops suitable for biofuels could also affect water resources and impact on biodiversity and energy security. There are also some technical challenges that have to be overcome in order to commercially produce biofuels from lignocellulose cost-effectively (Balat, 2011; Howard *et al*., 2003). Production of cellulosic ethanol is currently estimated at $1.80/ gallon, which is approximately twice as much as production of ethanol from starch (Merino and Cherry, 2007), mainly due to operational, equipment and enzyme costs and the need to pre-treat the lignocellulose. Production of bioethanol from lignocellulose involves four major steps, pre-treatment of biomass feedstock, enzymatic conversion of pre-treated material into simple sugars,

fermentation of sugars into ethanol by yeast/ bacteria, and lastly purification/ separation of ethanol from other wastes (Balat, 2011; Dashtban *et al*., 2009; Lin *et al*., 2010).

Future generation biofuels are therefore being proposed which involve production of hydrocarbon fuels (bioalcohol, biodiesel) from energy crops such as algae (third generation), and fuels from genetically modified organisms (fourth generation) (Lin and Tanaka, 2006). Although these methods of biofuel production have environmental and social benefits, the economic issues surrounding these technologies may hinder their commercialisation.

Commercial production of biofuels from lignocellulosic biomass is mainly hindered by the high cost of enzymes (Chen *et al*., 2008). Novozyme, Genencor, DSM Innovation Center and Verenium are currently the major enzyme research companies that produce commercial enzyme mixtures that can be used in industrial biofuel production. The research at Novozyme and Genencor is funded by the Department of Energy (DOE) in the USA (Banerjee *et al.*, 2010b; Merino and Cherry, 2007). These companies are mandated to produce cheap enzymes that will make commercialisation of bioethanol production from lignocellulose economically viable. Their approaches include strain improvement of bacteria or fungi, e.g. *T. reesei,* by molecular and conventional mutagenesis, screening of new organisms with better qualities and organisms which can act synergistically with other available enzymes (bioprospecting), improving enzyme properties by protein engineering and directed evolution, and lastly, to efficiently produce enzymes on an industrial scale (Banerjee *et al.*, 2010b; Dashtban *et al*., 2009; Howard *et al*., 2003; Merino and Cherry, 2007; Zhou *et al*., 2009).

Commercial enzyme mixtures are crude preparations containing different enzyme activities, present a cheaper option than individual enzymes and can be optimised in terms of combination and ratio to degrade lignocellulose. Optimisation of enzyme activities and ratios to relevant feedstocks is important for hydrolysis and reduction of enzyme costs (Gao *et al*., 2011; Kovacs *et al*., 2009). A number of commercial enzymes preparations are reported in literature, such as Accellerase 1500 (cellulase, hemicellulase, β-glucosidase), Optimash^{TM}BG (xylanase, β-glucanase) (Lin *et al*., 2010), Celluclast 1.5L (mainly cellulase), Ultraflo L and Viscozyme L (mainly β-glucanase and hemicellulase) (Merino and Cherry, 2007; Sorensen *et al*., 2003), Multifect, Spezyme and Biocellulase (Howard *et al*., 2003). Celluclast 1.5L,

Ultraflo L and Viscozyme L are used in large scale in food (brewing and baking) and animal feed production. The type and amount of enzymes required for lignocellulose hydrolysis depends on the type of feedstock, whether pre-treatment has been carried out and the type of pre-treatment. The use of commercial enzyme mixtures for lignocellulose degradation is mainly hindered by lack of characterisation of the enzymes in these mixtures (Van Dyk and Pletschke, 2012). It is assumed that the use of crude enzyme mixtures could be cheaper than using individual purified enzymes (Van Dyk and Pletschke, 2012) and that the presence of other proteins may contribute to the hydrolysis of lignocellulose biomass (Suwannarangsee *et al*., 2012; Yang *et al*., 2011). Important to note is that the properties of the selected enzymes should be compatible with other enzymes and proposed biotechnological applications.

1.1.1.3. Industrial bioethanol production processes and bioreactor design

Industrial conversion of lignocellulose to biofuels involves mainly four stages, namely pre-treatment of biomass feedstock, enzymatic hydrolysis/ conversion of pre-treated material into simple sugars by different enzymes, fermentation of sugars into ethanol or other alcohols by yeast/ bacteria, and lastly purification/ separation of ethanol/ alcohols from other wastes (Balat, 2011; Dashtban *et al*., 2009; Galbe and Zacchi, 2002; Lin *et al*., 2010). Pre-treatment and enzyme hydrolysis poses a major challenge for industrial commercial viability of ethanol production from lignocellulose biomass (Mills *et al*., 2009). Studies have been done on improving the characteristics of enzymes and production of enzymes at an industrial scale in order to reduce enzyme costs (Banerjee *et al*., 2010b; Dashtban *et al*., 2009; Howard *et al*., 2003; Merino and Cherry, 2007; Zhou *et al*., 2009).

The industrial production of biofuels can involve the use of different bioreactor systems, namely batch, fed-batch or semi/continuous systems. The design of bioreactors is based on mode of operation, geometry of the reactor and type of phases involved. The bioreactor must be safe and easy to control and monitor. Others factors to consider are diffusion, heat transfer, mass transfer, reactor size, residence time and friction. The design of the bioreactor should ensure maximum conversion of substrate, yield and use of low amount of enzymes (Andric *et al*., 2010b). Prior to the design of the reactor, kinetic and thermodynamic data are required to understand the rate of reaction under different operating conditions (Lyagin *et al*., 2012). The size of the reactor is also important and it depends on the required concentration of the

product and product inhibition. Larger reactors are associated with high conversion rates and high product concentration and are used in cases of product inhibition (Andric *et al*., 2010c). The cost of the reactor can be reduced by using simultaneous saccharification and fermentation (SSF) (Hodge *et al*., 2009). Bioreactor systems are sensitive to temperature, and there may be some local hot spots in the reactor if proper mixing is not achieved. An automated system is therefore important (Hodge *et al*., 2009). A number of mixing strategies can be used for SSF, e.g. shake flasks, pilot-scale helical ribbon impellers, horizontally mounted paddle-impellers, vertically mounted paddle-impellers, and horizontal revolving reactors (Hodge *et al*., 2009).

The classes of reactors designs include stirred tank reactors, tubular, packed bed and fluidised bed reactors. There are four types of reactors which include (a) batch reactors, where all reactants are added at once at the start of the reaction and products removed at the completion of the reaction. They are associated with rapid rates of hydrolysis at the initial stages, which then fall with time (Yang *et al*., 2011). Mixing can be by impellers, gas bubbles or pumps. They are normally used for small scale production with long incubation times. (b) Continuous stirred tank reactors, where reactants are introduced in the tank continuously and products removed from the effluent in-between, are suitable for large volumes of reactants (Andric *et al*., 2010c). They are associated with long residence times. The rate of product formation per given reactor volume is important in the continuous bioreactor design. In cases where production is low, it may be necessary to have large reactors in order to produce the required amount of product (Andric *et al*., 2010b). The reactors can be operated in parallel or in series, in vertical or horizontal position, and are suitable for large scale production. Mixing is normally effected by impellers. (c) Plug flow reactors, where reactants are pumped through a pipe or tube with a continuous concentration gradient. They alleviate product inhibition associated with batch reactions as well as minimize the volume associated with continuous reactors. They can have several tubes in parallel placed either in a horizontal or vertical position. (d) Fed-batch reactors, where reactants can be added as batch, but some reactants can be added or products removed in-between the process. The production volumes in fed-batch reactions are generally low (Andric *et al*., 2010b).

Some researchers have reported the use of batch, fed-batch and continuous reactors for the enzymatic conversion of lignocellulose (Andric *et al*., 2010b; Gupta *et al*., 2012; Lu *et al*., 2010; Rosgaard *et al*., 2007; Rudolf *et al*., 2005). In order to have high product concentration, high substrate loadings are recommended. However, the use of high substrate concentrations poses problems such as high viscosity, mass transfer limitations, unproductive binding of enzyme to the substrate, product inhibition and mixing difficulty, especially when using SSF (Gupta *et al*., 2012). These problems are mainly associated with batch processes, and to alleviate these problems, fed-batch and continuous reactors have been recommended. In fed-batch reactors, high amounts of substrate can be converted using the same enzyme concentrations and the viscosity is reduced (Andric *et al*., 2010c). Membrane reactors have been used for the continuous enzymatic hydrolysis of lignocellulose (Belafi-Bako *et al*., 2006; Gan *et al*., 2002; Liu *et al*., 2011). The reactors enable the fast removal of products, mainly glucose, to prevent product inhibition - yield, conversion rates, and volumetric productivity are normally higher in membrane reactors. The fast removal of products also aid to prevent transglycosylation (Andric *et al*., 2010b; Andric *et al*., 2010c). Membranes that retain the substrate and enzymes while allowing glucose to pass through have been used (Andric *et al*., 2010c). However, the method was reported to be unsuitable for lignocellulose hydrolysis due to low amounts of glucose in lignocellulose, slow reaction rates, the possibility of cellobiose leaching out, fouling, difficult in mixing, and build-up of unreacted substrate and enzymes on the membrane (Andric *et al*., 2010c). Fed-batch reactions are characterised with lower capital costs as a result of reduced volume, lower operating costs and lower downstream cost due to the high product concentration achieved (Gupta *et al*., 2012). The limitation of fed-batch is that there is a change in residence time distribution of substrate over time and changing reactivity of the substrate, which results in unequal accessibility of the substrate by the enzyme (Gupta *et al*., 2012).

Different bioconversion process strategies can be employed in bioreactors, namely separate hydrolysis and fermentation (SHF), where enzyme hydrolysis and fermentation are done separately in two different reactors; simultaneous saccharification and fermentation (SSF), where both enzymatic hydrolysis and fermentation are performed in the same reactor; simultaneous saccharification and co-fermentation (SSCF), which involves pentose fermentation in addition to hexose fermentation and lastly, consolidated bioprocessing (CBP),

where a microorganism is used in the same bioreactor to produce both hydrolytic and fermentative enzymes (Chandel *et al*., 2007; Lynd *et al*., 2005; Olofsson *et al*., 2008; Sipos, 2010; Van Dyk and Pletschke, 2012; Wyman, 2007; Xu *et al*., 2009). SHF offers the advantage of easy optimisation of reaction conditions for each process, easy recovery of products and recycling of yeast (Galbe and Zacchi, 2002). SSF offers biotechnological advantages by preventing end-product inhibition, increasing the hydrolytic rates of enzymes, and reducing cost due to the use of lower reaction volumes, and less or no β-glucosidase. However, the challenge lies in meeting conditions that are favourable for both enzymatic hydrolysis and fermentation (Andric *et al*., 2010a; Chandel *et al*., 2007; Mills *et al*., 2009; Van Dyk and Pletschke, 2012). In both SSF and CBP, inhibition by alcohols, organic acids, phenolic compounds and hydrolysis products pose a major challenge.

1.1.2. Fruit production in South Africa

The production of fruit is increasing world-wide and the fruit is generally processed for juice production. However, the greater part of the fruit ends up as waste (Marin *et al*., 2007; Pourbafrani *et al*., 2010). South Africa produces a wide variety of fruits ranging from deciduous fruits such as table grapes, pome fruit (apples, pears), stone fruit (apricots, peaches, nectarines, plums), citrus (oranges, lemons, grape fruit, limes, mandarins) and subtropical fruits (mangoes, litchis, melons, avocadoes, bananas, pine apples) (Figure 1.1). Deciduous fruits are produced all-year round. According to the National Marketing Council report (Greeff and Kotze, 2007), South Africa had 74 246 hectares of deciduous fruits (32 567 hectare of pome fruit, 22 653 of grapes and 19 279 of stone fruit), with the Western Cape being the largest producer (74%), followed by the Northern Cape (15%) and the Eastern Cape (8%). The Eastern Cape accounts for 19% and the Western Cape for 78% of apple production in South Africa. The report on South African fruit production in 2009 indicated production levels of 798 000 tons of apples, 50 000 tons of apricots, 1 821 000 tons of grapes, 163 000 tons of peaches and 348 000 tons of pears (Portocarrero, 2010). The fruits are exported or consumed locally, either as canned or dried fruit (Maspero and Van Dyk, 2004).

Figure 1.1: Fruit producing regions in South Africa (Maspero and Van Dyk, 2004).

1.1.3. Characteristics of fruit wastewater

Fruits are used mainly in the fruit juice and canning industry, which is growing rapidly in all regions of the world, especially in developing countries like South Africa. Fruit juice production generates large quantities of waste called pomace and fruit wastewater which can cause environmental pollution (Bhushan *et al.*, 2008; Marin *et al*., 2007; Perdih *et al*., 1991; Schieber *et al*., 2001; Van Schoor, 2005). Fruit pomace has a high moisture content (70-75%), a high chemical oxygen demand (COD, 10 000 mg/L) and a biological oxygen demand (BOD) (Bhushan *et al.*, 2008; Burton *et al*., 2008; Capek *et al.*, 1995) and this makes the disposal of fruit waste a major environmental and health problem. Dumping, landfilling and land application have serious health and environmental consequences. As organic decomposition occurs, organic matter percolates and contaminates groundwater resources or runs off into surface waters, causing diseases like cholera, typhoid and dysentery (Mahmood *et al.*, 2010; Perdih *et al*., 1991). Burning and incinerating agri-industrial waste releases carbon dioxide, and so increases greenhouse gas emissions (Malherbe and Cloete, 2002). Fruit wastewater is normally released into the sewerage systems, which can result in clogging

of pipes and eutrophication of water bodies as well as corrosion of sewage pipes due to the high concentration of organic acids (Capek *et al.*, 1995; Perdih *et al*., 1991). Waste disposal is also costly for industries in terms of disposal fees and transport of the waste, and landfills and land application incur additional land use fees (Pourbafrani *et al*., 2010). Water is also becoming increasingly scarce. Therefore, cost-effective techniques for generation of clean, reclaimable water from these industrial effluents are of great importance, especially in water-stressed regions such as South Africa (Van Schoor, 2005; WRC, 2007).

1.1.4. Fruit and fruit pomace

1.1.4.1. Apples and Apple pomace

Apples are deciduous fruit which belong to the family *Rosaceae*, genus *Malus* and are mainly produced in China and USA with world production volumes of about 46.1 million tons in 2006-2007 (Bhushan *et al*., 2008). Apples are normally grown in temperate regions with a world production of 58 million tons (FAO, 2012; Shalini and Gupta, 2010; Schieber *et al*., 2001). Apples contain polyphenolic compounds such as flavonols (e.g quercetin and glycosides), monomeric and oligomeric flava-3-ols, dihydrochalcones (e.g. phloridzin), anthocyanidins, *p*-hydroxycinnamic and *p*-hydroxy-benzoic acids (Bhushan *et al*., 2008; Cetkovic *et al.*, 2008; Queji *et al.*, 2010; Schieber *et al.*, 2001; Van Dyk *et al*., 2013; Virot *et al*., 2010), which influence the properties of fruits, e.g. colour, taste, astringency and antioxidative properties. Apples also contain 85.3% water, 0.3% protein, 0.4% lipids, 11.8% carbohydrates, 0.6% organic acids, 2.3% fibre (including lignin) and a variety of minerals (particularly potassium), vitamins (particularly vitamin A and C) and amino acids. The carbohydrates in apples are predominantly sugars (glucose, fructose and sucrose) and starch (Vendruscolo *et al*., 2008).

Apple pomace is a by-product of the apple juice processing industry which consists of seeds, core, calyx, skins, stalks, soft tissue and constitutes about 25-35% of the original fruit mass (Bhushan *et al.*, 2008; Joshi and Attri, 2006; Kolodziejczyk *et al*., 2009). It is a rich source of polyphenols, pectin, carbohydrates, minerals (P, K, Zn, Cu, Mn, Ca, Mg and Fe), organic acids and crude fibre, hence a good source of nutrients (Lavelli and Corti, 2011; Shalini and Gupta, 2010; Vrhosek *et al*., 2004). Apple slop 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 (Bhushan *et al.*, 2008; Perdih *et al*., 1991). However, it should be noted that different authors have reported different chemical compositions of apple pomace (Bhushan *et al.*, 2008; Vendruscolo *et al*., 2008), for example Bhushan *et al.* (2008) reported pectin concentrations of 10-15% on a dry weight basis while Joshi and Attri (2006) reported values of 3.5-14.32%. Dried apple pomace has been reported to have high carbohydrate content with a fermentable sugar content of up to 50% (Bhushan *et al.*, 2008). The composition of apple pomace varies depending on the apple variety, growth climates, maturity of the fruit and juice extraction method that was employed (Kennedy *et al.*, 1999; Schieber *et al.*, 2001; Taasoli and Kafilzadeh, 2008). As apples ripen, there is an increase in the amount of soluble pectin compared to insoluble pectin in apple pomace (Kennedy *et al.*, 1999).

Apple pomace is normally dumped on the land or discharged into water streams causing environmental pollution due to its biodegradability, high moisture content, high chemical oxygen demand (COD) and biological oxygen demand (BOD). A number of countries like India and the United States of America charge disposal fees for apple pomace (Bhushan *et al.*, 2008; Sato *et al*., 2010; Shalini and Gupta, 2010). Apple pomace can also be used as an animal feed, a source of dietary fibre, for pectin extraction and as a fuel for heating in processing plants (Figuerola *et al*., 2005; Gullon *et al*., 2008; Joshi and Attri, 2006). The use of apple pomace in food products, for example in jam and sauce, citric and lactic acid production, and apple powder for confectionery industry has been extensively reviewed by Shalini and Gupta (2010). However, apple pomace can also be used for ethanol production due to its high carbohydrate content (Kolodziejczyk *et al*., 2009; Sato *et al*., 2010; Taasoli and Kafilzadeh, 2008).

1.1.4.2. Citrus fruits and citrus waste

World production of citrus fruit is over 88 million tons per year, making it the most abundant fruit. Citrus fruits include predominantly oranges as well as lemons, grapefruits, mandarins, clementines, tangerines and limes (Lohrasbi *et al*., 2010). Citrus fruits are mainly used to make juice (50%), with the other 50% being waste which includes seeds, peels and segment membranes (Lohrasbi *et al*., 2010; Marin *et al.*, 2007). The other uses of citrus include

canning, production of marmalade, and the extraction of flavonoids and essential oils (Marin *et al.*, 2007; Schieber *et al.*, 2001). Citrus fruits are a rich source of flavonoids e.g. hesperidin, narirutin, naringin and eriocitrin (Schieber *et al.*, 2001). Citrus fruit also contain different carbohydrate polymers, high amounts of vitamin C, folate and other vitamins, potassium and other minerals. Citrus waste can also be used to produce products such as molasses, ethanol, fibre-pectin, citrus pulp, D-limonene, oils and essence (Marin *et al.*, 2007; Pourbafrani *et al*., 2010; Schieber *et al.*, 2001).

1.1.4.3. Grapes and grape pomace

Grapes (*Vitis* sp., Vitaceae) are abundantly grown in temperate regions around the world, and 80% of the crops are used in the wine industry with 20% becoming pomace (Schieber *et al.*, 2001). The residue remaining, after grapes have been used to make wine, is known as grape pomace and consists of skins, pulp, seeds and stems (Korkie *et al*., 2002; Meyer *et al.*, 1998). Grapes and grape pomace contain high concentrations of phenolic compounds, like anthocyanins, catechins, flavonol glycosides, phenolic acids and alcohols and stilbenes (Deng *et al*., 2011; Meyer *et al.*, 1998, Schieber *et al.*, 2001; Van Dyk *et al*., 2013). Grape pomace also contains lignocellulose (also known as dietary fibre) and products like ethanol, tartrates, citric acid, hydrocolloids, and grape seed oil can be produced from grape pomace (Schieber *et al.*, 2001). Grape pomace can also be used as animal feed, for conditioning of soils as organic fertilisers and for ethanol production (Korkie *et al*., 2002; Van Dyk *et al*., 2013).

1.1.5. Useful industrial products derived from conversion of lignocellulose waste

Large amounts of lignocellulose are generated through agricultural and forestry practices, timber and paper- pulp industries and many other agro-industries and they are regarded as waste which can cause environmental and health problems (Bhushan *et al.*, 2008; Howard *et al.*, 2003; Schieber *et al.*, 2001; Sun and Cheng, 2002; Vendruscolo *et al*., 2008)). Fruit pomace causes waste disposal problems due to its high water content, volume and chemical composition. Fruit wastes can be enzymatically degraded to clean up the environment and water sources, which may require a bioreactor situated at the juicing and canning factories. However, the processing costs may be high due to high cost of enzymes, equipment and operational costs. In order to make the process cost-effective, value-added products can be

produced, for example biofuels (e.g. ethanol, butanol, biogas), enzymes, single cell proteins, chemicals, organic acids (e.g. lactic acid, citric acid, acetic acid), cheap energy sources for fermentation, improved animal feeds and human nutrients (Das and Singh, 2004; Gullon *et al*., 2008; Howard *et al.*, 2003; Joshi and Attri, 2006; Kolodziejczyk *et al*., 2009; Marin *et al*., 2007; Mary *et al*., 2010; Shalini and Gupta, 2010). Although the objective of this study is to degrade lignocellulose from agricultural wastewater, production of valuable by-products will make the whole process more economically feasible. Some examples of products and speciality chemicals and products produced from lignocellulosic wastes are shown in Figure 1.2.

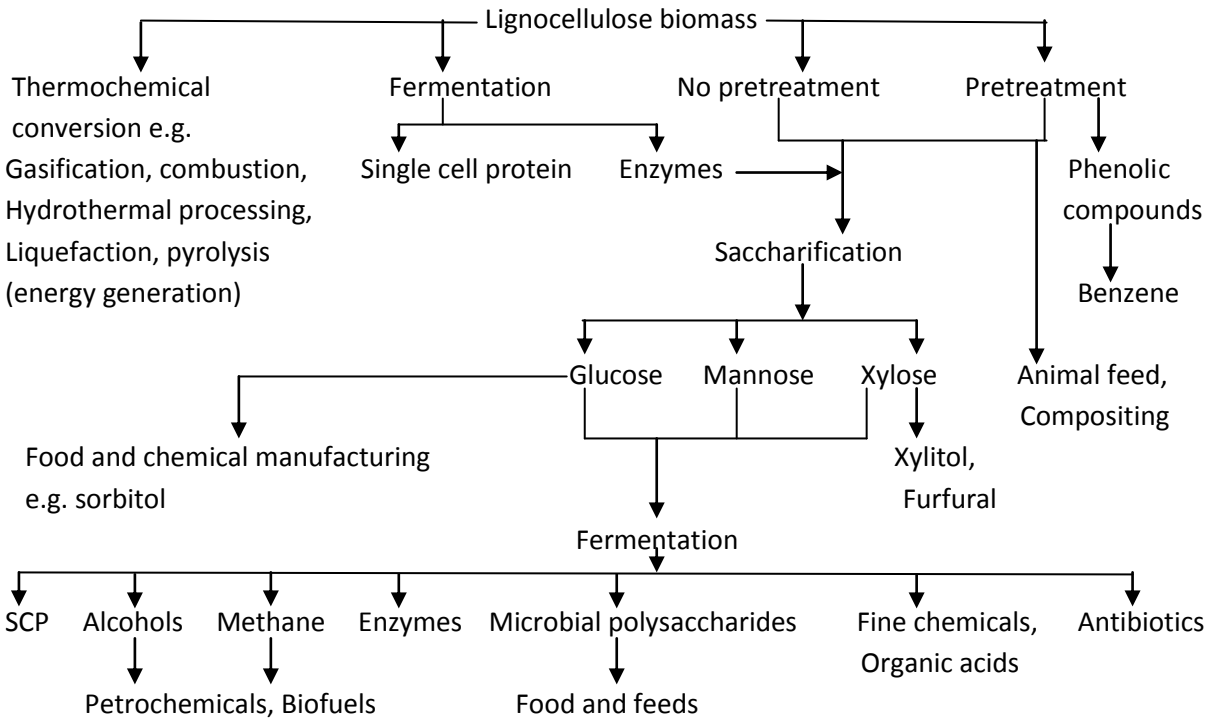


Figure 1.2: Generalised outline of stages in lignocellulose bioconversion into value-added by-products (modified from Howard *et al.*, 2003).

Some biorefinery chemicals/ products that can be derived from lignin degradation are vanillin, gallic acid, polymeric materials (e.g. polyurethanes) and aromatics e.g. phenol, toluene, benzene and xylene. Vanillin can be used to produce herbicides, anti-foaming agents or drugs, air-fresheners and floor polishes. Degradation of the hemicellulose component can yield products such as xylitol and furfural. Xylitol is mainly used as a sweetener in the food

industry and furfural is used in the production of pesticides, varnishes and plastics. Ethanol, butanol, acetone, levulinic acid, fumaric acid, citric acid, formic acid, acetic acid and glycerol, can be generated from fermentation or hydrolysis of sugars derived from cellulose and hemicellulose (Balat, 2011; Bozell and Petersen, 2010; Chen *et al*., 2008; Chundawat *et al*., 2011; Demirabas, 2008; Foyle *et al.*, 2007; Howard *et al.*, 2003; Van Dyk *et al*., 2013) and these products have a wide application in industry. Sorbitol can be produced from glucose. Cellulose can also be used for the industrial production of methylcellulose, hydroxypropylcellulose and carboxymethylcellulose which is used in different commercial applications (Bhushan *et al.*, 2008).

1.1.6 Enzymatic vs. chemical transformation

Enzyme reactions are more preferred than chemical methods due to the fact that enzyme reactions are carried out under mild conditions (pH and temperature), they are highly specific, have high reaction rates, equipment used is not complicated and is widely available, and reactions are easily controlled and less waste products are generated (Ferreira *et al*., 2009; Merino and Cherry, 2007). Enzyme hydrolysis is environmentally friendly and has low utility costs as compared to chemical hydrolysis (Rosgaard *et al*., 2007; Vanderghem *et al*., 2010). However, enzyme hydrolysis of lignocellulose is very slow due the presence of lignin and hemicellulose, the small surface area available for enzymes and cellulose crystallinity. Enzymatic hydrolysis of lignocellulose is limited by the structure of the substrate and the mechanism and activity of the enzymes involved. For example, removal of hemicellulose and lignin results in an increase in accessible volume and surface area for cellulose hydrolysis. Other limiting factors include end product inhibition, inactivation of the enzymes, unproductive binding of enzymes on the substrate and unavailability/ depletion of parts that are easily degraded (Palonen *et al*., 2004). On the other hand, chemical reactions result in poor product yield due to non-specific reactions. There are high energy costs due to high temperatures and pressures required to drive the reactions, high costs from specially designed equipment and control systems, environmental pollution due to disposal of harmful by-products and fouling and corrosion of pipes (Balat, 2011; Dashtban *et al*., 2009; Howard *et al*., 2003).

1.1.7. Lignocellulosic biomass composition and enzymes required for its degradation

There has been an increase in the production of agri-industrial waste biomass world-wide, consisting mainly of lignocellulose, which sometimes finds its way into water streams or dumped on the land. In order to reclaim the waste water from lignocellulose waste and to potentially produce other valuable by-products, it is important to understand the structural composition of lignocellulose biomass. Lignocellulose is composed of lignin, cellulose, hemicellulose, pectin, extractives, protein and ash (Das and Singh, 2004; Dashtban *et al*., 2009; Foyle *et al*., 2007; Lin *et al*., 2010; Merino and Cherry, 2007; Subramaniyan and Prema, 2002), and is resistant to enzymatic and microbial degradation (Himmel *et al*., 2007) (Figure 1.3). The lignocellulosic components interact with each other through different bonds. Cellulose, pectin and lignin are water insoluble, while hemicellulose is water soluble.

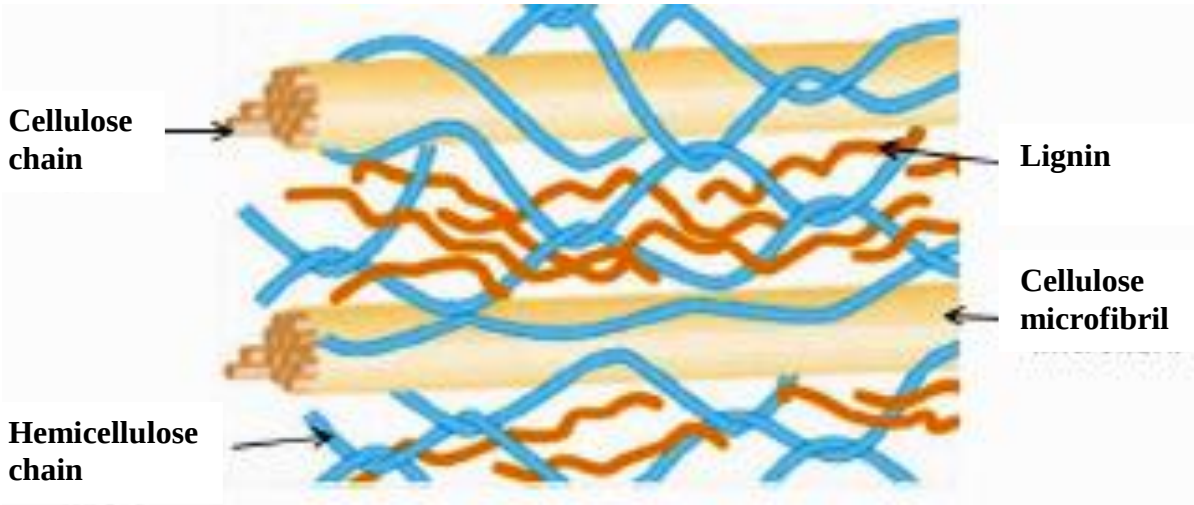


Figure 1.3: Schematic structure of plant cell wall, showing lignocellulosic components (Adopted from Beukes, 2011).

Cellulose is the major plant polymer of lignocellulose, constituting 40-50% of lignocellulosic biomass. Hemicellulose and lignin are present at approximately 25-35% and 15-25%, respectively, depending on the source (Foyle *et al*., 2007; Malherbe and Cloete, 2002; Merino and Cherry, 2007; Wyman, 2007). The chemical composition of these components varies according to species, age, growth conditions and treatment processes. Components such as cellulose and hemicellulose have higher levels in hardwoods than in softwoods, with

softwoods having a higher lignin content than hardwoods (Balat, 2011). Table 1.1, shows the chemical composition of different biomass residues, however, some authors reported different composition for the same biomass material (Van Dyk and Pletschke, 2012).

Table 1.1: Chemical composition of some selected biomass wastes (Balat, 2011; Das and Singh, 2004; Howard *et al*., 2003; Sun and Cheng, 2002).

Biomass residue	**Cellulose (%)**	**Hemicellulose (%)**	**Lignin (%)**
Wheat straw	30	50	15
Rice straw	32.1	24	18
Corn cobs	45	35	15
Switch grass	45	31.4	12
Bagasse	33.4	30	18.9
Paper	85-99	0	0-15
Nut shells	25-30	25-30	30-40
Cattle manure	1.6-4.7	1.4-3.3	2.7-5.7
Newspapers	40-45	25-40	18-30
Hardwood stems	40-55	24-40	18-25
Softwood stems	45-50	25-35	25-35

1.1.7.1. Cellulose composition and cellulose degrading enzymes

1.1.7.1.1. Composition of cellulose

Cellulose is a homopolymer comprising of linear chains of β-(1→4)-linked glucose units and is insoluble. The average degree of polymerisation of cellulose is about 10 000 glucose units, but this can be as low as 15 units, depending on the source (Eveleigh, 1987; Teeri, 1997). Two adjacent glucose units form a subunit called cellobiose. Cellulose has hydroxyl groups that interact with each other or other functional groups, forming hydrogen bonds. The hydroxyl groups may also interact with water, making the surface of cellulose hydrophilic. Cellulose chains are assembled into parallel sheets that stack on top of each other through hydrogen bondings, van der Waals forces and hydrophobic interactions, forming structures called microfibrils (Dashtban *et al*., 2009; Horn *et al*., 2012) (Figure 1.3). Hemicellulose is

found fitted in spaces between the microfibrils and lignin forms a sheath around hemicellulose and microfibrils forming an extended matrix. Microfibrils consist of two structural regions, crystalline regions with chains packed in a highly ordered fashion, forming structures of high tensile strength which are resistant to enzymatic degradation; and amorphous, or less orderly regions that are interspersed between crystalline regions and are less resistant to enzymatic degradation (Howard *et al.*, 2003; Walker and Wilson, 1991) (Figure 1.4). The amount of crystalline and amorphous cellulose in a structure depends on its biological source (Foyle *et al.*, 2007).

1.1.7.1.2. Enzymes required for cellulose degradation

Cellulose degrading enzymes are generally called cellulases. The efficient hydrolysis of cellulose to glucose requires glucanases (exo-glucanases and endo-glucanases) and β-glucosidases (Teeri, 1997) (Figure 1.4). Cellulases are glycoside hydrolases and their classification into families is according to the amino acid sequence similarities in their catalytic domains (Bayer *et al.*, 1998), hence similarities in structural folding and enzyme catalytic mechanism. Cellulases are used in various industries, such as food, brewery, animal feed, textile, paper and agriculture (Howard *et al.*, 2003).

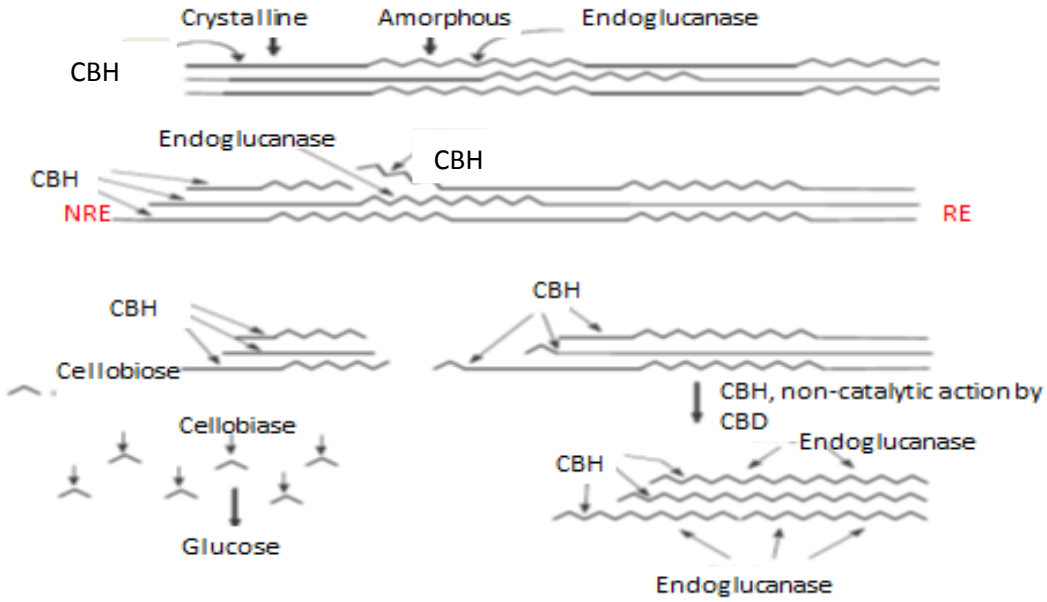


Figure 1.4: Schematic model of cellulose degrading enzymes and their action on cellulose (modified from Beldman, 2008, personal communication). NRE - non reducing end, RE - reducing end, CBH – cellobiohydrolase, CBD – carbohydrate binding domain.

Endoglucanases (EG, endo-1,4-D-glucanohydrolase, E.C. 3.2.1.4) depolymerise amorphous cellulose, such as the commercial substrate carboxymethyl cellulose (CMC), by hydrolysing internal β-1,4-glycosidic bonds randomly along the cellulose chain, which is aided by its open active site. Thus it creates new sites for exo-glucanases to act on (Dashtban *et al*., 2009; Ferreira *et al*., 2009; Howard *et al*., 2003). Their pH and temperature optima depend on the organism from which it is isolated. Fungal species produce multiple EGs, for example *Trichoderma reesei* (5 EGs) and *Phanerochaete chrysosporium* (3 EGs), with some enzymes having carbohydrate binding modules (CBMs) while others don't have CBMs (Dashtban *et al*., 2009).

Exoglucanases (or cellobiohydrolases) (CBH, 1,4-β-D-glucancellobiohydrolase, E.C. 3.2.1.91 and E.C. 3.2.1.176) cleave the glycosidic bonds from both reducing and non-reducing ends of crystalline cellulose (such as the commercial substrate Avicel®) and are considered processive enzymes as the degree of crystallinity does not affect its hydrolysis. In *T. reesei*, there are two forms of exoglucanases, with different specificities, that can work in synergy, resulting in efficient degradation. It has been reported recently that CBHII is bi-specific, as it can degrade both the amorphous and crystalline regions (Ganner *et al*., 2012). CBH I attacks reducing ends, while CBH II acts on the non-reducing ends, releasing cellobiose (Dashtban *et al*., 2009; Ferreira *et al*., 2009; Merino and Cherry, 2007; Teeri, 1997). It is proposed that the active site of CBH form a tunnel-like shape, where it binds the cellulose chain in the middle and hydrolyse the cellulose chain processively while it remains in the CBH tunnel (Dashtban *et al*., 2009; Teeri, 1997).

Lastly, cellobiases (β-glucosidases, E.C. 3.2.1.21) hydrolyse short cellooligosaccharides and cellobiose into glucose units which competitively inhibits this enzyme (Merino and Cherry, 2007; Qing *et al*., 2010). Cellobiases prevent product inhibition of other cellulases by cellobiose, and is very important for achieving complete enzymatic hydrolysis of cellulose (Chen *et al*., 2008; Dashtban *et al*., 2009). Cellobiases are produced by fungi, bacteria and plants and they belong to glycoside hydrolase families 1 and 3.

1.1.7.1.3. Synergy between cellulases

The activities of endo- and exo- glucanases lead to synergy, which is an enhanced activity by the combined enzymes, more than the sum of the activities of the individual enzymes (Andersen *et al.*, 2008; Capek *et al.*, 1995; Kleman-Leyer *et al*., 1996; Tomme *et al.*, 1995). There is also synergy between different exoglucanases, exoglucanase and β-glucanase and between cellulose binding modules (CBH) and the catalytic site (Bras *et al*., 2011; Ferreira *et al*., 2009; Lynd *et al*., 2002; Qing *et al*., 2010). Cellulose binding modules are required for effective hydrolysis of crystalline cellulose. The cellulase enzymes work synergistically by creating new accessible sites for each other, thereby removing hindrances and preventing end-product inhibition (Zhou *et al*., 2009). Cellulases, working together, display higher hydrolytic activity than the sum of the hydrolytic activities of individual enzymes. The degree of enzyme synergy is defined as the actual observed activity of enzymes in combination, divided by the theoretical sum of activities of individual enzymes (Andersen *et al.*, 2008; Merino and Cherry, 2007; Zhou *et al*., 2009).

1.1.7.2. Hemicellulose composition and enzymes required for its degradation

1.1.7.2.1. Composition of hemicellulose

Hemicellulose is a complex polymer which is branched and heterologous, consisting of different polysaccharides that have diverse interactions and are soluble in alkali. The polysaccharides in hemicellulose can include non-cellulose β-D-glucans, pectic substances (polygalacturonans), arabinogalactans, galactogluco- and glucomannans and arabinoglucurono- and glucuronoxylans depending on the source (Balat, 2011; Gray *et al*., 2006). The main sugar components of these hemicelluloses are D-glucuronic acid, D-mannose, D-xylose, D-glucose, L-arabinose, D-galactose, L-rhamnose, L-fucose, D-galacturonic acid, 4-O-methyl-D-glucuronic acid and various O-methylated sugars (Balat, 2011). The composition of these polysaccharides and their interaction varies depending on the hemicellulose source (Gray *et al*., 2006). The interaction of hemicellulose and cellulose is through hydrogen bonding with the outer surface of microfibrils, thus preventing microfibrils from interacting with one another (Gomez *et al*., 2008; Laureano-Perez *et al*., 2005). Hemicellulose is covalently attached to lignin in secondary plant cell walls and is associated with pectin and proteins in primary cell walls, thus conferring rigidity to plant cell walls (Shallom and

Shoham, 2003; Subramaniyan and Prema, 2002). Hemicellulose is soluble, amorphous and branched in nature making it more readily hydrolysed by enzymes compared to cellulose (Balat, 2011; Horn *et al*., 2012).

Xylan is the most abundant hemicellulose in lignocellulosic biomass (Capek *et al.*, 1995; Chen *et al*., 2011; Howard *et al.*, 2003; Dashtban *et al*., 2009). Xylan normally has a backbone comprising of a chain of D-xylose residues, with side chains containing mannose, arabinose, galactose, glucuronic acid and other sugars. L-Arabinofuranose is linked to the *O*-3 position of D-xylose, while D-glucuronic or 4-*O*-methyl-D-glucuronic acid is linked to the *O*-2 position and acetyl groups at either the *O*-2 or *O*-3 position. Xylan forms hydrogen bonds with cellulose microfibrils and is covalently linked to lignin (ester linkage to 4-*O*-methyl-D-glucuronic acid), thereby conferring stability to plant cell walls (Foyle *et al*., 2007; Subramaniyan and Prema, 2002). The arabinofuranosyl side chains in hemicellulose, that have been esterified by ferulic and *p*-coumaric acid residues, form ether linkages with lignin (Subramaniyan and Prema, 2002). Arabinoxylan is composed of a linear backbone of β-1,4-D-xylose residues that is partly substituted with arabinose residues. There are different commercial xylans available and they differ in composition, e.g. birchwood xylan contains 94.1% xylose residues, oat spelt xylan contains 52.5% xylose, 22.3% arabinose and 15.7% glucose residues (Li *et al*., 2000).

In softwoods and legumes, mannan forms a significant part. Mannan consists of a 1,4-β-D-mannopyranose backbone. There are different types of mannan e.g. galactomannans, glucomannans and galactoglucomannans, depending on the substituent residues on the mannan backbone (Hrmova *et al*., 2006; Shallom and Shoham, 2003).

1.1.7.2.2. Enzymes required for hemicellulose degradation

Hemicellulases are enzymes involved in hemicellulose degradation and are produced naturally by microorganisms (Shallom and Shoham, 2003). Efficient degradation of hemicellulose requires a variety of different enzymes that work synergistically, since it is a complex polymer with different types of sugar subunits (Bissoon *et al.*, 2002) (Figure 1.5). The enzymes include glucuronidase, galactosidase, acetylesterase, xylanase, β-xylosidase, mannanase and arabinofuranosidase that work in synergy (Subramaniyan and Prema, 2002).

Hemicellulases are classified into two groups, depending on the amino acid sequence of their catalytic domains, the glycoside hydrolases which hydrolyse glycosidic linkages, and carbohydrate esterase family which catalyse the hydrolysis of ester linkages of acetate and ferulic acid side chains (Dashtban *et al*., 2009; Howard *et al.*, 2003; Shallom and Shoham, 2003). Some hemicellulases have CBMs similar to cellulases and they are classified into 20 GH families. It has been reported that the removal of hemicellulose from lignocellulose enhances accessibility and hydrolysis of cellulose by cellulases (Qing *et al*., 2010). Hemicellulases are used in industry, such as the pulp and paper industry (biobleaching and biopulping), and xylanases are applied in baking (processing of flour), clarification of juices and the animal feed industry (Howard *et al*., 2003; Shallom and Shoham, 2003; Subramaniyan and Prema, 2002). Hemicellulases have a potential application in the conversion of lignocellulose wastes into valuable products.

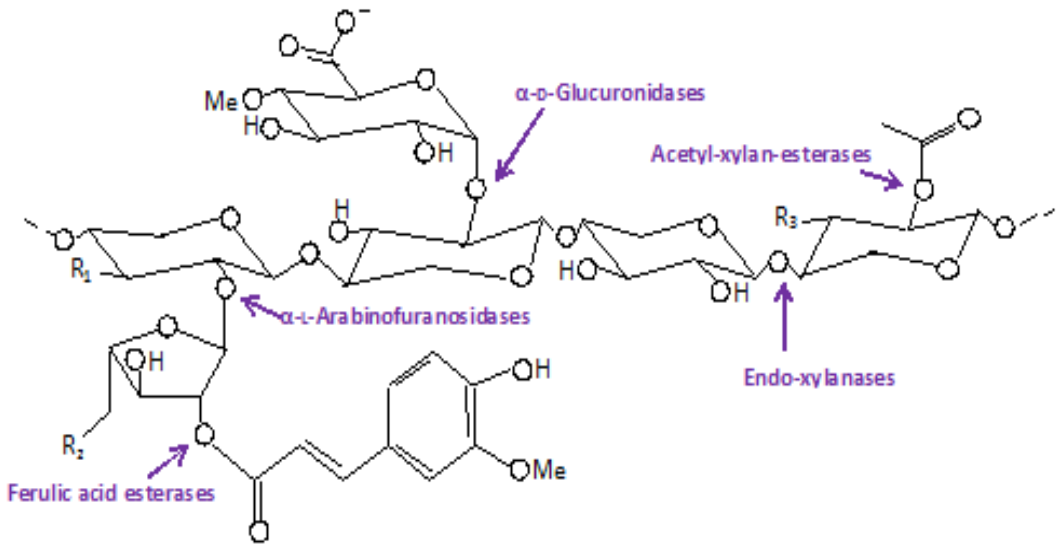


Figure 1.5: Model structure of xylan composed of D-xylose units and various side chains illustrating the enzymes that degrade these structures (Modified from Shallom and Shoham, 2003).

Endo-β-1,4-endoxylanases (E.C.3.2.1.8) randomly hydrolyse the β-1,4-glycosidic bonds in the xylan backbone yielding short unsubstituted or branched xylooligomers and most of these enzymes belong to GH 10 and 11 families. The presence of other groups on the side chains of xylan like 4-O-methyl-glucuronic acid and arabinofuranose tend to hinder the binding and hydrolysis of xylan by endo-β-1,4-endoxylanase (Shallom and Shoham, 2003; Subramaniyan

and Prema, 2002). Exo-β-1,4-xylosidase (E.C. 3.2.1.37) hydrolyse xylooligosaccharides and xylobiose from the non-reducing end to form xylose and they belong to GH families 3, 39, 43, 52 and 54 (Howard *et al.*, 2003; Saha, 2000; Shallom and Shoham, 2003; Sorensen *et al*., 2003) (Figure 1.5). Most xylosidases are inhibited by xylose. Endoxylanases are produced mainly by bacteria and fungi and some higher animals like fresh water molluscs. Fungal xylanases have an optimum pH of 5, but are stable in the range of pH 3-8, and are tolerant to temperatures below 50ºC. Bacterial xylanases have slightly higher pH optima than that of fungi. β-1,4-xylosidase can be produced by a number of organisms like fungi and bacteria (e.g. *Bacillus* sp.) (Subramaniyan and Prema, 2002).

Arabinofuranosyl-containing hemicellulose requires enzymes to cleave side chains and depolymerising enzymes, for example exo-α-L-arabinofuranosidase (EC.C. 3.2.1.55) and endo-1,5-α-L-arabinase (E.C. 3.2.1.99) and they are found in GH families 3, 43, 51, 54 and 62. α-L-Arabinofuranosidase act on branched arabinans, arabinoxylans and arabinogalactans and hydrolyse 1,3- and 1,5-α-arabinosyl bonds of arabinoxylan from non-reducing ends, while 1,5-α-L-arabinase act on linear arabinans (Howard *et al*., 2003; Saha, 2000; Subramaniyan and Prema, 2002). α-L-Arabinofuranosidase have a broad substrate specificity and act in synergy with other glycosyl hydrolases in the degradation of arabinose containing polysaccharides. Another enzyme important for xylan hydrolysis is α-D-glucuronidases (E.C. 3.2.1.139) which hydrolyse the α-1,2-glycosidic bond of the 4-O-methyl-D-glucuronic acid side chain of xylans, releasing glucuronic acid, and are found in GH family 67 (Howard *et al*., 2003; Shallom and Shoham, 2003; Subramaniyan and Prema, 2002).

Other enzymes that further assist in the depolymerisation of hemicellulose include hemicellulolytic esterases, e.g. acetyl esterases which hydrolyse the acetyl substitutions on xylose moieties, feruloyl esterase (E.C 3.1.1.73) and *p*-coumaric acid esterases which hydrolyse the ester bond between the arabinose substitutions and ferulic acid and *p*-coumaric acid respectively (Saha, 2000; Shallom and Shoham, 2003; Sorensen *et al*., 2003). Acetyl xylan esterases (E.C. 3.1.1.72) cleave acetyl groups at C-2 and C-3 of xylose residues in acetylxylan. Feruloyl esterases and *p*-coumaric acid esterases assist in the release of hemicellulose from lignin and hence leave hemicellulose exposed and easily degraded by the

other hemicellulases (Howard *et al.*, 2003; Prates *et al.*, 2001; Subramaniyan and Prema, 2002).

A number of enzymes are responsible for the degradation of mannan. There are endo-β-mannanases (E.C. 3.2.1.78) which randomly hydrolyse the mannan backbone to release short manno-oligomers and mannobiose and they belong mainly to GH families 5 and 26. Exo-β-mannosidases (E.C. 3.2.1.25) release mannose from short manno-oligomers and mannobiose (Howard *et al*., 2003; Shallom and Shoham, 2003), but can also release mannose from mannan polysaccharides and they belong to GH families 1, 2 and 5. There are also other enzymes like α-galactosidase (3.2.1.22), acetyl-mannan esterase and β-glucosidase which act on the mannan backbone, releasing galactose, acetyl groups and glucose respectively (Shallom and Shoham, 2003).

1.1.7.3. Pectin composition and enzymes required for its degradation

1.1.7.3.1. Composition of pectin

Pectin is a complex plant cell polysaccharide that contains many sugars found mainly in the middle lamellae of plants sugar beet and fruits like apples and citrus and commercial pectin is extracted from these sources. Fruit pectins are used in the food industry as gelling agents, thickeners, emulsifiers, stabilisers and texturizers, and they also have applications in the pharmaceutical and cosmetic industries (Bhushan *et al*., 2008; Donaghy and McKay, 1994; Emaga *et al*., 2008; Funami *et al*., 2011).

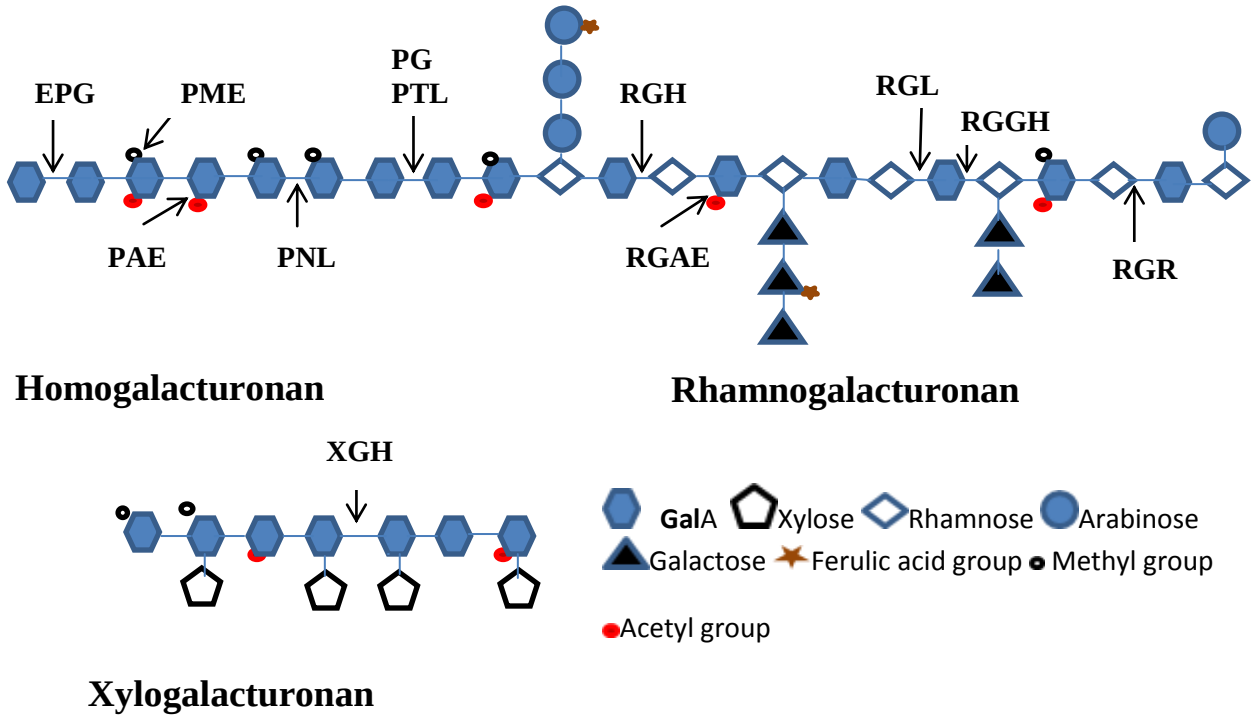
Pectin forms linkages that hold cellulose, hemicellulose and proteins together (Buga *et al*., 2010; Carpita and Gibeaut, 1993) through different bonds. Pectin can be divided into four major structural groups, homogalacturonan (HG), rhamnogalacturonan I (RGI), rhamnogalacturonan II (RGII) and xylogalacturonan (XGA). HG or the smooth region of pectin is a linear polymer of α-1,4-D-galacturonic acid which can be methyl-esterified at carboxylic acid group (C-6) and/or acetylated at C-2 and C-3 (O'Neill and York, 2003; Van Dyk *et al*., 2013; Vincken *et al*., 2003; Zandleven, 2006). RGI consists of repeated alternating monomers of α-1,2-L-rhamnose and α-1,4-D galacturonic acid residues. Rhamnose residues can have side chains of neutral sugars such as xylose, arabinose and galactose at C-4 and

galacturonic acid residues can be methylated and acetylated or both (Schols and Voragen, 1995). RGII is a homogalacturonan chain with galacturonic acid residues attached to complex side chains of rare sugars such as 2-keto-3-deoxy-D-manno-octulosonic acid, 2-keto-3-deoxy-D-lyxo-heptulosaric acid, aceric acid and apiose. Xylogalacturonan consists of β-D-xylose residues attached to a linear chain of α-1,4-D galacturonic acid residues through β-1,3 or β-1,4 linkages. XGA was reported to account for 4% apple pectin (Oechslin *et al*., 2003; Van Dyk *et al*., 2013; Voragen *et al*., 2001; Zandleven, 2006).

1.1.7.3.2. Enzymes required for degradation of pectin

Pectinases are enzymes that are able to break down pectin to its constituent monomers (El-Sheekh *et al*., 2009; Jayani *et al.,* 2005). Pectin enzymes are produced by many organisms such as fungi, bacteria, nematodes, protozoa, insects, yeasts and also in many plants (Benen *et al*., 2002; Buga *et al*., 2010). In plants, pectinases are involved in the change of texture during ripening, storage and processing of fruits and vegetables (El-Zoghbi, 1994). Pectinases are used commercially for different industrial applications e.g. in the textile and food industry, and particularly the fruit juice industry to clarify fruit juice (Buga *et al*., 2010). Pectinases are also used in the maceration of vegetables to produce pastes and purees, in wine making, coffee and tea fermentation, textile processing and in treatment of wastewater from the vegetable food industry (Donaghy and McKay, 1994; El-Sheekh *et al*., 2009; Pedrolli *et al*., 2009).

The complexity of pectin structure results in the need for many enzymes for its complete degradation and these include polygalacturonases, pectin methylesterases, pectin-, pectate- and rhamnogalacturonan lyases, pectin- and rhamnogalacturonan acetylesterases and rhamnogalacturonan hydrolyases (El-Sheekh *et al*., 2009; Pedrolli *et al*., 2009; Van Dyk *et al*., 2013) (Figure 1.6).



EPG (Exo-polygalacturonase), **PME** (Pectin methylesterase), **PG** (Endo-polygalacturonase), **PTL** (Pectate lyase), PAE (Pectin acetyl esterase), **PNL** (Pectin lyase**), RGH** (RG-hydrolase), **RGL** (RG-lyase), **RGGH** (RG-galacturonohydrolase), **RGAE** (RG-acetyl esterase), **RGR** (RG- rhamnohydrolase), **XGH** (Xylogalacturonan hydrolase)

Figure 1.6. Pectinases involved in the degradation of HG, XGA and RGI (Modified from Zandleven, 2006). RG – Rhamnogalacturonan.

Polygalacturonase cleaves α-1,4-D-galacturonosidic linkages in the homogalacturonan (HG) region and with a preference for non-methylesterified pectins. Polygalacturonases can be divided into two groups, endo-polygalacturonases (E.C. 3.2.1.15) and exo-polygalacturonases (E.C. 3.2.1.67 and 3.2.1.82). Endo-polygalacturonases act by randomly cleaving α-1,4-D-galacturonan linkages of the HG chain internally, producing galacturonic acid oligosaccharides. Exo-polygalacturonases cleave the HG chain from the non-reducing end in a zipper fashion, releasing mono- or digalacturonic acid (Benen *et al.*, 2002; Donaghy and McKay, 1994). Pectin and pectate lyases cleave α-1,4-D-galacturonan linkages of HG by β-elimination, introducing a double bond at C-4 and C-5 at the non-reducing end. Pectin lyases (E.C. 4.2.2.10) prefer highly methylated HG while pectate lyases (endo-, E.C. 4.2.2.2 and

exo-, E.C. 4.2.2.9) prefer low methylated HG (pectate) (Benen *et al*., 2002; Pilnik and Voragen, 1991).

Other pectinases are able to hydrolyse methyl and acetyl groups from pectin. Pectin methyl esterase (E.C. 3.1.1.11) cleaves the methyl ester bond at C-6 of galacturonic acid residues next to a non-esterified galacturonic acid in the HG region, releasing methanol. Pectin acetylesterases (E.C. 3.1.1.6) cleave the acetyl bond from C-2 and/ or C-3 of galacturonic acid residues in HG and rhamnogalacturonan acetylesterases cleave the acetyl bond from C-2 and/ or C-3 of galacturonic acid residues in rhamnogalacturonan (Benen *et al*., 2003; Buchholt *et al*., 2004; Voragen *et al*., 2001; Voragen *et al*., 2009). Enzymes that work on the rhamnogalacturonan region are rhamnogalacturonan hydrolase which cleaves α-galacturonic acid-(1, 2)-α-rhamnose bonds and rhamnogalacturonan lyase which cleaves α-rhamnose-(1, 4) - α-galacturonic acid bonds by β-elimination. There are also two other enzymes that have been reported, rhamnogalacturonan rhamnohydrolase and rhamnogalacturonan galacturonanhydrolase, which split off rhamnose and galacturonic acid from the non-reducing end respectively (Beldman *et al*., 1996; Benen *et al*., 2002; Mutter *et al*., 1998; Voragen *et al*., 2001; Zandleven, 2006).

1.1.7.4. Lignin composition and enzymes required for its degradation

1.1.7.4.1. Composition of lignin

Lignin is a hydrophobic, heterogeneous polyphenylpropane aromatic polymer (which accounts for its recalcitrant nature), and contributes 15-25% of the lignocellulose, depending on the source. Lignin is made from aromatic alcohol pre-cursors, coniferyl, sinapyl, and coumaryl, which are bonded together by groups such as hydroxyl, methoxyl and carbonyl groups to form a complex matrix that is resistant to enzymatic degradation (Balat, 2011; Hendriks and Zeeman, 2009). Lignin acts as a binding material in lignocellulose, encasing the cellulose-hemicellulose complex by cross-linking through ester or ether linkages e.g. via ferulic acid, and making it difficult for enzymes to reach these polysaccharides and degrade them (Dashtban *et al*., 2009; Horn *et al*., 2012; Howard *et al*., 2003). Lignin also reduces the catalytic efficiency of cellulases due to unproductive binding and steric hindrance (Lin *et al*., 2010; Merino and Cherry, 2007). It has been also reported in literature that lignin can inhibit

hydrolytic enzymes directly and may also block the processive hydrolysis of cellulose chain by cellulases (Van Dyk and Pletschke, 2012). In nature, lignin can be degraded by enzymes produced by some fungi and bacteria, releasing cellulose and hemicellulose. Due to the recalcitrant nature of lignocellulose, pre-treatment strategies have been employed to increase enzyme accessibility and hence make the production of biofuels economically viable.

1.1.7.4.2. Enzymes required for degradation of lignin

The enzymes responsible for degrading the lignin component of lignocellulose, ligninases, include peroxidases, oxidases and esterases (Cullen and Kersten, 1992; Howard *et al.*, 2003). This includes the extracellular oxidative enzymes: lignin peroxidases (LiP), phenol oxidase (laccases), manganese-dependent peroxidases (MnP) and horseradish peroxidases with non-specific activity. The white-rot basidiomycete, *Phanerochaete chrysosporium*, has been most extensively studied and is one of the fastest lignin degraders. It degrades lignocellulosic biomass non-selectively as it acts on both carbohydrates and lignin (Cullen and Kersten, 1992; Dashtban *et al*., 2009). Other fungi like *Phlebia radiate*, *P. floridensis* and *Daedalea flavida* were found to selectively degrade lignin in wheat straw, while *Ceriporiopsis subvermispora* was found to selectively degrade lignin in various woods (Dashtban *et al*., 2009). *Stropharia coronilla* produces manganese peroxidase and *Botrytis cinerea* produces laccase. Recently, a patented product known as Lignozyme® has been demonstrated to successfully delignify wood for the paper/pulp industry. Laccases can be used to treat wastewater from the textile industry (Howard *et al*., 2003) and this can be adapted to fruit wastewater treatment. The products produced from lignin degradation (free radicals, oligomers) can result in the inactivation of peroxidases (Hamid and Rehman, 2009). This inactivation can be overcome by the use of additives such as polyethylene glycol (Gomez *et al.*, 2008) or by immobilisation and using a dilute lignin dispersion (as found in fruit waste) and a low steady state supply of H_2O_2 (Cullen and Kersten, 1992; Hamid and Rehman, 2009).

1.1.7.5. Mechanism of action of enzymes

Cellulases and xylanases function via an acid-base reaction mechanism, involving two amino acid residues in their catalytic domains, and can cause inversion or retention of the anomeric carbon configuration. Hydrolysis by inversion involves a single step mechanism whereas retention involves a double displacement mechanism (Bayer *et al.*, 1998; Dashtban *et al*.,

2009). However, most cellulases and xylanases hydrolyse their substrates with retention of configuration using a double displacement mechanism (Subramaniyan and Prema, 2002). The first residue is an acid catalyst that protonates the oxygen of the glycosidic bond, splitting two cellulose or hemicellulose subunits and forming an oxocarbonium intermediate. The second residue binds with the oxocarbonium intermediate by acting as a nucleophile, promoting the formation of an OH^- from a water molecule which converts the intermediate into a free cellobiose or xylobiose subunit.

Cellulases and hemicellulases have both catalytic domains and non-catalytic domains, called carbohydrate binding modules (CBMs), which hold the enzyme's active site in contact with the substrate (targeting), and allow the enzyme to remain bound to the substrate during hydrolysis (Boraston *et al*., 2004; Howard *et al*., 2003; Igarashi *et al*., 2009; Shallom and Shoham, 2003). The presence of some ions, such as Na^+ and Ca^{2+}, aids in the binding of CBMs to its substrate. The purpose of CBMs is to bind and increase the concentration of the enzyme at the surface of the substrate and prolong interaction of the enzyme with the substrate, and thus increase the rate of hydrolysis (Boraston *et al*., 2004; Dashtban *et al*., 2009; Subramaniyan and Prema, 2002). Some CBMs target specific substrates, such as cellulose or xylan, within complex plant matter. It has been suggested that the CBM attaches to the substrate and 'unzips' the crystalline cellulose chains by lifting a single chain away from the crystalline structure and directing it to the catalytic domain where cellobiose or xylobiose units are removed (Subramaniyan and Prema, 2002). Some enzymes, at high concentrations, may adsorb non-productively on the substrate through CBMs (Palonen *et al*., 2004; Van Dyk and Pletschke, 2012). CBMs have a flat planar, twisted or sandwich binding surface that contain highly conserved aromatic amino acid residues (e.g. tryptophan, tyrosine, phenylalanine) that allow interaction with the surface of carbohydrates, for example crystalline cellulose (Boraston *et al*., 2004). A number of CBMs have been identified so far and are classified into 50 families, based on the similarity of the amino acid sequence, with 20 families belonging to fungi (Dashtban *et al*., 2009).

Recently, there was a discovery of proteins that are classified as GH61 from fungi, which have functional and structural similarity to CBM33 proteins from bacteria. It is believed that these proteins act synergistically with cellulases to degrade cellulose (Horn *et al*., 2012).

GH61 and CBM33 have cellulose binding modules that allow them to aatch to the crystalline surfaces of cellulose and cut the cellulose polymer. The proteins have been reported to oxidatively cleave cellulose at C1 and potentially, C4 and C6 carbon in the presence of an electron donor (e.g. gallic acid or lignin) and divalent metal ions (copper-dependent monooxygenases) (Horn *et al*., 2012). As a result of the oxidation products like aldonic, cellobionic and gluconic acid may be produced. It is believed that addition of these proteins to commercial cellulase cocktails may be of great benefit to lignocellulose degradation as this will aid in reducing enzyme loading and processing time (Horn *et al*., 2012).

Ligninases affect initial changes to the lignin substrate by employing diffusible reactive compounds of low molecular weight (Bhushan *et al.*, 2008; Call and Mucke, 1997). Lignin is oxidized by the radical products of lignin and manganese peroxidases. The interaction of LiP with its substrate is via a ping-pong mechanism. H_2O_2 oxidises the ferric enzyme to give compound I, which then oxidises the aromatic substrates to give compound II, which again oxidises the aromatic substrate to return the enzyme to its resting state. The resultant aryl radicals spontaneously degrade via many reactions. LiP catalyses the hydroxylation of benzylic methylene groups, phenol oxidation, benyzyl alcohols oxidation, cleavage of C_α -C_β bond of propyl side chains of lignin. Oxidation of non-phenols produces phenolics. MnP oxidises Mn^{2+} to Mn^{3+} using H_2O_2 as an oxidant. Laccases catalyses the oxidation of phenolic compounds which include aromatic amines and electron-rich substrates resulting in the reduction of O_2 to H_2O. Lignin phenolic units are oxidised to phenoxy radicals leading to aryl-C_α cleavage (Cullen and Kersten, 2004). The pathway for complete mineralisation of lignin to CO_2 and organic acids is well understood (Hofrichter, 2002).

1.1.7.6. Sources of lignocellulolytic enzymes

Lignocellulose can be naturally degraded in nature by enzymes released from a variety of microorganisms, including bacteria, fungi, protozoa, insects, molluscs and some plants (e.g. the avocado fruit) as free or multi-enzyme complexes (cellulosomes). Most organisms display several different cellulolytic and hemicellulolytic enzyme activities. Free enzymes are produced mainly by aerobic organisms e.g. *Trichoderma reesei* and *Aspergillus niger* (Zhang and Lynd, 2004). Cellulosomes are produced mainly by anaerobic microorganisms e.g. *Clostridium*, *Butyrivibrio*, *Acetivibrio*, *Bacteroides*, *Ruminococcus* (Bayer *et al*., 1998; Bras

et al., 2011; Dashtban *et al*., 2009; Doi *et al*., 2003), which have shown great synergistic activity on lignocellulose biomass. Examples of hemi/cellulolytic fungi include species of *Aspergillus, Penicillium, Schizophylum* and *Trichoderma* (Dashtban *et al*., 2009; Howard *et al.*, 2003; Sun and Cheng, 2002). Research has been carried out on the cellulases, hemicellulases and ligninases of *Trichoderma reesei* and *Phanerochaete chrysosporium* (Merino and Cherry, 2007). Many species of hemi/cellulolytic bacteria have also been isolated, belonging to *Clostridium, Cellulomonas, Bacillus, Thermomonospora, Ruminococcus, Bacteriodes, Erwinia, Acetovibrio, Microbiospora* and *Streptomyces* (Howard *et al*., 2003; Sun and Cheng, 2002). Screening for bacteria that produce better, more stable enzymes with broader pH and temperature ranges is on-going. A number of these naturally produced enzymes have been extracted and are commercially available. Isolated enzymes are preferred to whole cell organisms because they have greater specificity, are easier to handle and store, and the enzyme concentration used in the process is not dependent on microbial growth (Van Dyk and Pletschke, 2012).

1.1.7.7. Cellulosomes

Bacterial hemi/cellulolytic enzyme systems are usually very complex, comprising of many types of enzyme activities that can degrade cellulose, hemicellulose and pectin. In some bacterial cellulase systems (mainly anaerobic bacteria e.g. *Clostridium, Acetivibrio, Bacteroides, Ruminococcus*) the components are produced as aggregates or form aggregates of 20 or more different enzymes, and in aerobic fungi (e.g. *Neocallimastix frontalis* and *Piromyces*), 6-10 different enzymes. These aggregates are known as multi-enzyme complexes and the most studied example is the cellulosome (large extracellular enzyme complexes) (Bayer *et al*., 1998; Bras *et al*., 2011; Dashtban *et al*., 2009; Doi *et al*., 2003). The cellulosome consists of a number of hydrolytic enzymes attached to a non-catalytic protein scaffold, called a scaffoldin (CbpA, CipA, or CipC), which holds the enzymes together in a protein complex (Bayer *et al*., 1998; Merino and Cherry, 2007; Xu *et al*., 2003). The cellulosome concentrates enzymes on a substrate at specific sites which leads to improved cellulolytic hydrolysis. The organisation of the enzymes in a cellulosome increases the synergistic action of the enzymes (Bayer *et al*., 1998). Different microorganisms produce cellulosomes that vary in their enzyme composition and arrangement (Lu *et al*., 2006;

Subramaniyan and Prema, 2002; Teeri, 1997). Fungal cellulosomes produce mainly glucose and bacterial cellulosomes produce cellobiose as the main product (Dashtban *et al*., 2009).

The scaffoldin contains cohesin domains and a cellulose binding domain (CBD) while the enzymes contain dockerin domains (Dashtban *et al*., 2009; Doi *et al*., 2003). Some cellulosomes may also have more than one CBD and some have more than one scaffoldin, such as in the *Acetovibrio cellulolyticus* cellulosome (Doi *et al*., 2003). Free cellulases do not contain dockerin domains. Dockerins bind to cohesin domains (dockerin-cohesin interaction) and facilitate the assembly of the cellulosome (Figure 1.7), so that the entire cellulosome can attach to the substrate, as opposed to individual enzymes binding separately (Bayer *et al*., 1998; Dashtban *et al*., 2009; Doi *et al*., 2003). There are 50 dockerin domains identified in fungi which have different amino acid sequence as compared to dockerins in bacteria. In *C. thermocellum* and other bacteria, cellulosomes are packed together in polycellulosomal organelles, called protubozymes. These can be seen bound to the outside of the cells using electron microscopy. The protubozymes mediate adhesion of the cell to the cellulose substrate, and when binding occurs the cellulosome undergoes a conformational rearrangement.

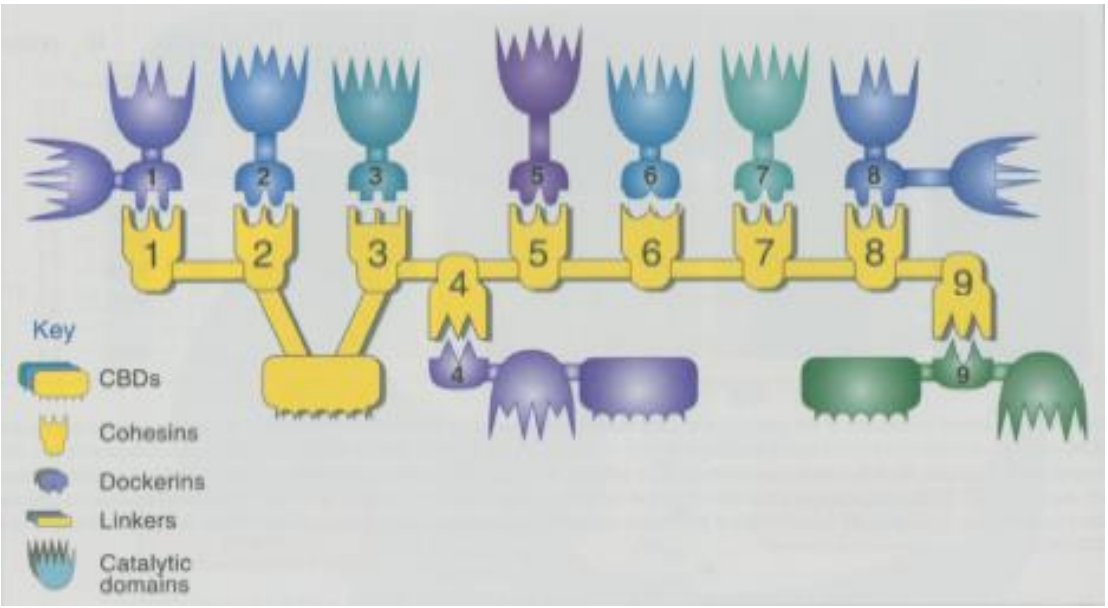


Figure 1.7: Simplified illustration of a cellulosome. Yellow structure represents the scaffolding subunit (scaffoldin) and the catalytic (cellulolytic) subunits are in blue, green and violet (Bayer *et al*., 1998).

Since the discovery of cellulosomes, extensive research has been carried out on these complexes. This has resulted in the construction of designer mini-cellulosomes in order to enhance an understanding of the synergistic relations between various enzymes in the cellulosome, leading to the improvement in degradation efficiency of different substrates (Doi *et al*., 2003). Cellulosomes can be constructed by incorporation of enzymes or non-catalytic components to existing cellulosomes to improve their function. Scaffoldin and dockerins expressed and isolated separately from cellulosomes can be used as the basis for constructing new cellulosomes with the desired combination and ratio of enzymes. The experiments indicated that native cellulosomes had higher activity than a combination of mini-cellulosomes, which may due to the fact that microorganisms in nature produce cellulosomes and a battery of non-cellulosomal enzymes that work together with cellulosomes to degrade the substrate (Doi *et al*., 2003). Recombinant DNA technology has also been used to insert cellulosomal genes in bacteria that have the capacity to produce value added products from lignocellulose wastes (Doi *et al*., 2003), hence reducing the cost of producing the value added products.

1.1.7.8. Enzyme synergy

Synergy can be defined as the interaction between two or more components that produces a combined effect that is greater than the sum of the effects of the individual components (Andersen *et al*., 2008; Hu *et al*., 2011; Merino and Cherry, 2007; Varnai *et al*., 2011; Zhou *et al*., 2009). Enzymes working together synergistically are more efficient in the deconstruction of lignocellulose than individual enzymes (De Vries *et al*., 2000). Synergy between enzymes has been observed between cellulases (Murashima *et al*., 2002; Teeri *et al*., 1997; Watson *et al*., 2002) and between hemicellulases (Beukes *et al*., 2008; Cerri e Silva *et al*., 2000; De Vries *et al*., 2000; Murashima *et al*., 2003; Renard *et al*., 1991; Subramaniyan and Prema, 2002). The factors that affect enzyme synergy are the ratio of the enzymes, characteristics of the enzyme and that of the substrate. Synergy can either be simultaneous or sequential. Enzyme synergy in lignocellulose degradation has been extensively reviewed by Van Dyk and Pletschke (2012).

1.1.8. Pre-treatment

Lignin needs to be degraded first so as to reduce the recalcitrance of lignocellulose and allow access of the cellulases and hemicellulases to their substrates. Chemical or physical pre-treatment strategies are employed before enzyme hydrolysis to address the challenge of lignocellulose recalcitrance due to the presence of lignin and crystallinity of cellulose. The pretreatment strategies include, pyrolysis, steam explosion, ammonia fibre explosion, solvent extraction, ozonolysis, acid and alkaline treatment (Berlin *et al*., 2006; Dashtban *et al*., 2009; Gao *et al*., 2011; Merino and Cherry, 2007; Sun and Cheng, 2002). The pre-treatment process results in removal or modification/ solubilization of hemicellulose and/ or lignin and in the disruption of cellulose crystallinity, hemicellulose and lignin structure, and increase porosity of the feedstock, hence allow cellulases to access cellulose (Boluda-Aguilar *et al*., 2010; Chang *et al*., 2011; Chen *et al*., 2011; Dashtban *et al*., 2009; Del Rio *et al*., 2012; Ferreira *et al*., 2009; Garcia-Aparicio *et al*., 2011). Pre-treatment also results in the redistribution of lignin, reduction in the degree of polymerization of cellulose and a reduction in lignocellulose particle size (Van Dyk and Pletschke, 2012). The pore volume and surface area accessible for cellulose degradation increases, and the removal of lignin results in a reduction in enzyme loading and hence cost.

Chemical pre-treatment processes are expensive processes in biofuel production (Balat, 2011) and they are not successful with all types of biomass (Wyman, 2007). Chemical pre-treatment may result in the loss of some sugars and in the release of inhibitors that subsequently affect enzymatic hydrolysis and fermentation e.g. phenolics, guaiacol, vanillic acid, vanillin, 4-hydroxybenzoic acid, *ρ*-coumaric acid, hydroxyl-cinnamic, ferulic acid, furfural and acetovanillone (Cho *et al*., 2009; Balat, 2011; Berlin *et al*., 2006; Hendriks and Zeeman, 2009; Kovacs *et al*., 2009; Okuda *et al*., 2008; Palmqvist and Hahn-Hagerdal, 2000; Qing *et al*., 2010; Van Dyk and Pletschke, 2012). Biological pre-treatment produces less waste and doesn't pollute the environment, while using less energy than chemical pre-treatment (Dashtban *et al*., 2009) and hence is being proposed in this study.

1.1.9. Immobilisation of cellulases and hemicellulases

Enzyme immobilisation involves attaching/ confining an enzyme to an inert support material (natural or synthetic) for repeated and continued use without loss of catalytic activity. There has been a growing interest over the years in immobilised enzymes for industrial, biomedical and analytical applications. The advantages of immobilised enzymes include convenient handling, high production volumes, low residence time, ease of separation of enzymes from the reaction mixture/product, reusability, resistance to degradation, and increased pH and temperature stability under operational and storage conditions (Buga *et al*., 2010; Schoevaart *et al*., 2004; Sheldon, 2007; Tischer and Wedekind, 2000). These advantages will lead to a reduction in the production costs due to a lowering of enzyme cost and improved enzyme performance.

A number of enzymes have been immobilised and used commercially e.g. glucose isomerase, glucoamylase and aminocyclase (Woodward, 1989), and penicillin G amidase (Sheldon, 2007). The critical step in the conversion of lignocellulose to bioethanol and other products is the hydrolysis of lignocellulose to sugars. Enzymes required for this bioconversion are expensive, hence the need to enhance bioactivity and reuse of the enzymes (Dalal *et al*., 2007; Vieira *et al*., 2011). Enzymes such as cellulases are reported to be difficult to recover from solution and are inactivated by organic solvents and extreme pH and temperature (Andriani *et al.*, 2012). However, this problem can be overcome by immobilisation of the cellulases. A number of enzymes that act on lignocellulose have been immobilised on different support materials and are reported in literature. Cellulase was immobilised on acrylamide membranes to enhance bioactivity and reusability (Yuan *et al.*, 1999). Polygalacturonase was immobilised by entrapment using calcium alginate, which resulted in improved temperature and pH stability (Buga *et al.*, 2010). Immobilisation of commercial pectolytic enzymes onto a porous anion exchange resin was carried out using electrostatic attraction and the enzyme‘s activity was found to be stable and reusable for 5 cycles (Demir *et al*., 2001; Sarioglu *et al.*, 2001). Commercial pectolytic enzyme was also immobilised onto Duolite-polystyrene magnetic particles (Demirel *et al.*, 2004; Demirel and Mutlu, 2005).

There are different methods that can used to immobilise enzymes, such as adsorption, covalent binding, entrapment and membrane confinement and, more recently, cross-linked enzyme aggregates (CLEAs) (Hanefeld *et al*., 2009; Schoevaart *et al*., 2004; Sheldon, 2007; Talekar *et al*., 2012).. The choice of the method depends on the properties of the enzyme, the substrate and the intended application (Buga *et al*., 2010). The preferred method should result in a high turnover rate of the enzyme and retention of high catalytic activity over time. The properties of the immobilised enzyme (chemical, biochemical, mechanical and kinetic) depend on the properties of the free enzyme and that of the support material. Mass transfer effect is the major challenge of immobilised enzymes in industrial application. This may be due to slow/ limited diffusion of the substrate and limited availability of the enzyme (Tischer and Wedekind, 2000). However, reuse and improved stability of the enzyme help to reduce the production costs.

The immobilisation method should have a limited effect on the active conformation of the enzyme and maintain the enzyme's catalytic flexibility. The enzyme should be easily recovered after hydrolysis. For immobilisation of lignocellulose degrading enzymes in a bioreactor, a single method and the same support material would have to be used for hemicellulases, cellulases and ligninases. Although there are different immobilisation methods available, a cheap method that will make the process cost effective is ideal. The other challenge with the immobilisation is that the apple pomace is insoluble, therefore a method that allows the substrate to access the enzyme is required. Since the process is a bioremediation process, high costs will be severely limiting, hence a cheaper method, together with production of value added products, is required. For the improved degradation of lignocellulose, combinations of enzymes with the highest synergy can be chosen and immobilised onto an insoluble support for use in a bioreactor.

1.1.10. Kinetic modelling

Kinetic modelling of enzymatic hydrolysis is important for understanding the hydrolytic process and for optimisation of production of intended products. Models are developed to understand hydrolysis patterns, which aid in process design, simulation and control (Hodge *et al*., 2009). Production of ethanol from lignocellulosic biomass is hampered by enzyme and

pretreatment costs and the saccharification step is the most rate limiting (Lynd *et al*., 2008; Zhang *et al*., 2009; Zhang *et al*., 2010). Optimising the process conditions for efficient conversion of lignocellulose to sugar monomers through kinetic modelling will help to improve the feasibility of commercial biofuel production. Mathematical models which describe the characteristics of enzymatic hydrolysis are important for the design of an efficient bioreactor system.

Most kinetic models reported in literature are based on the action of cellulases on cellulose or pretreated substrates to produce glucose and cellobiose (Bansal *et al*., 2009; Hodge *et al*., 2009; Sousa *et al*., 2011). However, modelling of the enzyme kinetics in hydrolysis of lignocellulose has not been fully understood and is very complex due to the heterogeneous and insoluble nature of the biomass, as well as the multiple enzyme system and synergistic action required to degrade lignocellulose material (Gan *et al*., 2003; Kadam *et al*., 2004; Sousa *et al*., 2011). The substrate starts off as insoluble, but gradually becomes solubilised through enzyme action. An understanding of enzyme properties is important, but equally important is the physical parameters of the substrate that impacts on the kinetics, e.g. surface area accessibility, available substrate, adsorption capacity, composition, degree of polymerisation, pore size and volume and crystallinity (Zhang and Lynd, 2004). Enzyme concentration, composition, and synergism should also be considered together with hydrolysis conditions. A number of factors that influence enzyme hydrolysis have been explored, for example, enzyme concentration, substrate concentration, substrate particle size, shear deactivation, product inhibition, time, crystallinity, temperature, pH and lignin content (Bansal *et al*., 2009; Gan *et al*., 2003; Gupta *et al*., 2012).

Hydrolysis of cellulose follows first order reaction kinetics, depending on either amorphous or crystalline regions. Reaction rates are normally high at the beginning as a result of hydrolysis of the easily hydrolysable amorphous regions. The depletion of the amorphous regions will result in a decrease in the reaction rate (Al-Zuhair, 2008; Andric *et al.*, 2010b). As the reaction proceeds, enzymes may become inactivated due to shear factor, adsorption on substrate and end-product inhibition (Bommarius *et al*., 2008; Converse, 1993; Movagarnejad *et al*., 2000; Zhang *et al*., 1999). Product inhibition can be alleviated by the use of excess β-glucosidase.

The enzymatic hydrolysis of lignocellulose involves a number of steps. The enzyme has to be transferred from the bulk solution to the surface of the substrate where it will adsorb through the binding domain (Bansal *et al*., 2009; Wang *et al*., 2011). The active site of the enzyme will then become properly oriented on the susceptible bond of the substrate, leading to the formation of an enzyme-substrate complex and hydrolysis. The formed product will then desorb from the surface of the substrate and is transferred to aqueous phase. The enzyme can also desorb and readsorb along the cellulose chain. Mass transfer effects play a very critical role in the kinetics of heterogeneous and insoluble substrates like lignocellulose (Sarkar and Etters, 2004). A profit rate model that combines effect of hydrolytic variables and cost is sought. Batch process data can be used to predict the effect on a continuous process. However, in a continuous process, enzyme is constantly lost in the outflow and there is a need for continuous enzyme addition. Fed-batch processes are difficult to model due to changes in volume and dilution effect due to addition of more reactants with time (Hodge *et al*., 2009).

A number of kinetic models to predict enzymatic hydrolysis of biomass have been developed and reviewed in literature and these include Michaelis-Menten based models, empirical models, mechanistic models, models accounting for adsorption and models for soluble substrates, functionally based and structurally based models (Bansal *et al*., 2009; Holtzapple *et al*., 1984; Movagarnejad *et al*., 2000; Sousa *et al*., 2011; Zhang *et al*., 2010; Zhang and Lynd, 2004). The classifications are based on the extent to which the models describe/define the different parameters that affect enzymatic hydrolysis. Most models can explain hydrolysis during the initial stages but not at the later stages. To gain more insight into lignocellulose hydrolysis, more parameters are required to be estimated, leading to complex models being formulated.

Empirical models seek to understand the initial rate of hydrolysis and how it is affected by different conditions, e.g. temperature, pH, enzyme and substrate properties. The different hydrolysis conditions are normally correlated with time or substrate structural properties (Bansal *et al*., 2009). Interaction between different parameters can be understood. Optimisation of reaction conditions can be statistically modelled using large empirical data sets. Empirical models look at few parameters (Holtzapple *et al*., 1984), which are easy to

understand, and therefore are suitable for industrial application. However, the models have limited use outside the conditions under which it was developed and do not fully describe the mechanisms underlying the hydrolysis (Wang *et al*., 2011).

Empirical models were adequately used to describe cellulase hydrolysis of cotton fibres (Sarkar and Etters, 2004; Wang *et al*., 2004), as well as waste paper hydrolysis to reducing sugars using different cellulase concentrations over time (Park *et al*., 2002). Olsen *et al*. (2011) investigated the effect of enzyme load, rate and time on hydrolysis of corn stover and Avicel by Celluclast and Novozyme 188. The work by Vasquez *et al*. (2007) considered pH, solid percentage, enzyme loading and temperature for sugarcane bagasse hydrolysis. They used a factorial design and developed a quadratic model. Ezhumalai and Thangavelu (2010) got maximum response for ethanol production from cellulase using temperature, pH and time as factors. Sasikumar and Viruthagiri (2010) optimised for ethanol concentration from pretreated sugarcane baggase by cellulase using pH, temperature and hydrolysis time. Table 1.2 shows work reported in literature where researchers used empirical models to optimise conditions for higher conversions of lignocellulose. More examples were reviewed and reported by Bansal *et al*. (2009). The designing of a bioreactor will need a robust and consistent model using a large experimental data set (Sousa *et al*., 2011).

Table 1.2: Examples of empirical models from literature for lignocellulose hydrolysis and their applications.

Substrate	Enzyme source	Predicted variable	Independent variable	Reference
Cotton fibers	Cellusoft (Novozymes NA)	Reducing sugar concentration	Substrate concentration, flow rate, time	Sarkar and Etters (2004)
Pretreated sugarcane bagasse	Cellulase, *Candida wickerhamii* MTCC*3013	Ethanol concentration	Temperature, pH, time	Ezhumalai and Thangavelu (2010)
Pretreated sugarcane bagasse	Cellulase, *Pachysolen tannophilus* MTCC*1077	Ethanol concentration	Temperature, pH, time	Sasikumar and Viruthagiri (2010)
Hardwood kraft pulp, pretreated poplar	Celluclast 1.5L, Novozyme 188	Glucose concentration	Substrate consistency	Zhang *et al*. (2009)
Steam-exploded corn stover	Celluclast	Hydrolysis yield	Substrate concentration, enzyme loading, time	Yang *et al*. (2009)
Pretreated wheat straw	Celluclast 1.5 FG L, Novozyme 188	Glucose conversion	Substrate loading, enzyme loading, mixing speed	Jorgensen *et al*. (2007)
Apple pomace	Celluclast 1.5L, Novozyme 188	Glucose, total monosaccharide concentration	Enzyme loading, time	Gullon *et al*. (2007)
Knot rejects	Cellulase (Novozyme)	Glucose concentration	Substrate loading, enzyme loading, time	Zhang *et al*. (2010)
Apple pomace	Cellulase, pectinase, β-glucosidase	Glucose concentration	Enzyme loading, pH, temperature, time	Parmar and Rupasinghe (2012)
Delignified *Prosopis juliflora* wood	Cellulase, β-glucosidase	Sugar concentration	Substrate consistency, time, feeding regime	Gupta *et al*. (2012)
Filter paper	Celluclast 1.5L, Novozyme 188	Conversion %, glucose concentration	Solid content, time,	Kristensen *et al*. (2009)
Pretreated corn stover	Spezyme CP	Cellulose conversion, glucose concentration	Feeding policy, time	Hodge *et al*. (2009)
Steam-exploded corn stover	Commercial cellulase	Glucose concentration, viscosity	Solid concentration, time	Lu *et al*. (2010)
oil palm empty fruit bunches fibre	Cellulase, Novozyme 188	Glucose concentration	Temperature, pH, substrate loading	Hamzah *et al*. (2011)

Michaelis-Menten models best describe soluble substrates, but are not sufficient to describe behaviour in insoluble heterogeneous substrates. The rate of hydrolysis decreases as the reaction proceeds and often approach zero before the substrate is completely hydrolysed. The model does not factor in the change in substrate structure, interaction between enzyme and substrate at the interface, enzyme inhibition and deactivation (Gan *et al*., 2003; Olsen *et al*., 2011). Michaelis-Menten models assume that the substrate concentration is higher than enzyme concentration, but this is not always the case as the substrate gets depleted over time (Brown *et al*., 2010). However, Michaelis-Menten models can predict conversion of cellobiose to glucose by β-glucosidase (Chauve *et al*., 2010; Sousa *et al*., 2011). Although the Michaelis-Menten model has limited application in lignocellulose hydrolysis, it has been used successfully to predict complex systems, e.g. in pure cellulose and lignocellulose reactions (Brown *et al*., 2010; Kadam *et al*., 2004, O'Dwyer *et al*., 2007). Michaelis-Menten models were reviewed by Bansal *et al*. (2009). Li *et al.* (2004) used a pseudo-homogeneous Michaelis-Menten equation for the hydrolysis of cellulosic pulps to release total reducing sugars and glucose. They factored in inhibition. A modified Michaelis-Menten model, HCH-1 was used to model non-competitive inhibition (Brown *et al*., 2010; O'Dwyer *et al*., 2007). Another modification of a Michaelis-Menten model is called jammed fractal Michaelis-Menten models, which seeks to model heterogeneous reactions and the effect of overcrowding of substrate or enzyme, was successfully applied (Bommarius *et al*., 2008; Xu and Ding *et al*., 2007). Jamming kinetics happens when there is high concentration of enzymes or substrate, such that the molecules interfere with each other.

Mechanistic models involve looking at factors such as enzyme-substrate interactions, deactivation and inhibition of the enzyme, enzyme system, substrate system and mass transfer effects. Mechanistic models look into the reaction mechanisms between the substrate and the enzyme. This depends on understanding characteristics of the enzyme (adsorption, synergy, end-product inhibition, mass transfer limitations) and substrate characteristics (composition, lignin, hemicellulose; particle and pore size, crystallinity, degree of polymerisation) (Brown *et al*., 2010; Levine *et al*., 2010). Considering all these factors in a model is quite complicated and involve too many parameters in the differential equations, making the estimation of the parameters for bioreactor design and scale-up difficult (Gan *et al*., 2003; Sousa *et al*., 2011). Therefore they are not practical for industrial application. Semi-mechanistic models have

been developed and successfully used to predict results by some researchers (Al-Zuhair, 2008; Kadam *et al*., 2004; Zhang *et al*., 2009; Zheng *et al*., 2009). The models look at interactions based on physical properties.

Functionally based models consider some important features of hydrolysis mechanism as well as substrate properties, such as pore size, degree of polymerisation, crystallinity, surface area and also hydrolysis mechanisms of different enzymes. Their application is rather difficult due to large amounts of data set required (Sousa *et al*., 2011). On the other hand, structural based models explore the relationship between structural features of the substrate and the function. Kinetic models involving adsorption of enzymes on lignocellulose substrates were also investigated. The adsorption models were based on a Langmuir-type isotherm (Bansal *et al*. 2009; Kadam *et al*. 2004; Sousa *et al*., 2011; Zheng *et al*., 2009). Some kinetic models were developed to accommodate different feeding regimes/profiles, e.g. batch, fed-batch, simultaneous saccharification and fermentation (SSF) and separate hydrolysis and fermentation (SHF) (Drissen *et al*., 2009; Hodge *et al*., 2009; Ko *et al*., 2010; Shao *et al*., 2009; Sousa *et al*., 2011; Zhang *et al*., 2009).

Non-kinetic models such as artificial neural networks (ANN) can be used for simulation of complex systems and nonlinear data like the conversion of lignocellulose to biofuel (O'Dwyer *et al*., 2008). ANNs have been successfully applied in medicine, economics, power systems, biotechnology, enzyme production and in food technology (prediction of food quality, properties, shelf life and processing) (Bhotmange and Shastri, 2011; Kalogirou, 2000). ANN, which is a computational mathematical network, can be used in the modelling of large empirical experimental data in complex systems involving the interaction of many parameters without derivatised mathematical equations (Wang *et al*., 2011). The model is evaluated for its ability to predict experimental data by relating input to output variables, simulating the way the human brain works (Nodeh, 2012; O'Dwyer *et al*., 2008). ANN has been used to solve modelling problems in situations where there is insufficient detailed knowledge of the underlying process and formulation of a reaction mechanism not possible (Nodeh, 2012). Experimental data (input) is used to train the ANN to predict/ estimate data (output) within a certain range under investigation, by identifying and learning the patterns input and output data. The advantage of ANN is that it allows for rational optimisation of

variables and processes using reduced data as compared to traditional empirical models. Besides having excellent data fitting and prediction, ANN doesn't depend on mathematical derivatization and it can fit non-linear data, making it applicable in industrial set ups. Informal experimental designs can be used due to the flexibility of ANN in relation to the amount and form of data. Complex interactions of variables can be evaluated at the same time as more data can be analysed at once. There is no need to transform the data output from ANN. If any new data is added, the system can relearn and new observations can be added anytime (Bhotmange and Shastri, 2011). ANN has been shown to perform better than quadratic polynomials (Sousa *et al*., 2011).

The computational network has processing elements called neurons/ nodes that are interconnected into layers through weights. There are many inputs and outputs in each processing element, which receive and process inputs to give an output. There are basically three layers, input, hidden and output layers, which are generally referred to as network topology or architecture (Figure 1.8).

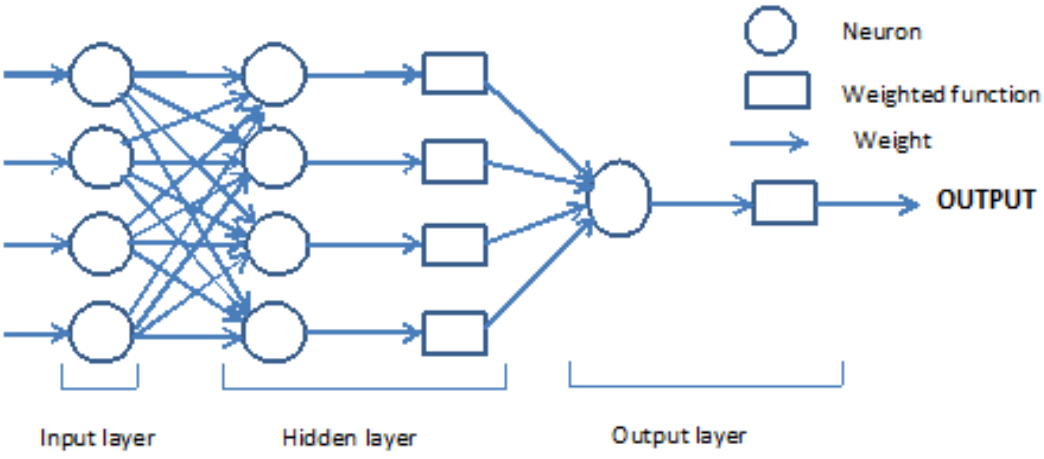


Figure 1.8: A schematic diagram of a four-layer feed-forward back –propagation artificial neural network (Modified diagram from Kalogirou, 2000; O'Dwyer *et al*., 2008; Wang *et al*., 2011).

The input is received at the input layer neurons, where it is weighted and summed and then transferred to subsequent layers/ hidden layers through nonlinear transfer function and finally to the output layer, which then processes and gives the output (Wang *et al*., 2011). The flow of information can be in one direction (feed-forward) or in both directions. There is a scalar weight associated with each connection that modifies the intensity of the signal (Sousa *et al*.,

2011). During training three sub data sets are used, the training set (optimisation of weights), test set and validation set evaluate the ability to predict and this assists in reducing overestimation (Sasikumar and Viruthagiri, 2010). A number of parameters have to be set and these include, function, algorithm and training parameters (Ezhumalai and Thangavelu, 2010). Training of model parameters include time of training, number of layers and neurons and rate of learning to estimate weights related to data inputs and their outputs. During training, the ANN recognises and learns the relationship/pattern between data input and output from different experimental data sets. It then uses the established data correlation to predict outputs from any new data inputs. Training is important to reduce mean square error between predicted/ target outputs and actual outputs of the network and this is achieved by adjusting biases and weights. This is achieved through a back-propagation, where, if there is an error it is sent back for modification in the hidden layer (O'Dwyer *et al*., 2008). Back-propagation is one of the most powerful learning algorithms used in multilayer networks with nonlinear differential transfer function (Kalogirou, 2000; O'Dwyer *et al*., 2008). Careful selection of data of neural network training is important to avoid undertraining or overtraining (Sasikumar and Viruthagiri, 2010). Overestimation results in low minimum square error but the network interpolation ability reduced. However, this can be corrected by regularization using an additional term, allowing for better performance and prediction of new data (O'Dwyer *et al*., 2008). MATLAB neural network toolbox can be used for the construction of ANN (Bhotmange and Shastri, 2011).

ANN has been used to model simultaneous saccharification and fermentation (SSF) of sugarcane bagasse using temperature, initial pH and fermentation time data (Ezhumalai and Thangavelu, 2010; Sasikumar and Viruthagiri, 2010). O'Dwyer (2008) used ANN to successfully predict the effect of acetyl and lignin contents and crystallinity indices on the digestibility of poplar wood. ANN was also successfully used to model the effect of pretreatment conditions (temperature, time, solid content and sulphuric acid concentration) on glucose production from biomass (Sousa *et al*., 2011). Glucose yield from different loadings of cellulase and β-glucosidase was predicted by Rivera *et al*. (2010). Zhang *et al*. (2009) used ANN to better predict glucose concentration by considering three hydrolysis parameters, substrate concentration, cellulase concentration and hydrolysis time. A combination of ANN

and a semi-mechanistic model provided the best option for bioreactor design and scale-up (Sousa *et al*., 2011).

Understanding enzyme kinetics will help in the design of bioreactors with optimal conditions for hydrolysis at reduced costs.

1.2. Problem Statement

The fruit juice industry in South Africa produces large amounts of fruit waste water and solid waste (pomace) which are difficult to degrade due to the presence of recalcitrant plant cell components. Due to the ever increasing scarcity of water, there is a need to treat the water cost-effectively and degrade the cell wall components into biotechnologically important monomers that can be utilised in other industries. Solid waste can be used to produce value-added products, thereby alleviating health and environmental problems associated with dumping the waste on land.

There has been little or no research performed on complete degradation of fruit pomace to its simple components, which makes this project novel. Complete degradation of fruit waste needs a variety of enzymes that work synergistically. This project will investigate a system which can allow the selection and variation of the enzymes employed. Commercial enzymes mixtures are preferred to whole cell organisms because they have greater specificity, and are easier to handle and store.

This project will assist in an improved understanding of the concept of synergy between cellulases, hemicellulases and ligninases and its application to the degradation of other complex substrates, thereby paving the way for effective waste beneficiation processes. Synergy studies have previously been performed between cellulases only and hemicellulases only and mainly on pre-treated substrates. It is hoped, in the long term, that by analysing the synergy in complex substrate degradation, bioreactors can be designed and become part of every fruit juice manufacturing plant. This will assist in fruit waste water beneficiation and hopefully reduce the impact of the fruit juice industry on the environment.

1.3. Hypothesis

We hypothesise that combinations of enzymes are required to effectively break down fruit wastes and that synergy occurs between commercially available ligninase, cellulase and hemicellulase. Synergy between lignocellulose enzymes will result in improved yields of glucose and other sugars. The degradation of apple pomace requires a specific combination and ratio of lignocellulolytic enzymes. As one enzyme acts on its substrate, the degree of complexity and inter-linking is reduced, thereby granting access to another enzyme to act on its particular substrate.

1.4. Aim and Objectives

1.4.1. General aims

The main aim of the project is to degrade apple pomace using a combination of commercial enzyme preparations using a sustainable and cost effective process. Based on the presented research, in future, the second aim would be to obtain value added products from the breakdown of lignocellulose components.

1.4.2. Specific Objectives

a) To determine the most feasible commercial enzyme mixtures required for complete degradation of apple waste based on the waste composition.

b) To design optimal ratios and combinations of enzymes and optimal conditions required to degrade apple pomace biomass effectively.

c) To determine the sugar yield and synergistic associations between commercial enzyme mixtures for apple pomace degradation.

d) To design a simple and inexpensive bioreactor for apple pomace hydrolysis and determine the bioreactor kinetics.

CHAPTER 2: CHARACTERISATION OF COMMERCIAL ENZYME MIXTURES AND APPLE POMACE SUBSTRATE

2.1. Introduction

The main substrate used in this study was apple pomace, which is one of the major fruit wastes generated from the juice and canning industry in the Western region of South Africa, and hence, was targeted in this research. The disposal of apple pomace poses a major environmental and health problem due to its composition (Mahmood *et al.*, 2010, Malherbe and Cloete, 2002) (section 1.1.4). Apple pomace is lignocellulosic in nature, containing mainly cellulose, hemicellulose, pectin and lignin and also proteins, ash, salt and minerals, depending on the plant source (Balat, 2011; Das and Singh, 2004; Dashtban *et al*., 2009; Foyle *et al*., 2007; Lin *et al*., 2010; Malherbe and Cloete, 2002; Merino and Cherry, 2007; Subramaniyan and Prema, 2002; Wyman, 2007). The composition of fruit waste makes it a feasible target for treatment and re-utilisation. However, different authors have reported different chemical compositions for apple pomace in terms of its sugar, cellulose, hemicellulose and pectin content (Bhushan *et al*., 2008; Joshi and Attri, 2006; Nawirska and Kwasniewska, 2005; Parmar and Rupasinghe, 2012; Van Dyk *et al*., 2013; Vendruscolo *et al*., 2008). The composition of apple pomace varies depending on the apple variety, growth climate, maturity of the fruit and the juice extraction method that is employed (Kennedy *et al*., 1999; Schieber *et al*., 2001; Taasoli and Kafilzadeh, 2008). It was therefore, important to characterize the apple pomace used in this study to determine the feasibility of its treatment and utilisation to produce products like bioethanol. Chemical composition can be used to determine the enzymes required for its degradation, enzyme loadings and the ratios to be used. Sugar yields can be easily calculated to measure the extent of degradation and whether additional/ accessory enzymes are required for efficient apple pomace degradation and for feasible biofuel production. The type and amount of enzymes required for lignocellulose hydrolysis depends on the type of feedstock, whether pre-treatment has been carried out and the type of pre-treatment employed.

A number of commercial enzymes preparations, produced mainly by Novozyme and Genencor, are reported in literature, e.g. Accellerase 1500 (cellulase, hemicellulase, β-glucosidase), Optimash^{TM}BG (xylanase, β-glucanase) (Lin *et al*., 2010), Celluclast 1.5L

(mainly cellulase), Ultraflo L and Viscozyme L (mainly β-glucanase and hemicellulase) (Merino and Cherry, 2007; Sorensen *et al*., 2003), Multifect, Spezyme and Biocellulase (Howard *et al*., 2003). This study involved the selection of commercially available enzymes, based on the knowledge of the kinetics, synergies, manufacturer's data sheet and cost (Beukes *et al*., 2008; Murashima *et al*., 2003), and physicochemical properties of the key enzymes. Information from the manufacturer indicated the main enzymes present in the commercial mixtures with no additional information on their concentrations within the mixture and optimal hydrolysis conditions. The use of commercial enzyme mixtures for lignocellulose degradation is hindered by lack of characterisation of the enzymes in these mixtures (Van Dyk and Pletschke, 2012). It was therefore important for this study to establish the enzyme activities and their ratios in two commercial enzyme mixtures, namely Viscozyme L and Celluclast 1.5L. Synergy between these two enzyme preparations in the degradation of apple pomace was of key importance. This information will give insight into what other additional purified enzymes or mixtures could be added for specific substrates. It is assumed that the use of crude enzyme mixtures is cheaper than using individual purified enzymes (Van Dyk and Pletschke, 2012) and that the presence of other proteins may contribute to the hydrolysis of lignocellulosic biomass (Yang *et al*., 2011; Suwannarangsee *et al*., 2012). It is important to note that the properties of the selected enzymes should be compatible with other enzymes and the proposed biotechnological application. The presence of potential enzyme inhibitors was also investigated.

Chemical pre-treatment may result in the loss of some sugars and in the release of inhibitors/ compounds that subsequently affect enzymatic hydrolysis and fermentation (section 1.1.8). To the best of our knowledge, the influence the compounds on commercial enzyme mixtures has never been reported. The industrial production of biofuels can make use of different bioreactor systems: batch, fed-batch or semi/continuous systems (Chandel *et al*., 2007). Different bioconversion process strategies can be employed: separate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF), consolidated bioprocessing (CBP) (Andric *et al*., 2010b; Chandel *et al*., 2007; Lynd *et al*., 2005; Mills *et al*., 2009; Van Dyk and Pletschke, 2012; Xu *et al*., 2009) (section 1.1.1.3). In both SSF and CBP, inhibition by alcohols, organic acids, phenolic compounds and hydrolysis products poses a major challenge.

2.2. Aims and Objectives

a) To determine the chemical composition of apple pomace. Chemical composition will provide insight into the feasibility and possible utilisation of the apple pomace. Knowing the composition of the apple pomace will aid in the selection of enzymes, their combinations and ratios required for its degradation. Sugar yields can also be calculated.
b) To characterise the commercial enzyme preparations, in order to provide insight into the hydrolysis conditions required. Knowing which enzymes are present in the mixtures will aid in the optimisation of enzyme combinations and ratios for efficient hydrolysis.

2.3. Materials and Methods

2.3.1. Apple pomace biomass preparation

Golden Delicious apples, obtained from a local supermarket (His Majesty's Fruit and Vegetables, Grahamstown, South Africa), were cut into small pieces and slowly added to a kitchen juicer to separate the pomace from the juice. The pomace was then homogenised in a food processor. The pomace was washed several times with distilled water using muslin cloth, until no sugars were detected in the wash using the 3,5-dinitrosalicylic acid (DNS) assay. The apple pomace was stored at -20°C until used in assays. Apple pomace, 5% (wet w/v) concentration (0.5%, dry w/v), was used as the natural substrate in the assays. Sodium azide was added as a microbial preservative to buffers and apple pomace to a final concentration of 0.03% (w/v).

2.3.2. Characterisation of apple pomace – polysaccharide and lignin composition

Apple pomace was characterised using a modified sulphuric acid method by Moxley and Zhang (2007) and Sluiter *et al*. *(*2010) (National Renewable Energy Laboratory -NREL).

2.3.2.1. Determination of sugar composition of apple pomace

Freeze-dried and ground apple pomace was used. Avicel PH101 and carboxymethylcellulose (CMC) were used as controls. A volume of 3 mL of 72% (v/v) sulphuric acid was added to 300 mg of ground pomace in a test-tube. Reactions were carried out in triplicate. The samples were mixed using a glass rod and incubated at 30°C for 60 min with frequent mixing and then placed on ice. The hydrolysate was diluted down to 4% sulphuric acid by adding 84 mL of

dH_2O. One set of reactions were further diluted to 1% by adding 3 mL dH_2O to 1 mL of 4% hydrolysate. The samples were autoclaved at 121°C for 1 h. Upon cooling of the hydrolysate, calcium carbonate was added to neutralise samples to pH 6.0. The samples were then centrifuged at 13 000 *x g* for 5 min and the supernatant stored in the freezer. The 4% hydrolysate was used to measure glucose, galactose, fructose and galacturonic acid and the 1% hydrolysate to measure xylose and arabinose (using a Megazyme kit and HPLC methods). A Shimadzu HPLC (Shimadzu Scientific, Japan) equipped with a Reflective Index detector and Shodex column (8.0 mm ID x 300 mm L, SP-0810, Japan) was used for analysis. The mobile phase was water, with flow rate of 1.0 mL/min and a column temperature of 80°C. Reducing sugars (as glucose equivalents) were measured using a modified 3,5-dinitrosalicylic acid (DNS) assay method (Miller, 1959) and total sugars using a phenol sulphuric acid method (Dubois *et al*, 1956). A volume of 300 µL concentrated sulphuric acid was added to 100 µL of each sample and then 60 µL of 5% phenol. The mixture was vortexed and heated at 90°C for 10 min. After cooling to room temperature absorbance was measured at 490 nm using a Powerwave X microplate reader (Biotek Instruments with KC Junior software). Total sugars were determined using a glucose standard curve with concentrations ranging from 0.0125–0.4 mg/mL (see Appendix 1).

Correction coefficients (CR) for each sugar were determined using known samples of each sugar that were treated in the same way as the samples above.

CR = Concentration measured/Concentration known

The concentration (C_s, %) of the sugars in the polysaccharide was calculated as follows:

$C_s = ((C_iV/CR)*(MW_p/MW_m)/Wt)*100$

Where; C_i represents the measured sugar concentration, MW_p the molecular weight of hexose or pentose polysaccharides (162.14 or 132.11 g/mol), MW_m the molecular weight of monomeric hexose or pentose (180.16 or 150.13 g/mol), Wt the weight of sample and V the volume of hydrolysate (mL).

2.3.2.2. Determination of acid insoluble lignin

A volume of 86 mL each of the 4% sulphuric acid hydrolysed samples was quantitatively transferred into filtering sintered crucibles, porosity 3, using 50 mL of warm deionised water. Filtration was performed and the crucible with solid residues were dried at 105°C for 4 h or until a constant weight was achieved. Samples were then cooled in a desiccator. The weight was recorded (W1). The crucibles were then placed in a muffle furnace at 580°C for 24 h,

cooled in a desiccator and then weighed (W2). The amount of acid insoluble lignin was then calculated (W1-W2).

2.3.3. Chemicals

Chemicals used in this study were of analytical reagent grade and were purchased mainly from chemical companies (see Appendix 2).

2.3.4. Enzymes

Two commercial enzyme preparations were used, Viscozyme L (an enzyme complex from *Aspergillus aculeatus*) and Celluclast 1.5L (a commercial *Trichoderma reesei* ATCC 26921 cellulase preparation). Both were obtained from Sigma (South Africa). Various concentrations were prepared in appropriate buffers depending on the application.

2.3.5. Enzyme assays

Enzyme activity was determined by measuring reducing sugars released (as glucose equivalents) using a modified 3,5-dinitrosalicylic acid (DNS) assay method (Miller, 1959). The assay mixture comprised of 150 µL enzyme, 300 µL substrate and 750 µL buffer unless stated otherwise. Assays were performed using a final concentration of 5% (wet w/v) (0.5% dry w/v) apple pomace (20% wet w/v, 2% dry w/v initial concentration) with different concentrations of Viscozyme L and Celluclast 1.5L (depending on application) in a citrate buffer (pH 5.0, 0.05 M). Reactions were incubated at 37°C (unless otherwise stated) for different time periods on a 360° rotary shaker at 50 rpm. The enzyme reaction was terminated by heating the assays at 100°C in a digital dry bath for 5 min and then cooling on ice for 5 min. The assay mixture was then centrifuged at 13 000 *x g* for 5 min. The DNS assay was performed by adding 150 µL of the supernatant to 300 µL DNS reagent, heating for 5 min in a digital dry bath, then cooling on ice for 5 min. Absorbance was measured at 540 nm using a Powerwave X microplate reader (Bio-Tek instruments with KC Junior software). The reducing sugars released were determined as glucose equivalents using a glucose standard curve (see Appendix 1). Activity (U) was defined as µg glucose equivalents released per mL of substrate per min under the assay conditions specified.

2.3.6. Characterisation of commercial enzyme mixtures

2.3.6.1. Protein concentration determination

Protein concentration was determined using a modified Bradford method (Bradford, 1976), based on the manufacturer's protocol. Absorbance readings were taken at 595 nm using Powerwave X microplate reader (Bio-Tek instruments with KC Junior software). Protein concentration was estimated using bovine serum albumin (BSA) as a standard (see Appendix 1).

2.3.6.2. pH and temperature optima and stabilities of commercial enzyme mixtures

The pH optimum was determined using 5% (wet w/v) apple pomace in 0.05 M universal buffer (boric, acetic and phosphoric acid) (Britton and Robinson, 1931) at pH values ranging from pH 3.0-10.0. The temperature optima was determined using 5% (wet w/v) apple pomace in citrate buffer (pH 5.0, 0.05 M) at temperature values ranging from 20–70°C. The assays were performed in triplicate under standard assay conditions at 37°C for 1 h. Enzyme concentration of 4 µL/mL, 0.304 mg/mL stock (0.038 mg/mL final concentration) was used for each enzyme, except for pH optima and stability where a concentration of 10 uL/mL (0.76 mg/mL) of Celluclast 1.5L was used. The pH stability of Viscozyme L and Celluclast 1.5L was determined by pre-incubating the enzyme solution in universal buffer (0.05 M) at pH 3.0, 4.0, 5.0 and 6.0 over a period of 24 h at 37°C. Aliquots were removed at various time intervals and stored on ice. The standard assay was then performed in triplicate for the different time intervals for each pH. An assay control using enzyme at each pH, which had not been pre-incubated, was included. The temperature stability of Viscozyme L and Celluclast 1.5L was determined by incubating the enzyme solution at temperatures of 20, 28, 37 and 50°C and a pH of 5.0 over a period of 24 h. Aliquots were removed at various time intervals and stored on ice. The standard assay was then performed in triplicate for the different time intervals for each temperature. pH and temperature stability was also determined for a period of up to 15 days using a buffer at pH 5.0 and a temperature of 37°C. Enzymes were pre-incubated in buffer (pH 5.0) at 37°C, with aliquots taken at 1, 3, 6, 10 and 15 days. Enzyme activity was then determined at 37°C for 1 h under standard assay conditions. The residual activity for each enzyme and each pH and temperature was calculated by using the activity of the control (not pre-incubated) as 100%.

2.3.6.3. Identification of enzyme activities in commercial mixtures

The different enzyme activities found in Viscozyme L and Celluclast 1.5L were determined using various substrates; namely carboxymethyl cellulose (low viscosity, amorphous) for endoglucanase activity, birchwood xylan for endoxylanase activity, locust bean gum for endomannanase activity, polygalacturonic acid for polygalacturonase activity and apple pectin was used for pectinase activity. A 2% (w/v) substrate stock solution in citrate buffer (pH 5.0, 0.05 M) was prepared. An enzyme concentration of 4 µL/mL (0.304 mg/mL) stock solution was prepared for each enzyme in citrate buffer. The DNS assay was then carried out under standard assay conditions in triplicate at 37°C for 30 min. Activity was estimated using a glucose standard for endoglucanase and endomannanase; a xylose standard curve for endoxylanase; and a galacturonic acid standard curve for polygalacturonase and pectinase. Xylose and galacturonic acid standard curves were prepared using concentrations ranging from 0–1.0 mg/mL (see Appendix 1).

Whatman no. 1 filter paper (98% cellulose, Kristensen *et al*., 2009) was used for total cellulase activity using a modified NREL method (Adney and Baker, 1996). The filter paper was cut into identical small pieces to fit into 1.5 mL eppendorf tubes. An enzyme stock solution of 5 µL/mL (0.38 mg/mL) in citrate buffer (pH 5.0, 0.05 M) was used for each enzyme mixture (Viscozyme L and Celluclast 1.5 L). The assay mixture comprised of 50 mg filter paper, 0.5 mL enzyme and 1 mL buffer. The reaction mixture and controls were incubated at 37°C for 30 min, after which the DNS assay was performed to determine the total cellulase activity. Activity was defined as the µmole of glucose equivalent liberated per min per mg of enzyme under assay conditions.

A modified 4-nitrophenol assay (Berghem and Pettersson, 1974) was used to determine the other activities of the enzymes. 4-nitrophenyl-β-D-cellobioside was used for cellobiohydrolase, 4-nitrophenyl-β-D-glucopyranoside was used for β-D-glucosidase, 4-nitrophenyl-β-D-xylopyranoside was used for β-D-xylosidase, 4-nitrophenyl-β-D-mannopyranoside was used for β-D-mannosidase and 4-nitrophenyl- α-L-arabinofuranoside was used for α-L-arabinofuranosidase. A 4-nitrophenol standard curve was prepared using 4-nitrophenol in concentrations ranging from 0.001–0.08 µmol/mL (see Appendix 1).

A substrate concentration of 0.5 mM was prepared in 50 mM citrate buffer (pH 5.0, 0.05 M). A 50 µL volume of 2 µL/mL (0.152 mg/mL) enzyme was added to 450 µL of substrate and

the mixture was incubated at 37°C for 30 min. The enzyme reaction was terminated by the addition of 500 µL, 2 M Na_2CO_3. The absorbance of the resultant 4-nitrophenolate was measured at 405 nm using a Powerwave X microplate reader (from Bio-Tek instruments with KC Junior software).

2.3.6.4. SDS-PAGE analysis

The enzymes present in each commercial enzyme mixture were determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using a 10% (w/v) resolving gel and a 4% (w/v) stacking gel (see Appendix 3). Electrophoresis was performed using apparatus from BioRad, after which the gel was stained using Coomassie Brilliant Blue. A pre-stained molecular weight marker (20–120 kDa) was used to estimate the size of different enzymes present in the commercial enzyme mixtures.

2.3.6.5. Determination of optimal substrate loading, K_m and V_{max} on apple pomace

Optimal apple pomace concentration was determined by using concentrations ranging from 2–120% (20–1 200 mg/mL) (wet w/v) and enzyme concentrations of 3 µL/mL (0.228 mg/ mL) (50:50 Viscozyme L to Celluclast 1.5L) stock solution at 37°C for 24 h under standard assay conditions. A Lineweaver-Burk plot was prepared and used to calculate K_m and V_{max} values.

2.3.6.6. The effect of alcohols, sugars and lignin on Viscozyme L and Celluclast 1.5L

The effect of ethanol, propanol and butanol on Viscozyme L and Celluclast 1.5L was determined at concentrations of 10–60 g/L; the effect of glucose, cellobiose, xylose and xylobiose at concentrations of 0–2 mM; organic acids and phenolic compounds at 1, 5 and 10 g/L; lignin at concentrations of 0–2 g/L and metal ions at 10 and 50 mM in citrate buffer (pH 5.0, 0.05 M). Assays were performed under standard conditions using a 50:50, Viscozyme L: Celluclast 1.5L enzyme combination (2 µL/mL, 0.152 mg/mL, optimal protein concentration) stock concentration of each enzyme and also with individual enzymes for 24 h at 37°C. An assay control with the enzymes and substrate without the inhibitor was included. The residual activity for each enzyme or combination at each inhibitor concentration was calculated using the activity for enzymes and substrate without the presence of an inhibitor as 100%.

2.3.6.7. Type of inhibition exhibited by alcohols and sugars on Viscozyme L and Celluclast 1.5L

The type of inhibition exhibited by alcohols and sugars on Viscozyme L and Celluclast 1.5L was investigated using concentrations of 0, 20 and 40 g/L for alcohols and 0, 1.0 and 1.5 mM for sugars versus an increase in apple pomace concentration from 0–60 % (wet w/v). Assays were performed under standard conditions using a 50:50, Viscozyme L: Celluclast 1.5L, enzyme combination (2 µL/mL, 0.152 mg/mL, optimal protein concentration) stock concentration (0.019 mg/mL final concentration) of each enzyme and also with individual enzymes for 24 h at 37°C. Lineweaver-Burk plots were plotted to identify the type of inhibition.

2.4. Results and Discussion

2.4.1. Chemical composition of apple pomace

The chemical composition of apple pomace after 72% sulphuric hydrolysis followed by analysis of individual sugars using Megazyme kit assays (Megazyme, Ireland) and HPLC are shown in Table 2.1.

Table 2.1: Composition (%) of some selected sugars in apple pomace. Values are presented as mean values ±SD (n=3).

	Glucose	**Arabinose**	**Xylose**	**Galactose**	**Galacturonic acid**	**Fructose**
Megazyme kit	20 (±0.8)	6.2(±0.6)	1.1(±0.4)	-	5 (±0.4)	-
HPLC	22.3(±2.0)	12.5(±1.3)	-	5.1(±0.3)	-	0.8 (±0.05)

The composition for total sugars, reducing sugars and acid soluble lignin was 46.4 (±2.1), 47.8 (±0.5) and 19.8%, respectively. The remainder is likely to consist of mainly galacturonic acid and other components in low concentrations, e.g. ferulic acid, extractives, proteins, minerals and other sugars like rhamnose. The galacturonic acid content of apple pomace in the literature vary widely from 49-64% (Bhushan *et al*. 2008), to 11.7% (Nawirska and Kwasniewska, 2005). Galacturonic acid content could not be directly measured using the HPLC method that was employed in this study, so the actual percentage galacturonic acid could not be determined.

The amount of acid soluble lignin in apple pomace was 19.8%, which falls in the range reported by other researchers, 15.2–20.4% (Bhushan *et al.*, 2008, Nawirska and Kwasniewska, 2005). Of the sugars in apple pomace, glucose was the highest at 22.3%, followed by arabinose (12.5%) and galactose (5.1%). Very little xylose was detected (1.1%) (Table 2.1). The amount of glucose, arabinose and galactose obtained were similar to results reported by other researchers (Bhushan *et al*., 2008; Joshi and Attri, 2006; Parmar and Rupasingle, 2013). However, there is still insufficient and inconsistent information in literature about apple pomace composition (Dhillon, 2012; Nawirska and Kwasniewska, 2005; Van Dyk *et al*., 2013; Vendruscolo *et al*., 2008). The difference in composition of apple pomace from literature may be due to the method employed in the estimation, apple variety used, growth climate, maturity of the fruit and juice extraction method that was employed (Kennedy *et al.*, 1999; Schieber *et al.*, 2001; Taasoli and Kafilzadeh, 2008).

Correct estimation of apple pomace composition is important as composition of sugars can be used to calculate yield, aiding in the selection of enzymes to be used, their loading and ratios. Yield shows the extent of hydrolysis and whether additional/ accessory enzymes are required for efficient apple pomace degradation and for feasible biofuel production. The amount of total sugars released from apple pomace by sulphuric acid method was close to the expected range as compared to literature values of 48–62% (Bhushan *et al.*, 2008; Joshi and Attri, 2006). In another experiment, the time for 72% sulphuric acid hydrolysis was increased to 3 and 6 h to ascertain whether more sugars could be released. The results did not show differences in the amount of sugar released when apple pomace was hydrolysed for different time periods, which suggest that 3 h was sufficient for total hydrolysis. A large difference was observed between total sugars measured and the expected literature value, which may be attributed to different treatments of the sample before hydrolysis and reaction conditions which was not clear from literature, but can also be due to degradation of monomeric carbohydrates during the whole process (Moxley and Zhang, 2007). Pentose sugars like arabinose and xylose are reported to be more acid-labile than hexose sugars like mannose, galactose and glucose.

All polysaccharides should be converted to oligosaccharides during hydrolysis by 72% sulphuric acid, with cellodextrins converted to glucose by use of 4% sulphuric acid and autoclaving, and hemicellulose oligosaccharides converted to sugar monomers by use of 1% sulphuric acid and autoclaving (Gubitz *et al.*, 1998; Moxley and Zhang, 2007). The method

by Moxley and Zhang (2007) allows better estimation of acid labile carbohydrates in lignocellulose than other methods previously used. Besides sulphuric acid hydrolysis, trifluoroacetic acid (TFA) (Fengel and Wegener, 1979) can also be used, but this method resulted in the release of less sugars (results not shown). Use of different methods or acid concentration for hydrolysis and analysis of lignocellulose composition result in different concentrations obtained, as indicated by the work of Foyle *et al*. (2007). The method for sugar quantification can also lead to some variations in composition e.g. quantification of sugars as alditol acetates using GC is more sensitive for low sugar concentrations, but is prone to errors. On the other hand, HPLC is less sensitive to low sugar concentration but is faster than GC (Agblevor *et al*., 2004). The use of kits like Megazyme kits is more specific and rapid, but can be subject to interference. The obtained results showed that glucose concentration was similar using the HPLC and the Megazyme kit, however a large variation was observed for arabinose, with HPLC yielding a result closer to reported literature value of 14-23% (Bhushan *et al.*, 2008).

The amount of total sugars released was lower than that of reducing sugars due to the fact that galacturonic acid reacts less with the phenol sulphuric acid method (5x less than glucose), but reacts at the same level as glucose with the DNS assay. Hexoses are measured at 490 nm and pentoses at 480 nm, therefore estimating sugar composition at 490 nm using the DNS method may underestimate the pentoses and hence the final sugar composition (Gubitz *et al.*, 1998). The presence of galacturonic acid in apple pomace suggested a high pectin content in apple pomace, however, the result obtained was low as compared to that reported in literature, 49-64% (Bhushan *et al.*, 2008).

Galacturonic acid is an important product for value addition (Baciu and Jordening, 2004; Boluda-Aguilar *et al*., 2010; Van Dyk *et al*., 2013). The amount of glucose in apple pomace and other sugars make apple pomace a feasible feedstock for biofuel production. Arabinose is also a useful product for industrial use (Baciu and Jordening, 2004). The presence of substantial amounts of lignin in apple pomace poses a challenge for its complete degradation, not only due to recalcitrance, but also unproductive binding and steric hindrance of cellulases and hemicellulases. However, if chemical pre-treatment is employed to remove lignin, a number of enzyme inhibitors will be produced, thereby reducing the hydrolysis rate (Balat, 2011; Berlin *et al*., 2006; Cho *et al*., 2009; Hendriks and Zeeman, 2009; Kovacs *et al*., 2009;

Okuda *et al*., 2008; Palmqvist and Hahn-Hagerdal, 2000; Qing *et al*., 2010; Van Dyk and Pletschke, 2012). Enzyme pre-treatment using ligninases may therefore be a feasible option.

2.4.2. Characterisation of commercial enzyme mixtures

Two commercial enzymes preparations, Viscozyme L and Celluclast 1.5L were selected based on their reported enzyme composition and therefore potential to degrade lignocellulose biomass. Local availability and cost were also considered factors. The commercial enzyme preparations are mixtures of different enzymes and using them in combination may result in enhanced hydrolysis of fruit pomace than can be achieved by a preparation of individual enzymes. Limiting the number of mixtures will be important to keep the enzyme cost low. Synergy studies were first performed using Biocip Membrane, Viscozyme L and Celluclast 1.5L enzyme preparations (section 3.4.1). A 50:50 combination of Viscozyme L and Celluclast 1.5L displayed a higher degree of synergy and release of sugars than any other combination, hence characterisation was carried out on these two enzyme preparations.

2.4.2.1. Protein concentration determination

The protein concentration for the two commercial enzyme mixtures were determined by the Bradford method and the following protein concentrations were obtained: Viscozyme L – 78.1 mg/mL and Celluclast 1.5L – 75.5 mg/mL. The protein concentrations are important for calculating specific activities, enzyme loadings and enzyme ratios.

2.4.2.2. pH and temperature optima and stabilities

The pH optima of Viscozyme L and Celluclast 1.5L enzyme mixtures were determined using apple pomace as a substrate and an universal buffer. The results are shown in Figure 2.1.

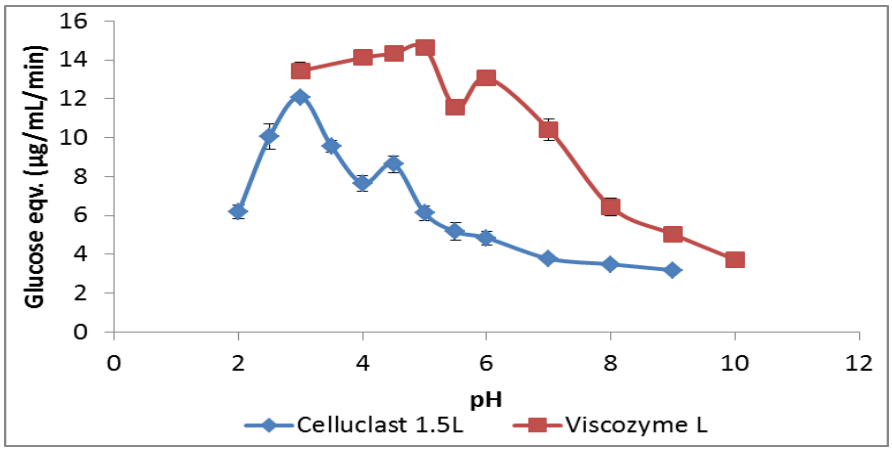


Figure 2.1: Viscozyme L and Celluclast 1.5L activities at different pH values and incubation temperature of 37°C. Activities were measured on 5% (wet w/v) apple pomace using Britten-Robinson buffer pH 3.0 – 10.0. Data points are presented as mean values ±SD (n=3).

The pH optima were found to be pH 5.0 (pH 3.0–6.0) for Viscozyme L and pH 3.0 (3.0–4.5) for Celluclast 1.5L, respectively. There were two peaks at pH 5.0 and 6.0 for Viscozyme L and pH 3.0 and 4.5 for Celluclast 1.5L, which could be attributed to the different enzymes present in the commercial mixtures having different pH optima (see later Table 2.2, page 67). Since Celluclast 1.5L showed a peak at pH 3.0, a pH of 2.0 and 2.5 was also included to determine the activity in this range. A pH range of pH 3.0–6.0 was chosen for stability studies as high levels of activity were present in this range which was also reported by many studies related to hydrolysis of corn stover and wheat arabinoxylan (Boussaid and Saddler, 1999; Kristensen *et al*., 2009; Merino and Cherry, 2007; Ohgren *et al*., 2007; Sorensen *et al*., 2003). The results of the pH stability studies are shown in Figure 2.2. Viscozyme L had a residual activity of more than 80% at pH 4.0, pH 5.0 and pH 6.0 after 24 h incubation. However, it was less stable at pH 3.0 with the residual activity falling to around 60% with fluctuations in activity measured. Celluclast 1.5L maintained an activity above 80% at pH 3.0 – pH 6.0 after 24 h incubation. The highest stability for the two enzyme mixtures was at pH 4.0. Generally, the two enzyme mixtures were shown to be quite stable over a period of 24 h and can be used in combination in a bioreactor at a pH of 4.0-6.0. Celluclast 1.5L was used at a concentration of 10 µL/mL (0.76 mg/mL) as the amount of reducing sugar released at a concentration of 4 µL/mL (0.304 mg/mL) fell below the detectable limits of the DNS assay at some pH values.

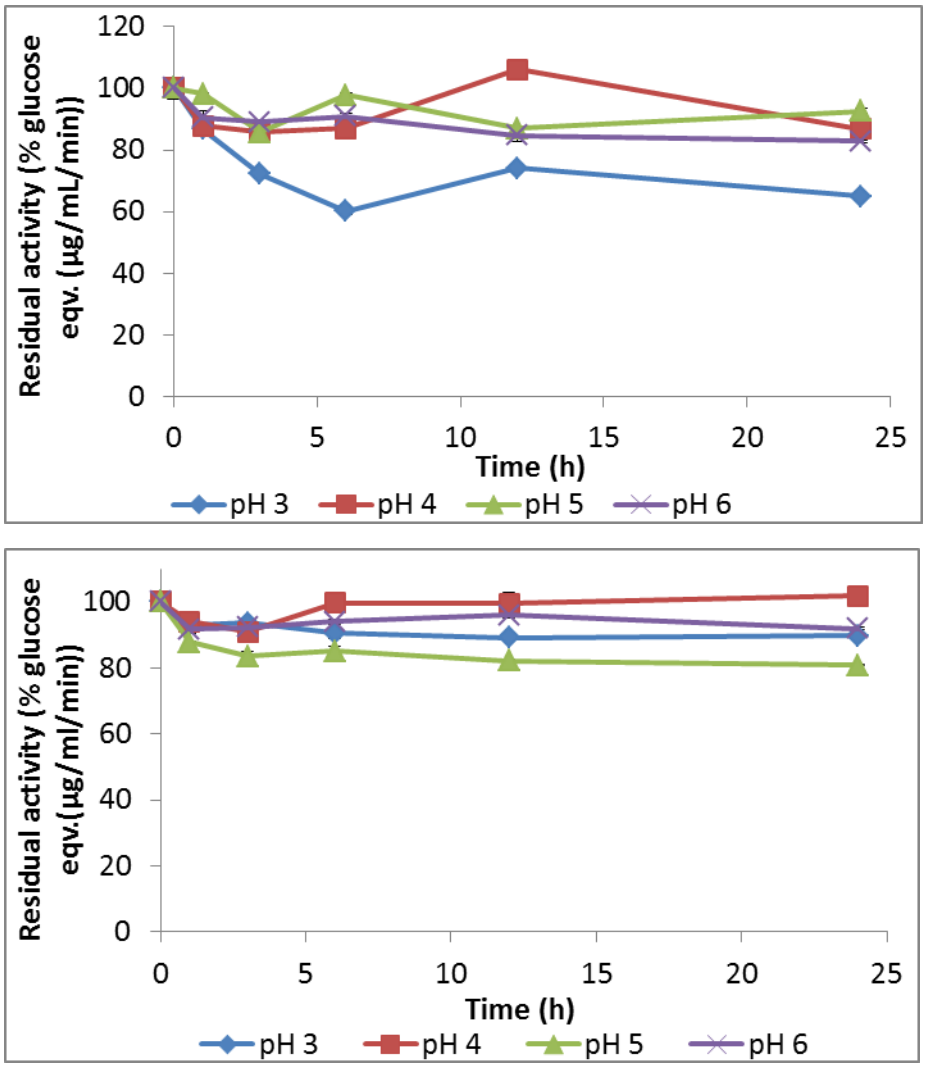


Figure 2.2: Residual activity of Viscozyme L (A) and Celluclast 1.5L (B) at pH 3.0 – 6.0 and temperature of 37°C**. Residual activity was measured on 5% (wet w/v) apple pomace using** Britten-Robnson **buffer (pH 3.0–6.0)**. Residual activity was calculated as the activity obtained as a result of pre-incubation at a given pH value, divided by activity without pre-incubation at the same pH value, which was then multiplied by 100. Data points are presented as mean values ±SD (n=3).

The temperature optima were determined on the two commercial enzymes using apple pomace and citrate buffer (pH 5.0). The results are shown in Figures 2.3.

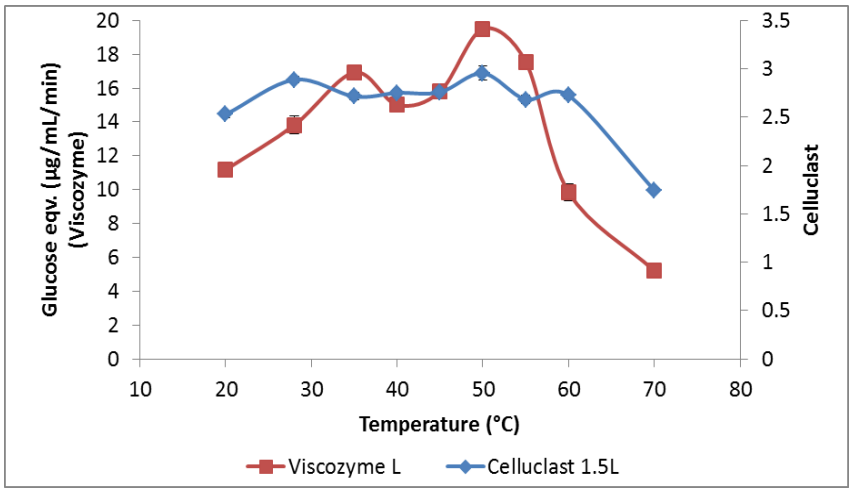
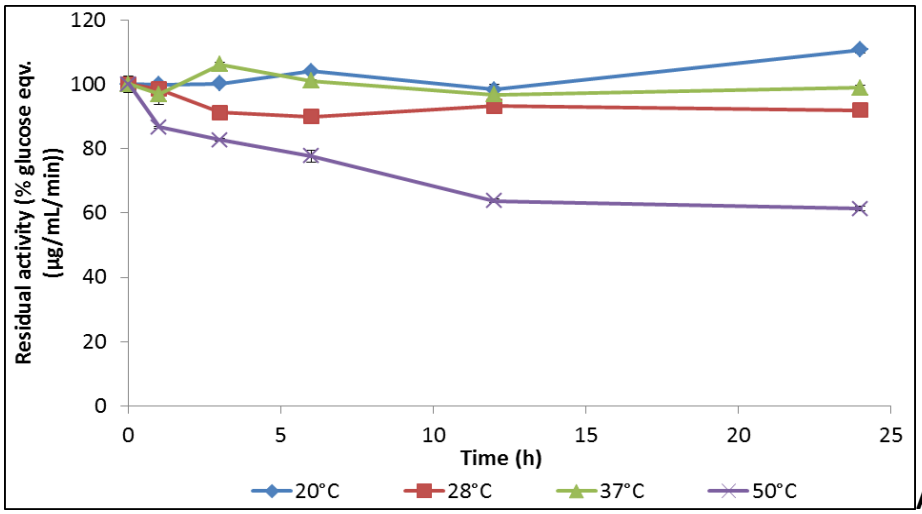


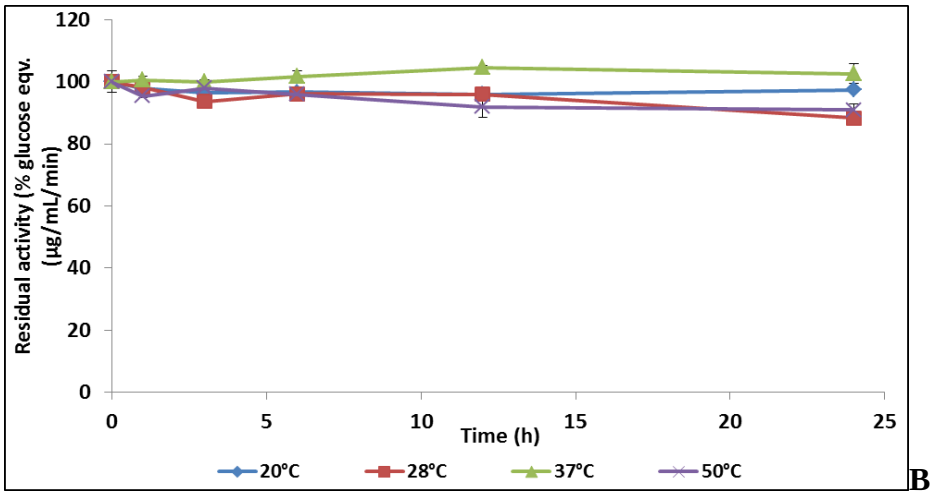
Figure 2.3: Temperature optima for Viscozyme L and Celluclast 1.5L activity. Activity was measured on 5% (wet w/v) apple pomace using a temperature range of 20-70°C and a citrate buffer at pH 5.0. Data points are presented as mean values ±SD (n=3).

The temperature optima for both enzyme mixtures were at 50°C, however Celluclast 1.5L had a broad temperature range from 25–60°C, while Viscozyme L displayed two peaks at 37°C and 50°C. The presence of two peaks may signify different optima for different enzymes in the commercial mixtures. Activity was drastically reduced for both enzymes at 70°C, which may be due to enzyme denaturation. The enzymes displayed the same temperature optima which allows for the enzymes to be used together in a bioreactor for lignocellulose degradation.

The results of the temperature stabilities are shown in Figure 2.4 (A and B) below. Residual activity of Viscozyme L and Celluclast 1.5L was measured at 20, 28, 37 and 50°C as high levels of activity were retained in this range. Viscozyme L maintained residual activity above 90% after 24 h incubation at 20, 28 and 37°C, however, the residual activity dropped to about 60% at 50°C. Celluclast 1.5 L showed higher temperature stability than Viscozyme L, maintaining activities above 90% at 20, 28, 37 and 50°C.



A



B

Figure 2.4: Residual activity for Viscozyme L (A) and Celluclast 1.5L (B) at temperature, 20, 28, 37 and 50°C. Residual activity was measured on 5% (wet w/v) apple pomace using citrate buffer pH 5.0. Residual activity was calculated as the activity obtained as a result of pre-incubation at a given temperature value divided by activity without pre-incubation at the same temperature value, which was then multiplied by 100. Data points are presented as mean values ±SD (n=3).

It was therefore concluded that the two enzyme preparations can be used together in a single bioreactor at a temperature between 20-37°C and pH 5.0, as both enzyme preparations exhibited high activity and stabilities under these conditions. These reaction conditions do not only offer high activity and stability, but are also important for industrial application in SSF and CBP bioreactor systems, where a temperature between 30-37°C is used for fermentation (Andric *et al*., 2010a, Van Dyk and Pletschke, 2012) which lowers energy costs as less or no heating will be required. Using the two enzyme preparations together in a bioreactor will help to lower industrial production costs and simplify operations. Therefore, the stability of Viscozyme L and Celluclast 1.5L was tested at pH 5.0 and temperature of 37°C for 15 days.

Enzymes can be used for a long period of time in a bioreactor, while products can be constantly removed from the bioreactor and fresh substrate added. Residual activities of Viscozyme L and Celluclast 1.5L at 37°C and pH 5.0 were measured on apple pomace using citrate buffer. The results for Celluclast 1.5L and Viscozyme L stabilities over a period of 15 days are shown in Figure 2.5. Celluclast 1.5L was used at a concentration of 10 µL/mL (0.76 mg/mL) as the amount of reducing sugar released at a concentration of 4 µL/mL (0.304 mg/mL) fell below the detectable limits of the DNS assay. A combination of Viscozyme L and Celluclast 1.5L was more stable than the individual enzyme mixtures and displayed more than 90% residual activity after 15 days. Both individual enzymes maintained residual activity of about 80% after 15 days, which made these enzyme mixtures very suitable for bioreactor applications.

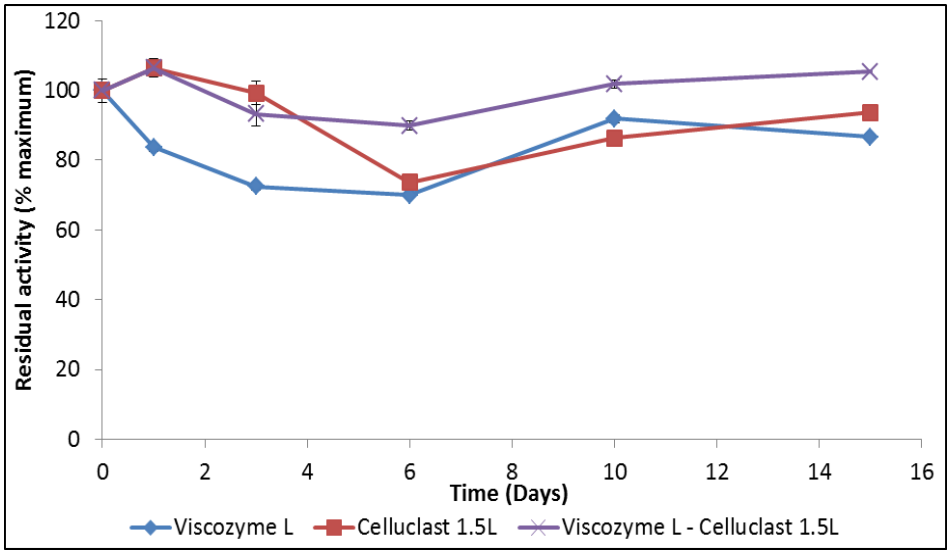


Figure 2.5: Residual activity of Viscozyme L and Celluclast 1.5L at 37°C and pH 5.0. Residual activity was measured on 5% (wet w/v) apple pomace using citrate buffer and with different enzyme concentrations of Viscozyme L and Celluclast 1.5L. Residual activity was calculated as the activity obtained as a result of pre-incubation at a given temperature value divided by activity without pre-incubation at the same temperature value, which was then multiplied by 100. Data points are presented as mean values ±SD (n=3).

The obtained results were in agreement with reaction conditions used in other studies, i.e. pH 4.5–6.0 and a temperature of 45-50°C for activity assays with Celluclast 1.5L (Boussaid and Saddler, 1999; Kristensen *et al*., 2009; Merino and Cherry, 2007; Ohgren *et al*., 2007; Suwannarangsee *et al*., 2012). Merino and Cherry (2007) and Sorensen *et al*. (2003) reported using a temperature of 50-60°C and pH 3.5-5.5 for assays with Viscozyme L. The selected

pH and temperature conditions for both Viscozyme L and Celluclast 1.5L were pH 5.0 and 37°C, respectively. NREL, a leading research group in lignocellulose hydrolysis, regularly employ pH 4.8–5.0 and temperature of 50°C as reaction conditions (Selig *et al*., 2008).

According to information obtained from the BRENDA enzyme database (http://www.brenda-enzymes.info/), enzymes from *Aspergillus aculeatus* show different pH and temperature optima; e.g. feruloyl esterase has a temperature optimum of 50–60°C and temperature stability 50°C, a pH optimum of pH 4.0-6.0 and a pH stability of pH 5.0; cellulase has a temperature optimum of 40°C and a pH optimum of pH 5.0; endo-1,5 arabinan endo-1,5-α-L-arabinosidase has a pH optimum of pH 5.5 and a pH stability of pH 5.5-6.3; pectin esterase has a pH range pH 4.5-8.0 and a temperature optimum of 22°C; polygalacturonase has a pH optimum of pH 3.0, a pH maximum of pH 4.5, a pH range pH 2.0-6.0, a temperature optimum of 60°C; β-glucosidase has a pH optimum of pH 3.0 (β-glucosidase 3), a pH 4.5 (β-glucosidase 1 and 2), pH 5.0, a temperature optimum of 60°C (β-glucosidase 2), 55°C (β-glucosidase 1), 65°C (β-glucosidase 3) and α-L-rhamnosidase has a pH optimum, pH 4.5–5.0, a temperature optimum of 30°C. This may explain the different peaks obtained by the two enzyme preparations, with Viscozyme L originating from *Aspergillus aculeatus.* The pH optimum range of pH 3.0–6.0 may be attributed to the presence of some of these enzymes, depending on the amounts available.

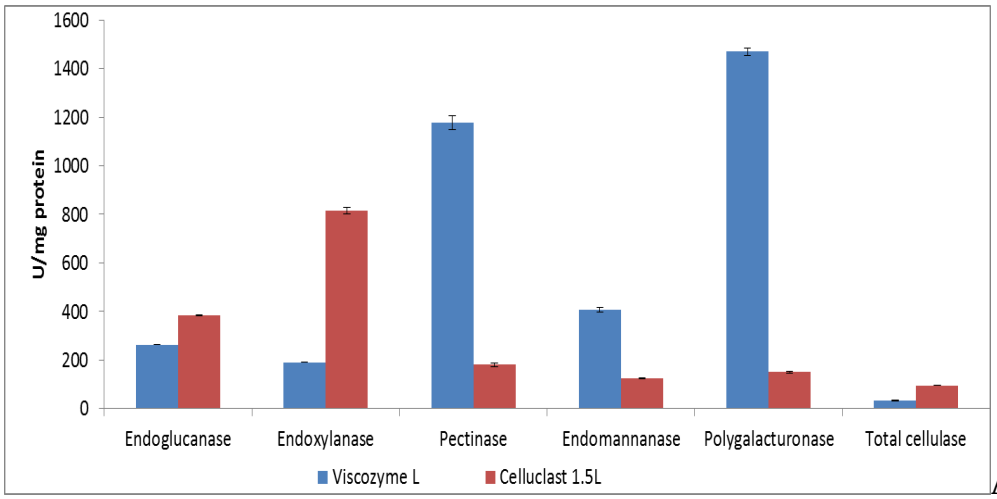
According to product data sheets obtained from the supplier, Viscozyme L contains mainly hemicellulase, arabanase, cellulase, β-glucanase, and xylanase and Celluclast 1.5L contain mainly cellulase.

2.4.2.3. Identification of enzyme activities in Viscozyme L and Celluclast 1.5L preparations

The different enzyme activities present in Viscozyme L and Celluclast 1.5L were determined using appropriate substrates and results are shown in Table 2.2 and Figure 2.6. The activities tested were selected based on the approximate structural composition of apple pomace (cellulose - 43.3%, hemicellulose -24.4% and pectin - 11.6% on a dry weight basis) and availability of enzyme substrates (Bhushan *et al*., 2008; Gullon *et al*., 2007; Joshi and Attri, 2006).

Table 2.2. Activities of Viscozyme and Celluclast on different substrates. Activities are expressed as reducing sugar equivalents released (µg/mL/min) per mg protein (endoglucanase, endoxylanase, endomannanase, pectinase, polygalacturonase and total cellulose) and 4-nitrophenol liberated (µmol/mL/min) per mg protein (cellobiohydrolase, β-D-glucosidase, β-D-xylosidase, β-D-mannosidase, α-L-arabinofuranosidase). Values are presented as mean values ±SD (n=3).

Substrate	Activity measured	Viscozyme	Celluclast
Carboxymethylcellulose	Endoglucanase	263.6(±1.6)	385.1(±2.4)
Birchwood xylan	Endoxylanase	191.1(±1.5)	813.9(±12.5)
Pectin	Pectinase	1177.3(±28.3)	180.6(±7.9)
Locust bean gum	Endomannanase	406.5(±10.7)	124.4(±0.8)
Polygalacturonic acid	Polygalacturonase	1470.7(15.6)	149.6(±2.7)
Filter paper	Total cellulase	33(±0.3)	95.2(±0.9)
4-nitrophenyl-β-D-cellobioside	cellobiohydrolase	0.004(±0.0002)	0.03(±0.001)
4-nitrophenyl-β-D-glucopyranoside	β-D-glucosidase	0.2(±0.001)	0.3(±0.002)
4-nitrophenyl-β-D-mannopyranoside	β-D-mannosidase	0.006(±0.0002)	0.0003(±0.0001)
4-nitrophenyl-β-D-xylopyranoside	β-D-xylosidase	0.005(±0.0002)	0.4(±0.002)
4-nitrophenyl-α-L-arabinofuranoside	α-L-arabinofuranosidase	0.4(±0.002)	0.06(±0.001)



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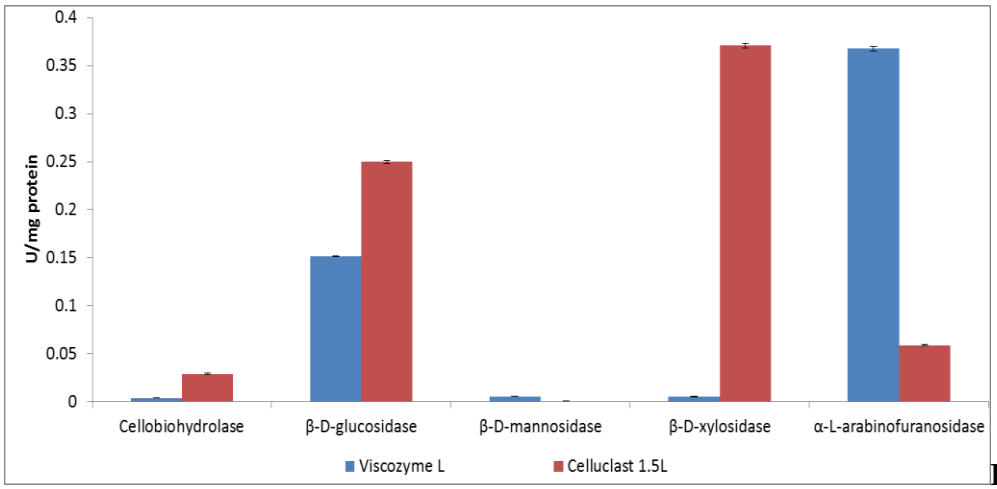


B

Figure 2.6: Activity of Viscozyme L and Celluclast 1.5L on different substrates. Activities are expressed (A) as glucose equivalents released (µg/mL/min) (U) per mg protein (endoglucanase, endoxylanase, endomannanase, pectinase, polygalacturonase and total cellulase), (B) 4-nitrophenyl liberated (µmol/mL/min) (U) per mg protein (Cellobiohydrolase, β-D-glucosidase, β-D-xylosidase, β-D-mannosidase, α-L-arabinofuranosidase). Data points are presented as mean values ±SD (n=3).

The results indicated that both Viscozyme L and Celluclast 1.5L contain many enzymes in different proportions. However, Viscozyme L and Celluclast 1.5L preparations may also contain other enzymes that were not tested in this study. Viscozyme L had higher pectinase, polygalacturonase, endomannanase and α-L-arabinofuranosidase activity than Celluclast 1.5L (Table 2.2 and Figure 2.6). These results indicate that Viscozyme L contains mainly hemicellulases and will act more on the hemicellulose and pectin component of apple pomace, releasing mainly mannose, arabinose and galacturonic acid. On the other hand, Celluclast 1.5L had more β-xylosidase, endoxylanase, β-glucosidase and total cellulase activity than Viscozyme L. This is an indication that Celluclast 1.5L will hydrolyse the

cellulose and xylose chains of apple pomace, releasing mainly cellobiose, glucose and xylose. Combining Viscozyme L and Celluclast 1.5L will give an enhanced hydrolysis of both the cellulose and hemicellulose components of apple pomace. Hemicellulases will act on the hemicellulose part, hence opening up the structure and enabling cellulases to act on cellulose. SDS-PAGE results (Figure 2.7) also showed the presence of different proteins in both Viscozyme L and Celluclast 1.5L. Although some of the proteins were of the same size, the majority of the proteins were of different sizes, suggesting the presence of different enzymes in different amounts in the two enzyme preparations. Rahikainen *et al*. (2011) reported the molecular weights of some enzymes in a cellulase system of *T. reesei*, Cel7B (EG I) 50-55 kDa, Cel5A (EG II) 48 kDa and Cel6A (CBH II) 50-58, which corresponded to the bands found in Celluclast 1.5L (Figure 2.7). The presence of different enzymes and their amounts in both Viscozyme L and Celluclast 1.5L enable their activities to complement one another, which may lead to increased efficiency and synergism in biomass degradation.

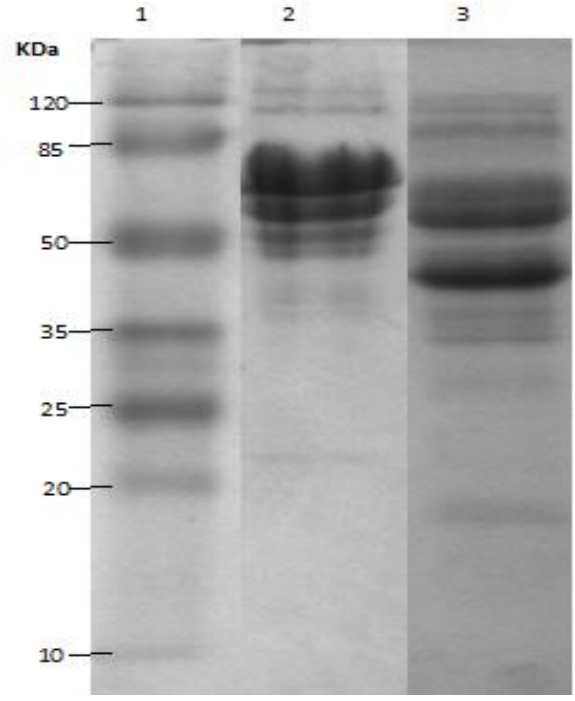


Figure 2.7: SDS-PAGE photograph of Viscozyme L and Celluclast 1.5L with 10% resolving gel and 4% stacking gel. Lanes: 1 – Marker protein, 2 – Celluclast 1.5L, 3 – Viscozyme L.

The amount of cellobiohydrolase in the two enzyme preparation mixtures was generally low and a cause of concern. Celluclast 1.5L had more cellobiohydrolase activity than Viscozyme L. Cellobiohydrolase is required in the processive cleavage on glycosidic bonds from both reducing and non-reducing ends and is not affected by the degree of crystallinity (Teeri,

1997). There may be a need to supplement cellobiohydrolase activity in these enzymes in order to maximally degrade the crystalline cellulose in apple pomace, hence releasing more reducing sugars.

The obtained results agreed with Merino and Cherry (2007), who suggested that Celluclast 1.5 L had more β-xylosidase activity than Viscozyme L. The results are also in agreement with Garcia-Aparicio *et al*. (2006) and Berlin *et al*. (2007) who indicated that Celluclast 1.5L contained mainly cellulase activity, but also had β-xylosidase activity, hence it is capable of catalysing the hydrolysis of xylobiose and xylotriose to xylose. Many authors reported supplementation of β-glucosidase activity in Celluclast 1.5L with Novozyme 188 (cellobiase) on different substrates (Bansal *et al*., 2009; Boussaid and Saddler, 1999; Hsu *et al*., 2010; Pryor and Nahar, 2010; Romani *et al*., 2010; Van Dyk and Pletschke, 2012; Varnai *et al*., 2010; Zheng, 2006), which indicates that the amount of β-glucosidase in Celluclast 1.5L was low. Combining both Celluclast 1.5L and Viscozyme L will complement the amount of β-glucosidase to amounts that may be sufficient to prevent inhibition by cellobiose. Ohgren *et al*. (2007) also reported that xylanase supplementation with Celluclast 1.5L increased initial rates of xylose and glucose hydrolysis. Increasing hemicellulose and pectin hydrolysis results in improved cellulose hydrolysis. However, the conditions maximising the individual sugar yields are often not the same as those maximising the total sugar yield.

A recent study by Suwannarangsee *et al*. (2012) identified a number of enzymes and some proteins in Celluclast using LC/MS/MS. The identified enzymes included two exoglucanases, five endoglucanases, one xyloglucanase, two β-xylosidases, two endoxylanases and non-hydrolytic proteins such as Cip1 and 2 and swollenin, which cannot be detected using enzyme assays. However, they did not identify any pectinases and mannanases, which were found through activity assays in our study. These enzymes were possibly only present in low concentrations and thus could not be detected. However, Kovacs *et al*. (2009) reported the presence of mannanase and mannosidase activities in Celluclast. Swollenin is believed to play an active role in weakening cellulose in lignocellulose biomass, and Cip1 and 2 contain cellulose binding modules which are critical for cellulose hydrolysis (Suwannarangsee *et al*., 2012). These proteins can contribute to the degradation of a substrate, although they themselves don't cleave hydrolytic bonds. One type of activity, identified by Suwannarangsee *et al*. (2012), but not identified in our study, was xyloglucanase activity. No specific enzyme assay was performed to identify this enzyme in our study. Xyloglucanases

act on xyloglucan - a polysaccharide component in primary cell walls, but which is also present in fruit such as apple. Some endoglucanases may also display activity on xyloglucan, which complicates identification in assays. However, the presence of a xyloglucanase could certainly enhance apple pomace hydrolysis.

The obtained results were consistent with other researchers, namely that there was low β-glucosidase activity, but high cellobiohydrolase activity in Celluclast 1.5L (Berlin *et al*., 2007; Suwannarangsee *et al*., 2012). Viscozyme L is likely to contain multiple proteins as well. Knowing the protein composition of the commercial enzyme mixtures will assist in optimisation of the enzyme mixtures and ratios, for example, accessory enzymes and lacking activities can be added using commercial available enzymes. Berlin *et al*. (2007) reported that addition of β-glucosidase to Celluclast 1.5L resulted in the increase in glucan-to-glucose conversion by 38% in pre-treated corn stover. Optimisation of the enzyme mixture will also depend on the targeted yield e.g. glucose, xylose or galacturonic acid. Celluclast 1.5L was also reported to contain acetyl-xylan esterase, which is important in the removal of acetyl groups on xylose residues of acetylxylan (Sipos, 2010). According to Pettitt *et al*. (2004), Viscozyme L contains cellulase, endo-β-glucanase, feruloyl esterase, endo-1,4-β-xylanase and endo-α-L-arabinosidase, which are all important for lignocellulose degradation. The reported information is in agreement with the results obtained in our study as some of these activities were also identified.

The use of commercial enzyme preparation mixtures will be easy and cost-effective as the mixtures are already optimized to some extent, rather than optimising mixtures of different individual enzymes. Optimisation of the enzyme mixtures will not only enhance hydrolysis but also reduce enzyme loadings for a specific biomass and prevent product inhibition. This will result in the reduction of enzyme and process costs for industrial applications.

2.4.2.4. Determination of substrate loading, K_m and V_{max} on apple pomace

Optimal apple pomace concentration was determined using Viscozyme L: Celluclast 1.5 L (50:50) at a concentration of 2 µL/mL (0.019 mg/mL). There was a more or less a linear relationship between the amount of reducing sugars released and the increase in apple pomace concentration up to 800 mg/mL (Figure 2.8). At concentrations above 800 mg/mL apple pomace, the rate of reducing sugar release slowed down. It was important to include higher concentrations of apple pomace until the amount of reducing sugar released became

constant, however, it was difficult to pipette higher concentrations of the apple pomace. As substrate concentration was increased, little buffer was used in the total volume of 1.2 mL used in the standard assay. This would not only affect the final pH of the mixture, as the buffering capacity may be reduced, but also the accessibility of the enzyme to the substrate. At high substrate concentrations, the mixture became viscous and mixing became difficult, which may have resulted in less interaction between the lignocellulose and the enzymes, due to reduced enzyme transport mechanisms. There would also be reduced mass transfer for intermediates and end-products (Andric *et al*. 2010b; Brown *et al*., 2010; Kristensen *et al*., 2009). High initial substrate concentrations, though they may increase the product, may lead to substrate inhibition and problems associated with product inhibition, which will eventually lower the rate of hydrolysis (Jorgensen *et al*., 2007; Sipos, 2010; Sun and Cheng, 2002). This is a challenge especially in batch reactions. Therefore, low to medium substrate concentrations are recommended (Yang *et al*., 2009).

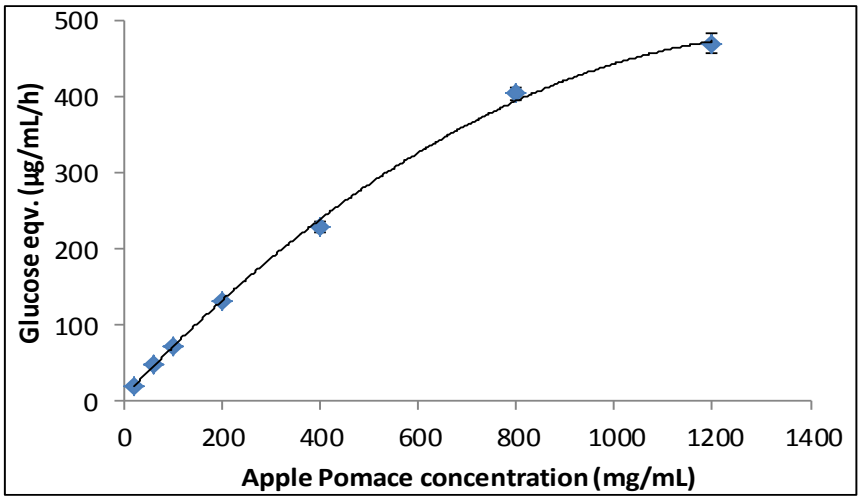


Figure 2.8: Activity of Viscozyme L - Celluclast 1.5L (50:50) on different apple pomace concentrations (mg/mL, wet w/v). Data points are presented as mean values ±SD (n=3).

The obtained results appear to follow a Michaelis-Menten pattern. The Michaelis-Menten model has been reported to have limited application in lignocellulose hydrolysis, however it has been used successfully to predict complex systems e.g. in pure cellulose and lignocellulose reactions by some researchers (Brown *et al*., 2010; Kadam *et al*., 2004, O'Dwyer *et al*., 2007).

A Lineweaver-Burk plot was prepared to determine the V_{max} and K_m apparent values for the apple pomace substrate (Figure 2.9). The V_{max} apparent was calculated to be 416.7 mg/mL/min and the K_m was 409.3 mg/mL. These figures were probably high due to insolubility and the highly heterogeneous nature of apple pomace. The enzyme kinetics in lignocellulose degradation is not completely understood and a number of models have been proposed in literature, such as empirical models, Michaelis-Menten based models, adsorption in cellulose based models and models on soluble cello-oligosaccharides (Bansal *et al*., 2009). Michaelis-Menten based models are best suited to homogenous and soluble substrates, and not to insoluble, heterogeneous lignocellulose substrates. Although there may be an excess of substrate initially, the substrates become depleted over time, and therefore the conversion rate changes, and therefore K_m. Enzymatic hydrolysis also occurs first on the substrate surface in heterogeneous substrates such as lignocellulose (Bansal *et al*., 2009) and then the liquid phase for the soluble intermediates and cellobiose. The substrate is therefore, hydrolysed at different rates, depending on the site that the enzymes will be attacking. The catalytic rates also changes as the more easily hydrolysed parts of the substrate are hydrolysed first and the complex parts later and more slowly, e.g. the amorphous cellulose and crystalline cellulose regions, respectively (Andric *et al*., 2010b).

In this study, not only the insolubility and heterogeneous nature of the apple pomace complicated the kinetics, but also the fact that multiple enzymes were used. Although the concentration of the substrate is known, it's difficult to determine the true concentration of the accessible substrate. The substrate concentration used in experimental analyses should ideally be 10x K_m so that there will be excess substrate, but this couldn't be attained practically in this study. At high substrate concentrations, enzyme activity doesn't change with change in substrate concentration as substrate saturation would have been achieved (Ong *et al*., 2012). In order to keep the hydrolysis conditions constant, a stock concentration of 200 mg/mL (20%) (5% wet, w/v in final reaction) apple pomace was used in all subsequent experiments.

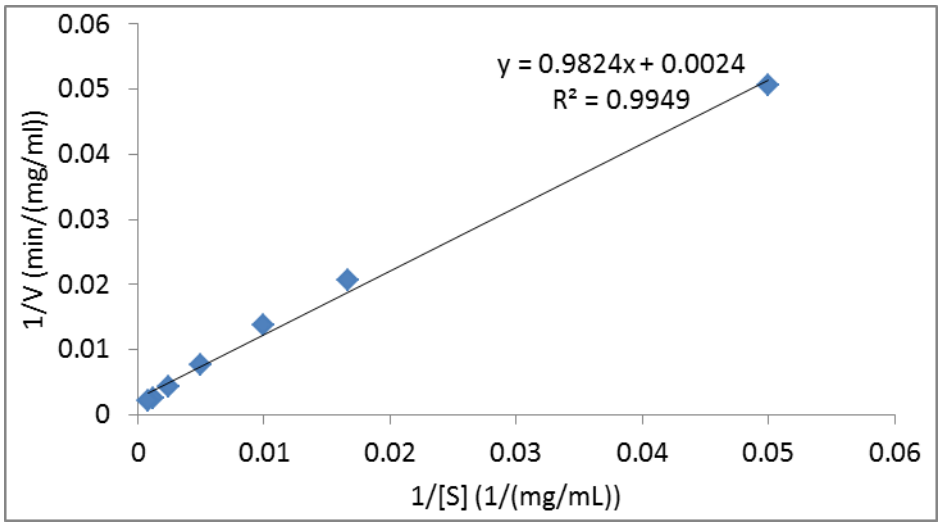


Figure 2.9: A Lineweaver-Burk plot using activity of Viscozyme L - Celluclast 1.5L (50:50) and different apple pomace concentrations.

2.4.2.5. Influence of alcohols, sugars, organic acids, lignin, phenolic compounds and metal ions on Viscozyme L and Celluclast 1.5L

The optimal combination and synergy for Viscozyme L and Celluclast 1.5L was found to be at a ratio of 50:50 and therefore this combination was used in subsequent experiments. Hydrolysis of cellulose and hemicellulose results in the production of sugars such as glucose, cellobiose, cello-oligomers, xylose, xylo-oligomers, mannose, galactose and arabinose. The sugars are then fermented by yeast or bacteria to produce alcohols (Banerjee *et al*., 2010a; Sun and Cheng, 2002; Tejirian and Xu, 2011; Van Dyk *et al*., 2013). The main alcohols produced are ethanol, butanol and propanol. Dien *et al*. (2003) reported alcohol concentrations in the range of 23–63 g/L resulting from fermentation. These alcohols may cause inhibition of cellulases and hemicellulases in the bioreactor if using simultaneous hydrolysis and fermentation (SSF) or to the organisms if using the consolidated bioprocessing (CBP) approach.

Enzymatic hydrolysis of polysaccharides results in the production of small sugars that may result in an inhibition of the enzymes. Glucose and cellobiose are the main products of cellulose degradation, while xylobiose and xylose are the main products of hemicellulose degradation in apple pomace and arabinose and galactose arise from the pectin component of the apple pomace (section 2.4.1). These products may cause end-product inhibition of the enzymes in the cellulose and xylose degradation pathways, respectively. The concentrations of the sugars tested may be lower than what can possibly be released from apple pomace,

however, use of high sugar concentrations interfered with the DNS assay. The effect of sugars on activity of Viscozyme L, Celluclast 1.5L and the Viscozyme L -Celluclast 1.5L combination were therefore determined using apple pomace as a substrate and results are shown in Figure 2.10. The selection of concentrations was based on literature values for formation of products from lignocellulosic biomass hydrolysis. The Viscozyme L: Celluclast 1.5L combination activity was more inhibited in the presence of cellobiose as compared to glucose. Xylobiose had a higher inhibitory effect on the enzymes than xylose and at 1.5 and 2 mM concentration it was more inhibiting than glucose and at 0.25 mM it was more inhibiting than cellobiose. The activity of Viscozyme L and the combined enzymes decreased with an increase in xylobiose concentration, resulting in the activity of the enzyme combination decreasing to around 42% at a 2 mM xylobiose concentration. This is probably due to the presence of higher β-xylosidase activity in Celluclast 1.5L than in Viscozyme L, which results in the hydrolysis of the xylobiose to xylose. This is in agreement with the report by Garcia-Aparicio *et al*. (2006) which indicated that as β-xylosidase activity is present in Celluclast 1.5L, it can hydrolyse xylobiose and xylotriose to xylose. A decrease in Celluclast 1.5L activity after 1.5 mM xylobiose concentration may be due to xylose accumulation.

The Viscozyme L and Viscozyme L- Celluclast 1.5L combination displayed a reduction in activity with increased cellobiose concentration, resulting in residual activities decreasing to around 40% at 2 mM cellobiose concentration. Celluclast 1.5L has more β-glucosidase activity than Viscozyme L, therefore Celluclast 1.5L may be hydrolysing more cellobiose into glucose. The marked decrease in activity by the enzyme combination may be due to the inhibitory effect of glucose, as more glucose will be produced from the combined effect of the two β-glucosidase activities. Kristensen *et al*. (2009) reported that cellobiohydrolases are indirectly inhibited by glucose, as high concentrations of glucose inhibits β-glucosidase, there will be an accumulation of cellobiose, which in turn strongly inhibits cellobiohydrolases. Cellobiose was reported to directly inhibit both cellobiohydrolases and endoglucanases, while glucose inhibited β-glucosidase (Andric *et al*., 2010c; Teeri, 1997). Holtzapple *et al*. (1990) reported that glucose inhibited cellobiohydrolases and endoglucanases directly. However, individual enzyme preparations were affected differently in the presence of sugars compared to the combined enzyme preparations, due to the different enzymes found in each mixture.

The combined enzyme mixtures were inhibited to a greater degree in the presence of glucose with activity decreasing to around 53% at 2 mM, while Celluclast 1.5L and Viscozyme L

decreased to 67% and 71%, respectively. Generally, the activity of both enzymes and the combination decreased with an increase in xylose concentration, with Viscozyme L being more resistant to xylose inhibition, maintaining activity above 80% even at a 2 mM xylose concentration (data not shown). Celluclast 1.5L was not inhibited by xylobiose concentrations of up to 1 mM, but concentrations above this resulted in a marked decrease in activity to about 60% at 2 mM (data not shown). The results were in agreement with reports by Corazza *et al*. (2005), Garcia-Aparicio *et al*. (2006), Kumar and Wyman (2009), Qing and Wyman (2011), Qing and Wyman (2010) and Shi *et al*. (2011), who reported that xylobiose and xylo-oligomers inhibited cellulases strongly and more so than xylose, glucose and cellobiose. Bezerra *et al*. (2006) reported that ethanol and cellobiose are inhibitors of exoglucanase and that cellobiose inhibited cellulase more than glucose. However, the influence of the sugars on individual enzymes is not known, since a combination of the two commercial enzyme preparations was used.

There was an inhibition of the enzymes by both arabinose and galactose at a level of 45-50% residual activity in the presence of 2 mM concentrations for the combined mixtures and Celluclast 1.5L. Galactose and arabinose have also been reported to inhibit cellulases (Garcia-Aparicio *et al*., 2006; Xiao *et al*., 2004), which was in agreement with the results obtained in this study. The obtained results indicate that galactose inhibit the enzymes at concentrations above 0.5 mM. However, the concentration of these sugars is low in apple pomace as compared to glucose (section 2.4.1). Other sugars like fructose may also be released during apple pomace hydrolysis, but their levels are too low to exert a significant effect on the enzymes.

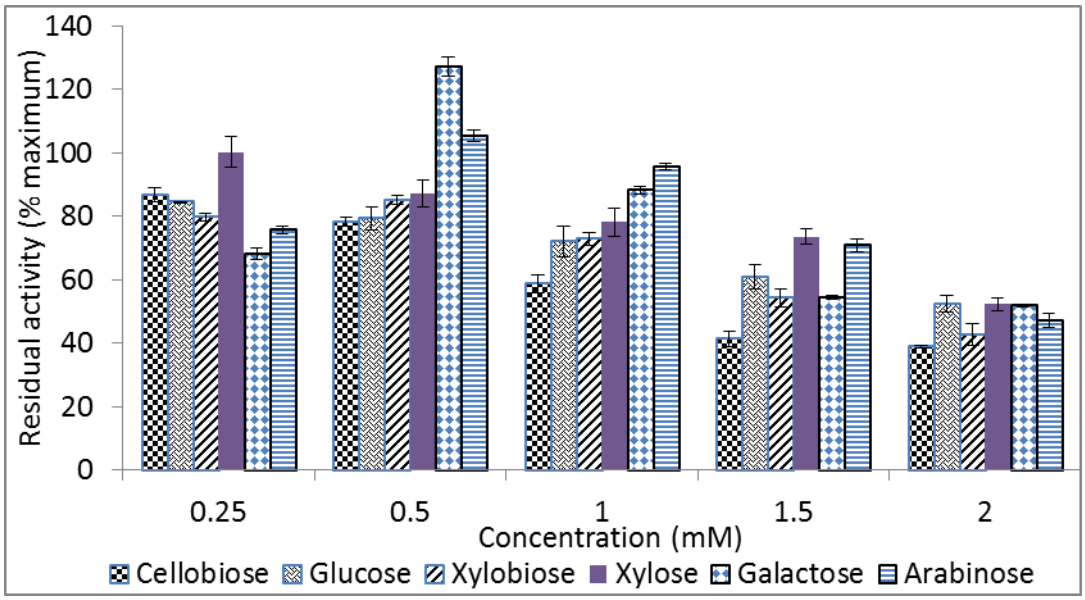


Figure 2.10: Viscozyme L and Celluclast 1.5L (50:50 combination) residual activity in the presence of different sugar concentrations - cellobiose, glucose, xylobiose, xylose, arabinose and galactose. Residual activity was calculated as the activity obtained at each sugar concentration divided by the activity without sugar included, which was then multiplied by 100. Activity was measured using 5% (wet w/v) apple pomace as substrate. Data points are presented as mean values ±SD (n=3).

Hydrolysis products may also cause a reduction in enzyme hydrolysis by inhibiting the binding of enzymes to their substrates or by the non-productive binding/adsorption of the enzymes (Kumar and Wyman, 2009). Use of simultaneous saccharification and fermentation bioreactors will assist in preventing inhibition of hydrolysis/ saccharification enzymes by sugars formed, as the sugars will be directly fermented. This does not only increase the hydrolysis rate of the lignocellulose and prevent end-product inhibition, but also a reduction in reactor volumes (as one reactor will be used instead of two reactors) and a reduction in the amount of β-glucosidase required. However, this bioreactor set-up has its own challenges in terms of finding reaction conditions (e.g. temperature and pH) for both hydrolytic enzymes and fermentation microbes.

Saccharification of polysaccharides may be carried out with the objective of biofuel production. Where the fermentation into alcohols is carried out in the same bioreactor, the production of alcohols may display an effect on enzyme activity. Investigations in this regard have mainly focused on the effect of alcohol production on the organism involved in fermentation. However, studies have indicated that alcohols may have an impact on enzymatic activity (Morrison *et al*. 2011; Van Dyk *et al*. 2009). The influence of butanol,

propanol and ethanol on the enzymatic hydrolysis of apple pomace by Viscozyme L and Celluclast 1.5L was examined at 10-60 g/L alcohol concentrations and the results are shown in Figure 2.11. Butanol exhibited the highest inhibitory effect and ethanol was the least inhibitory to Viscozyme L and Celluclast 1.5L. Their inhibition was proportional to the concentration of alcohol used. Celluclast 1.5L was more sensitive to the presence of butanol with activity falling below 60% at 40–60 g/L. Morrison *et al*. (2011) reported a high inhibitory effect on XynA (endoxylanase) activity by butanol compared to ethanol and propanol, which was in agreement with the obtained results in our study since Celluclast 1.5L has more β-xylosidase and xylanase activities (Table 2.2). The activity of the enzymes generally decreased with an increase in propanol concentration, with Viscozyme L being less sensitive to the presence of propanol than Celluclast 1.5L and the Viscozyme L-Celluclast 1.5L combination.

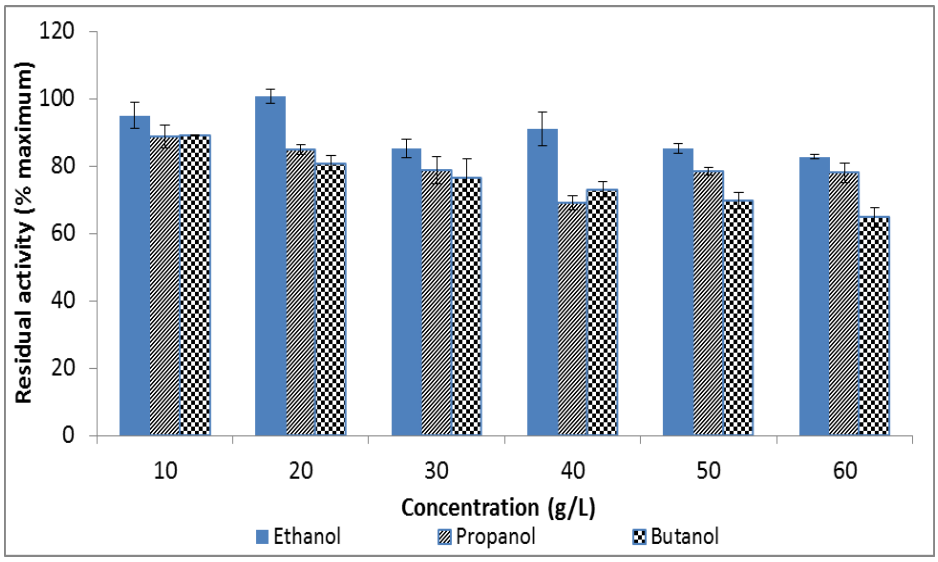


Figure 2.11: Viscozyme L and Celluclast 1.5L (50:50 combination) residual activity in the presence of different alcohol concentrations - ethanol, propanol and butanol. Residual activity was calculated as the activity obtained at each alcohol concentration divided by the activity with no alcohol included, this was then multiplied by 100. Activity was measured using 5% (wet w/v) apple pomace as substrate. Data points are presented as mean values ±SD (n=3).

Wu and Lee (1997) reported that cellulases were inhibited by 64% at concentrations of 60 g/L ethanol. With more than 80% activity remaining at a concentration of 60 g/L ethanol in our study, it demonstrates that the enzymes have a good tolerance to the presence of ethanol, an advantage in bioreactor applications where hydrolysis is coupled with fermentation (SSF), since ethanol will be the main alcohol produced in the bioreactor. A minimum ethanol

concentration of 5% (v/v) is considered economically viable for industrial application (Chen and Jin, 2006). Ethanol is reported to uncompetitively inhibit cellulases due to ethanol forming a complex with enzyme-substrate complex as ethanol may affect the non-catalytic sites of the enzymes, resulting in conformational changes (Chen and Jin, 2006; Ghosh *et al*., 1982; Holtzapple *et al*., 1990). Cellulase inhibition by ethanol was found to be reversible at low concentrations (1-7%) (Chen and Jin, 2006). According to Ghosh *et al*. (1982), ethanol showed a distinct inhibition of cellulases at 7.5 g/L concentration and at 75 g/L half of the enzyme activity was lost, which was similar to the results obtained in this study. They also reported that cellobiose was most inhibitory to cellulases, followed by glucose and then ethanol, which is in agreement with that obtained in our study (Bezerra *et al*., 2006; Ghosh *et al*., 1982).

The results showed that Celluclast 1.5L was inhibited to a greater degree in the presence of ethanol than Viscozyme L, which can be attributed to the fact that ethanol is reported to inhibit cellulases (Kristensen *et al*., 2009; Podkaminer *et al*., 2011). Chen and Jin (2006) reported that β-glucosidase activity increased with an increase in ethanol concentration between 1 and 9% (10-90 g/L), which may be due to ethanol acting as a nucleophile to β-glucosidase, affecting its flexibility. The results may explain why Celluclast 1.5L (more β-glucosidase activity than Viscozyme L) had more than 100% residual activity at 10% ethanol concentration. Ethanol has also been reported to have an inhibitory effect on cellulases and xylanases (Holtzapple *et al*., 1990; Morrison *et al*., 2011; Taherzadeh and Karimi, 2007; Van Dyk *et al*., 2009).

Organic acids may be present in a bioreactor for different reasons. They may be produced through a fermentative pathway or through hydrolysis of the substrate, for example galacturonic acid is released from the degradation of apple pomace (Balat, 2011; Chen *et al*., 2008; Chundawat *et al*., 2011; Foyle *et al*., 2007; Howard *et al*., 2003; Lavelli and Corti, 2011; Shalini and Gupta, 2010). The influence of selected organic acids on the enzymatic hydrolysis of apple pomace by Viscozyme L and Celluclast 1.5L was investigated at 1, 5 and 10 g/L organic acid concentrations and the results are shown in Figure 2.12. Viscozyme L and Celluclast 1.5L maintained activities above 80% in the presence of acetic acid, butyric acid and citric acid at all the concentrations tested (1, 5 and 10 g/L). The most inhibitory acids were malic acid, formic acid and lactic acid, with enzyme activities falling below 40% at a 10 g/L concentration. Inhibition was concentration dependent. Inhibition by organic acids

is probably due to denaturation of the enzymes as a result of changes in pH (Mills *et al.*, 2009). The initial pH at 10 g/L organic acid ranged from pH 3.2 to pH 3.75 for formic acid, malic acid, lactic acid and citric acid. Though pH in this range may be inhibitory to some enzymes, it may be best for other enzymes in the enzyme mixtures (section 2.4.2.2). Lactic acid maintained more than 90% activity even at 10 g/L concentration, which may imply that the drop in pH may not be the only reason for the drop in activity for the other acids.

Galacturonic acid was less inhibitory to the Viscozyme L and Celluclast 1.5L combination and maintained the enzyme activity above 70% at all concentrations tested. There was a slight decrease in enzyme activity with increase in galacturonic acid concentration. This is an advantage for apple pomace hydrolysis since galacturonic acid is one of the main products of apple pomace degradation.

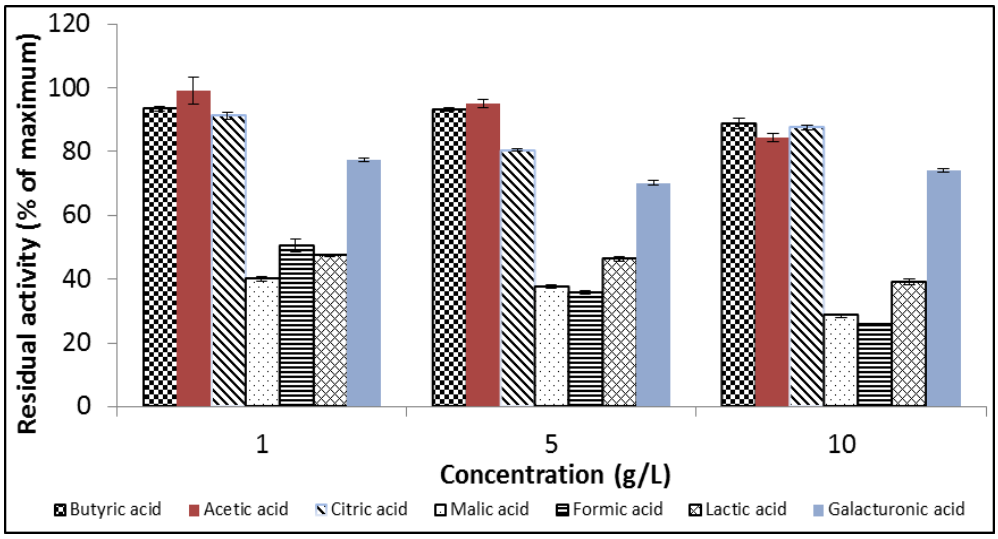


Figure 2.12: Viscozyme L and Celluclast 1.5L (50:50 combination) residual activity in the presence of different organic acid concentrations. Residual activity was calculated as the activity obtained at each acid concentration divided by activity with no acid included, which was then multiplied by 100. Activity was measured using 5% (wet w/v) apple pomace as substrate. Data points are presented as mean values ±SD (n=3).

The individual enzyme preparations are affected differently in the presence of different acids, but the Viscozyme L - Celluclast 1.5L combination was more stable than the individual enzyme preparations. At 10 g/L formic acid, both Viscozyme L and Celluclast 1.5L had lost all their activity, while the combination exhibited 26% activity. Malic acid, lactic acid and galacturonic acid were more inhibitory to Viscozyme L than Celluclast 1.5L, e.g. in the

presence of 10 g/L malic acid, the activity of Viscozyme L was reduced to 20% and that of Celluclast 1.5 L to 25%. The fact that galacturonic acid inhibited Viscozyme L more than Celluclast 1.5L may be due to the fact that Viscozyme L contains mainly hemicellulases and pectinases (Table 2.2), which results in the release of more galacturonic acid, hence increasing the concentration of galacturonic acid in the reaction media. The results indicate that hemicellulases and pectinases were more prone to organic acid inhibition than cellulases. Inhibition by organic acids is probably due to denaturation of the enzymes as a result of changes in pH (Mills *et al.*, 2009). The organic acids also inhibit microbial fermentation by inhibiting their growth and metabolism e.g. formic acid and acetic acid have been reported to have an inhibitory effect on microbial fermentation (Cho *et al*., 2009; Okuda *et al*., 2008; Parmar and Rupasinghe, 2012; Takahashi *et al*., 1999; Zaldivar and Ingram, 1999). Considering these results, separate hydrolysis and fermentation will be more ideal to prevent enzyme inhibition. In our study, the effect of galacturonic acid, which is produced during hydrolysis, could have an impact on the performance of a bioreactor.

Lignin is recalcitrant in nature and hinders the degradation of cellulose and hemicellulose and it also bind to cellulases and hemicellulases unproductively, thereby lowering their activity. The effect of lignin at concentrations between 0.05-2 g/L on Viscozyme L and Celluclast 1.5L activity was determined using apple pomace as a substrate and the results are shown in Figure 2.13. Generally, Celluclast 1.5L, Viscozyme L and their combination was not very sensitive to the presence of lignin, with activities maintained over 80% at 2 g/L lignin concentration. Enzymes that contain CBMs have been reported to have a higher affinity for lignin than enzymes without CBMs, as the CBMs will aid enzyme-lignin interaction. However, domains on the enzyme surface are also important for binding to lignin (Rahikainen *et al*., 2011). The presence of lignin seems to have a slight stimulatory or no effect on the activity of Viscozyme L, which may be due to low levels of cellulase and CBMs.

The Celluclast 1.5L- Viscozyme L combination was inhibited to a greater degree by lignin than the individual enzyme preparations, which may be linked to individual enzymes being present in the mixtures and unproductive binding/ adsorption of the enzymes on lignin. It has been reported that some enzymes have a higher binding affinity to lignin than others e.g. cellobiohydrolases have a higher binding affinity for lignin as compared to endoglucanases (Boussaid and Saddler, 1999). Endoglucanase, CBH and β-glucosidase activity were found to

be inhibited by both enzyme and acid hydrolysed lignin residues, with β-glucosidase showing high adsorption. However, all enzymes present in Celluclast 1.5 L were found to bind to lignin residues to a similar extent (Rahikainen *et al*., 2011). Enzymes such as cellulases, xylanases and β-glucosidase have been found to be inhibited by lignin, but β-glucosidase was the least affected (Berlin *et al*., 2006; Senior *et al*., 1991; Sewalt *et al*., 1997). Low soluble lignin concentrations were found to activate xylanases (Kaya *et al*., 2000), but Morrison *et al*. (2011) reported a 25% reduction in XynA activity at 0.075 g/L lignin concentration, with no further inhibition at higher lignin concentrations. In contrast to the obtained results, Lin *et al*. (2010) reported that the presence of free lignin had no inhibitory effect on cellulose and hemicellulose enzymatic hydrolysis and Palonen *et al*. (2004) reported no adsorption of cellulases on lignin.

The cellulases and hemicellulases are inhibited by lignin through adsorption onto the lignin surface (unproductive binding and steric hindrance), through hydrophobic and ionic interactions (lignin and enzyme surfaces contain COOH, OH and in some cases CO groups), hence the enzymes can't partake in hydrolysis (Berlin *et al*., 2006; Lin *et al*., 2010; Liu and Zhu, 2010; Rahikainen *et al*., 2011). In contrast, other factors that influence enzyme adsorption on lignin are surface area and the temperature used during adsorption. Low temperatures (4°C) have been found to reduce cellulase adsorption on lignin compared to high temperatures (45°C) (Rahikainen *et al*., 2011). The fact that Viscozyme L and Celluclast 1.5L are less inhibited by lignin offers an advantage to apple pomace degradation, since there is a considerable amount of lignin in apple pomace (Bhushan *et al*., 2008; Perdih *et al*., 1991).

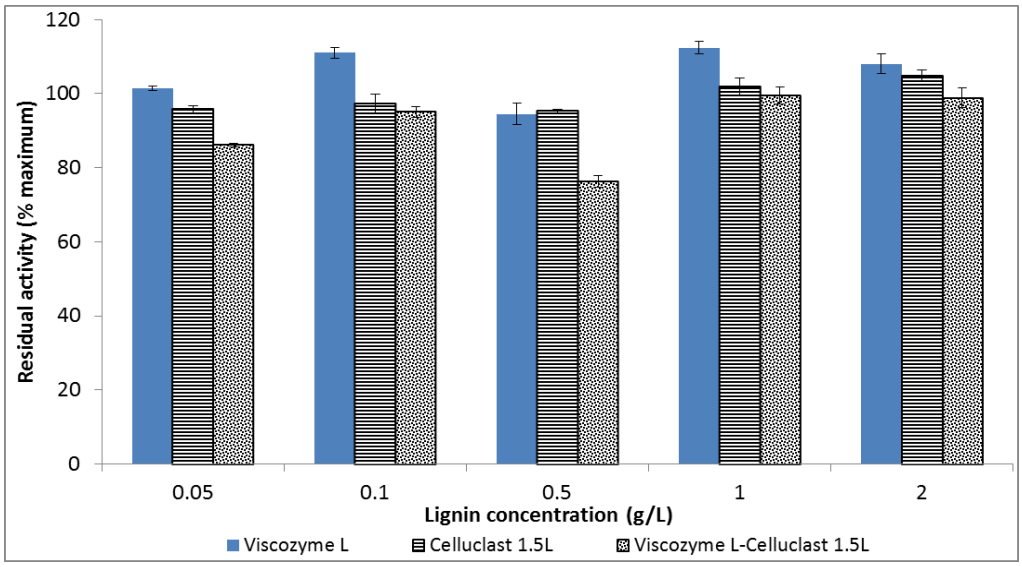


Figure 2.13: Viscozyme L and Celluclast 1.5L (50:50 combination) residual activity in the presence of different lignin concentrations. Residual activity was calculated as the activity obtained at each lignin concentration divided by the activity with no lignin included, which was then multiplied by 100. Activity was measured using 5% (wet w/v) apple pomace as substrate. Data points are presented as mean values ±SD (n=3).

Degradation products of lignin may cause more inhibition to the enzymes (Ximenes *et al*., 2010) than lignin itself. These only become important when there is a pre-treatment step for lignin degradation (Hendriks and Zeeman, 2009; Jonsson *et al*., 2013; Palmqvist and Hahn-Hagerdal, 2000) and when both hydrolysis and fermentation takes place at the same time in the same bioreactor. A number of possible inhibitors can be produced during pre-treatment and hydrolysis of the lignocellulose biomass, e.g. phenolics, guaiacol, vanillic acid, vanillin, 4-hydroxybenzoic acid, ρ-coumaric acid, hydroxyl-cinnamic, ferulic acid, furfural and acetovanillone and are fermentation inhibitors (Cho *et al*., 2009; Hendriks and Zeeman, 2009; Okuda *et al*., 2008; Palmqvist and Hahn-Hagerdal, 2000; Van Dyk and Pletschke, 2012). Phenolic aldehydes like syringaldehyde and vanillin are found in higher concentrations in lignocellulose hydrolysates. Apple pomace contains less phenolics as compared to other fruit wastes such as grape pomace (Arnous & Meyer, 2008; Bhushan *et al*., 2008; Watt *et al*., 1999). The influence of selected phenolic compounds at concentrations of 1, 5 and 10 g/L on Viscozyme L and Celluclast 1.5L are shown in Figure 2.14.

The Viscozyme L and Celluclast 1.5L combination activity was inhibited most by gallic acid with only 40% activity observed at 10 g/L, followed by syringic acid. The presence of syringic acid, vanillin, guaiacol and ρ-coumaric acid result in the enzymes maintaining more

than 80% activity at all concentration tested, with guaiacol being less inhibitory. However, individual enzyme preparations were also affected differently in the presence of the phenolic compounds. Celluclast 1.5L was less inhibited by gallic acid than Viscozyme L and their combination, with activity of 53%, 38% and 41% respectively at a 10 g/L concentration (data not shown). Viscozyme L was less inhibited by syringic acid and guaiacol than Celluclast 1.5L and their combination, with activity of 83%, 65% and 81% and 82%, 65% and 86%, respectively, using 10 g/L syringic acid and guaiacol concentration. These differences can be attributed to the different enzymes present in each enzyme preparation. The results that degradation products of lignin have a higher inhibitory effect on hydrolysis enzymes than lignin, agree with literature (Ximenes *et al*., 2010). Lignin degradation compounds such as gallic acid and vanillin have been found to have a 20-80% inhibitory effect on cellulases and β-glucosidase (Ximenes *et al*., 2011), which partly agrees with the obtained results. However, the source of the enzyme also has an effect on its inhibition.

Cho *et al*. (2009) tested six phenolic compounds which showed complete inhibition of butanol production at 1 g/L by *Clostridium beijerinckii* due to their inferences with conversion of Acetyl-CoA to butyryl-CoA. Acetone and ethanol production was also affected. The phenolic compounds were reported to inhibit enzymes by causing an increase in biological membrane fluidity and loss of cellular integrity resulting in leakage of cellular contents, hence affecting membranes and enzyme matrices (Cho *et al*., 2009; Mills *et al*., 2009; Palmqvist and Hahn-Hagerdal, 2000). Oligomeric phenolics were reported to inhibit cellulases more than simple phenolics and they reversibly inhibited cellulases by complexing them. They also inhibited cellulases by adsorbing onto cellulose (Tejirian and Xu, 2011). Phenolic compounds have also been reported to inhibit fermentative organisms like fungi and bacteria (Tejirian and Xu, 2011). Vanillin has been reported to inhibit fermentation at very low concentrations (Endo *et al*., 2008).

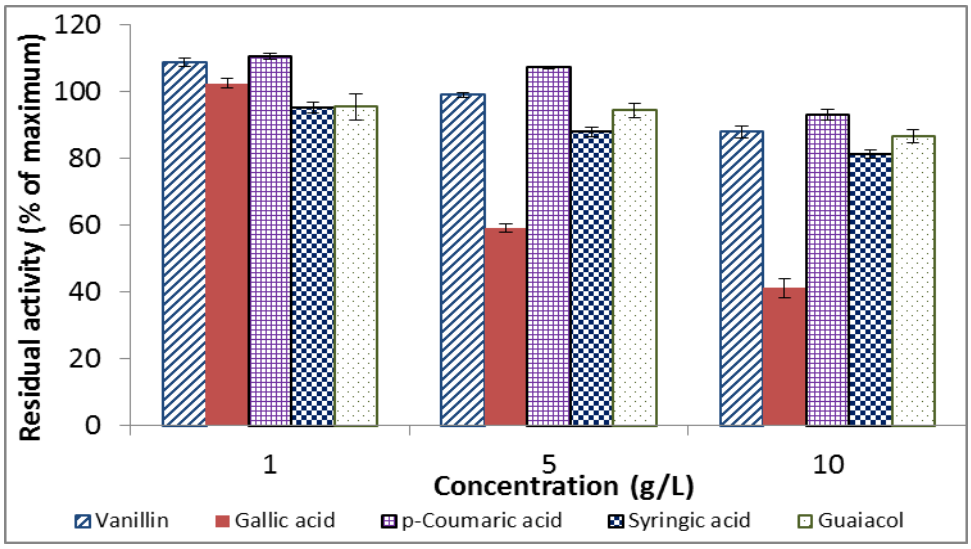


Figure 2.14: Viscozyme L and Celluclast 1.5L (50:50 combination) residual activity in the presence of different phenolic compounds concentrations. Residual activity was calculated as the activity obtained at each lignin concentration divided by the activity with no lignin included, which was then multiplied by 100. Activity was measured using 5% (wet w/v) apple pomace as substrate. Data points are presented as mean values ±SD (n=3).

Cellulosic enzymes are prone to interference by non-cellulosic substances such as metal ions, which may be present in biomass feed stocks or caused by upstream treatments, dissolved in water, or microbial activities. Metals ions can also come from corrosion of reaction vessels that are used during hydrolysis (Mussatto and Roberto *et al.*, 2004; Parajo *et al.*, 1996; Watson *et al.*, 1984). The effect of metal ions on Viscozyme L and Celluclast 1.5L was determined in the presence of 10 mM and 50 mM metals ions, with the results shown in Figure 2.15. The presence of NaCl and $CaCl_2$ had very little effect on the activity of Viscozyme L and Celluclast 1.5L combination. Activity was reduced to 98.2% at both 10 and 50 mM NaCl concentrations, $CaCl_2$ resulted in 101.3 % activity at 10 mM, but an increase in concentration resulted in a decrease in activity. Inhibition was concentration dependent for all the other metal ions tested. The most inhibiting metal ion was NH_4Cl with 46 and 40% residual activity at 10 and 50 mM, respectively. Celluclast 1.5L and Viscozyme L were affected by metals ions differently due to different enzymes that they contain (data not shown), e.g. Celluclast 1.5L had a residual activity of 135% and 53% at 10 and 50 mM $FeSO_4$, and 99 and 97% in the presence of 10 and 50 mM $MgSO_4$, respectively. In other cases, activity increased with an increase in metal ion concentration, e.g. in the presence of 10 and 50 mM NaCl, Viscozyme L residual activity was 85 and 90%, respectively. Viscozyme L maintained more than 50% residual activity in the presence of both 10 and 50 mM NH_4Cl. Celluclast 1.5L residual activity increased from 42 to 51% in the presence of 10

and 50 mM $CuSO_4$, respectively. Celluclast 1.5L residual activity was 98% at 10 mM $ZnSO_4$, but activity was lost at 50 mM concentration. Ca^{2+}, Mg^{2+}, Zn^{2+}, Fe^{2+}, Mn^{2+} and Cu^{2+} are normally required for enzyme stability and may form part of the active site in some enzymes (Tejirian and Xu, 2010), which may explain why activities were high in the presence of these ions. The obtained results were in agreement with some results reported in literature, for example, Kim *et al*. (2001) reported that Mn^{2+}, B^{2+} and Ca^{2+} had a stimulatory effect on CBHI and CBHII, while Hg^{2+} was most inhibiting, but the levels of stimulation and inhibition was different between the two enzymes. Ca^{2+} and Mg^{2+} were found to stimulate cellulase activity (Johnson *et al*., 1982; MacKenzie *et al*., 1987). Ca^{2+} and Co^{2+} were found to slightly activate cellulase activity at 1 mM concentration, but Pb^{2+} and Hg^{2+} caused inhibition (Ferchak and Pye, 1983). Cu^{2+} and Fe^{2+} have been reported to exhibit stronger inhibition on CBH, EG and BG (Tejirian and Xu, 2010), but the mechanisms are not clearly understood. $FeSO_4$ and $CuSO_4$ resulted in a 70% loss of initial cellulase activity, and $FeSO_4$ displayed concentration dependent inhibition, which was in agreement with results obtained in this study. $MgCl_2$ and $CaCl_2$ were found to have a slight enhancement of cellulase activity, whereas $CoSO_4$, $MnSO_4$, $NiCl_2$ and $ZnSO_4$ were found to have slight or moderate inhibition at 10 mM concentration (Tejirian and Xu, 2010).

Some metals are also found naturally in apple pomace e.g. P, Zn, Cu, Mn, Ca, Mg and Fe (Lavelli and Corti, 2011; Shalini and Gupta, 2010). Depending on the amounts present in apple pomace, addition of the metal ions tested would have resulted in concentrations of some ions to levels that are inhibitory to the enzymes. Inhibition by some metal ions may be probably due to denaturation of the enzymes as a result of changes in pH (Mills *et al.*, 2009). The initial pH at 50 mM concentration for $CaCO_3$ and $NaHCO_3$ was pH 6.8 and 6.1, respectively. pH in this range may be inhibitory to some enzymes especially in Celluclast 1.5L, though it may be best for other enzymes in the enzyme mixtures (section 2.4.2.2). The effects of metal ions on other glycoside hydrolase enzymes have received little attention in literature and may be completely different from the observed effects of cellulases.

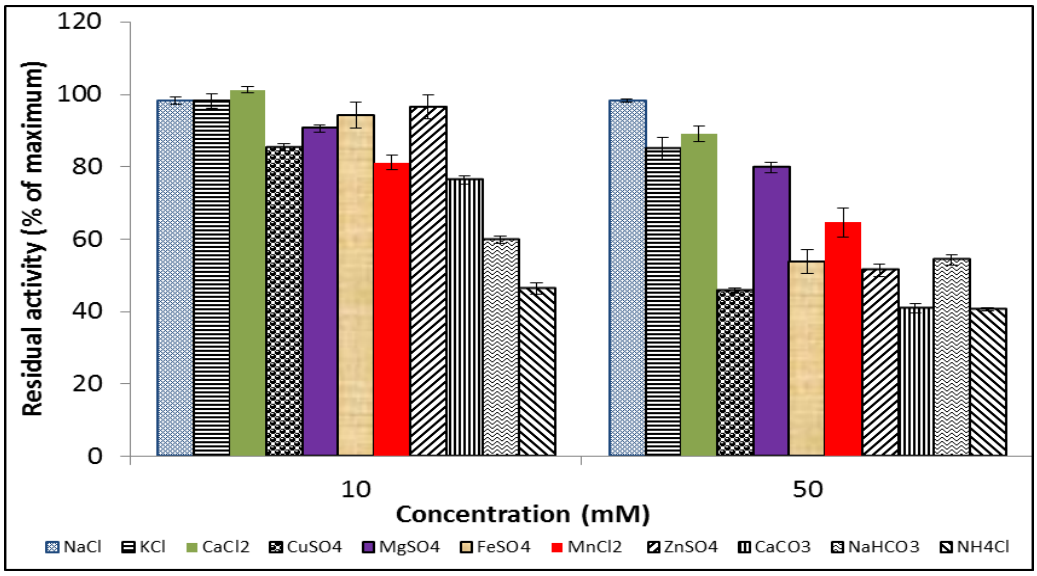


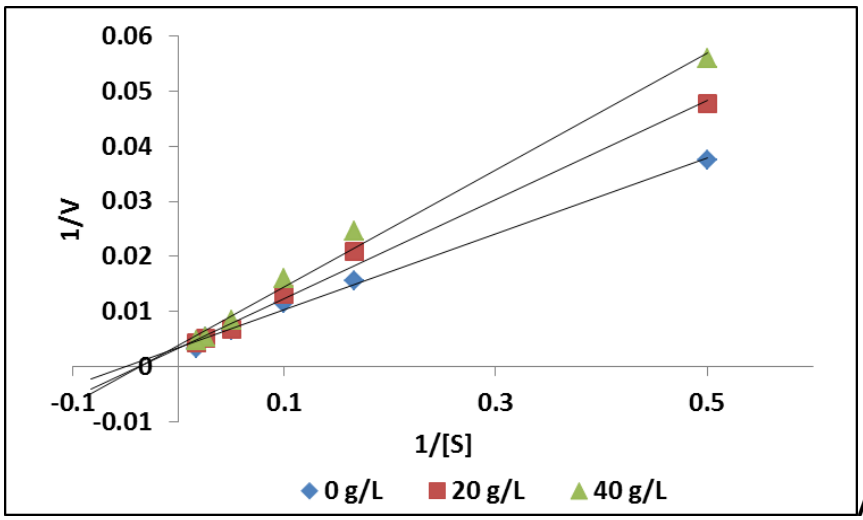
Figure 2.15: Viscozyme L and Celluclast 1.5L (50:50 combination) residual activity in the presence of different metal ions concentrations. Residual activity was calculated as the activity obtained at each lignin concentration divided by activity with no lignin included, this was then multiplied by 100. Activity was measured using 5% (wet w/v) apple pomace as substrate. Data points are presented as mean values ±SD (n=3).

Metal ions can have an effect on both the enzymes and the substrate. Metal ions may exert an effect on enzymes by changing the conformation of the enzyme or by replacing the native metal cofactors. Metal ions may also block the accessibility of the substrate to the enzymes. The presence of metal ions affects both adsorption affinity and tightness of binding of the enzyme on its substrate and there is a direct correlation between the amounts of sugar released by enzyme hydrolysis and the adsorption affinity and tightness (Kim *et al*., 2001). It has been reported that an enzyme with a strong adsorption affinity and tightness for microcrystalline cellulose resulted in the disruption of the hydrogen bonds network, resulting in the weakening of the cellulose structure (Kim *et al*., 2001). Fe^{2+} and Cu^{2+} have been found to inhibit enzyme activity through a redox mechanism, e.g. cellulose can be oxidised by these ions at the reducing end, resulting in cellulose being less reactive to cellulases. Fe^{2+} can be oxidised by air to Fe^{3+}, which is more inhibitory (Tejirian and Xu, 2010). Since the enzymes used were commercial mixtures, the metal ions may be affecting some enzymes to a greater degree than others. In industrial bioreactors, citric acid, oxalic acid, EDTA can be added to chelate the Fe^{3+}. Inhibition of enzymes by metal ions also depends on the charge and/or size of the metal ion. Addition of some metal ions like Cu^{2+} and Mg^{2+} may have some beneficial effects in lignocellulose hydrolysis as they can form complexes with soluble lignin, thereby reducing the affinity of lignin to cellulases (Liu and Zhu, 2010). This does not only eliminate

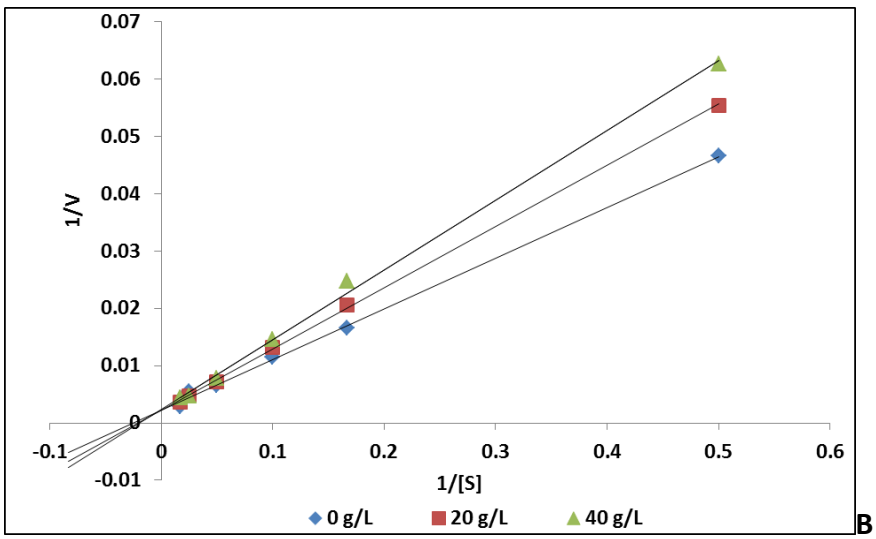
lignin inhibition on cellulases, but also the need for washing the substrate after pre-treatment, which can reduce industrial production costs. Cu^{2+} and Mg^{2+} are also beneficial in fermentation.

2.4.2.6. Type of inhibition

The mechanism by which different alcohols and sugars influence the activity of enzymes may vary. Knowing the inhibitory mechanism will aid in finding ways to control the effect of inhibitors on the enzymes. Figure 2.16 shows Lineweaver-Burk plots for some alcohols and sugars. Type of inhibition was determined on Viscozyme L and Celluclast 1.5L.



A



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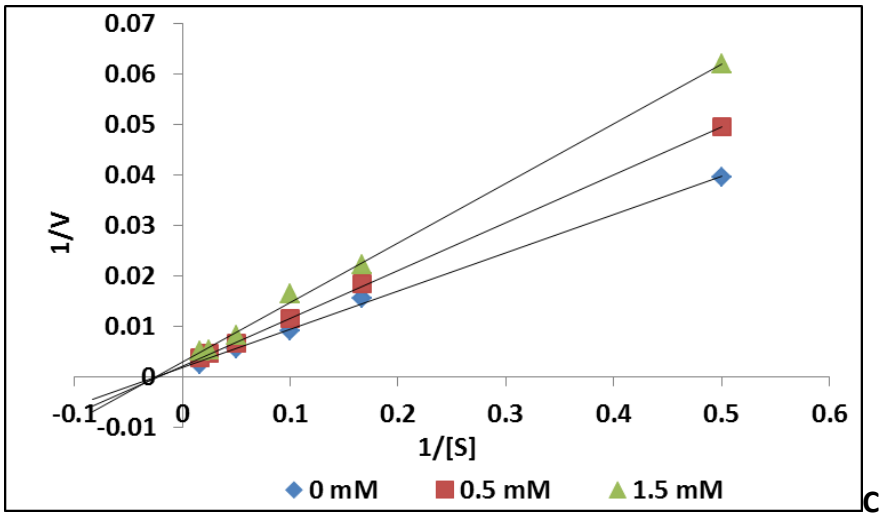

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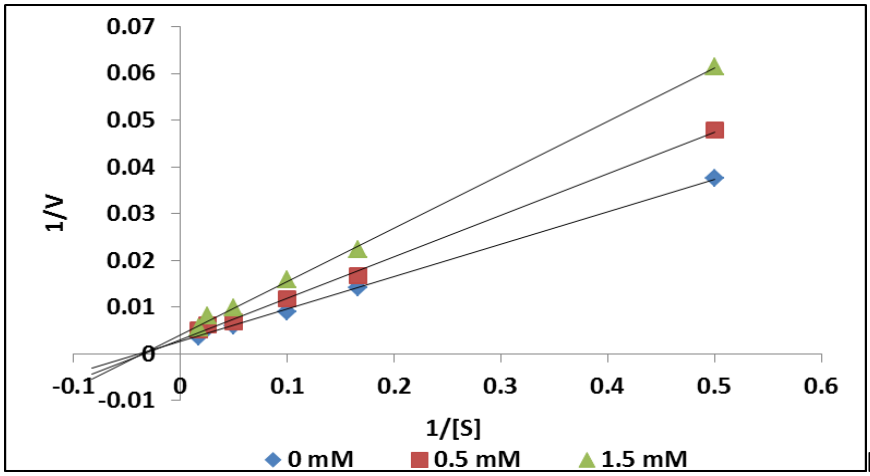
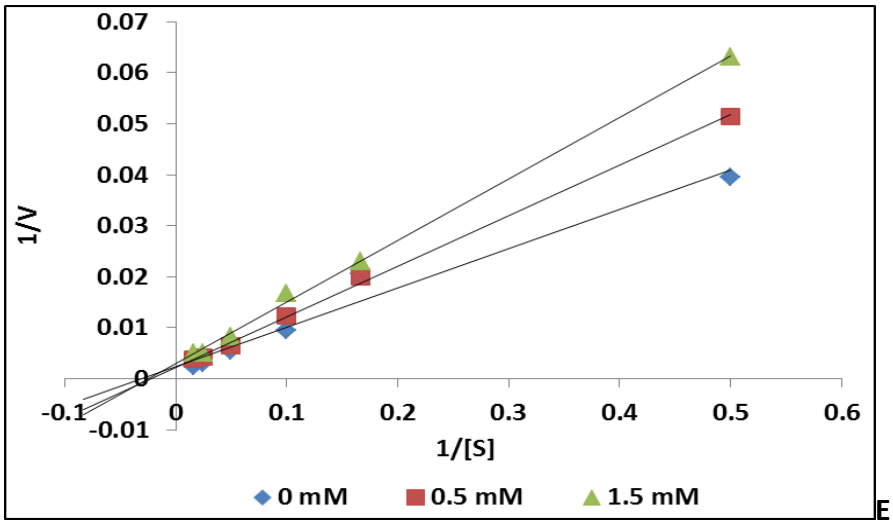


D



E

Figure 2.16: Lineweaver-Burk plot using a mixture of Viscozyme L- Celluclast 1.5L (50:50) and different apple pomace concentrations. A - ethanol, B - butanol, C - glucose, D - cellobiose and E - xylose. S - substrate concentration in mg/mL (wet w/v) and V – mg/mL per min.

All the results above were inconclusive as to the type of inhibition by ethanol, butanol, glucose, cellobiose and xylose on the enzymes. They showed mixed to non-competitive and

competitive inhibition. Competitive inhibition will show common intercepts on the *y* axis and non-competitive on the *x* axis (Chauve *et al*., 2010). The failure to exhibit a distinct type of inhibition may be attributed to apple pomace which is both heterogenous and insoluble, and the presence of many enzymes in the commercial mixtures (section 2.4.2.3).

Due to the complexity of lignocellulose and its kinetics, different researchers have reported different types of inhibition for similar enzymes (Andric *et al*., 2010b). The concentration of the inhibiting sugars changes during the course of hydrolysis. Andric *et al*. (2010b) also reported the limitation with inhibition studies in that the hydrolysis and attack on the substrate with an inhibitor added, may not be the same as that with the substrate on its own. The site at which the inhibitor attaches on the enzyme (active or remote) will determine the type of inhibition. There is the possibility of transglycosylation taking place which will also influence inhibition. This can be reduced by the immediate removal of the sugars produced (Andric *et al*., 2010c). Ethanol has been reported to inhibit cellulases uncompetitively (Chen and Jin, 2006; Ghosh *et al*., 1982; Holtzapple *et al*., 1990). Glucose has been reported to competitively inhibit β-glucosidase (Chauve *et al*., 2010). Morrison *et al*. (2011) reported that ethanol displayed a mixed type of inhibition on XynA (an endoxylanase from *C. cellulovorans*), which agree with the obtained results. However, Van Dyk *et al*. (2010) reported that ethanol competitively inhibited the activity of xylanase from *B. licheniformis* SVD1.

2.5. Conclusions

Characterisation of apple pomace as substrate used in this work was important in order to identify the sugar composition, to assist in the selection of appropriate commercial enzymes, and calculations of yield. The main sugars in apple pomace were glucose, arabinose and galactose, which can be utilised for biofuel production. There are substantial amounts of pectin and galacturonic acid in apple pomace, which can also be utilised for value addition. The presence of substantial amounts of lignin in apple pomace poses a challenge for its complete degradation, not only due to recalcitrance, but also unproductive binding and steric hindrance of cellulases and hemicellulases. If chemical pre-treatment is employed, a number of enzyme inhibitors will be produced, thereby reducing the hydrolysis rate. Enzymatic pre-treatment is therefore an attractive option.

The use of commercially available enzyme mixtures with the right combinations and proportions will result in effective apple pomace hydrolysis. This study demonstrated that there were many enzymes present in both Viscozyme L and Celluclast 1.5L. Viscozyme L contained mainly hemicellulases and pectinases, while Celluclast 1.5L contained mainly cellulases and xylanases, with both enzymes containing very little β-glucosidase. These two enzyme preparations can be used in combination to effectively degrade apple pomace. Hydrolysis conditions of these enzymes are also similar and the enzymes are very stable for a long period of time, allowing their application together in industrial bioreactors with minimal costs. The temperature optimum was 50°C for both enzymes, and pH optima were observed at pH 5.0 and pH 3.0 for Viscozyme L and Celluclast 1.5L, respectively.

Viscozyme L and Celluclast 1.5L were affected by the presence of alcohols, sugars, organic acids, lignin, phenolic compounds and metal ions to different extents, depending on their concentrations. Oligosaccharides were more inhibitory than monomers, indicating that there is need to hydrolyse the apple pomace into sugar monomers to reduce end product inhibition; this can be achieved by using the right proportions of enzymes. Ethanol was less inhibitory to the enzymes than other alcohols, making it feasible to use a SSF bioreactor system for bio-ethanol production. However, when considering the effect of organic acids on the enzymes, a SHF bioreactor system is recommended. The effect of various inhibitors on Viscozyme L and Celluclast 1.5L can be reduced by using enzymes for pre-treatment, using a SHF bioreactor system, and using water that is not contaminated with metal ions e.g. deionised water.

Having identified the composition of apple pomace and also the enzymes present in Viscozyme L and Celluclast 1.5L preparations, further investigation was performed on the synergistic associations between Viscozyme L and Celluclast 1.5L for the efficient degradation of apple pomace.

CHAPTER 3 – SYNERGISTIC STUDIES OF CELLULASES, HEMICELLULASES AND LIGNINASES ON APPLE POMACE

3.1. Introduction

Initial screening of three selected commercial enzyme mixtures, Biocip Membrane, Viscozyme L and Celluclast 1.5L, was performed to determine their synergistic action on apple pomace. The two enzymes with the highest degree of synergy were used in subsequent experiments. Having characterised the enzymes (chapter 2), it was important to understand how the enzymes cooperate to degrade apple pomace and optimise their ratios in order to reduce enzyme costs. Enzyme costs remain one of the key challenges to commercial biofuel production from lignocellulosic feedstocks like apple pomace.

Lignocellulosic biomass, e.g. apple pomace, is a complex substrate and recalcitrant to enzymatic degradation. The deconstruction of apple pomace to its monomers requires a number of enzymes that work synergistically (section 1.1.7). Lignin degradation requires lignin peroxidase, manganese-dependent peroxidase and laccase (Dúran and Esposito, 2000). Degradation of cellulose to glucose requires cellulases, namely glucanases (exo-glucanases, or cellobiohydrolases, and endo-glucanases) and β-glucosidases (Dashtban *et al*., 2009; Ferreira *et al*., 2009; Howard *et al*., 2003; Merino and Cherry, 2007; Qing *et al*., 2010; Teeri, 1997). Degradation of hemicellulose requires several different enzymes, which include α-glucuronidase, α-galactosidase, acetylxylan esterase, *p*-coumaric acid esterase, ferulic acid esterase, endo-xylanase, β-xylosidase, endo-mannanase, β-mannosidase and α-L-arabinofuranosidase that work in synergy (Bissoon *et al*., 2002; Dashtban *et al*., 2009; Howard *et al*., 2003; Shallom and Shoham, 2003; Subramaniyan and Prema, 2002; Van Dyk and Pletschke, 2012). Pectin deconstruction requires a number of enzymes that include polygalacturonases, pectin methyl esterases, pectin-, pectate- and rhamnogalacturonan lyases, pectin- and rhamnogalacturonan acetylesterases and rhamnogalacturonan hydrolyases (El-Sheekh *et al*., 2009; Pedrolli *et al*., 2009) (section 1.1.7). These enzymes have to work in synergy for the complete hydrolysis of lignocellulose. However, there has been little work done on how these enzymes can work synergistically to degrade fruit waste.

The factors that affect enzyme synergy are the ratio of the enzymes, characteristics of the enzymes and characteristics of the substrate. Synergy can either be simultaneous or sequential. Enzyme synergy in lignocellulose degradation has been extensively reviewed by Van Dyk and Pletschke (2012). Synergy between enzymes has been observed between cellulases (Murashima *et al.*, 2002; Teeri *et al*., 1997; Watson *et al*., 2002) and between hemicellulases (Beukes *et al*., 2008; Cerri e Silva *et al*., 2000; De Vries *et al*., 2000; Murashima *et al*., 2003; Renard *et al*., 1991; Selig *et al*., 2008; Subramaniyan and Prema, 2002) on many substrates, and especially on pre-treated substrates, but not fruit pomace.

This study involved the determination of synergy between the selected commercial enzyme mixtures, Viscozyme L, Celluclast 1.5L and Biocip Membrane. These commercial enzyme mixtures are crude preparations containing different enzyme activities (section 2.4.2.3), and they present a cheaper option than individual enzymes and can be optimised in terms of combinations and ratios to degrade lignocellulose**.** Optimisation of enzyme activities and ratios to relevant feedstocks is important for biomass hydrolysis and reduction of enzyme costs (Gao *et al*., 2011; Kovacs *et al*., 2009). The type and amount of enzymes required for lignocellulose hydrolysis depends on the type of feedstock, whether pre-treatment has been carried out and the type of pre-treatment. Synergistic action between these commercial enzyme preparations in apple pomace degradation was of paramount importance. This information will provide insight into what other additional purified enzymes or mixtures could be added for complete hydrolysis of apple pomace. This study will aid in a better understanding of the concept of synergy between cellulases, hemicellulases and ligninases and its application to the degradation of other complex substrates, thereby paving the way for waste beneficiation processes. It is hoped, in the long term, that by analysing the synergy in complex substrate degradation such as apple pomace, bioreactors can be designed and become integral parts of fruit juice manufacturing plants. This will aid in fruit waste beneficiation and hopefully reduce the negative environmental effects of the fruit juice industry.

3.2. Aims and objectives

a) To identify the most feasible commercial enzyme mixtures that can work synergistically for complete degradation of apple pomace biomass.

b) To design optimal ratios and combinations of these enzyme mixtures and optimal conditions required to degrade apple pomace biomass.

3.3. Materials and Methods

3.3.1. Apple pomace biomass preparation

This was performed as described in section 2.3.1.

3.3.2. Commercial enzyme preparations stock solutions

The commercial enzyme preparations used in this work included Biocip Membrane (Novozymes A/S, Bagsvaerd, Denmark), Viscozyme L (an enzyme complex from *Aspergillus aculeatus)*, Celluclast 1.5L (a commercial *Trichoderma reesei* ATCC 26921 cellulase preparation) and Novozym 188 (a commercial *Aspergillus niger* - β-glucosidase preparation). All the enzymes were obtained from Sigma (South Africa). Various concentrations were prepared in appropriate buffers depending on the application. Ligninases used in the experiments included laccase (from *Trametes versicolor*) (0.2 mg/mL), lignin peroxidase (0.4 mg/mL) and manganese peroxidase (0.4 mg/mL) (both from *Phanerochaete chrysosporium*).

3.3.3. Enzyme assays

This was performed as described in section 2.3.5.

3.3.4. Enzyme Synergy studies

3.3.4.1. Determination of best enzyme combination and optimal synergy between commercial enzymes

Synergy between Biocip Membrane, Viscozyme L and Celluclast 1.5L was determined using apple pomace stock solution, 20% (wet w/v) (final 5% wet w/v) and 1 µL/mL (0.076 mg/mL) enzyme stock solution (final 0.0095 mg/mL) of each enzyme in citrate buffer (pH 5.0, 0.05 M). The assay mixture comprised of 150 µL enzyme, 300 µL substrate and 750 µL buffer. Enzyme mixtures were assayed individually, and in combinations of two and three enzymes. Controls with individual enzymes in buffer only were also included. The assay was performed in triplicate at 37°C for 1 h under standard assay conditions. The standard DNS assay was performed to determine the amount of reducing sugar liberated. The degree of

synergy was calculated as the actual observed activity of the enzymes in combination, divided by the theoretical sum of activities of the individual enzymes.

3.3.4.2. Determination of optimal enzyme ratios

Following the screening of commercial enzymes based on their activity and synergistic action, Viscozyme L and Celluclast 1.5L were selected (section 3.4.1), hence they were used for characterisation (section 2.3.6) and subsequent experience. The optimum ratio for Viscozyme L and Celluclast 1.5 L was determined by using an apple pomace suspension of 5% (wet w/v) final concentration and various percentage ratios of Viscozyme L and Celluclast 1.5L according to the scheme in Table 3.1. The total protein concentration used in each enzyme combination was kept the same.

Table 3.1: Enzyme protein ratios (%) for Viscozyme L (V) and Celluclast 1.5L (C) and their corresponding volumes used to determine the optimal enzyme ratios. A ratio of 100% represents 0.0912 mg protein (0.076 mg/mL).

Enzyme protein ratio (%)	**Viscozyme L (V), mg**	**Celluclast 1.5L (C) mg**
V100: C0	0.0912	0
V87.5: C12.5	0.0798	0.0114
V75: C25	0.0684	0.0228
V62.5: C37.5	0.057	0.0342
V50: C50	0.0456	0.0456
V37.5: C62.5	0.0342	0.057
V25: C75	0.0228	0.0684
V12.5: C87.5	0.0114	0.0798
V0: C100	0	0.0912

Two sets of experiments were performed for each ratio, one with 0.16 mg of Novozym 188 (β-glucosidase) (2 µL/mL stock) and the other set without Novozym 188. The controls included individual enzymes at different concentrations with the substrate. The assay was performed for 48 h at 37°C under standard assay conditions. Specific activity was recorded as µg/mL/h glucose equivalents per mg protein.

3.3.4.3. Determination of optimal enzyme loading

Optimal enzyme and substrate concentrations were determined for hydrolysis experiments to assist in obtaining maximum yields with minimal enzyme loading and maximum substrate. The optimal enzyme concentration for Viscozyme L and Celluclast 1.5 L was determined at 37°C using an optimal enzyme ratio of 50:50 and an optimal time of hydrolysis of 24 h. Assays were performed using different enzyme concentrations ranging from 0–0.19 mg/mL, using 5% (wet w/v) apple pomace final concentration in citrate buffer (pH 5.0, 0.05 M).

3.3.4.4. Optimal time of apple pomace hydrolysis

The optimal time of hydrolysis for Viscozyme L and Celluclast 1.5L was determined using an enzyme ratio of 50:50 Viscozyme L to Celluclast 1.5L at final concentration of 0.019 mg/mL) each (based on the results from the optimal enzyme ratio experiment, section 3.3.4.2). A final substrate concentration of 5% (wet w/v) apple pomace solution was used. Assays were performed under standard conditions over a period of 48 h at 37°C. Reactions were terminated at different time intervals.

3.3.4.5. Determination of the type of synergy

The type of synergy was determined by having two experimental designs, one in which the two enzymes were added simultaneously using the 50:50 optimal enzyme ratio (0.019 mg/mL each) and optimal time of hydrolysis of 24 h at 37°C. The other design involved Viscozyme L added first and hydrolysis allowed for 24 h at 37°C, after which the assay was terminated by heating at 100°C on a heating block for 5 min and cooled on ice for 5 min. Celluclast 1.5L was then added and hydrolysis continued for 24 h at 37°C. The same design was performed with Celluclast 1.5L added first and Viscozyme L last. Assays were performed under standard conditions after which the DNS assay was performed to determine the amount of reducing sugars released.

3.3.4.6. Analysis of hydrolysis products using thin layer chromatography (TLC)

The extent of apple pomace hydrolysis was determined after 24 h using a modified TLC method (Van Dyk, 2009). Apple pomace (5%, wet w/v) (final concentration) was hydrolysed using Viscozyme L and Celluclast 1.5L (0.019 mg/mL each) and their combination (50:50) at 37°C under standard assay conditions. After 24 h the reaction was terminated by heating at 100^{o}C for 5 min, cooled and then centrifuged at 13 000 *x g* for 5 min to remove insoluble carbohydrates. The samples were first precipitated using acetone by adding 750 µL of ice

cold acetone to 300 µL sample supernatant, mixing by vortexing and incubation at -20°C for 10 min. The precipitated protein and polysaccharides were removed by centrifugation at 13 000 *x g* for 5 min. The components of the hydrolysate were identified using 5 mg/mL glucose, cellobiose, xylose, xylobiose, xylotriose and xylotetrose standards. The samples and standards were concentrated using a Vacutec Centrivap DNA concentrator at 45°C for 45 min. Volumes of 1 µL standard and 2 µL samples were applied to Silica Gel 60 F254 HPTLC plates (Merck, Darmstadt, Germany). The plate was developed twice with acetone: ethyl acetate: acetic acid (2:1:1, v/v). The carbohydrates were visualised and identified by immersing the plates in 10% sulphuric acid in ethanol (v/v), after which plates were air-dried and then heated at 110°C for 10 min. A digital photograph of the results was taken using Fujifilm FinePix SL 1000 camera (Japan).

3.3.4.7. Synergy between Viscozyme L, Celluclast 1.5L and ligninases

3.3.4.7.1. The effect of ligninase pre-treatment on enzymatic hydrolysis

The synergy between ligninases and the Viscozyme L – Celluclast 1.5L combination was determined using apple pomace at a 5% (wet w/v) final concentration. Apple pomace was first pre-treated with different ligninases and their combinations and then the Viscozyme L – Celluclast 1.5L combination was added. The ligninases used and their concentrations were laccase (from *Trametes versicolor*) (0.2 mg/mL), lignin peroxidase (0.4 mg/mL) and manganese peroxidase (0.4 mg/mL) (both from *Phanerochaete chrysosporium*). Hydrogen peroxide (H_2O_2) at 0.05 M was used for peroxidases as it was required for peroxidase activity. The assay mixture consisted of 150 µL enzyme, 300 µL substrate, 300 µL H_2O_2 for peroxidases, and citrate buffer to make up to a total volume of 1 200 µL. Hydrolysis was carried out at 37°C for 7 and 15 days. After hydrolysis, the samples were centrifuged at 13 000 *x g* for 10 min and 450 µL supernatant removed and the pellet re-suspended in 450 µL buffer. A volume of 150 µL 50:50 Viscozyme L - Celluclast 1.5L combination (0.038 mg/mL final concentration) was then added and the final volume was made up to 1 200 µL with citrate buffer. The assay was run for 24 h at 37°C, after which the DNS assay was performed to determine the amount of reducing sugars released by each enzyme treatment.

3.3.4.7.2. Effect of washing after ligninase pre-treatment

Laccase was chosen as the most efficient ligninase as it displayed the highest amount of reducing sugars released as compared to peroxidases. The same experimental design was

repeated as in section 3.3.4.7.1 above, using laccase for 7 and 15 days. Two sets of experimental assays were performed for each time period, one with a washing step and the other set without a washing step. Viscozyme L and Celluclast 1.5L were used at a final concentration of 0.019 mg/mL each (optimal enzyme concentration) and hydrolysis was performed at 37°C for 24 h under standard assay conditions.

3.4. Results and Discussion

3.4.1. Synergy between Biocip Membrane, Viscozyme L and Celluclast 1.5L

Biocip Membrane, Viscozyme L and Celluclast 1.5L are commercial enzyme mixtures that were commercially available, affordable and displayed great potential to degrade lignocellulose based on the manufacturer's product data sheets and were used to determine the best combination and degree of synergy. The commercial enzyme preparations are mixtures of different enzymes and using these commercial preparations in combination may result in enhanced hydrolysis of fruit pomace, compared to what can be achieved by an individual enzyme preparation.

Enzyme synergy between Biocip Membrane, Viscozyme L and Celluclast 1.5L was determined on apple pomace using 1 µL/mL (0.0095 mg/mL) of each enzyme. Combinations of Viscozyme L and Celluclast 1.5L and that of Biocip Membrane, Viscozyme L and Celluclast 1.5L displayed the highest amounts of reducing sugars released than other combinations and individual enzymes, but the combination of Biocip Membrane, Viscozyme L and Celluclast 1.5L (BVC) had a lower degree of synergy of 1.03, while the combination of Viscozyme L and Celluclast 1.5L (VC) had a higher degree of synergy of 1.6. Biocip Membrane and Viscozyme L (BV) had a degree of synergy of 0.74 and Biocip Membrane and Celluclast 1.5L (BC) had a degree of synergy of 1.5 after 1 h incubation at 37°C (Figure 3.1). Viscozyme L and Biocip Membrane share many similar enzyme activities, although there are more pectinase and hemicellulase activities in Viscozyme L than in Biocip Membrane, which would have resulted in enzymes competing for the same sites on apple pomace when used together, which might explain the degree of synergy of less than 1 (competition - no synergy) obtained.

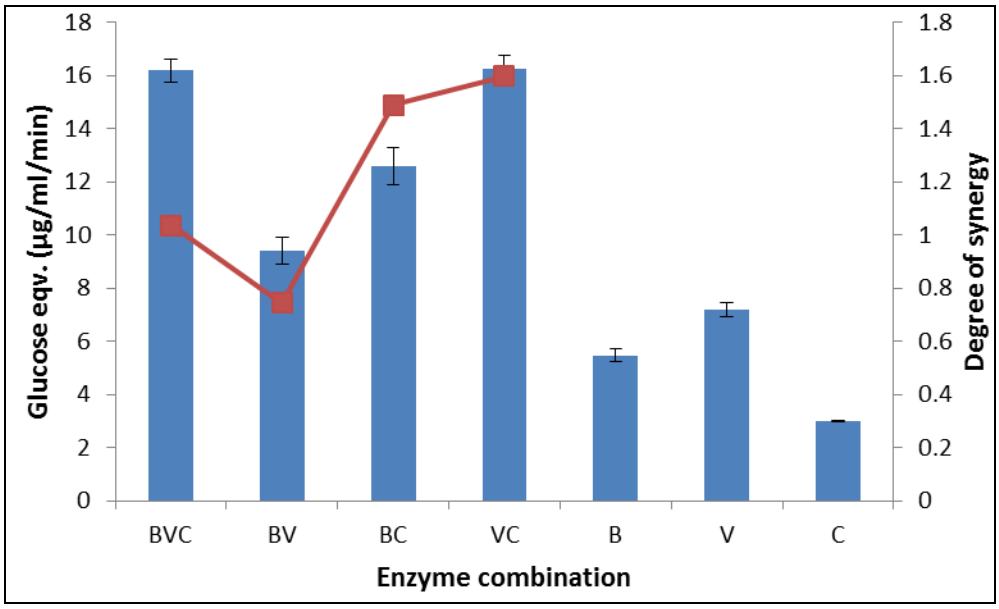


Figure 3.1: Activities (solid bars) and degree of synergy (line) of Biocip Membrane (B), Viscozyme L (V) and Celluclast 1.5L (C) individually and their combinations (equal ratios) on 5% (wet w/v) apple pomace after 1 h incubation at 37°C. Degree of synergy was calculated as the activity of the enzyme combination divided by the sum of the individual enzyme activities. Data points are presented as mean values ±SD (n=3).

Celluclast 1.5L on the other hand has more cellulase, β-xylosidase and endoxylanase activities (section 2.4.2.3) than the other enzymes, hence cannot easily degrade the pectin component to open up the structure for cellulases to act on cellulose. When Celluclast 1.5L was used in combination with either Biocip Membrane or Viscozyme L, there was enhanced activity as shown by the amount of reducing sugars released and a higher degree of synergy (more than 1), showing cooperation between the enzymes and the enzymes present in these mixtures complementing each other, resulting in enhanced hydrolysis of apple pomace as compared to individual enzyme preparations. The degree of synergy between the three enzyme preparations was lower than that of Biocip Membrane and Celluclast 1.5L and Viscozyme L and Cellulcast 1.5L. This may have been due to competition between the enzymes for the same sites on the substrate.

A combination of Biocip Membrane, Viscozyme L and Celluclast 1.5L (BVC) resulted in the release of 16.2 µg/mL/min reducing sugars, BV (9.4 µg/mL/min), BC (12.6 µg/mL/min), VC (16.3 µg/mL/min) reducing sugars. Celluclast 1.5L on its own resulted in the release of less reducing sugars (3 µg/mL/min) than Biocip Membrane (5.5 µg/mL/min) and Viscozyme L (7.2 µg/mL/min). Celluclast 1.5L has less hemicellulases and pectinases compared to Biocip

Membrane and Viscozyme L. The results suggest that the removal of the hemicellulose and pectin component of the apple pomace resulted in enhanced hydrolysis of the cellulose component by Celluclast 1.5L as indicated from the enzyme activity assays in chapter 2 (section 2.4.2.3). It is important to note that BVC and VC produced almost the same amount of sugars, but differed in the degree of synergy, indicating that the degree of synergy did not always correspond to yield and that less enzyme could be used to degrade apple pomace. Viscozyme L and Biocip Membrane act mainly on the hemicellulose and pectin components of apple pomace, hence freeing the cellulose component from steric hindrances for hydrolysis by Celluclast 1.5L, which contain mainly cellulases and xylanases.

The presence of Cip1 and 2 (which contain CBMs) and swollenin in Celluclast 1.5L (Suwannarangsee *et al*., 2012), was expected to cause an enhanced apple pomace hydrolysis. Swollenin has been reported to play a role in cellulose weakening (Saloheimo *et al*., 2002). However, other studies reported that the presence of CBMs and their action did not result in an increase in hydrolytic activity, which may be due to non-productive binding on lignin (Boraston *et al*., 2004; Gilbert *et al*., 2010; Shoseyov *et al*., 2006; Van Dyk and Pletschke, 2012). It has also been reported that cellulases employ a processive mechanism of hydrolysis as they remain bound to the substrate while hydrolysis of the bonds along the cellulose chain takes place (Boraston *et al*., 2004; Van Dyk and Pletschke, 2012). This may lead to a lower sugar yield by Celluclast 1.5L, due to steric hindrances from other components of the complex lignocellulose substrates. The presence of lignin may also have affected the hydrolysis of apple pomace as it shields cellulose, making its accessibility by cellulases difficult and also due to non-productive adsorption of cellulases and hemicellulases onto lignin (Dashtban *et al*., 2009; Howard *et al.*, 2003; Lin *et al*., 2010; Merino and Cherry, 2007; Van Dyk and Pletschke, 2012) (See section 2.4.2.5). It is important to note that the release of reducing sugars was monitored in this work, but that short oligomers may also be released as well. Measuring oligomers released may give a good idea of the extent of apple pomace degradation. The presence of oligomers would mean that other additional enzymes may be required to release the sugar monomers.

The presence of soluble inhibitory products, e.g. cellobiose, may reduce synergy and would require the addition of β-glucosidase (Zhang and Lynd, 2004). The results also indicated that there was a considerable amount of pectin and hemicellulose in apple pomace (Bhushan *et al*., 2008; Gullon *et al*., 2008; Joshi and Attri, 2006). Bhushan *et al*. (2008) reported that

apple pomace consisted of approximately 10–15% pectin and 18% xyloglucan (fucogalactoxyloglucans), although this differs from values reported by other authors. The presence of similar enzymes/ activities in both Viscozyme L and Celluclast 1.5L may suggest that there could have been competition between the enzymes for the same active sites on the apple pomace or simple adsorption of the enzymes on the substrate due to enzyme loadings exceeding saturation level.

The high synergy obtained may be also as a result of other enzymes present in the two mixtures having broad substrate specificity, which may be important in the reduction of costs as less enzyme will be used. Enzymes like cellulase, cellobiohydrolase, β-glucosidase, arabinofuranosidase and xylanase have been found to have broad substrate specificity as reviewed by Van Dyk and Pletschke (2012). Qing and Wyman (2011) reported that supplementing cellulase with β-xylosidase resulted in better glucose and xylose yields than that achieved by adding β-glucosidase, as it resulted in removal of the inhibitory xylooligomers which are converted to xylose. β-glucosidase had a lower adsorption capacity and affinity for most lignocellulose substrates than cellulase, and xylanase and β-xylosidase had a high adsorption on higher xylan content substrates. However, higher glucan content did not necessarily result in stronger cellulase binding. This was an indication that the cellulase was competitively binding to xylan and xylooligomers, which may explain why xylan or xylooligomers inhibited cellulase.

It is also important to note that synergy depends, amongst other things, on the characteristics of the substrate (physical and chemical heterogeneity and accessibility of the binding sites) and enzymes, the ratio and concentration of the enzymes and the assay conditions (Jeoh *et al*., 2006; Van Dyk and Pletschke, 2012). Synergy between enzymes is important for biotechnological industrial applications as it can lower the enzyme loading, leading to low enzyme cost and hence production costs. Many studies have reported on the synergy between different enzymes mainly on pre-treated lignocellulose substrates such as sugarcane bagasse, rice straw and corn stover (Beukes and Pletschke, 2011; Van Dyk and Pletschke, 2012), but, to the best of our knowledge, no reports have been documented for apple pomace.

A combination of Viscozyme L and Celluclast 1.5L displayed a higher degree of synergy than other enzyme combinations; hence this enzyme combination was used in all subsequent experiments.

3.4.2. Determination of the optimal enzyme ratio

The optimal enzyme ratio for degradation of apple pomace was determined for Viscozyme L and Celluclast 1.5L by combining different enzymes in different ratios, while keeping the final protein concentration the same. However, other researchers use molar ratios when using purified enzymes. Specific enzyme ratios are required for optimal hydrolysis of lignocellulose as well as reducing enzyme cost for biofuel production. Hydrolysis of apple pomace was performed at 37°C for 48 h and the results are shown in Figure 3.2. In another set of experiments, Novozyme 188 was added in excess to Viscozyme L and Celluclast 1.5L combinations (results not shown). The enzyme ratio that resulted in the highest release of reducing sugars (84.4 µg/mL/h) was V50:C50, followed closely by V62.5:C37.5 and V75:C25 with 81.1 and 79.0 µg/mL/h, respectively. The degree of synergy was also higher (approximately 1.1) on these three ratios as compared to the other ratios. A ratio of V50:C50 released high amount of reducing sugar and had a higher degree of synergy. The results displayed that a higher degree of synergy corresponded to a higher amount of sugars released, however this is not always the case (Van Dyk and Pletschke, 2012).

The degree of synergy was low in this experiment as compared to section 3.4.1, which indicated that the degree of synergy was affected by enzyme loading and hydrolysis time. In section 3.4.1 a low enzyme loading was used (0.019 mg/mL) for a short time period (1 h) as compared to the current results using 0.076 mg/mL enzyme load and 48 h hydrolysis time. The results were in agreement with findings of Jeoh *et al*. (2006) and Van Dyk and Pletschke (2012), which indicated that the degree of synergy depended on the molar ratios of the enzymes used and total enzyme concentration. Addition of excess Novozyme 188 (mainly β-glucosidase), resulted in a slight increase in the amount of reducing sugar released when added to Viscozyme L and Celluclast 1.5L or their different combinations and ratios (data not shown). In the presence of Novozyme 188, Viscozyme L and Celluclast 1.5L displayed a low degree of synergy, but in the absence of Novozyme 188 a high degree of synergy was displayed. This may be attributed to adsorption of the enzymes on the substrates and excess β-glucosidase when the three enzymes were added together. Hydrolysis of cellobiose and cellooligomers to glucose assist in the prevention of inhibition of cellulases by cellobiose (Merino and Cherry, 2007; Qing *et al*., 2010).

A ratio of V50:C50 released a high amount of reducing sugar and had a higher degree of synergy (above 1) without Novozyme 188 than with Novozyme 188 (degree of synergy less

than 1). It has been reported that enzyme ratios do not only depend on the type of substrate, enzymes used, but also enzyme loadings, as other enzymes may be more important at low loadings (Gao *et al*., 2010; Van Dyk and Pletschke, 2012). However, optimisation of enzyme ratios in commercial enzyme mixtures for lignocellulose degradation is complex and difficult. This is due to differences in the concentrations of enzymes and lignocellulose degrading components, and in the anatomy of the cell wall and microstructure of the substrate (Banerjee *et al*., 2010b; Duncan and Schiling, 2010; Gao *et al*., 2010; Van Dyk and Pletschke, 2012). The available commercial enzyme mixtures have generally been optimised for acid-pretreated corn stover and grasses, hence may be limited in terms of the number and variety of enzymes required for fruit pomace degradation (Banerjee *et al*., 2010c). The most efficient enzyme combination chosen for subsequent experiments was therefore V50:C50.

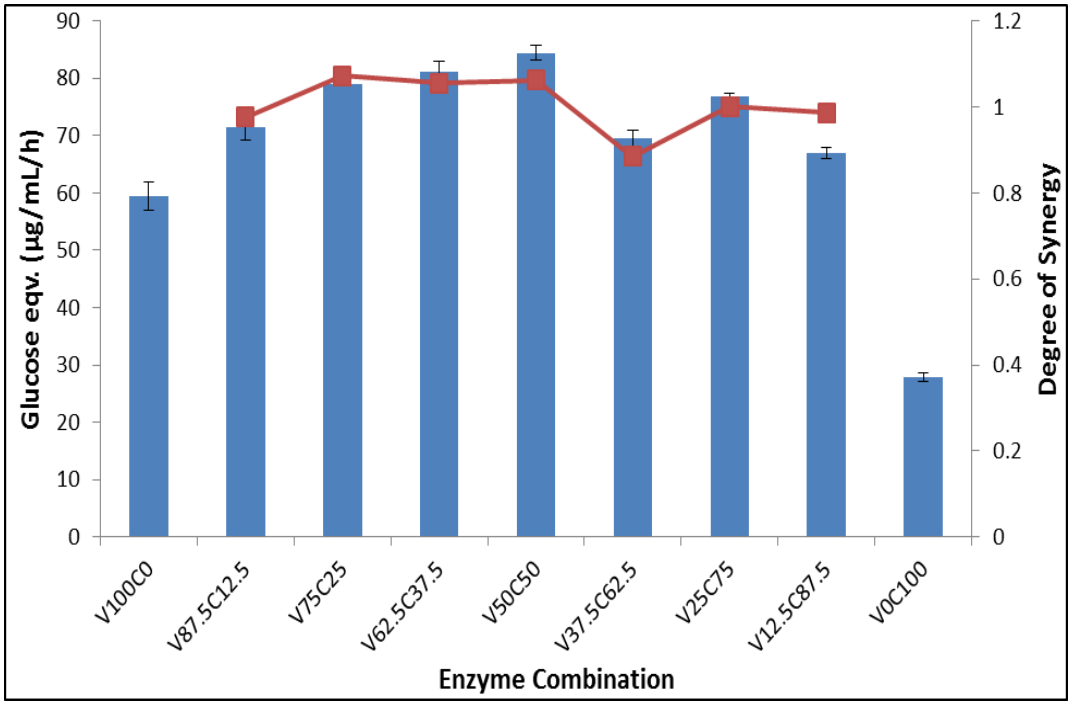


Figure 3.2: Activity (solid bars) and degree of synergy (line) of different ratios of Viscozyme L (V) and Celluclast 1.5L (C) on 5% (wet w/v) apple pomace at 37°C, pH 5.0 for 48 h. Data points are presented as mean values ±SD (n=3).

3.4.3. Determination of optimal enzyme loading

The optimal enzyme loading was determined using an enzyme ratio of V50:C50. There was a steady increase in the amount of reducing sugars released with an increase in enzyme concentration up to 0.038 mg/mL (Figure 3.3). At low enzyme concentrations, the rates of hydrolysis were low as all hydrolysis sites on the substrate are not yet saturated, and the

degree of synergy is normally low. An increase in enzyme concentration beyond 0.038 mg/mL resulted in very low increases in the amount of reducing sugar being released. This may be due to the limited surface area on the substrate for the enzymes to bind. As a result, excess enzyme molecules are adsorbed on the substrate to form multiple layers. Another reason for this phenomenon may be the fact that the enzyme's surface area is composed of active and inactive sites and only the substrate molecules adsorbed onto the active sites will take part in hydrolysis (Lin *et al*., 2010). The relative number of binding sites may be reduced at high protein loading, leading to enzymes competing for the same binding sites on the substrate and a reduction in hydrolysis rate (Banerjee *et al*., 2010a; Van Dyk and Pletschke, 2012). This also results in a low degree of synergy observed (Converse and Optekar, 1993). The reduction in the amount of reducing sugars released at an enzyme concentration above 0.114 mg/mL may be due to inhibition of enzyme adsorption on the substrate by hydrolysis products (Kristensen *et al*., 2009). At high enzyme concentrations, the reaction may be fast initially and quickly forms products such as glucose, cellobiose, xylose and xylobiose which may inhibit lignocellulose degrading enzymes. The optimal enzyme loading not only depends on the nature and type of substrate, but also on the pre-treatment strategy. The use of lower enzyme loadings will assist in the reduction of enzyme costs. The optimal enzyme concentration for V50:C50 was 0.038 mg/mL in total for the two enzymes.

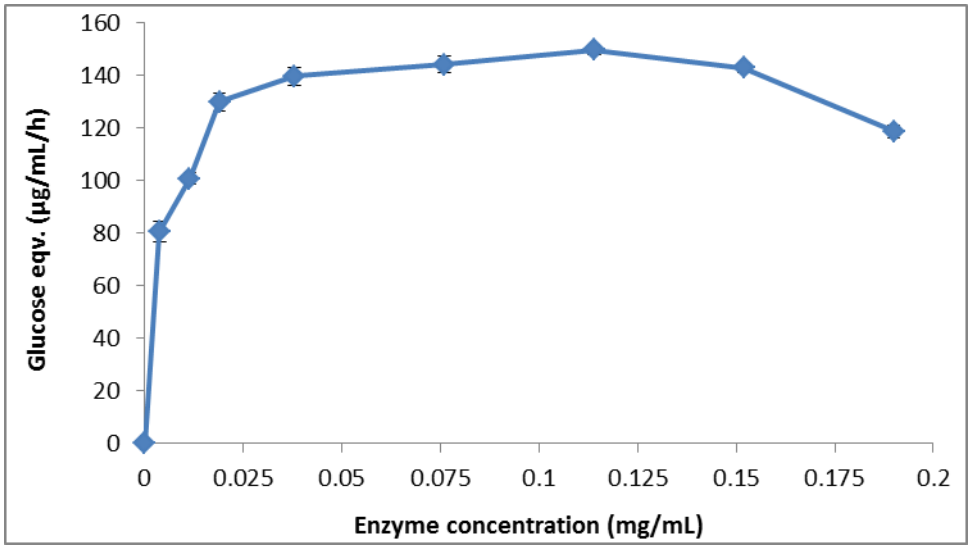


Figure 3.3: Activity of Viscozyme L: Celluclast 1.5 L (50:50) on 5% (wet w/v) apple pomace at different enzyme concentrations. Data points are presented as mean values ±SD (n=3).

3.4.4. Determination of optimal time of hydrolysis

Optimal hydrolysis time was determined as this would reduce incubation time and production costs. In an industrial setting it is important to know when to start removing the hydrolysis products, where the bioreactor is used for simultaneous hydrolysis and fermentation. The optimal time of V50:C50 hydrolysis was determined using 0.038 mg/mL enzyme concentration and 5% (wet w/v) apple pomace. There was a steady increase in the amount of reducing sugars released from 1–6 h, after which there was a very slight increase in the amount of reducing sugars released up to 18 h (Figure 3.4). Figure 3.5, shows rate of product formation per hour. The amount of reducing sugar released increased after 18 h, but remained constant at 24 and 48 h hydrolysis time. The degree of synergy decreased during the course of hydrolysis over time, although the amount of sugars released was increasing, which indicates that a high degree of synergy does not always correspond to high sugar yield (Andersen *et al*., 2008; Van Dyk and Pletschke, 2012). It was high (around 1.6) during the first 3 h, then decreased over time to around 1 at 48 h, which agreed with results obtained above (section 3.4.1 and 3.4.2).

The results demonstrated that a high degree of synergy was observed at initial stages of hydrolysis as the substrate structure became modified and opened up, requiring greater cooperation between enzymes. During the initial stages a maximal number of sites on the substrate will be available for enzyme activity (Boisset *et al*., 2001; Van Dyk and Pletschke, 2012). As the hemicellulases and pectinases hydrolyse the hemicellulose and pectin components, respectively, the substrate opens up, enabling cellulases to gain access to the cellulose component. The reduction in the degree of synergy, with an increase in the reducing sugars released, suggests that, as time progresses, other enzymes may become less active or important (for example pectinases) as others take over (cellulases). There may be also a drop in pH, due to the release of galacturonic acid and other acids, to levels that inhibit some enzymes. Lin *et al*. (2010) reported that the hydrolysis rate of hemicellulose was faster than that of cellulose during the first 24 h, which can be attributed to structural differences between hemicellulose and cellulose. The crystallinity of cellulose may pose resistance to hydrolysis, while hemicellulose, although branched, can easily be hydrolysed. During the initial stages of hydrolysis the more readily available parts of the apple pomace (e.g. amorphous regions) will be hydrolysed and later the less accessible parts (i.e. crystalline regions) will be hydrolysed (Andric *et al*., 2010b; Palonen *et al*., 2004).

The results show that the initial reaction rates were high, but decreases with time and may approach zero, which is characteristic of a batch mode kind of reaction (Yang *et al.*, 2009). As the reaction proceeds, the substrate becomes depleted and there may also be end product inhibition. The optimal time for Viscozyme L- Celluclast 1.5L hydrolysis was 24 h. An increase in hydrolysis time beyond 24 h did not result in any significant increase in the amount of reducing sugar being released, which may indicate that the enzymes had reached their maximal hydrolysis potential.

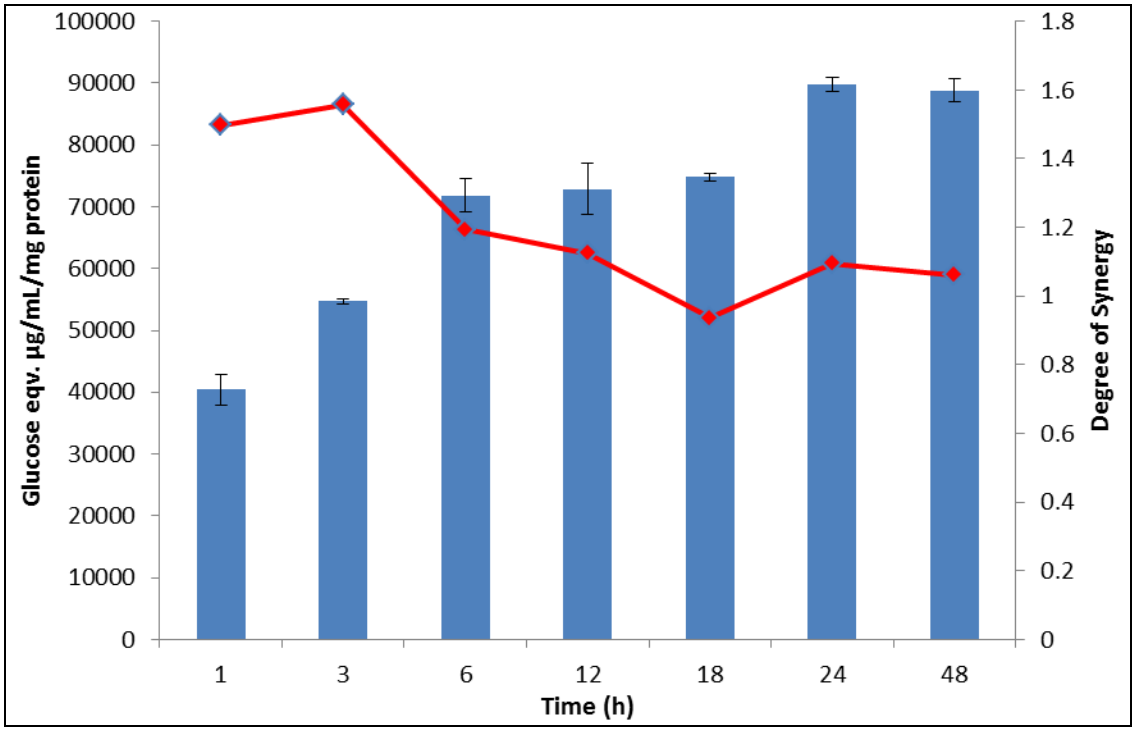


Figure 3.4: Activity (solid bars) and degree of synergy (line) of Viscozyme L and Celluclast 1.5L over different time periods. Activity was measured using 5% (wet w/v) apple pomace as substrate. Data points are presented as mean values ±SD (n=3).

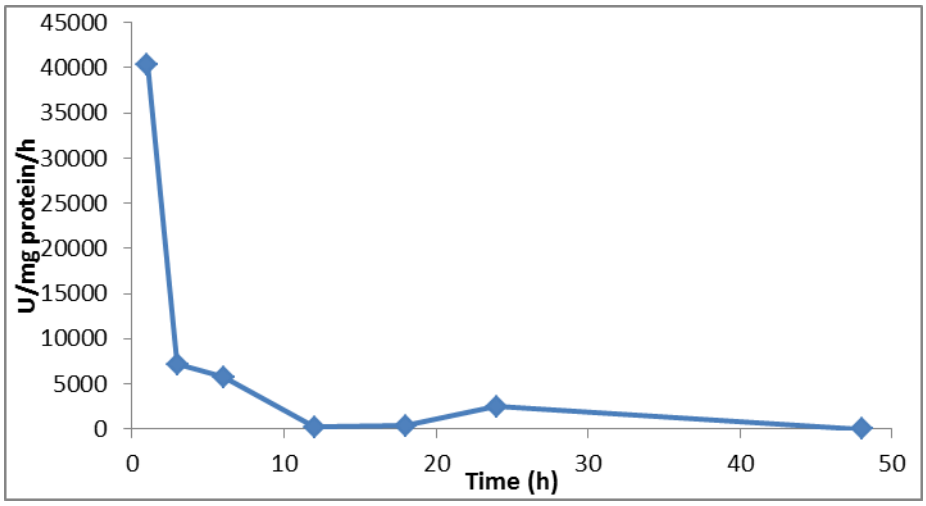


Figure 3.5: The rate of hydrolysis for Viscozyme L and Celluclast 1.5L on apple pomace over time. The rate is shown as the amount of glucose eq. (µg/mL/mg protein/h.)

3.4.5. Determination of the type of synergy

The type of synergy exhibited by Viscozyme L and Celluclast 1.5L was determined by adding the enzymes sequentially as well as simultaneously to apple pomace and the results are shown in Figure 3.6. There was a small difference in the amount of reducing sugar released when Viscozyme L and Celluclast 1.5L were added sequentially, regardless of the order of enzyme addition. This may be due to the overlapping activities in both Viscozyme L and Celluclast 1.5L. However, it was expected that starting with Viscozyme L followed by Celluclast 1.5L would result in the release of more reducing sugars than vice versa, as Viscozyme L contains hemicellulases and pectinases which would result in the opening of the substrate structure, exposing cellulose for hydrolysis by Celluclast 1.5L. The results may be due to inhibitory effects of some hydrolysis products on cellulases e.g. xylo-oligomers (Kumar and Wyman, 2009). The two enzyme mixtures displayed the best results when they were added simultaneously, which may be due to enhanced cooperation of enzyme activities. Simultaneous addition of enzymes will also simplify industrial application. The degree of synergy was around 0.96 for sequential enzyme addition and 1.1 for simultaneous enzyme addition. The results agreed with that obtained by Murashima *et al*. (2003) where a synergistic effect was found when xylanase and cellulase were added simultaneously. Other authors found different results (see review by Van Dyk and Pletschke, 2012). Qing and Wyman (2011) suggested that addition of xylanases and hemicellulases first, followed by cellulases in multiple steps could result in enhanced cellulose hydrolysis in lignocellulose biomass and the use of less enzymes, as this will not only help to enlarge the pore size for

cellulases, but also remove possible cellulase inhibitors and the physical barrier posed by hemicellulose and lignin.

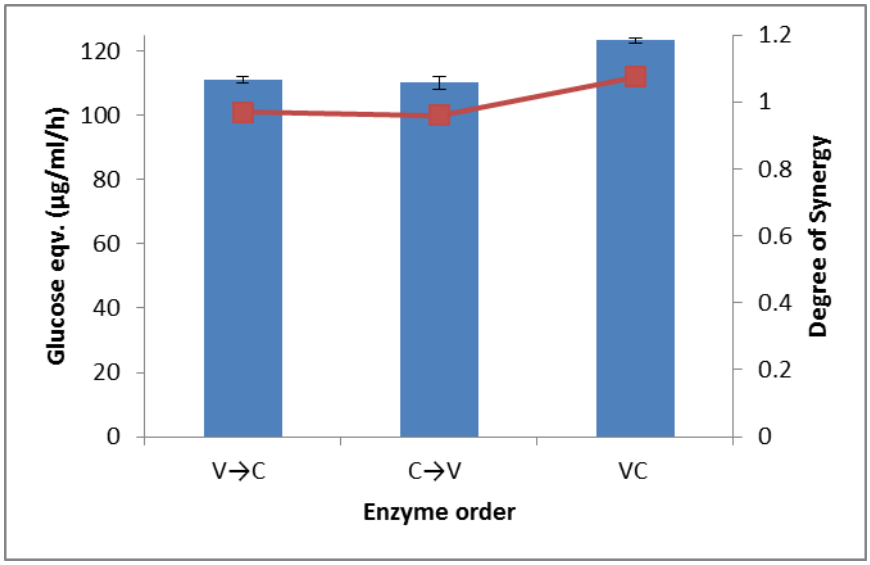


Figure 3.6: Effect of order of addition of Viscozyme L and Celluclast 1.5L on activity (solid bars) and degree of synergy (line). V→C – Viscozyme L first, Celluclast 1.5L second in sequence, C→V – Celluclast 1.5L first, Viscozyme L second in sequence, VC – Viscozyme L and Celluclast 1.5L added at the same time, simultaneously. Activity was measured using 5% (wet w/v) apple pomace as substrate. Data points are presented as mean values ±SD (n=3).

3.4.6. Analysis of hydrolysis products using TLC

Although total reducing sugars give an indication of yield from lignocellulose degradation, it doesn't display the extent of depolymerisation, as products may be in the form of oligosaccharides. This could indicate that supplementation of some enzymes, lacking in the commercial preparations, is required. Hydrolysis products were analysed after 24 h hydrolysis of apple pomace and the results are shown in Figure 3.7.

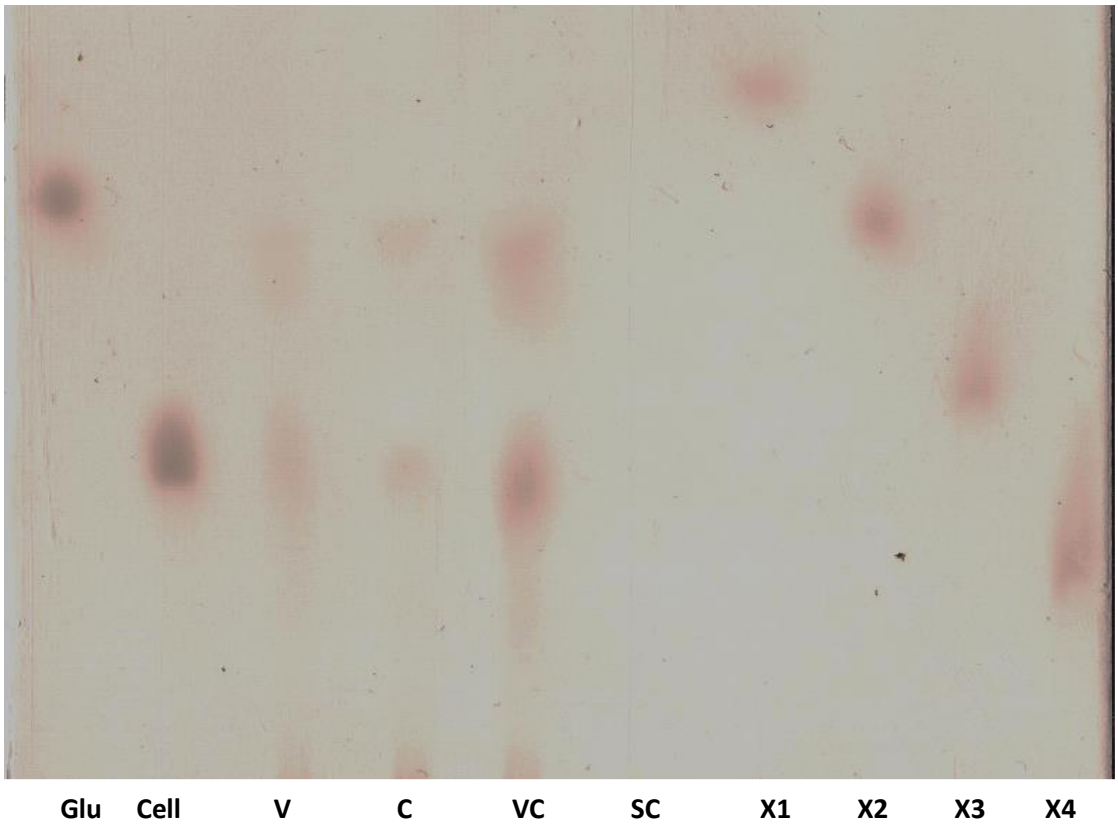


Figure 3.7: Thin layer chromatography of the products formed from the hydrolysis of apple pomace when incubated with Viscozyme L and Celluclast 1.5L. Glu (glucose), Cell (cellobiose), V (Viscozyme L), C (Celluclast 1.5 L), SC (control with apple pomace only), X1 (xylose), X2 (xylobiose), X3 (xylotriose) and X4 (xylotetraose).

The action of Viscozyme L, Celluclast 1.5L and their combination on apple pomace produced both glucose and cellobiose. Viscozyme L - Celluclast 1.5L combination showed the presence of more glucose and cellobiose than the individual enzymes, followed by Viscozyme L and lastly Celluclast 1.5L. The presence of cellobiose indicated that there was insufficient β-glucosidase in the enzyme mixtures and addition of β-glucosidase may be required to degrade cellobiose to glucose or the reaction time may have to be extended. The conversion of cellobiose to glucose not only helps to prevent inhibition of cellulases by cellobiose (Merino and Cherry, 2007; Qing *et al*., 2010; Teeri, 1997), but also increases the glucose yield, which is important for biofuel production. The results showed that other cellooligomers, xylose and xylooligomers were not present in the reaction mixture. This may be attributed very low amounts of xylose in apple pomace (section 2.4.1) Cellobiose reacts with the DNS reagent, which may explain why addition of β-glucosidase (section 3.4.2) resulted in a slight increase of reducing sugars liberated. Other sugar monomers like

arabinose and galactose which are also components of apple pomace (section 2.4.1) could also have been liberated.

3.4.7. The effect of ligninases on enzyme hydrolysis

3.4.7.1. Synergy between ligninases and Viscozyme L and Celluclast 1.5L

The effect of pre-treatment of apple pomace by ligninases on the activity and synergy of Viscozyme L and Celluclast 1.5L was determined and the results are shown in Figure 3.8. Laccase on its own resulted in the subsequent release of slightly higher amounts of reducing sugars by Viscozyme L and Celluclast 1.5L compared to other ligninases or their combinations. The individual ligninases and their combinations had a small difference in the amount of reducing sugars released after Viscozyme L and Celluclast 1.5L addition (around 60 µg/mL/h), which might not be significant, with the combination of all the enzymes showing the lowest sugar release. A combination of Viscozyme L and Celluclast 1.5L only resulted in the release of more sugars and a higher degree of synergy compared to treatments where ligninases were added. It was anticipated that the ligninases would enhance the hydrolysis of lignocellulose by cellulases and hemicellulases as the removal/ weakening of lignin would give greater access to hemicellulases and cellulases to hydrolyse the hemicellulose and cellulose components. It was therefore expected that the addition of ligninases would have resulted in an improved release of reducing sugars. However, the results indicated that the ligninases were not contributing to the amount of reducing sugars released by Viscozyme L and Celluclast 1.5L. Viscozyme L and Celluclast 1.5L may have been able to gain some access to its substrate despite the presence of lignin (section 3.4.1.-3.4.5 and chapter 2, section 2.4.2.5). The presence of ligninases could have contributed to a lower sugar release due to ligninases adsorbing on to the surface of the substrate, hence limiting the access of Viscozyme L and Celluclast 1.5L. Pre-treatment with ligninases may have resulted in the production of phenols or other products which could also have inhibited cellulases or hemicellulases (Hendriks and Zeeman, 2009; Palmqvist and Hahn-Hagerdal, 2000; Ximenes *et al.*, 2010).

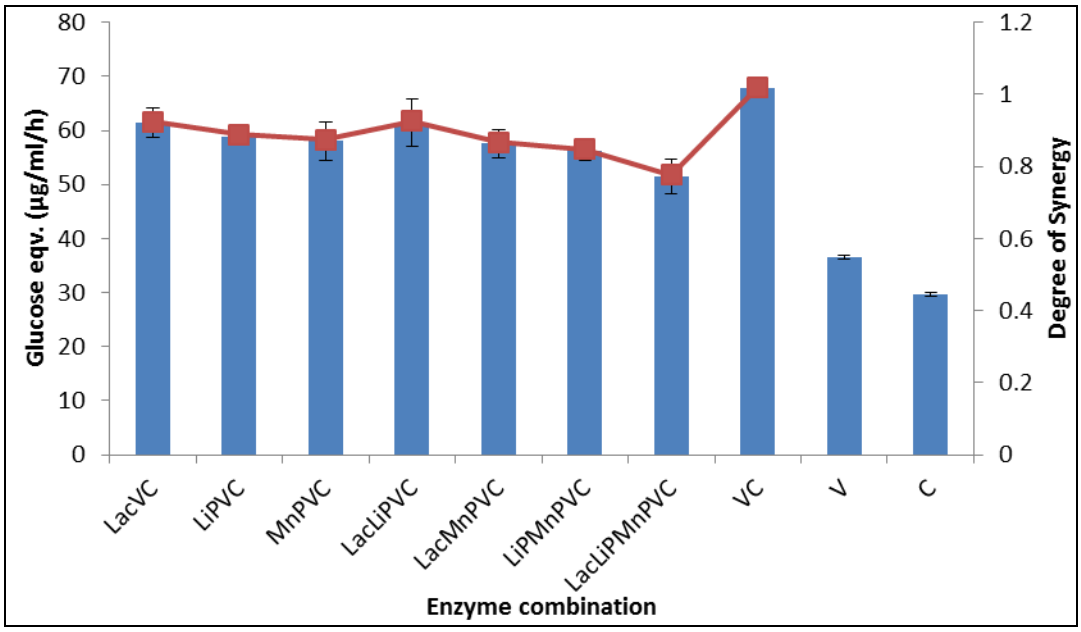


Figure 3.8: Activity (solid bars) and degree of synergy (line) of Viscozyme L (V) and Celluclast 1.5L (C) on apple pomace after pre-treatment with different ligninases. Lac – laccase; LiP – lignin peroxidase;

MnP – Manganese peroxidase. Activity was measured using 5% (wet w/v) apple pomace as substrate. Data points are presented as mean values ±SD (n=3).

3.4.7.2. Effect of washing after ligninase pre-treatment

The effect of washing apple pomace after pre-treatment with laccase for 7 and 15 days on Viscozyme L and Celluclast 1.5L activity is shown in Figure 3.9. There was a slight decrease in the amount of reducing sugars released after 7 and 15 days pre-treatment with laccase when the washing step was performed. There may have been some degradation of phenolic compounds by laccase as time progressed, resulting in the release of some products which might have inhibited Viscozyme L and Celluclast 1.5L. Laccase has been reported to degrade phenolic compounds in apple pomace in order to prevent their inhibitory effect on fermentative microorganisms (Parmar and Rupasinghe, 2012). Unwashed samples showed a slightly higher amount of reducing sugar released as compared to the washed samples. This was unexpected as the washing step is supposed to remove inhibitory compounds from lignin degradation (Hendriks and Zeeman, 2009 and Palmqvist and Hahn-Hagerdal, 2000; Ximenes *et al.*, 2010). However, the results obtained may be due to substrate losses during the washing step because the ligninases may have released soluble sugars that had been trapped within the cell wall matrix. However, a combination of Viscozyme L and Celluclast 1.5L without laccase pre-treatment resulted in the release of higher amounts of reducing sugars than when

there was a laccase pre-treatment. This may suggest that laccase pre-treatment was not removing any lignin from the apple pomace, which may indicate that laccase pre-treatment was not required for apple pomace degradation or that the conditions of laccase hydrolysis have to be optimised further. Viscozyme L and Celluclast 1.5L enzymes seemed to access the cellulose and hemicellulose components due to the presence of hemicellulases in these two mixtures. Degradation of hemicellulose gives cellulases access to degrade cellulose (Howard *et al.*, 2003; Sun and Cheng, 2002).

The results also give an indication that accessory enzymes play an integral part in apple pomace degradation and that cellulose and hemicellulose in apple pectin was not masked by lignin. Accessory enzymes such as arabinanases, pectinases, lyases and several types of esterases act on less abundant linkages in lignocellulose, but are very critical in releasing the cellulose from lignin and hemicellulose (Banerjee *et al.*, 2010c; Howard *et al.*, 2003; Sun and Cheng, 2002). Although some of these accessory enzyme activities were not determined, they may be present in the Viscozyme L and Celluclast 1.5L mixtures. The laccase may also be too large to penetrate the cell wall of apple pomace, hence failure to degrade the lignin in apple pomace (Howard *et al.*, 2003).

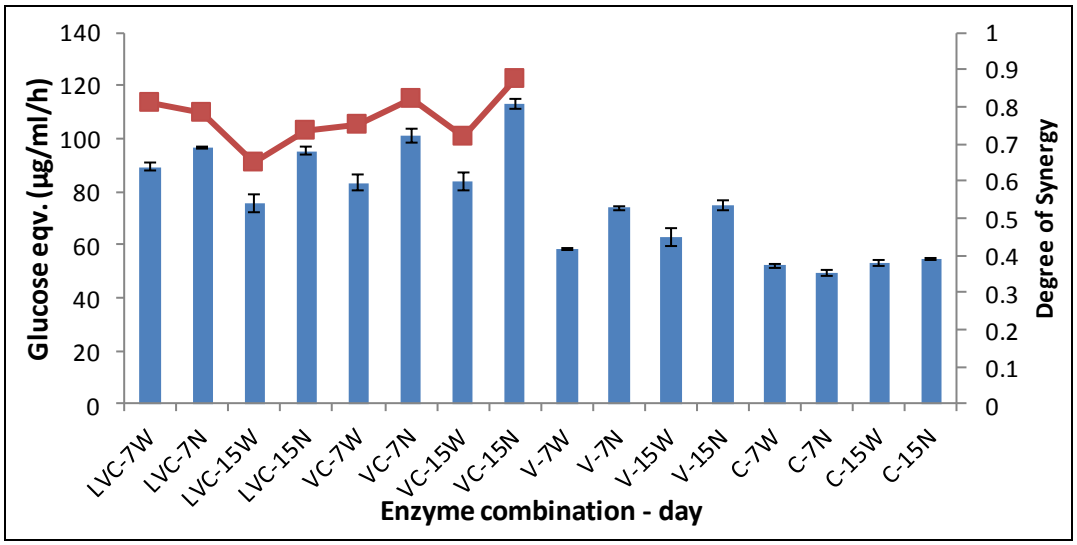


Figure 3.9: Effect of laccase (L) and pre-washing step on Viscozyme L (V) and Celluclast 1.5L (C) activity (solid bars) and degree of synergy (line) on apple pomace. Activity was measured after pre-treatment of 5% (wet w/v) apple pomace by laccase for 7 and 15 days, then washing (W) or not washing (N) the pomace before hydrolysis by Viscozyme L (V) and Celluclast 1.5L. Data points are presented as mean values ±SD (n=3).

3.5. Conclusions

These results demonstrated that a combination of Viscozyme L and Celluclast 1.5L achieved a high degree of synergy and release of sugars. At a ratio of 50:50, the enzyme mixture can be used to effectively degrade apple pomace, thereby assist in reducing enzyme costs and more convenient industrial application. These two enzyme preparations can be used in low concentrations for 24 h and can work simultaneously, which will not only reduce enzyme costs, but also operational costs and time. During apple pomace hydrolysis, the degree of synergy changed over time, being high at the beginning of incubation and low at later stages of incubation. It was also observed that the degree of synergy did not always correspond to yield. Pre-treatment of apple pomace with ligninases did not improve sugar release by Viscozyme L and Celluclast 1.5L and therefore addition of ligninases was not required as they would only contribute to high enzyme and production cost. Further investigation will involve bioreactor studies using the Viscozyme L and Celluclast 1.5L combination to degrade apple pomace.

Immobilisation of the enzymes on different support materials to increase stability and enable reuse and recovery was investigated, but was not successful (results not shown). Since the immobilisation of the enzymes was unsuccessful and the free enzymes showed about 90% stability over a period of 15 days, free enzymes were used in bioreactor optimisation studies (Chapter 4) and kinetic modelling (Chapter 5). Optimisation of apple pomace hydrolysis conditions, as well as the effect of scale-up and bioreactor design will be investigated in the next chapter, Chapter 4.

CHAPTER 4 – BIOREACTOR STUDIES AND OPTIMISATION OF APPLE POMACE HYDROLYSIS USING FREE VISCOZYME L AND CELLUCLAST 1.5L

4.1. Introduction

Chapter 2 and 3 have dealt with characterisation and synergy between the commercial enzyme mixtures, Viscozyme L and Celluclast 1.5L, which provided an insight into the nature of the enzymes. Different immobilisation strategies for the commercial enzyme mixtures, Viscozyme L and Celluclast 1.5L were attempted, however these were not very successful (results not shown). Since Viscozyme L and Celluclast 1.5L have shown good stability (Chapters 2 and 3), free enzymes were therefore used in subsequent experiments.This chapter will discuss optimising hydrolysis conditions, scaling up to larger volumes, bioreactor design, methods of mixing and substrate feeding regimes using free enzymes.

Apple pomace can be enzymatically degraded to clean up the environment and water sources, which may require a bioreactor situated at juicing and canning factories. However, the processing costs may be high due to the high cost of enzymes, equipment and operational costs. In order to make the process cost-effective, value-added products can be produced, for example biofuels (Balat, 2011; Das and Singh, 2004; Gullon *et al*., 2008; Howard *et al*., 2003; Joshi and Attri, 2006; Kolodziejczyk *et al*., 2009; Marin *et al*., 2007; Shalini and Gupta, 2010). Industrial conversion of lignocellulose to biofuels involves mainly four stages, namely pre-treatment of biomass feedstock, enzymatic hydrolysis/ conversion of pre-treated material into simple sugars by different enzymes, fermentation of sugars into ethanol or other alcohols by yeast/ bacteria, and lastly purification/ separation of ethanol/ alcohols from other wastes (Balat, 2011; Dashtban *et al*., 2009; Lin *et al*., 2010) (section 1.1.1.3).

The industrial production of biofuels can be carried out using different bioreactor systems, such as batch, fed-batch or semi/continuous systems. Different bioconversion process strategies can be employed, for example, separate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF) and consolidated bioprocessing (CBP) (Lynd *et al*., 2005; Van Dyk and Pletschke, 2012; Xu *et al*., 2009). SHF offers the

biotechnological advantages of preventing end product inhibition, increasing the hydrolytic rate of enzymes, and a reduction in cost due to the use of smaller reaction volumes and less/ no β-glucosidase. However, the challenge lies in finding suitable conditions that are favourable for both enzymatic hydrolysis and fermentation (Andric *et al*., 2010b; Mills *et al*., 2009; Van Dyk and Pletschke, 2012). In both SSF and CBP, inhibition by alcohols, organic acids and phenolic compounds poses a major challenge (see section 2.4.2.5).

It is envisaged, in the long term, that bioreactors for waste conversion, can be designed and become part of every fruit juice manufacturing plant. This will help in fruit waste beneficiation and hopefully reduce the negative environmental effects of the fruit juice industry. The bioreactor design should promote high productivity, reduce costs, produce consistent product quality, while being easily monitored and controlled. Addition of new substrate, mixing and product removal should also be easily achieved. Bioreactors can be used in sequence, which could be an advantage for separating pre-treatment by ligninases and hydrolysis by cellulases and hemicellulases, since these enzymes have different hydrolysis conditions. The properties of the selected enzymes should be compatible with other enzymes and proposed biotechnological application. However, from Chapter 2, it was seen that the enzymes can be used under same hydrolysis conditions for apple pomace degradation, which is an advantage.

Bioreactor studies were carried out using free Viscozyme L and Celluclast 1.5L. Different immobilisation strategies for the enzymes were attempted but were not very successful (results not shown) and therefore could not be implemented in this study.

4.2. Aims and Objectives

a) To optimise the bioreactor conditions in terms of temperature, pH, type of water, volume and mixing;
b) To design a simple and cheap bioreactor for industrial application and up-scaling;
c) To investigate the effect and efficiency of different substrate feeding regimes;
d) To determine the effect of using high substrate concentrations on enzymatic hydrolysis.

4.3. Materials and Methods

4.3.1. Apple pomace biomass preparation

Apple pomace was prepared as described in chapter 2 (section 2.3.1).

4.3.2. Commercial enzyme preparation stock solution

This was carried out as described in section 3.3.2.

4.3.3. Enzyme assays

The DNS assay was used for the determination of reducing sugars as explained in chapter 2 (section 2.3.5). Individual sugars e.g. glucose, xylose, arabinose, galacturonic acid were analysed using commercial kits (Megazyme, Ireland) according to the manufacturer's instruction manual (Appendix 1). Total sugars were analysed using a phenol sulphuric acid assay (Dubois *et al*., 1956).

The glucose and reducing sugar yield (%) from cellulose and total pomace, respectively, were calculated using the following formulas;
Glucose yield (%) = Glucose liberated (g) x 0.9 x 100/ Initial cellulose (g)
Reducing sugar yield (%) = reducing sugar liberated (g) x 100/ Initial polysaccharides (g)
Calculations were based on dry weights.

4.3.4. Total suspended solids

Total suspended solids were measured according to the modified NREL method (Sluiter *et al*., 2008). A volume of 10 mL from the 15 mL aliquots collected at different time intervals was centrifuged at 4 300 *x g* for 20 min. The supernatant was discarded and the remaining solids transferred to aluminium drying pans. The samples were then dried over night at 105°C after which the dry weight of the sample was determined.

4.3.5. Determining the effect of temperature and buffering capacity on the activity of Viscozyme L - Celluclast 1.5L

The effect of temperature and pH on the activity of Viscozyme L - Celluclast 1.5L (50:50) (0.019 mg/mL of each) combination was determined using 5% apple pomace (wet w/v) (0.5% dry, w/v) final concentration and tap water. Three different incubation temperatures were investigated, namely room temperature (22-26°C), 28°C and 37°C. The effect of

buffering capacity was investigated using tap water, deionised water and citrate buffer (0.05 M, pH 5.0) at 28°C. The assays were performed in 1 L shake flasks and reactions were carried out for 72 h on a platform shaker at 165 rpm with 15 mL aliquots taken at different times and the pH measured. The DNS assay was performed to determine the amount of reducing sugars, as glucose equivalents, released by each treatment. The amount of glucose released after 24 h hydrolysis was measured using a glucose kit from Megazyme (Megazyme, Ireland) (Appendix 1).

4.3.6. Determining the effect of scaling up on the activity of Viscozyme L and Celluclast 1.5L

4.3.6.1. A comparison between 1.2 mL (Eppendorfs) and 480 mL (shake flasks) volumes

The effect of scaling up from 1.2 mL to 480 mL was investigated using un-buffered tap water at room temperature (average 25°C) and 37°C on the activity of Viscozyme L - Celluclast 1.5L (50:50) (0.019 mg/mL each) was determined using 5% apple pomace (wet w/v) final concentration. The assays performed using eppendorfs (1.2 mL) were mixed using a 360° rotary shaker at 25 rpm and that for shake flasks (480 mL) on a platform shaker at 165 rpm. The reaction was run for 24 h with aliquots taken at different time intervals. The DNS assay was performed to determine the amount of reducing sugars, as glucose equivalents, released by each treatment.

4.3.6.2. Comparison between 1, 2 and 5 L reaction volumes (shake flasks)

The experiment was performed as described above in section 4.3.5., using different reaction volumes and deionised water. Hydrolysis was for 72 h at a temperature of 28°C, with 15 mL aliquots taken at different times and pH measured. NaN_3 was used to a final concentration of 0.03% (w/v) to avoid microbial contamination. The supernatants were analysed for reducing sugars (the DNS assay), total sugars (phenol sulphuric acid assay - Dubois *et al.*, 1956), glucose, xylose, arbinose and galacturonic acid content (Megazyme kit, Ireland) as well as total suspended solids. Figure 4.1 shows the set-up for the shake flask reactors.

Figure 4.1: Set up for the 1 L volume platform shake flasks.

4.3.7. Effect of mixing with compressed air and nitrogen on free Viscozyme L and Celluclast 1.5L activities

A comparison on the effect mixing with compressed air and nitrogen on Viscozyme L and Celluclast 1.5L activity was performed using the 1 L Schott bottles. Mixing using platform shaking is not feasible at high substrate loadings and is not applicable for industrial scale use. It was important to determine whether oxygen had a negative effect on the enzymes and the hydrolysis of the substrate. The initial substrate concentration was 5% apple pomace (wet, w/v). Bioreactors of 1 L volumes were designed using 1 L Schott bottles as shown in Figure 4.2. The assay was performed at room temperature for 132 h using compressed air and nitrogen gas for mixing. The other procedures were as described in section 4.3.6.2 above.

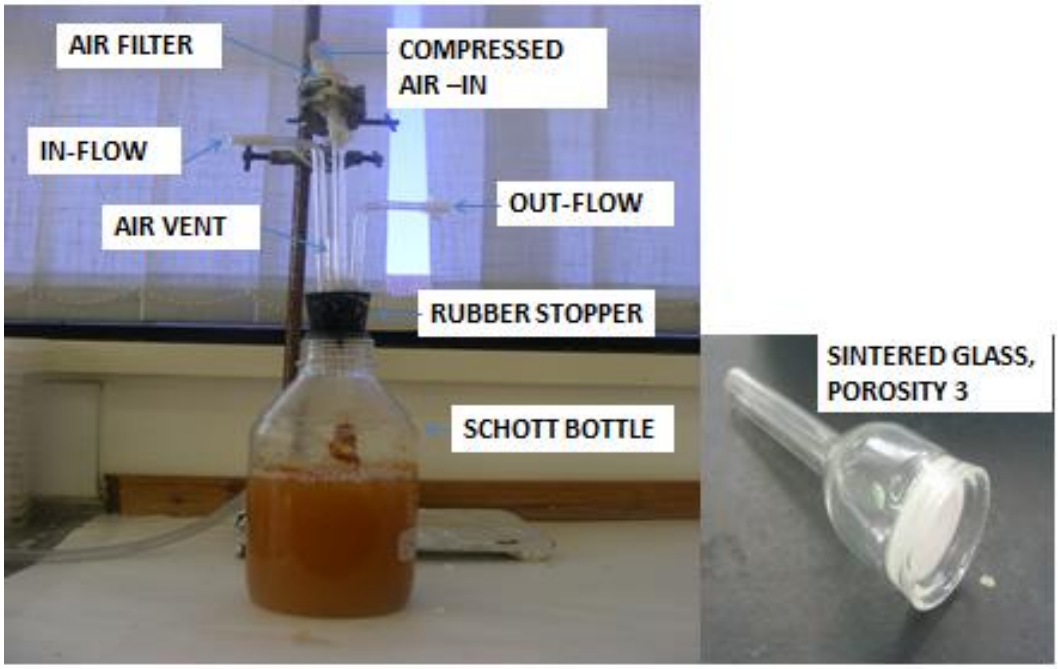


Figure 4.2: A photograph of a Schott bottle bioreactor.

The bioreactor comprised of an in-let, which was used to introduce substrate in the reactor; an out-flow, which was used for collecting samples and which could also be used to connect to another reactor in series; an air-vent to release pressure from the bioreactor; an air in-let fitted with an air filter, for air from the pump. An air sparger was fitted to the end of the air in-let tube for uniform distribution of air and mixing. The sparger was a sintered crucible with porosity 3.

4.3.8. Fed-batch reactions

4.3.8.1. Fed-batch reactions with different initial substrate concentrations and supplementing with the same amount of substrate

Bioreactors of 1 L volumes were designed using 1 L Schott bottles and mixing with compressed air. NaN_3 was added to reactors at a final concentration of 0.03% (w/v) to avoid microbial contamination. The reactors were operated in fed-batch mode at room temperature for 200 h. Two bioreactors were operated with different initial substrate loadings, 5% and 12.5% apple pomace (wet w/v) final concentrations in deionised water. Fresh apple pomace 2.5% (wet, w/v) was added at 50, 100, and 150 h, these were chosen because after 50 h there will be no further increase in sugar production (see Fig 4.3). The rest of the procedures were as described in section 4.3.7 above.

4.3.8.2. Fed-batch reactions with the same initial substrate concentration and varying additional substrate concentrations

An initial substrate loading of 5% apple pomace (wet w/v) was used, with fresh substrate added at 50 h intervals (50, 100, 150 h) using different substrate loadings, 2.5%, 5% and 10%. The rest of the procedures were as described in section 4.3.7 above.

4.3.8.3. Effect of β-glucosidase supplementation

An initial substrate loading of 5% apple pomace (wet w/v) was used, with fresh substrate (10% apple pomace) added at 50 h intervals (50, 100, 150 h). One reactor had only Viscozyme and Celluclast, while the other reactor also included Novozyme 188 (0.025 µL/mL, 0.0012 mg/mL). The rest of the procedures were as described in section 4.3.7 above.

4.3.9. A comparison between fed-batch and batch reactors at high substrate loadings

A comparison between a fed-batch and a batch process was performed. Apple pomace was added in different ways to reactors, but each reactor had a final substrate concentration of 20% apple pomace (wet, w/v). The scheme of substrate addition is demonstrated in Table 4.1. Three reactors were run, one starting with 5% substrate, then 5% additions at 6, 24 and 50 h, the other reactor started with 10% substrate, then 5% additions at 24 and 50 h and the last reactor started with 20% initial substrate loading with no further substrate additions. Viscozyme and Celluclast (50:50) (0.019 mg/mL each) were added together with Novozyme 188 (0.05 µL/mL, 0.0024 mg/mL) to all the reactors. pH was measured at each time interval. The amount of reducing sugars released was measured using the DNS assay, while glucose, galacturonic acid, galactose, arabinose and xylose were measured using Megazyme assay kits.

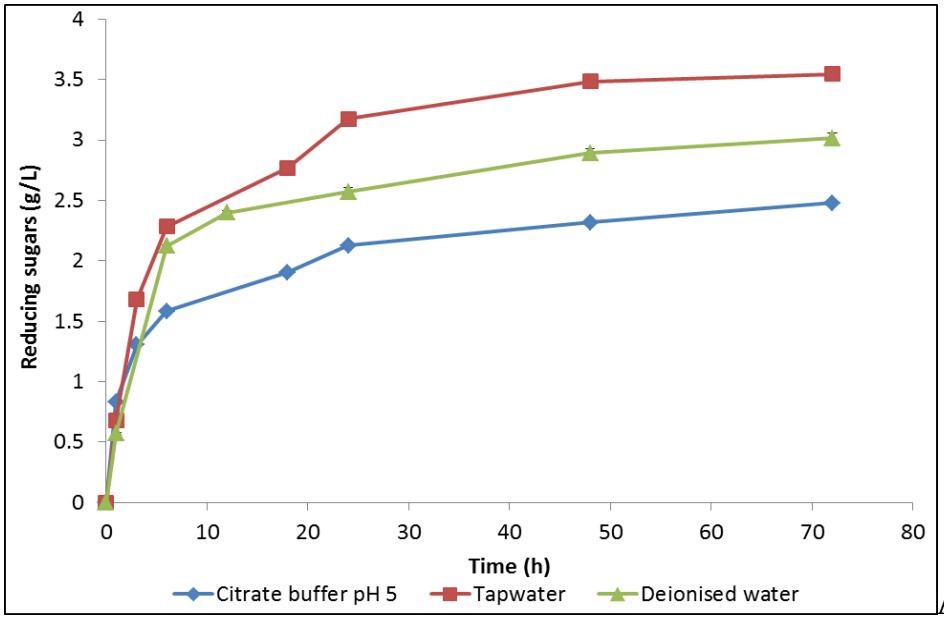
Table 4.1: **Substrate addition mode at different time intervals**

Substrate addition mode	Time(h)			
	0	**6**	**24**	**50**
5+5+5+5%	5	5	5	5
10+5+5%	10	---	5	5
20%	20	---	---	---

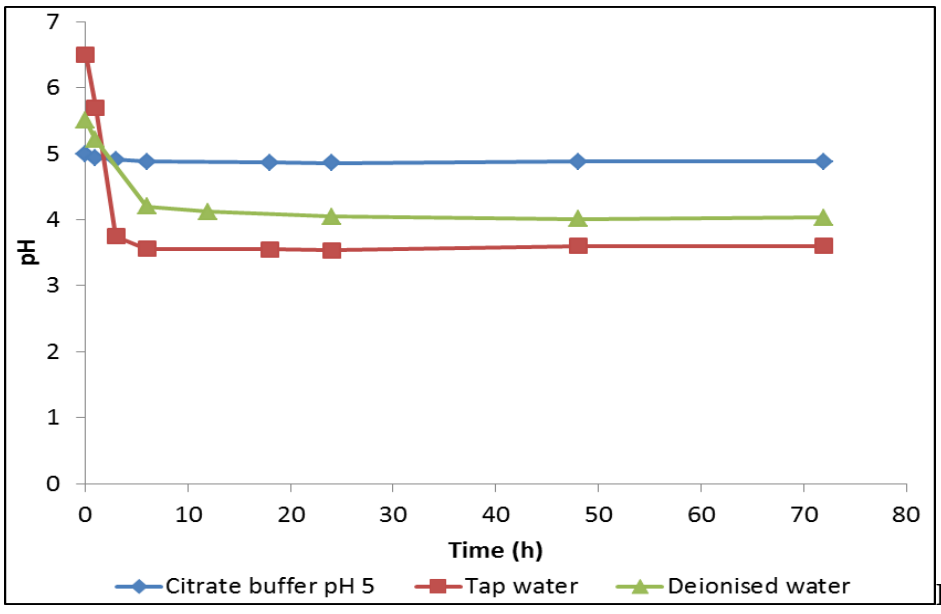
4.4. Results and discussion

4.4.1. Effect of temperature and pH (buffering)

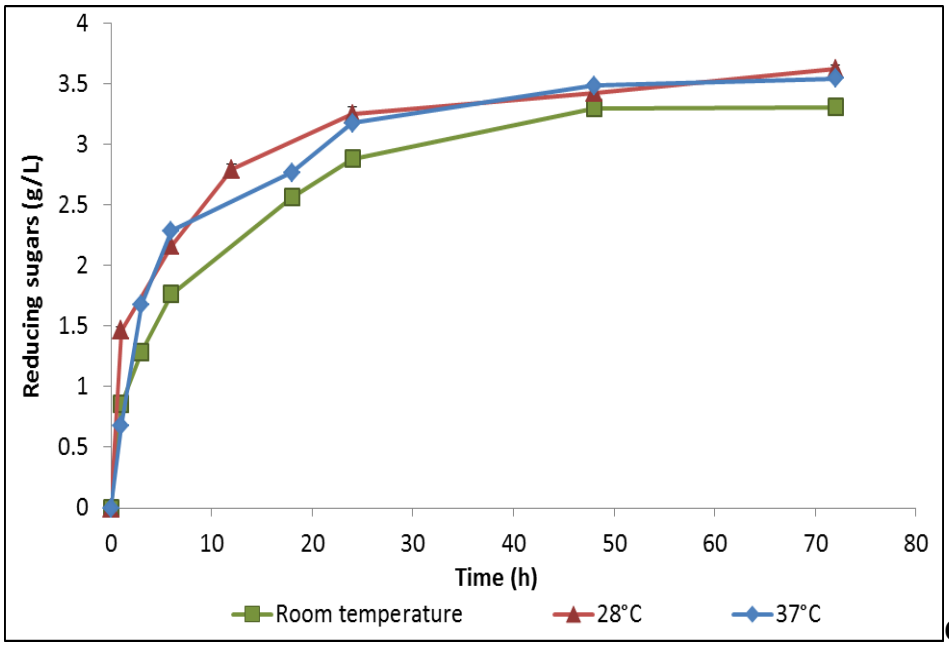
The amount of reducing sugars released and the change in pH was measured over 72 h of incubation of apple pomace using citrate buffer, tap water and deionised water and at different temperatures. The results are shown in Figure 4.3. There was a rapid release of reducing sugars during the first 6 h and the release of sugars was highest in the unbuffered reactors where a sharp decrease in pH was observed during this time (Figure 4.3A and B). The drop in pH in the unbuffered reactors can be attributed to the release of acids such as galacturonic acid from the pectin component.



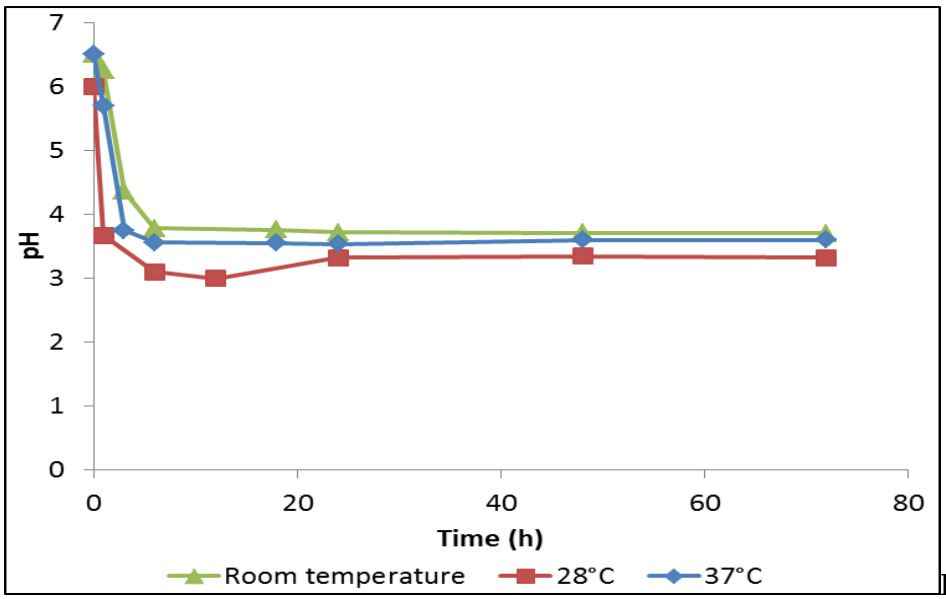
A



B



C



D

Figure 4.3: **The amount of reducing sugars released and pH measured at different time intervals using citrate buffer pH 5.0, tap water and deionised water at 28°C (A and B); and different incubation temperatures using tap water (C and D), respectively.** Apple pomace 5% (wet, w/v) was used as substrate in shake flasks of 1 L reaction volumes and was mixed by shaking on a platform shaker for 72 h. Data points are presented as mean values ±SD (n=3).

Tap water showed the highest release of sugars and lowest pH values, followed by deionised water and lastly citrate buffer (pH 5.0). Citrate buffer maintained the pH around pH 5.0, while in the case of tap water and deionised water, the pH dropped to around pH 3.6 (Figure 4.3 A and B). The enzymes showed activity over a broad range of pHs, Viscozyme L (pH 3.0-6.0) and Celluclast 1.5L (pH 3.0-4.5) (section 2.4.2.2), indicating that the different

enzymes in the mixtures may be active at different pH values. The change in pH may result in some enzymes in the commercial mixtures being inhibited, while others may display enhanced or stimulated activity at lower pH values. The difference between tap water and deionised water may be due to the presence of metal ions in tap water that may have a stimulatory effect on the enzymes, e.g. Ca^{2+}, Mn^{2+}, Mg^{2+} and K^{+} (Ferchak and Pye, 1983; Johnson *et al*., 1982; Tejirian and Xu, 2010). Results from our previous work indicated that these ions had a stimulatory effect on the activity of Viscozyme L and Celluclast 1.5L (section 2.4.2.5). Some ions dissolved in tap water such as $CaCO_3$ and $NaHCO_3$ may result in the change in pH (Mussatto and Roberto, 2004; Parajo *et al*., 1996; Watson *et al*., 1984) (section 2.4.2.5). The dissolved metal ions in water will not only affect the pH and the enzyme, but also the substrate, as they can interact with the substrate in different ways. However, the type and amount of dissolved ions in tap water will differ from batch to batch, and from one source to another and also from one season to another, which may lead to inconsistency from one set of reactions to another. Further experiments were performed with deionised water to exclude the effects of these ions. However, it is clear that tap water would be very suitable for hydrolysis of apple pomace using these enzymes.

The three different temperatures investigated did not display much difference in terms of sugar release and pH, although 28°C and 37°C had slightly higher amounts of sugar release and lower pH values than that of room temperature (Figure 4.3C and D). The amount of reducing sugars released after 72 h at 25°C, 28°C and 37°C were 3.3, 3.6 and 3.5 g/L, respectively. Previous results indicated that Celluclast 1.5L maintained a high activity even at 20°C, but Viscozyme L has slightly lower activity at 20-25°C, which may explain why the activity at room temperature was slightly lower than at 28 and 37°C (section 2.4.2.2). As the temperature increased, the speed of molecules and activation energy also increased up to the temperature optima, rate constants increased and mass transfer resistance decreased. Beyond the temperature optima, the enzyme may become denatured due to intra- and intermolecular bonds breakdown and lose activity (Al-Zuhair, 2008; Bommarius and Broering, 2005; Peterson *et al*., 2007).

The use of a buffered system in industrial applications is not only expensive but operationally difficult. Tap water at room temperature can be used for bioreactor applications, although the reaction will be slower compared to the reaction at 37°C. However, room temperature can fluctuate and that may produce inconsistencies in results. Running an industrial bioreactor

without buffering the system and use of ambient temperature for incubation will reduce operational costs associated with hydrolysis of lignocellulose. From the results it can be concluded that an unbuffered system at room temperature conditions can be used for the bioreactor application since there is no input of energy and cost of buffering, with a sufficiently high level of sugars still being produced.

Previous results showed that, during initial stages of hydrolysis, the substrate structure was opened up and modified, requiring extensive cooperation of enzymes (high synergy) (section 3.4.4), while a maximal number of easily hydrolysable sites on the substrate was available for enzyme activity (Bansal *et al*., 2009; Boisset *et al*., 2001; Van Dyk and Pletschke, 2012). As time progressed, the rate of sugar release decreased and levelled off, indicating that the number of hydrolysis sites for the enzymes was becoming low and also that the reactor conditions may have changed. This decrease in rate can also be attributed to product inhibition (Yang *et al*., 2009). The results were characteristic of a reaction run in batch mode, where the initial reaction rates are high, but decrease with time and may even decrease to zero (Bansal *et al*., 2009; Yang *et al*., 2009). The remaining solids will consist of lignin and unhydrolysed substrate such as crystalline cellulose and the enzyme may become unavailable due to unproductive binding. In addition to the above mentioned reasons, Gan *et al*. (2003) also mentioned a decrease in active enzyme molecules (loss of original catalytic power) as the reaction progressed. Shrinking of cellulose particles as the reaction proceeds may also lead to reduced hydrolysis (Brown *et al*., 2010).

4.4.2. The effect of scaling up to bigger volumes on the activity of Viscozyme L and Celluclast 1.5L

4.4.2.1. Comparison of 1.2 mL (Eppendorfs) and 480 mL (shake flasks) volumes.

The effect of scaling up from 1.2 mL (Eppendorfs) to 480 mL (shake flasks) reaction volumes was investigated. The amount of reducing sugars released after hydrolysis of apple pomace using tap water at different temperatures was determined and the results are shown in Table 4.2.

Table 4.2: Shows the amount reducing sugars (glucose equivalents, g/L) released after 24 h hydrolysis and corresponding yield (%) in brackets using tap water at different temperatures. Values are presented as mean values ±SD (n=3).

Treatment	1.2 mL	480 mL
Tap water (37°C)	2.58±0.05 (51.5±1.0%)	3.17±0.02 (61.2±0.73%)
Tap water (25°C)	2.22±0.04 (44.3±0.71%)	2.88±0.03 (55.5±0.86%)

The amount of reducing sugars released and yield after 24 h hydrolysis were higher using 480 mL reaction volume than a 1.2 mL reaction volume for both 37°C and 25°C. The amount of reducing sugars (yield) using 480 mL at 37°C and 25°C was 3.17 g/L (61.2%) and 2.88 g/L (55.5%), respectively compared to 2.58 g/L (51.5%) and 2.22 g/L (44.3%), respectively.

Differences between 1.2 mL and 480 mL using citrate buffer can be attributed to mixing. Mixing using shake flasks may not be efficient as compared to rotational mixing used with eppendorfs. The high release of reducing sugar and yield at 480 mL as compared to 1.2 mL may be related to the inconsistency in the composition of tap water with each batch or daily variations. Another reason may be the fact that enzymes can be adsorbed on the surface of the eppendorfs, thereby reducing the amount of enzyme that gets involved in the hydrolysis. Eppendorfs tubes (polypropylene) were found to absorb too much cellulase as compared to glass vessels (Bommarius *et al*., 2008). The amount of reducing sugars at 37°C were higher than that at 25°C, which was in agreement with results on Figure 4.3. A comparison between 480 mL and 1 L reaction volume was also performed (results not shown) and the reactors performed in a similar way.

4.4.2.2. A comparison between 1, 2 and 5 L (shake flasks) volumes

The effect of up scaling from 1- 5 L reactor volume was investigated at 28°C using deionised water (1 L) and tap water (2 and 5 L). The amount of sugars released and pH measured at different time points using different reaction volumes are shown in Figure 4.4.

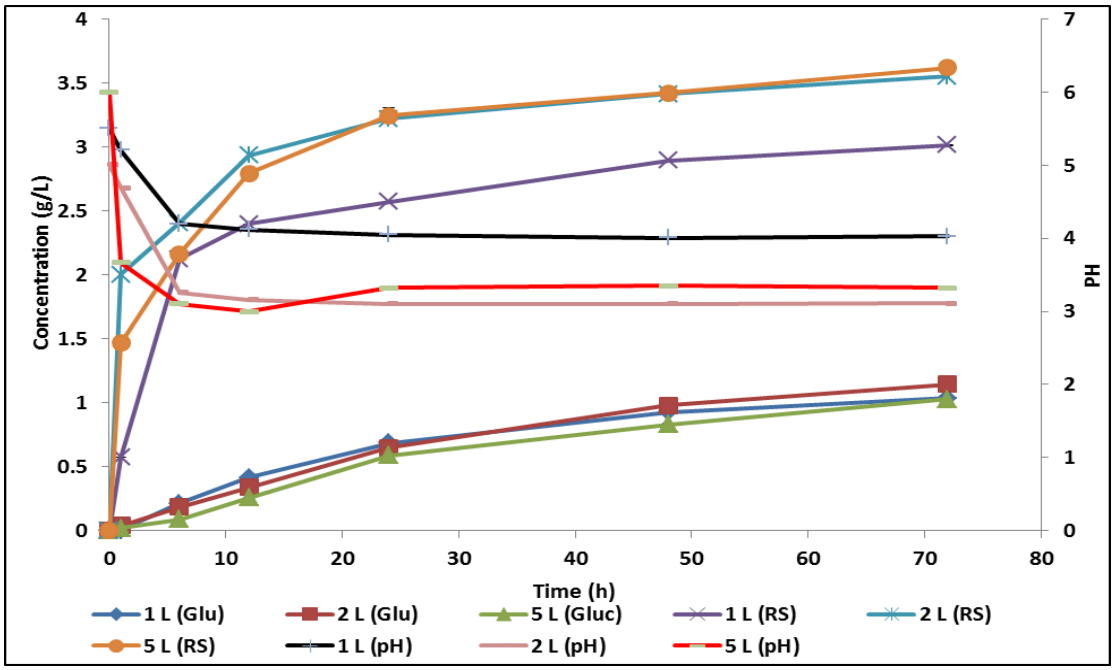


Figure 4.4: The amount of glucose (Glu) and reducing sugars (RS) released after 72 h at 28°C using deionised water and corresponding pH values using different reaction volumes. Data points are presented as mean values ±SD (n=3).

A comparison of hydrolysis between the different reactor volumes showed generally the same trend of pH and sugars released, although there were some minor variations at different time points. The major difference, however, was the low amounts of reducing sugars released in 1 L volumes and this was also related to pH. In order to easily monitor and optimise the reactions, 1 L bioreactors were used in all subsequent experiments. From the results it can be concluded that room temperature conditions can be used for the bioreactor application since there is no input of energy, and sugars released were not much different as compared to that at 28°C and 37°C.

The amount of sugars released and pH measured at different time points for the 2 L reaction volume is shown in Figure 4.5. The amount of sugars released using 2 L reactors after 72 h were 3.5 g/L reducing sugars, 1.1 g/L glucose, 0.1 g/L xylose and 1.2 g/L galacturonic acid. The amount of reducing sugars liberated began to plateau after 24 h. The pH decreased very rapidly in the first 6 h, which corresponded to the release of galacturonic acid. The rapid drop of pH may also be due to the absence of buffering. Galacturonic acid is the major component of pectin and its rapid release in the first 6 h as compared to glucose and xylose indicate that the enzyme hydrolyse the pectin component first. Hemicellulases and pectinases were more

active, opening up the structure for cellulases to hydrolyse the cellulose component. There seemed to be a direct correlation between the release of galacturonic acid, total sugars and reducing sugars with the decrease in pH, however, other organic acids may be released as well. The change in pH may also result in some enzymes in the commercial mixtures being suppressed while others may be enhanced/ stimulated. The two enzyme mixtures displayed a broad pH range with two optima between pH 3.0 and pH 6.0; it is possible that different enzymes may be active at different pH values (section 2.4.2.2). The results showed that during initial stages of hydrolysis the substrate structure was opened up and modified, requiring extensive cooperation of enzymes (high synergy) while a maximal number of easily hydrolysable sites on the substrate was available for enzyme activity (Bansal *et al*., 2009; Boisset *et al*., 2001; Van Dyk and Pletschke, 2012).

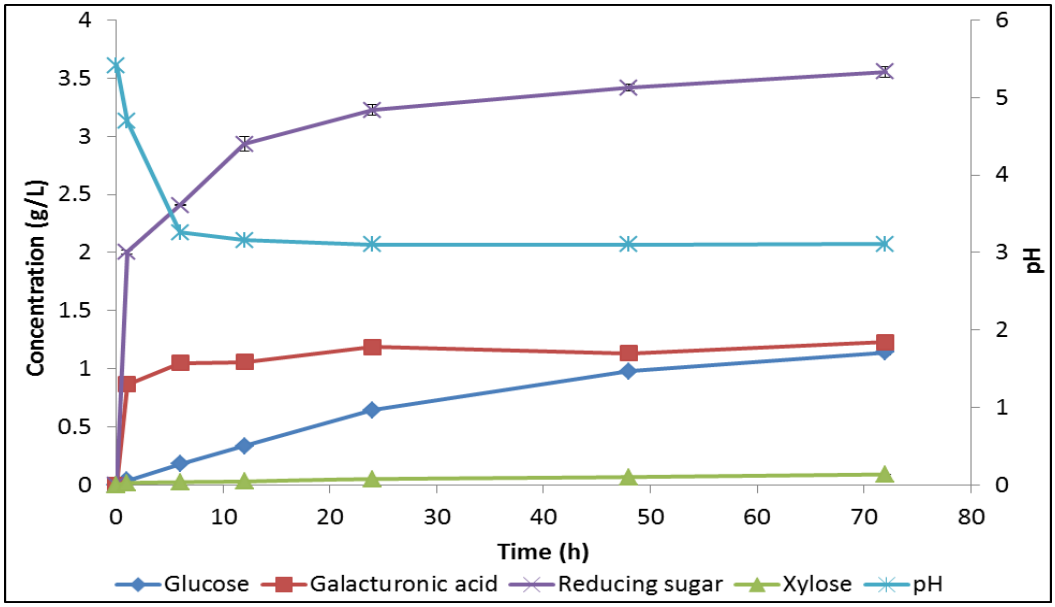


Figure 4.5: Concentrations of different sugars released by Viscozyme L – Celluclast 1.5L combination and pH values recorded at various time intervals. Hydrolysis was performed at 28°C using tap water. Apple pomace 5% (wet, w/v) was used as substrate and in a reaction volume of 2 L. Data points are presented as mean values ±SD (n=3).

As time progressed, the rate of sugar release was low and amounts of sugars produced began to plateau, indicating that the number of hydrolysis sites for the enzymes was becoming limiting and that the reactor conditions may have changed. Another reason, for this observation in the case of glucose release, may be that at the initial stages of hydrolysis the easily digestible parts of the substrate (e.g. amorphous regions) will be hydrolysed first and later the difficult parts (i.e. the crystalline regions) will be hydrolysed at a slower rate (Andric

et al., 2010b, Hall *et al*., 2010; Zhang *et al*., 1999). Enzymes can also be irreversibly/ reversibly adsorbed to the unconverted residue. Although thermal inactivation has also been suggested by other authors, this may not apply to this situation since the enzymes used in this experiment were shown to be very stable for long periods, even at higher temperatures (section 2.4.2.2). It seems that between 24 and 72 h the maximum hydrolysis potential of apple pomace by the enzyme would have been attained, as there was were no significant changes in terms of sugars released between these times. This can also be due to product inhibition (Yang *et al*., 2009). The results were also characteristic of a reaction run in batch mode, where the initial reaction rates are high initially, but decreases over time and may even approach zero (Bansal *et al*., 2009; Rosgaard *et al*., 2007; Yang *et al*., 2009). The remaining solids may be partly lignin and unconverted substrate which may involve crystalline cellulose, and enzyme may be lost by adsorbing to these materials. In addition to the above mentioned reasons, Gan *et al*. (2003) also reported a decrease in active enzyme molecules (loss of original catalytic power) as the reaction progresses. Shrinking of cellulose particles as the reaction proceeds may also lead to reduced hydrolysis (Brown *et al*., 2010).

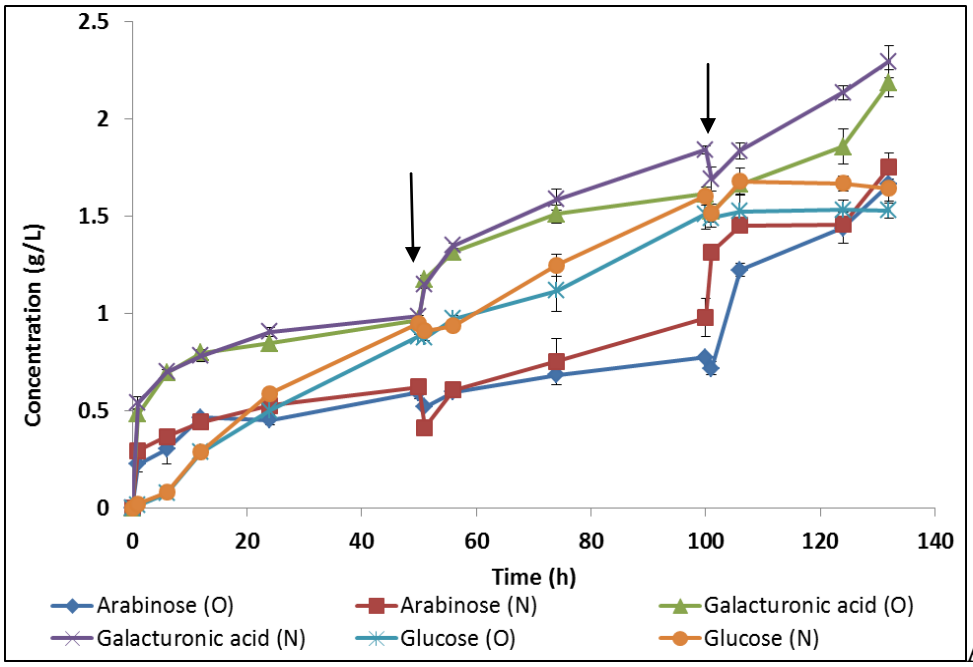
The liberation of high amounts of reducing sugars, greater than the sum of glucose, xylose and galacturonic acid, demonstrated that there were other sugars being liberated, for example arabinose, galactose, fructose, mannose or the presence of oligomers with reducing ends. It was also found that galacturonic acid reacted with the DNS assay in the same way as glucose, thereby adding to the reducing sugar component. Low amounts of xylose released are related to their low levels in apple pomace (Table 2.1). The galacturonic acid, glucose and xylose released from apple pomace are important for value addition, making the industrial process economically viable. However, there may be other sugars and products in the hydrolysate that were not measured and these may add to the value-addition of apple pomace degradation.

The addition of other enzymes such, as β-glucosidases, may result in the improvement in apple pomace hydrolysis, as it would prevent accumulation of cellobiose that is inhibitory to cellulases (Teeri, 1997; Zhang and Lynd, 2004). This could, however, add to the industrial costs of apple pomace degradation. The specific release of cellobiose over time could be measured with and without the addition of β-glucosidases to determine the extent to which this may result in improved performance.

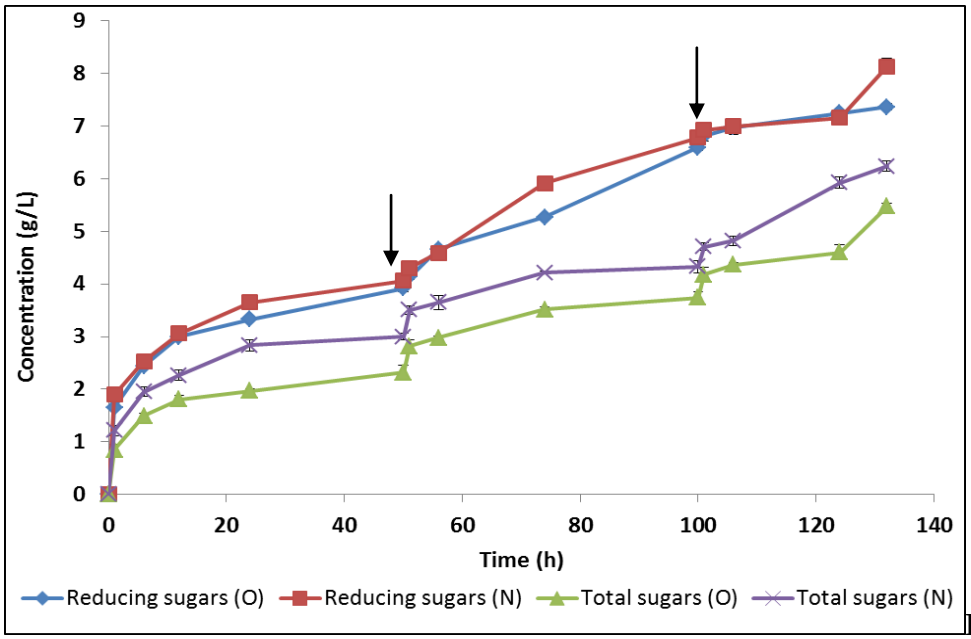
Tap water was also found to give inconsistent results due to variations in the initial pH values. Therefore, deionised water was used in this experiment. However, mixing by shaking was not efficient at bigger volumes. In order to optimise the hydrolysis conditions, the following experiments involved 1 L volumes.

4.4.3. Comparison of mixing with compressed air and nitrogen on the hydrolysis of apple pomace by Viscozyme L and Celluclast 1.5L

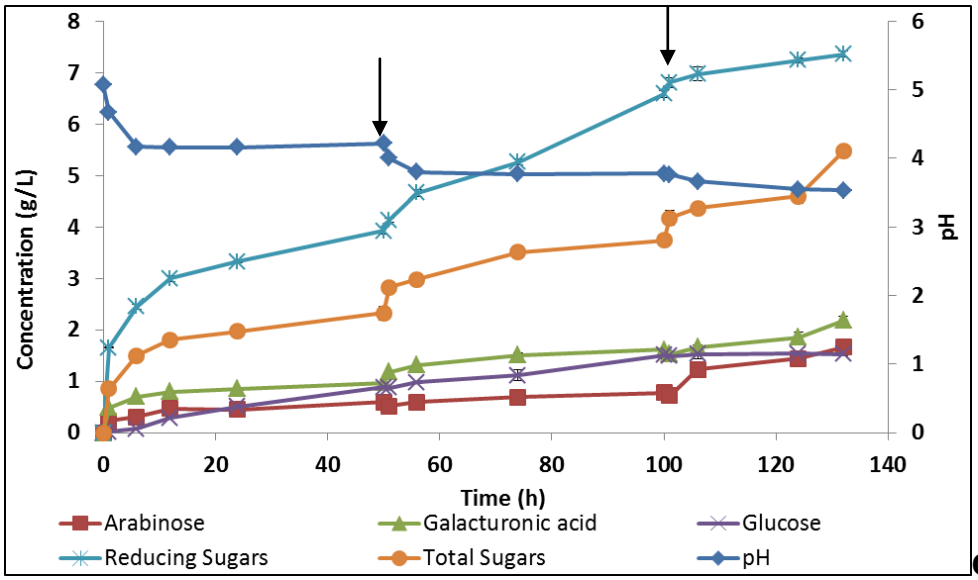
The effect of mixing bioreactors with compressed air and nitrogen was investigated to determine whether they could be used for mixing, and whether oxygen was required by the enzymes. As reported above (section 4.4.2), mixing using shake flasks is not very efficient at high substrate loading. An alternative mixing method was therefore sought. The amounts of different sugars released from the apple pomace (5% wet w/v) and the change in pH was recorded over time during hydrolysis by Viscozyme L and Celluclast 1.5L and the results are shown in Figure 4.6 with spiking at 50 and 100 h with 2.5% of the substrate concentration (half of the initial substrate loading).



A



B



C

Figure 4.6: Concentration of different sugars released at different time intervals for the two reactors, A (arabinose, galacturonic acid and glucose), B (reducing sugars and total sugars) and C (sugar profile and pH for the compressed air reactor). O – compressed air, N – nitrogen. Reactions were performed at room temperature for 132 h. Values are presented as mean values ±SD (n=3). Arrows indicate the point of addition of new substrate.

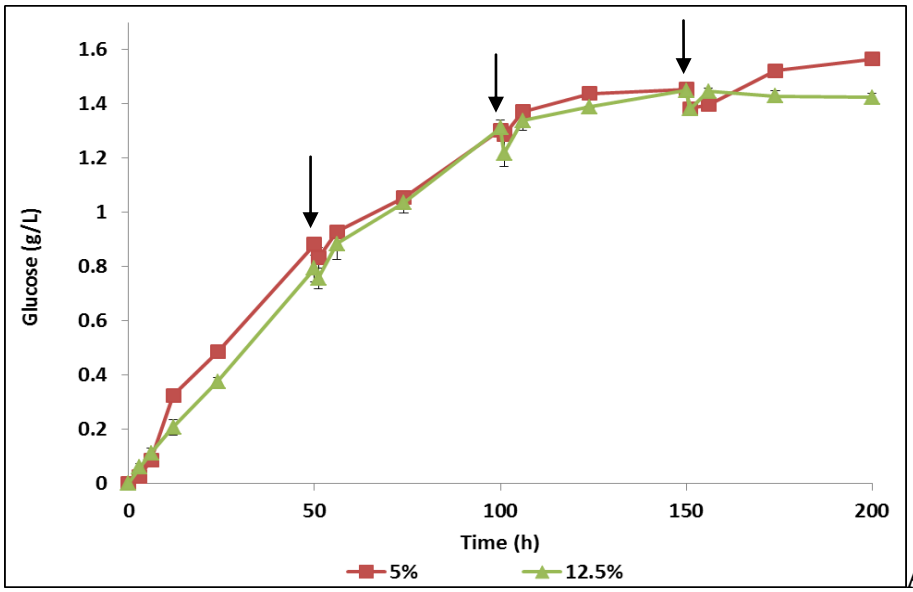
The two reactors generally displayed the same pattern in terms of sugar release, but the bioreactor mixed with nitrogen showed a higher level of sugar production. The total sugars (phenol sulphuric acid method) liberated should be higher than the reducing sugars (DNS assay), however, it was found out that galacturonic acid reacted with the DNS at the same

intensity as glucose, but was 5x less sensitive to the phenol sulphuric acid assay as compared to glucose (results not shown). It has been reported in literature that anaerobic conditions reversibly inhibit commercial cellulase activity (Podkaminer *et al*., 2012). However, the obtained results indicate that Viscozyme L and Celluclast 1.5L were not inhibited by anaerobic conditions. Rodriguez (1991) reported that xylanolytic and filter paper activity of *Cellulomonas* enzymes were inactivated/ inhibited in the presence of high concentration of oxygen, but that CMC-ase activity was not affected by different oxygen concentrations. The obtained results show that there might have been a slight inhibition of some enzymes in the commercial enzyme mixtures used. It has also been reported that xylanase, FPase, CMC-ase and β-glucosidase activities of *Chaetomium thermophile* were high at very low oxygen supply (Hayat *et al*., 2001). As nitrogen gas is more expensive than compressed air, it was decided that compressed air be used for mixing in the bioreactors. However, the use of compressed air may also be expensive for industrial application and may cause loss of water from the bioreactor, making calculations of yield difficult. Oxygen is required by ligninases, which may be important if ligninases are used together with cellulases and hemicellulases to degrade lignocellulose (Cullen and Kersten, 2004; Duran and Esposito, 2000). However, the previous results (section 3.4.7) indicated that ligninases were not important for the hydrolysis of apple pomace.

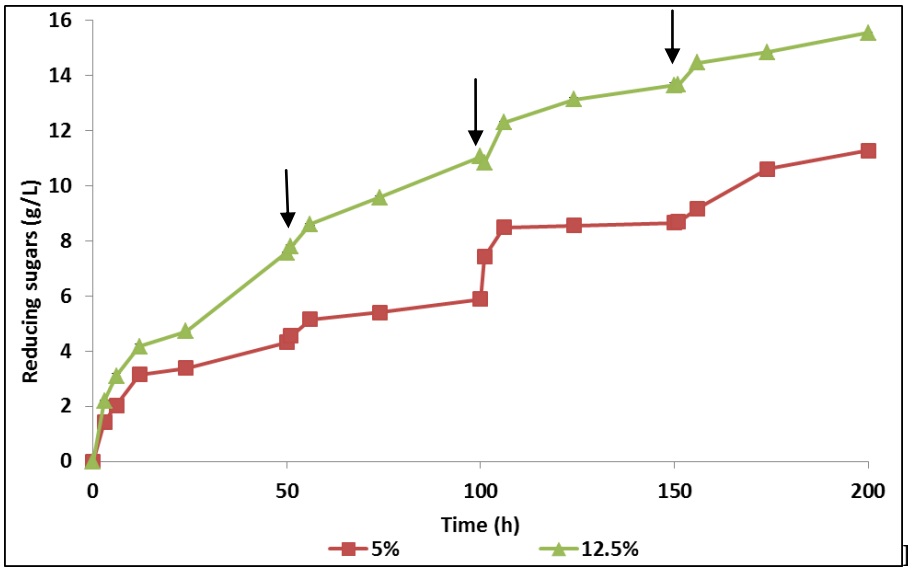
4.4.4. Fed batch reactions

4.4.4.1. Varying initial substrate concentration and supplementing with the same amount

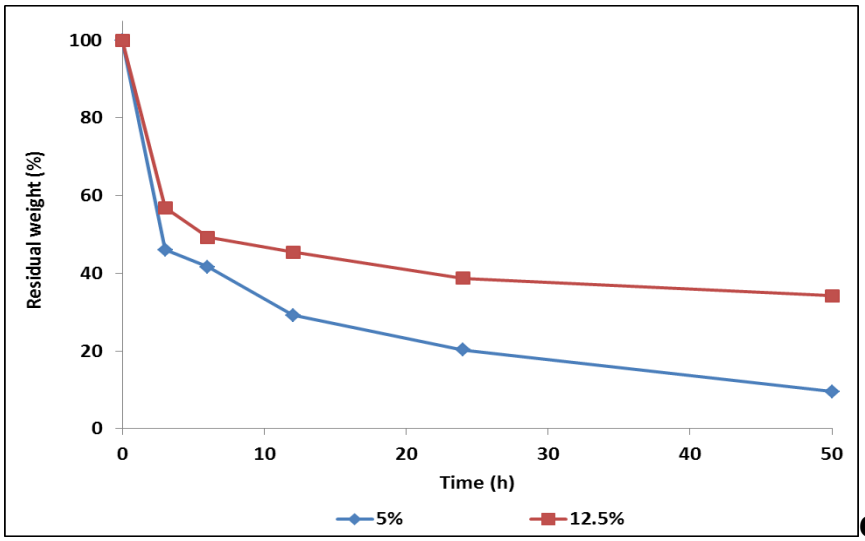
Different initial substrate loadings were used to determine the optimal substrate loading, and determine whether the enzyme or the substrate was limiting. Three bioreactors were run (i.e. triplicate) at room temperature using the designed bioreactor system and mixing with compressed air. The amounts of different sugars released from the apple pomace over time and the change in pH was recorded following different initial apple pomace loadings of 5% and 12.5%. Spiking with new substrate took place every 50 h with 2.5% substrate concentration and results for the released sugars and residual solids are shown in Figure 4.7. The glucose and reducing sugar yields were as shown in Figure 4.8. The final substrate loadings were 12.5% and 20% (wet w/v) for the reactors that were started with 5% and 12.5% substrate loading, respectively.


Glucose (g/L)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
50
100
150
200
Time (h)
5%
12.5%

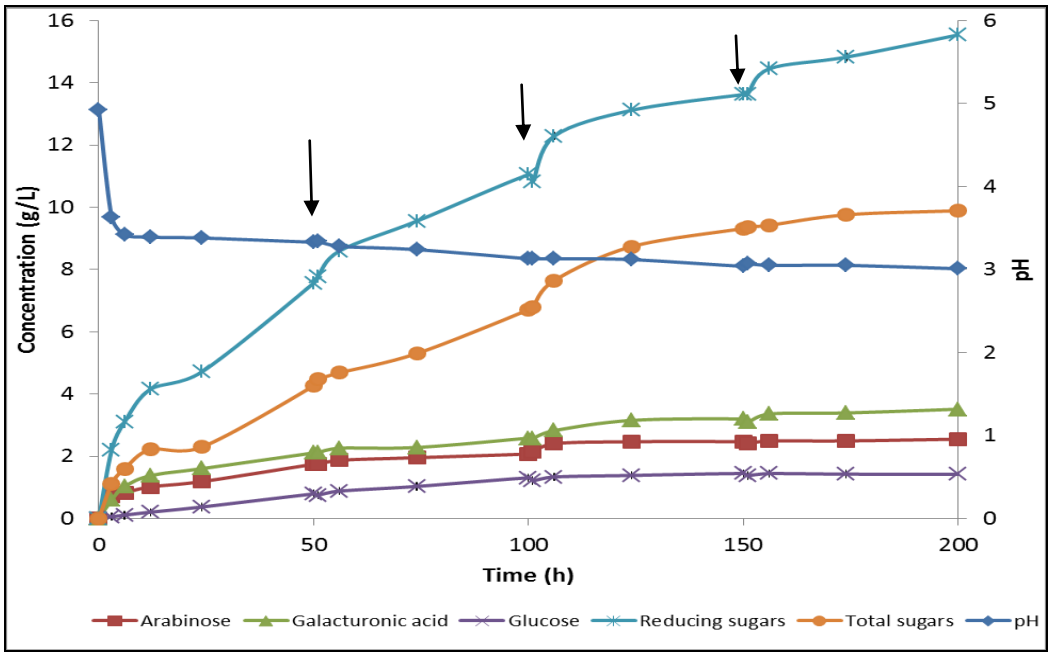
A


Reducing sugars (g/L)
16
14
12
10
8
6
4
2
0
0
50
100
150
200
Time (h)
5%
12.5%

B


Residual weight (%)
100
80
60
40
20
0
0
10
20
30
40
50
Time (h)
5%
12.5%

C



D

Figure 4.7: Comparison of release of different sugars from the two fed-batch bioreactors with different initial substrate concentration: A (Glucose), B (Reducing sugars), released over time, C (residual suspended solids) and D (sugar and pH profile for 12.5% reactor) at room temperature using compressed air for mixing. Apple pomace 5% and 12.5% (wet, w/v) was used as initial substrates and additional 5% substrate was added at 50 h intervals (indicated by arrows) with 1 L reactor volume. Data points are presented as mean values ±SD (n=3).

Multistage addition of substrate resulted in an overall increase in sugar concentrations suggesting that the enzymes were still active (section 2.4.2.2) and also the presence of hydrolysable sites from the fresh substrate (Andric *et al*., 2010b; Zhang *et al*., 1999). However, the overall yield decreased and the hydrolysis slowed down. Addition of new substrate increased the reaction rate, but did not result in a recovery of the initial reaction rate. Enzyme costs will be also low as fewer enzymes will be used to degrade large amounts of pomace. The reduction in hydrolysis rate may be due to end-product inhibition, mass transfer limitation and adsorption of the enzyme on the residual substrate which contain mainly lignin. There was a build-up of solids over time due to the addition of new substrate. The slight drop in observed sugars, immediately after addition of new substrate, may be due to a dilution effect and also insufficient time for the hydrolysis of the additional substrate, which is in agreement to what has been reported in literature (Andric *et al*., 2010c; Rosgaard *et al*., 2007). The use of high initial substrate concentrations resulted in a higher release of sugars compared to the reactors with lower starting substrate concentration, as more

hydrolysis sites were available for enzyme activity. The two reactors that were started with a 5% apple pomace concentration displayed almost identical results with respect to the sugars produced. The bioreactor with an initial apple pomace concentration of 12.5% displayed a higher release of reducing sugars (15.6 g/L), compared to the reactor with an initial substrate concentration of 5% which produced 11.3 g/L of reducing sugars (Figure 4.7). The amount of glucose released from the two reactors was almost the same, reaching around 1.5 g/L after 200 h. The slightly lower amount of glucose released from the 12.5% bioreactor as compared to the 5% bioreactor may be attributed to product inhibition at high substrate loadings. At 50 h, the 5% bioreactor showed about 9% residual solids as compared to 38% residual solids for the bioreactor with an initial substrate concentration of 12.5% (Figure 4.7). The amount of suspended solids decreased rapidly in the first 6 h, which corresponded to the high initial rate of reaction and release of sugars. Comparing the residual % of suspended solids to the original solids for both reactors, it was found that higher initial substrate concentrations resulted in higher amounts of suspended solids remaining after incubation.

Glucose yield was highest at 50 h (68.6%) and dropped to 33.6% after 200 h, while the yield for reducing sugars were 80.3% and 69%, respectively, for the bioreactor with the 5% starting load (Figure 4.8). The 12.5% bioreactor had a glucose yield of 24% (at 50 h) and 23.6% (at 200 h), while reducing sugars were 55.3% and 66%, respectively. The low yields associated with high initial substrate loadings could be due to low enzyme: substrate ratios and mass transfer limitations.

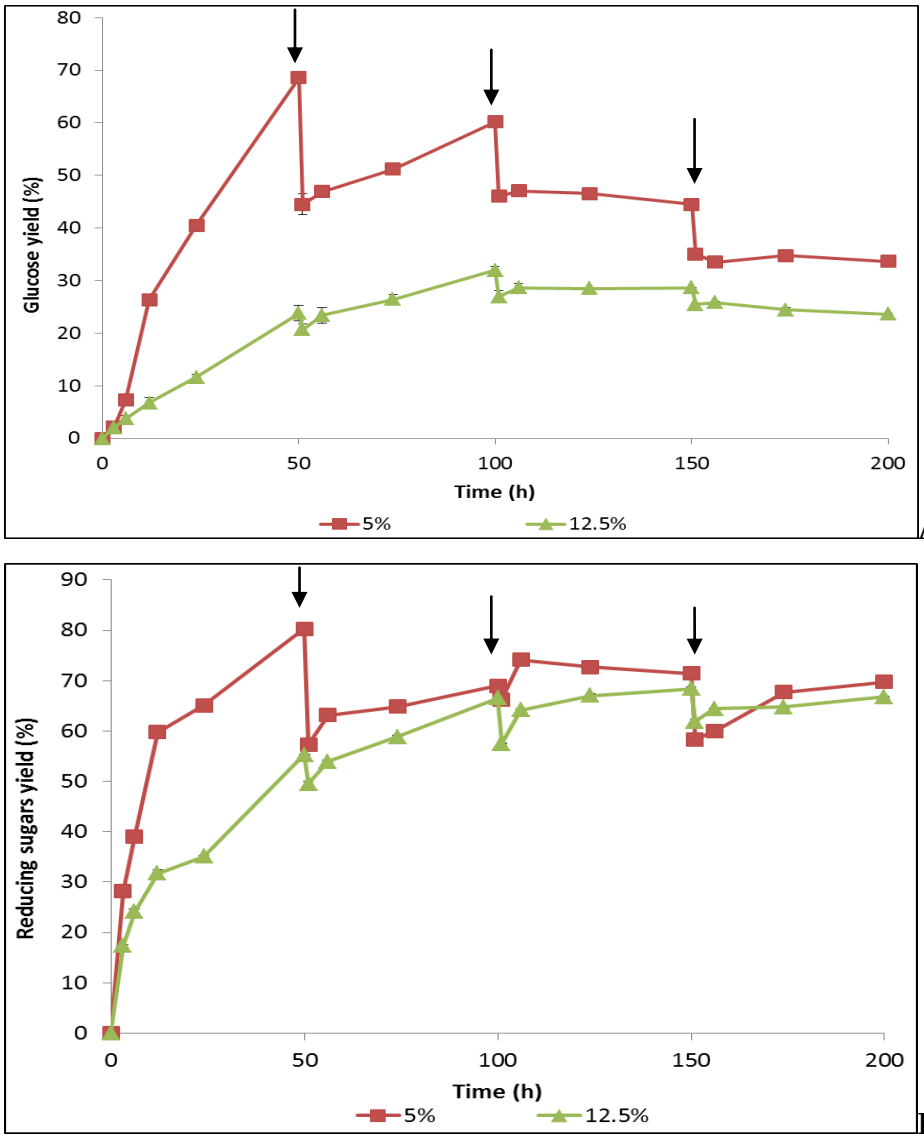


Figure 4.8: Comparison of sugars yields for the two bioreactors with different initial substrate concentrations: A (Glucose yield %) and B (reducing sugar yield%) over time and E (residual suspended solids) at room temperature using compressed air for mixing. Apple pomace 5% (wet, w/v) was used as substrate and additional substrate added 50 h intervals (indicated by arrows) with 1 L reactor volume. Data points are presented as mean values ±SD (n=3).

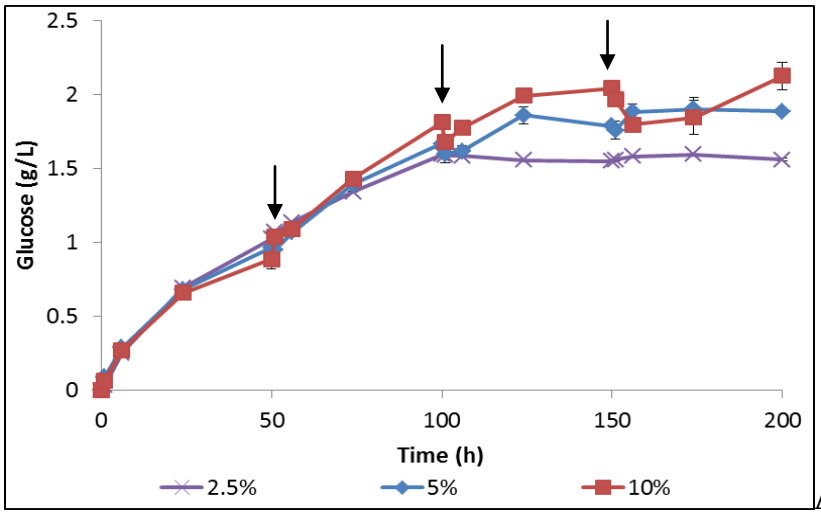
The results indicate that there was better utilisation of substrate at lower concentrations than at higher concentration. At high substrate concentration, substrate and product inhibition may play an important role, which will eventually lower the rate of hydrolysis (Jorgensen *et al*., 2007; Sipos, 2010; Sun and Cheng, 2002). However, the release of glucose was very similar between the two reactors, indicating that the higher concentration of reducing sugars was due to an increase in the release of other sugars in apple pomace. Another possibility for the drop in glucose concentration may be transglycosylation. At high glucose levels, equilibrium

levels may be shifted towards the formation of various glucose oligomers by the action of β-glucosidase (Andric *et al*., 2010a).

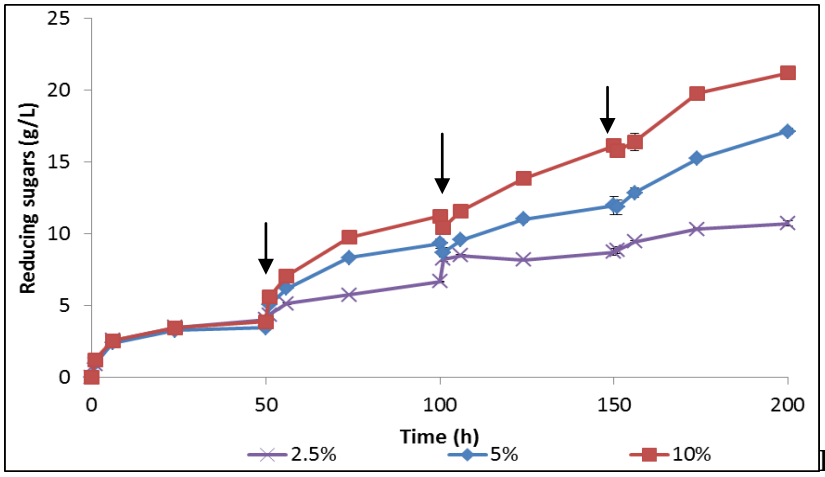
It was interesting to note that the amount of reducing sugars produced using compressed air bioreactors was almost 3x more than the bioreactors using platform shaking (11.3 and 3.2 g/L, respectively) although the amount of glucose was the same at 1.6 g/L (data not shown). This indicated that mixing the bioreactor with compressed air was more efficient than using the platform shaker. Efficient mixing is more important in the initial stages of the reaction to distribute the enzymes (Palmqvist *et al*., 2011). Rodriguez (1991) reported that mixing by shaking resulted in the shear inactivation/ inhibition of fungal cellulases, which could be a possible reason for the reducing sugars being lower when using platform shaking than when using compressed air. Lu *et al*. (2010) reported that shake-flask mixing systems pose heat and mass transfer problems. Mixing by compressed air may improve the mass transfer of heat, substrate and product, thus allowing uniform environmental conditions around the active site of the enzyme and in the bioreactor in general. The mixing by shaking may have been rigorous compared to mixing by air, which could have resulted in some shear deactivation of the enzyme. On the contrary, Gan *et al*. (2003) found that different impeller agitation speeds had little effect on production of reducing sugars. Aeration also increases the interface between the liquid and gas phases. However, the use of compressed air for mixing may not be practical due to cost considerations. Alternative methods of mixing like impeller agitation will have to be explored for the larger scale bioreactors.

4.4.4.2. Fed-batch reactions with the same initial substrate concentration and varying the amounts of additional substrate

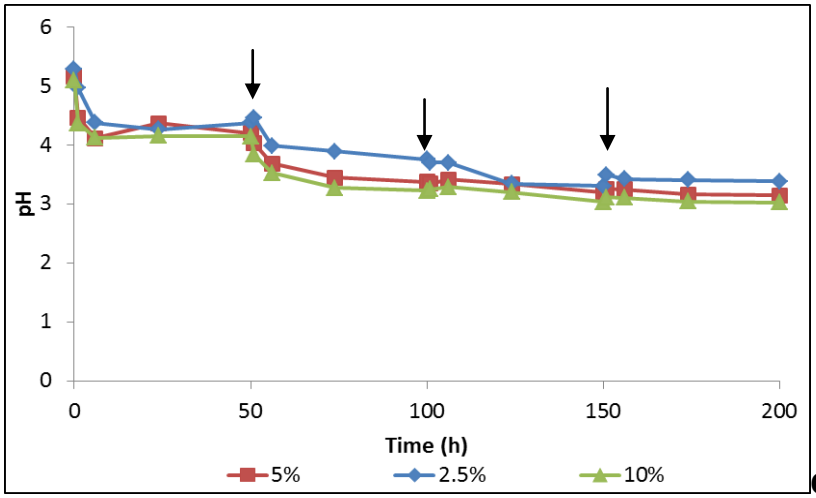
Three bioreactors were studied using the same initial substrate (5%, wet w/v) concentration, but with different levels of substrate addition (2.5, 5 and 10%) at 50 h intervals. The concentration of different sugars released over time and the pH was recorded as displayed in Figure 4.9. The total final substrate loadings for each reactor was 12.5, 20 and 35% apple pomace (wet w/v), respectively.



A



B



C

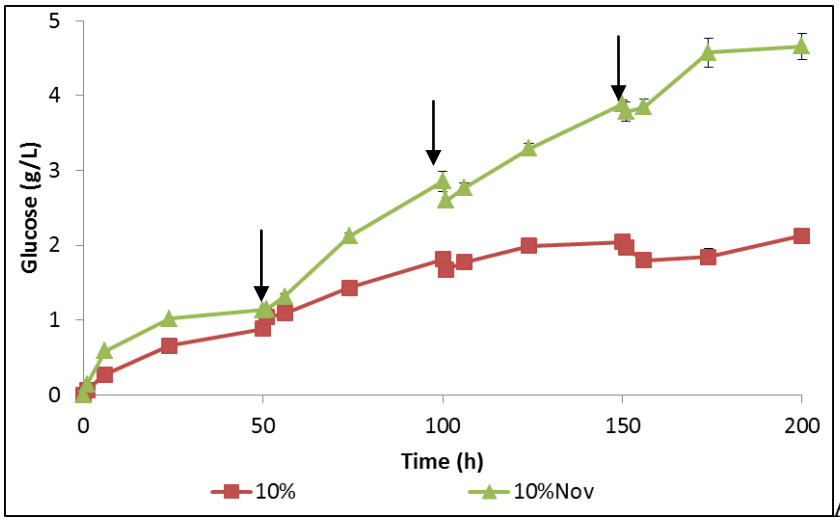
Figure 4.9: Concentration of sugars released and pH measured at different time intervals for the fed batch reactor mixed with compressed air at room temperature with different supplementary substrate concentration (A – glucose, B -reducing sugars, C - pH). All reactors started with 5% (wet, w/v) substrate and different amounts of substrate were then added every 50 h (indicated by arrows). Data points are presented as mean values ±SD (n=3).

The three bioreactors initially performed the same before addition of different amounts of fresh apple pomace after 50 h (Figure 4.9A and B). When new substrate was added, the amount of sugars released, especially reducing sugars increased with the amount of substrate added, while the pH decreased with every substrate addition, with a bigger decrease associated with the higher concentration of substrate added (Figure 4.9C). The increase in sugars released displayed a correlation with the decrease in pH (Figure 4.9C). The pH values recorded for the three reactors followed basically the same trend. The amount of glucose released from bioreactors with 2.5%, 5 and 10 substrate loadings after 200 h were 1.6, 1.9 and 2.1 g/L, respectively, with an overall glucose yield of 79 (40), 74 (30.6) and 69 (19), respectively, at 50 h (200 h). The amount of reducing sugars released from 2.5%, 5 and 10 bioreactors were 10.7, 17.11 and 21.2 g/L, respectively, which corresponded to yields of 76.2 (68.5), 65.7 (68%) and 74.5 (47.1), respectively, for 50 h (200 h) (Figure 4.9). An increase in substrate loading resulted in an increase in the amount of sugars released, but yields may be low at high substrate loadings. Operation at high solid concentrations have the advantage of increasing sugar concentration, which will lower capital operational costs associated with reduced reactor volume, reduced energy and reduced operational costs (Hodge *et al*., 2009; Zhang *et al*., 2009; Zhang *et al*., 2010). However, a high solid loading requires more energy for mixing and results in the build-up of higher levels of residual solids. Comparing the results of the 12.5% bioreactor (Figure 4.7) and 5% bioreactor (Figure 4.9), which had the same final substrate concentration of 20%, but loaded differently, it is apparent that starting with a low substrate loading will improve yield. The 12.5% bioreactor in Figure 5.7, produced 1.5 g/L glucose and 15.6 g/L reducing sugars, while the 5% bioreactor in Figure 4.9, produced 1.9 and 17.1 g/L, respectively. The yields at 50 h hydrolysis time in the 12.5% bioreactor was 24% and 55.3% for glucose and reducing sugars, respectively, while that for a 5% reactor was 74% and 65.7%, respectively.

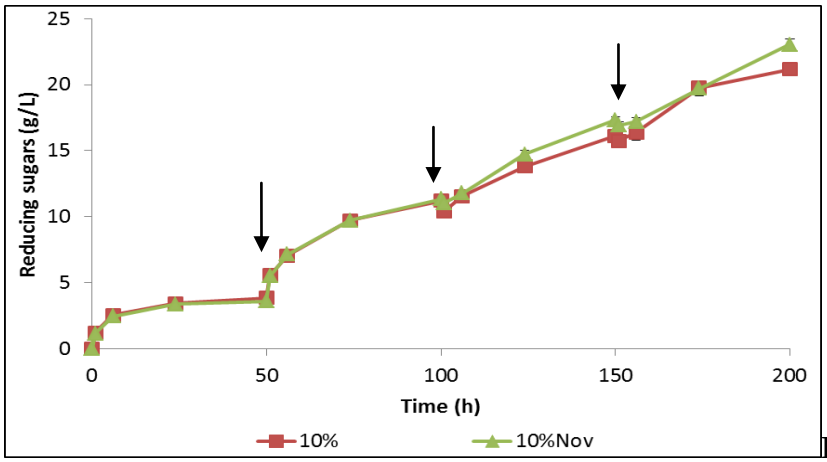
Continuing hydrolysis beyond 200 h did not result in significant increase in sugar release (results not shown) and resulted in reduction in the amount of glucose which could be attributed to transglycosylation or microbial contamination. Though NaN_3 was used, there is a possibility that sodium azide could have broken down overtime, resulting in the required concentration not maintained. There is however, no information in literature or from the manufacturer's product data sheet about the shelf life of sodium azide in water. Reducing the hydrolysis time will also serve on the energy input costs and time as well as avoiding loss of glucose. At high substrate concentration, more energy is required for mixing.

4.4.4.3. Effect of β-glucosidase supplementation

The effect of supplementing with additional β-glucosidase activity was determined by adding Novozyme 188 to a reactor and comparing it with a reactor without Novozyme 188. The initial substrate concentration for both reactors was 5% and additional substrate of 10% was added every 50 h. The amount of glucose and reducing sugars released are shown in Figure 4.10.



A



B

Figure 4.10: Concentration of sugars released at different time intervals for the fed batch reactor mixed with compressed air at room temperature, with (10%Nov) and without Novozyme 188 supplementation (10%) (A – glucose, B -reducing sugars). Both reactors were started with 5% (wet, w/v) substrate and 10% substrate added every 50 h (indicated by arrows). Data points are presented as mean values ±SD (n=3).

The amount of glucose released from bioreactors with 10% and 10Nov% substrate loadings after 200 h were 2.1 and 4.7 g/L, respectively, with an overall glucose yield of 69 (19) and 88% (46%), respectively at 50 h (200 h). The amount of reducing sugars released from 10%

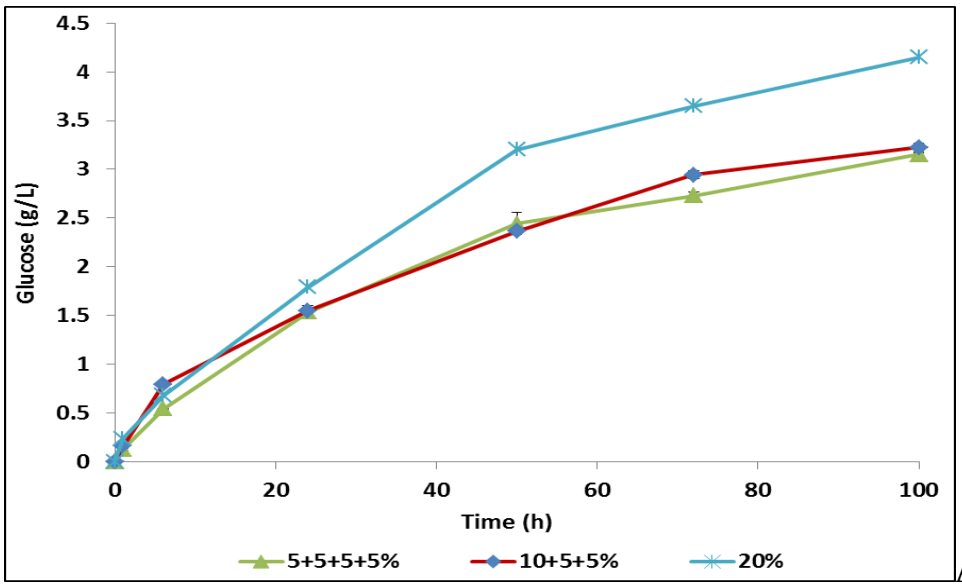
and 10Nov% bioreactors were 21.2 and 23 g/L, respectively, which corresponded to yields of 74.5 (47.1) and 69% (56%), respectively, for 50 h (200 h). The bioreactor with Novozyme 188 displayed the highest glucose release due to higher levels of β-glucosidase activity, which supplemented the β-glucosidase found in Viscozyme L and Celluclast 1.5L. The supplementation of β-glucosidase increased glucose yield due to cleavage of cellobiose into two glucose units, and also prevent end-product inhibition of cellulases by cellobiose (Teeri, 1997). Our previous results indicated significant inhibition of Viscozyme L and Celluclast 1.5L in the presence of cellobiose (2.4.2.5).

The reactor with Novozyme 188 released more than double the amount of glucose compared to the reactor without Novozyme 188. Though there was a big difference in glucose concentration with addition of Novozyme 188, only a small difference was observed in reducing sugars produced (Figure 4.10). HPLC results (data not shown) indicated that the amount of cellobiose was reduced by half following addition of Novozyme 188. However, there were still small amounts of cellobiose remaining in the reactor in spite of Novozyme 188 supplementation. Most researchers have supplemented Celluclast 1.5 L with Novozyme 188 for hydrolysis of different lignocellulose substrates (Bansal *et al*., 2009; Rosgaard *et al*., 2007; Van Dyk and Pletschke, 2012). Results from our previous work performed using 5% (wet, w/v) apple pomace for 24 h, indicated that Novozyme 188 supplementation had no effect on the release of reducing sugars (section 3.4.2). In this study, the amounts of reducing sugars were very similar with or without Novozyme 188. However, the effect on glucose release became significant after 50 h hydrolysis. This indicated that Novozyme 188 supplementation was required for longer hydrolysis times and when the substrate loading was high. However, a balance has to be struck as increased glucose concentrations can also inhibit β-glucosidase and may also cause enzyme jamming on the substrate (Bommarius *et al*., 2008). This will result in low yields and increased enzyme costs.

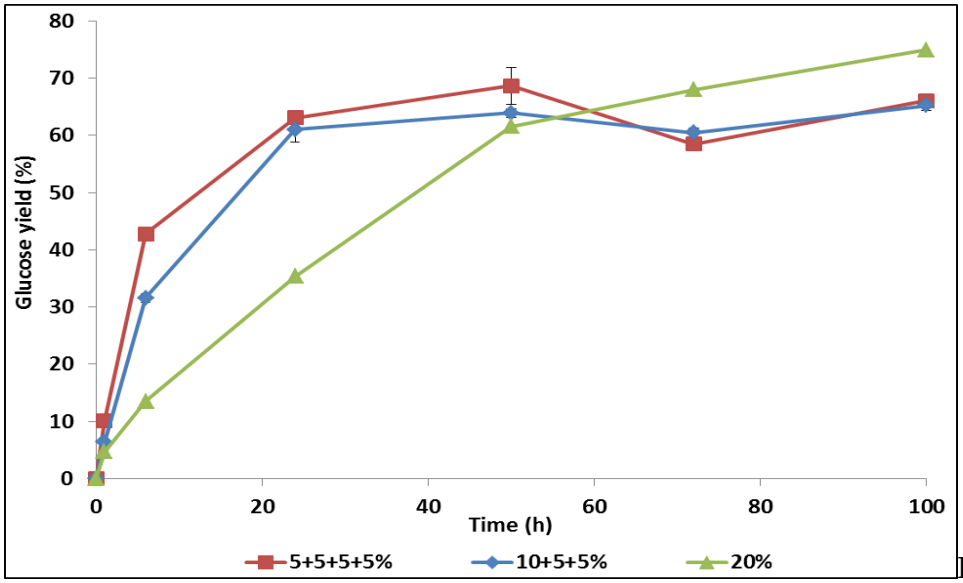
4.4.5. A comparison between fed-batch and batch reactors at high substrate loadings

Further studies were carried out to compare a fed-batch system with a batch process at a higher substrate loading (20%). Three reactors were compared, one reactor starting with 5% substrate and addition of a further 5% fresh substrate at 6, 24 and 50 h to give a total of 20% substrate. The second reactor was started with 10% substrate with further addition of 5% substrate at 24 and 50 h to also give 20% total substrate. The third reactor was started with

20% substrate with no further addition of substrate. The results are shown in Figure 4.11. The final substrate loading for each reactor was therefore 20%. A detailed sugar profile for the batch reactor is shown in Figure 4.12.



A



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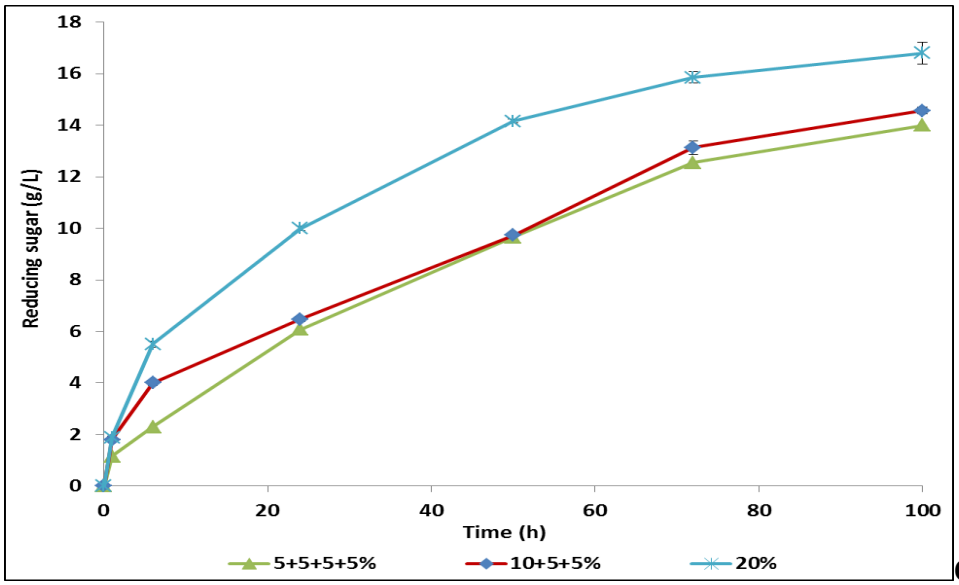
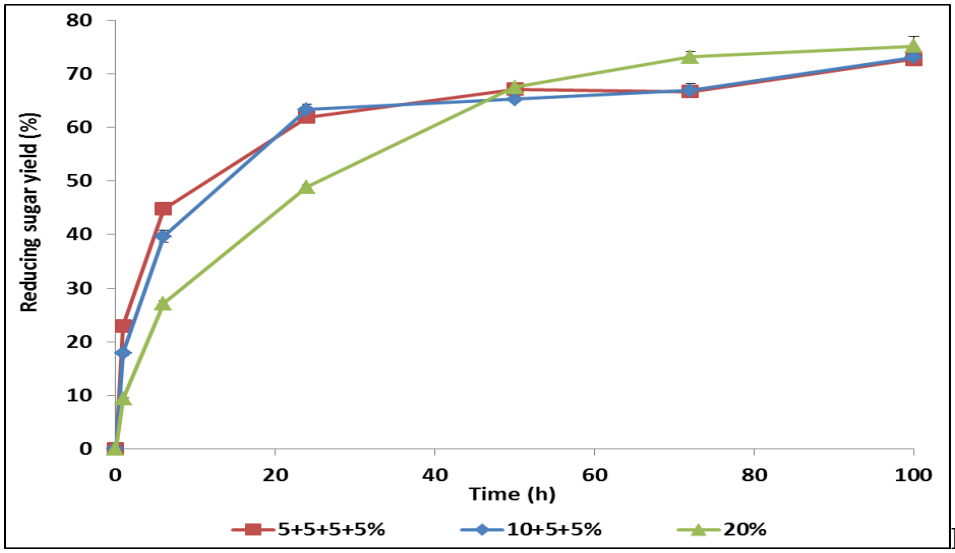


C



D

Figure 4.11: Concentration of sugars released at different time intervals for the reactor mixed with compressed air at room temperature with different substrate loading regimes. Reactors are identified according to their initial substrate loading (5%, 10% and 20%). A (Glucose), B (glucose yield), C (reducing sugars) and D (reducing sugar yield). Data points are presented as mean values ±SD (n=3).

The amount of glucose (% yield) released by the different reactor treatments (5%, 10% and 20% starting substrate concentration) after 100 h were 3.2 (66.1%), 3.2 (65.5%) and 4.2 g/L (75%), respectively. The bioreactors (5%, 10% and 20%) produced 14 (72.7%), 14.6 (73.1%) and 16.8 g/L (75%) reducing sugars, respectively. Reactors that started with 5 and 10% substrate performed almost identically. However, the reactor operated in batch mode, starting with 20% substrate, produced a higher sugar concentration and yield after 100 h (Figure 4.11). Low sugar yields obtained in bioreactors operated in fed-batch mode may be caused by

enzyme jamming, as enzyme molecules interfere with each other. Another reason for this may be due to a dilution effect and additional pomace at each new substrate addition. The enzyme: substrate ratio will initially be high as compared to a batch process. High initial substrate loading resulted in high sugar release, although the yield was lower for high initial substrate loading up to 50 h, which agrees with Ioelovich and Morag (2012). Higher initial substrate concentrations required a longer hydrolysis time for the reaction to be complete, which was in agreement with studies on CMC and corn stover using cellulase (Al-Zuhair, 2008; Imai *et al*., 2003; Lu *et al*., 2010). Viscosity will be high at the beginning of hydrolysis, thereby posing mass transfer limitations. Enzymes may bind unproductively to the substrate and, together with end-product inhibition, this may lead to a low rate of glucose release at high substrate loadings (Lu *et al*., 2010; Mussatto *et al*., 2008; Rosgaard *et al*., 2007). This is characteristic of a batch reactor and fed-batch reactors are reported to reduce these effects (Andric *et al*., 2010b).

The fed-batch reactors resulted in maximal conversion of substrate and higher yields up to 50 h as compared to a batch process. Though the substrate loading was the same for all reactors from 50 h, yields were higher for the batch process with an initial loading of 20% substrate, which can be attributed to lengthier interaction between the substrate and the enzymes. The obtained results agreed with work done on saccharification and fermentation of steam pre-treated spruce, which showed no major performance differences between fed-batch and batch process, although high yields were obtained for a fed-batch process at short incubation periods (Rudolf *et al*., 2005). Rosgaard *et al*. (2007) also reported a high sugar release using a batch strategy, but high initial yields with fed-batch reaction. However, Gupta *et al*. (2012) indicated that a fed-batch strategy performed better than a batch process. There was a correlation between initial substrate loading, yield and pH. High substrate loadings resulted in lower pH values and higher yields as compared to low substrate loadings. pH displayed a correlation with the amount of galacturonic acid released (Figure 4.12). The results indicated that there was greater synergy between the enzymes in the initial stages of hydrolysis, as the structure of the apple pomace was opened up. However, at higher substrate loadings, conversion efficiency decreased. It is therefore important to draw a balance between conversion ratio, substrate concentration and productivity by comparing the production costs.

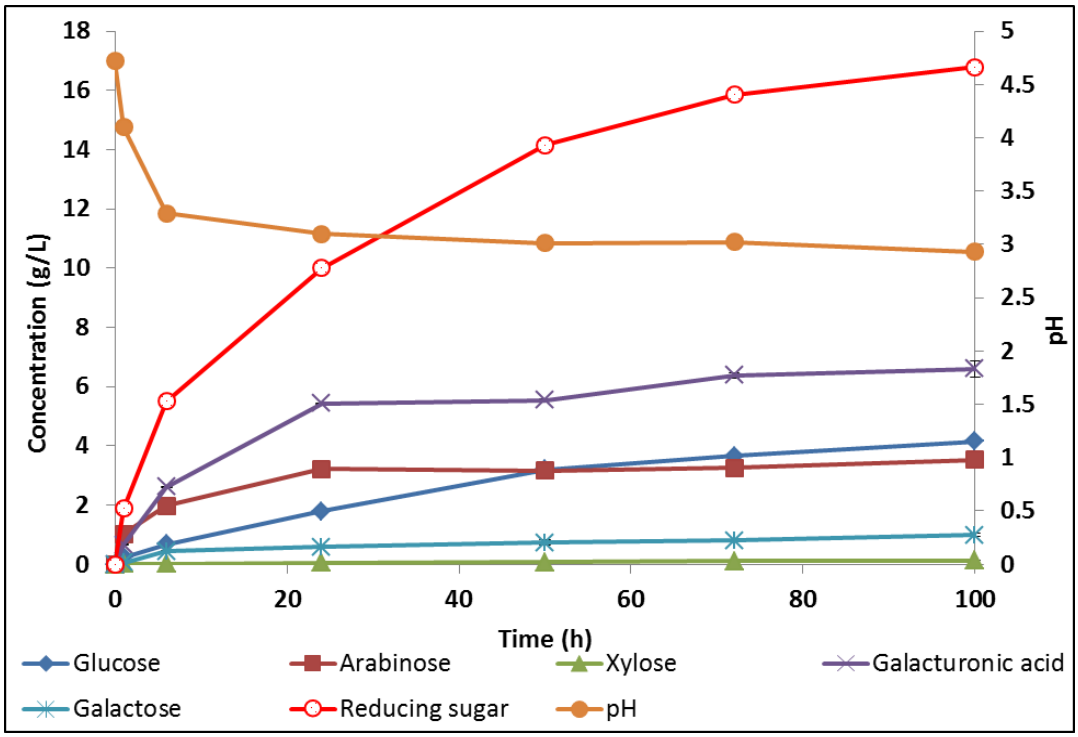


Figure 4.12: Concentration of sugars released at different time intervals for the batch reactor mixed with compressed air at room temperature for 100 h. The initial substrate loading 20% (wet, w/v). Data points are presented as mean values ±SD (n=3).

Galacturonic acid, followed by arabinose and glucose, were the major products released from apple pomace (Figure 4.12). The pattern of sugar release was more or less the same as shown previously, Figures 4.5, 4.6 and 4.7. Galacturonic acid and arabinose are components of pectin and their rapid release in the first 6 h, as compared to glucose and xylose, indicated that the enzymes hydrolysed the pectin component first (Figure 4.12). Hemicellulases and pectinases appeared to be more active at the initial stages, modifying and opening up the structure for cellulases to hydrolyse the cellulose component. There seemed to be a direct correlation between the release of galacturonic acid and the decrease in pH, however, other organic acids may be released as well. Pectin degradation was due to the presence of pectinases in the enzyme mixtures. Arabinose is part of the neutral side-chains in rhamnogalacturonan I, where it is attached to the rhamnose residues and is also associated with galactose (Oechslin *et al*., 2003; Voragen *et al*., 2001). The release of arabinose was an indication of the presence of arabinase and arabinofuranosidase activities in Celluclast 1.5L and Viscozyme L (as identified in previous work, section 2.4.2.3). Cleaving of arabinose residues probably resulted in increased hydrolysis of the rhamnogalacturonan I in pectin. This resulted in improved hydrolysis of pectin, hence opening up the pomace structure to allow increased accessibility of cellulose by cellulases. The results (Figure 4.12) indicate that most

of the sugars are released within the first 50 h, therefore operating the reactors for a shorter time in a batch mode will be a better option. Reducing the residence time will result in the reduction of costs and easy control of microbial contamination. Therefore a batch reactor is recommended as it also easy to operate.

Scanning electron microscope (SEM) images were taken to show the extent of enzyme degradation as time progressed. Figure 4.13 shows a SEM image of samples in the batch process (20% apple pomace) at 0 and 100 h.

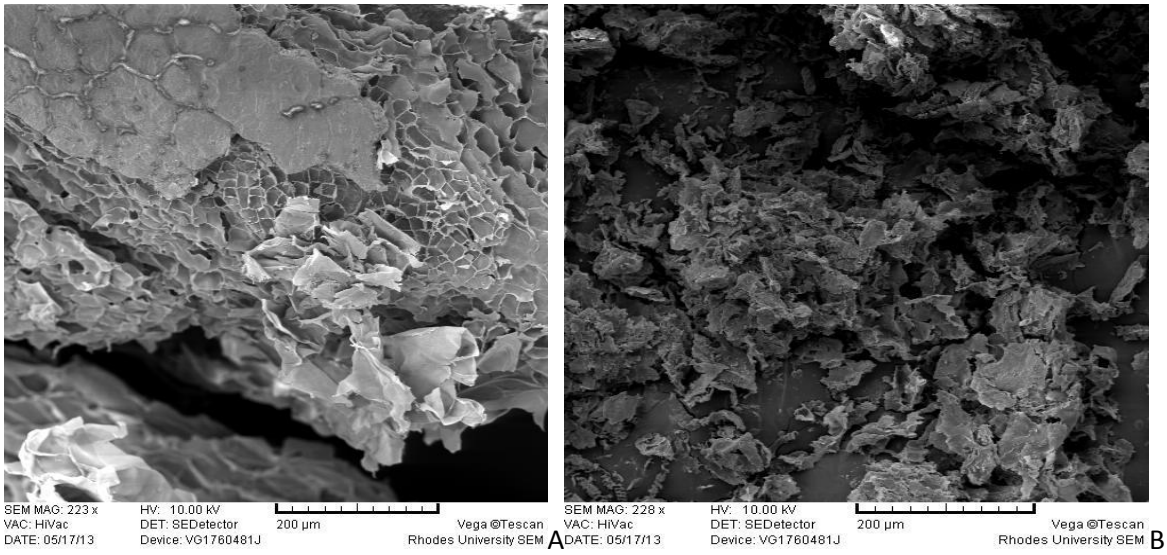


Figure 4.13: SEM image of apple pomace (A) before enzymatic hydrolysis and (B) after 100 h enzymatic hydrolysis. Reactions were run at room temperature using batch reactor (20%, wet w/v).

The SEM results (Figure 4.13) show that apple pomace cellwalls were broken down into small pieces by the hydrolytic action of Viscozyme L and Celluclast 1.5L after 100 h. The remaining solid material may contain mainly lignin and some unhydrolysed components of crystalline cellulose.

The produced sugars can be further converted to bioethanol through fermentation. Glucose can be fermented to ethanol by *Saccharomyces cerevisiae* and *Zymomonas mobilis*, while arabinose, xylose and galacturonic acid can be fermented using genetically modified organisms, e.g. *E. coli* K011 and *Erwinia* species (*E. crysanthemi* EC16 and *E. carotovora* SR38) (Dien *et al*., 2003; Grohmann *et al*., 1995; Grohmann *et al*., 1998; Van Dyk *et al*., 2013). Other possible utilisations of arabinose include: use in bacteriology for diagnostics;

derivatisation to 5-deoxy-L-arabinose, which has anti-Parkinson properties; and as a precursor of L-fructose and L-glucose that are used as sweeteners. Galacturonic acid can be used for synthesis of tensioactive agents through esterification with various fatty acids for pharmaceutical and cosmetic purposes; as acid agent; and for production of vitamin C (Baciu and Jordening, 2004; Boluda-Aguilar *et al*., 2010; Pourbafrani *et al*., 2010). Xylose and glucose can be used in the production of food sweeteners, xylitol and sorbitol, respectively (Bhushan *et al*., 2008; Demirabas, 2008). However, there may be other sugars and products in the hydrolysate that were not measured and these may add to the value-addition of apple pomace degradation.

4.4. Conclusions

The obtained results indicated that hydrolysis of apple pomace using Viscozyme L and Celluclast 1.5L can be successfully performed at room temperature without buffering, hence lowering the operational costs. The method of mixing was found to have a significant effect on enzyme activity. Sugars released using compressed air for mixing was 3x higher than that using a platform shaker for mixing. Using compressed air, however, could result in added operational costs and different mixing methods will be further investigated for larger volumes.

Increased substrate loadings resulted in increased sugar release, but a decrease in yield. The addition of new substrate resulted in a subsequent increase in the amount of sugars released, which indicated that Viscozyme L and Celluclast 1.5L were still active for longer periods of time under the assay conditions used. Supplementing β-glucosidase in Viscozyme L and Celluclast 1.5L resulted in the doubling of glucose released after 200 h. Novozyme 188 addition became more significant at high substrate loadings and after 50 h hydrolysis. The highest sugar concentrations (glucose, 4.7 g/L and reducing sugar, 23 g/L) were obtained using 35% final substrate concentration with Novozyme 188 supplementation. However, a balance has to be struck between the sugar concentration, residence time and enzyme cost. Operating the bioreactors in batch mode resulted in a better performance (4.2 g/L glucose and 16.8 g/L reducing sugar) than fed-batch in 72 h. Besides glucose, other products such as galacturonic acid, xylose and arabinose were released from the hydrolysis of apple pomace. These products can be utilised for value-addition, making the apple pomace degradation

process cost-effective. The produced sugars can be converted to bioethanol through fermentation. It will be important to measure the release of oligomers in the enzyme reactions in future studies, as this will give an idea of the extent of hydrolysis and whether additional enzymes are required to release monomers. The summary for the optimal hydrolysis conditions for apple pomace are shown in Table 4.3.

Table 4.3: Optimal hydrolysis conditions for degradation of apple pomace. V- Viscozyme L, C- Celluclast 1.5L and Nov- Novozyme 188.

Hydrolysis parameter	**Optimal conditions**
Temperature	Room temperature
pH	Unbuffered
Substrate concentration	20% (wet, w/v)
Enzyme concentrations	V (0.019 mg/mL), C (0.019 mg/mL), Nov (0.0024 mg/mL)
Time	100 h
Substrate feeding regime	Batch
Mixing	Compressed air
Reactor volume	1 L

Having investigated the effect of temperature and pH at low substrate loading and using the same amount of enzymes for all the reactions in this study, it is important to further optimise reactions at high substrate loading. The interaction of different hydrolysis parameters and their influence on enzyme kinetics are dealt with in Chapter 5, together with a kinetic model that can predict sugar production from apple pomace.

Chapter 5 – Artificial neural network (ANN) modeling of enzymatic hydrolysis of apple pomace for predicting sugar release

5.1. Introduction

Having designed the bioreactor, determined feeding regimes and conditions for apple hydrolysis in the Chapter 4, this chapter seeks to model apple pomace hydrolysis in order to predict sugar release. One of the ways to improve the economic feasibility of commercial biofuel production from lignocellulose biomass is to optimise the process conditions for the saccharification step (Lynd *et al*., 2008; O'Dwyer *et al*., 2008). Efficient hydrolysis of apple pomace into monomeric sugars will have effect on capital and operational costs for the production of biofuels and biorefinery chemicals. Therefore, there is a need to understand the hydrolysis process and be able to predict sugar production for an industrial process. There are many factors that influence the enzymatic hydrolysis of lignocellulose biomass, including enzyme and substrate properties and concentrations, as well as hydrolysis conditions such pH, temperature and time (Bansal *et al*., 2009; Gan *et al*., 2003; Gupta *et al*., 2012). The development of a kinetic model for enzymatic hydrolysis process can be used to describe the interaction of these different factors and their influence on apple pomace hydrolysis to enable the prediction of yields under varying operating conditions.

Lignocellulosic biomass such as apple pomace is heterogeneous in nature, as well as insoluble, and its hydrolysis requires a number of enzymes working in synergy (see section 3.3.1). These factors make kinetic modeling of apple pomace hydrolysis very complex (Al-Zuhair, 2008; Andric *et al.*, 2010b; Kadam *et al*., 2004; Sousa *et al*., 2011). Some of the kinetic models that have been used for lignocellulose hydrolysis have been reviewed (see section 1.1.10), which include Michaelis-Menten models, empirical models, mechanistic models, models accounting for adsorption and models for soluble substrates, as well as functionally and structurally based models (Bansal *et al*., 2009; Holtzapple *et al*., 1984; Movagarnejad *et al*., 2000; Sousa *et al*., 2011; Zhang and Lynd, 2004). Most of the models reported in literature are based on cellulase action on cellulose or pretreated substrates to produce glucose and cellobiose and have not been able to adequately explain lignocellulose hydrolysis (Bansal *et al*., 2009; Hodge *et al*., 2009; Sousa *et al*., 2011). In this study, apple pomace was used which contains cellulose, hemicellulose, pectin and lignin components

(section 2.3.1) while Celluclast 1.5L and Viscozyme L are multi-enzyme preparations. As a result of this complexity, none of the models can be used to model the hydrolysis of this substrate and it was decided to develop an artificial neural network (ANN) to assist in prediction of bioreactor performance based on certain variables.

In this study, the production of sugar monomers from apple pomace using commercial enzyme preparations; Celluclast 1.5L, Viscozyme L and Novozyme 188, was investigated. The proposed model focused on the main factors that influence apple pomace hydrolysis by commercial enzyme mixtures, namely variation in substrate concentration, enzyme concentration, temperature and pH over time in a batch reactor system (section 2.4.2, 3.4.3, 3.4.4, 4.4.1). It was envisaged that these parameters would be important for an industrial bioreactor process. The enzymes were found to be very stable (section 2.4.2.2) and mixing by compressed air showed no sign of deactivation of the enzymes (section 4.4.3). The use of excess Novozyme 188 alleviated inhibition of cellulases by cellobiose. The objective was to develop a robust kinetic model with a single set of kinetic parameters as it was important to find the conditions that produced the highest yields with the least enzyme for the shortest hydrolysis time.

Based on these factors, it was proposed to develop an artificial neural network (ANN) based on empirical data. ANNs seek to understand the relationship between inputs and outputs and have advantages over the traditional kinetic models. ANN, which is a computational mathematical network, has the potential to model large sets of non-linear, empirical experimental data in complex systems involving the interaction of many parameters without derivatised mathematical equations (O'Dwyer *et al*., 2008; Wang *et al*., 2011). ANNs have been successfully applied in fields such as medicine, economics, power systems, biotechnology, enzyme production and in food technology (prediction of food quality, properties, shelf life and processing) (Bhotmange and Shastri, 2011; Kalogirou, 2000). It has also been applied to lignocellulose hydrolysis (Ezhumalai and Thangavelu, 2010; O'Dwyer *et al*., 2008; Sasikumar and Viruthagiri, 2010). Details of this model were described in section 1.1.10. The model will be used for the prediction/ simulation of the amount of glucose and reducing sugars produced under certain conditions. The model will also allow for evaluation of the effect of different feeding regimes. A successful model for apple pomace hydrolysis is important for the design of an efficient and cost-effective bioreactor which will reduce capital

and operational costs for the production of biofuels and biorefinery chemicals. It is also important for process optimisation and control.

5.2. Aims and Objectives

a) To determine the influence of substrate concentration, enzyme concentration, temperature and pH over time on apple pomace hydrolysis.
b) To develop an artificial neural network to model the effect of substrate concentration, enzyme concentration, temperature and pH over time on apple pomace hydrolysis.
c) To predict sugar concentration and yield under different hydrolysis conditions using the ANN.
d) To determine the hydrolysis conditions for optimal sugar release that is also cost-effective.

5.3. Materials and methods

5.3.1. Apple pomace biomass preparation

This was performed as described in section 2.3.1.

5.3.2. Enzymes

Commercial enzyme preparations, Viscozyme L (an enzyme complex from *Aspergillus aculeatus),* Celluclast 1.5L (a commercial *Trichoderma reesei* ATCC 26921 cellulase preparation) and Novozyme 188 (a commercial *Aspergillus niger* - β-glucosidase preparation) were obtained from Sigma (South Africa). All the enzymes were stored at 4°C (section 3.3.2).

5.3.3. Enzyme assays

This was performed as described in section 2.3.5.

The glucose and reducing sugar yield (%) from cellulose and total pomace, respectively, were calculated using the following formulas;
Glucose yield (%) = Glucose liberated (g) x 0.9 x 100/ Initial cellulose (g)
Reducing sugar yield (%) = reducing sugar liberated (g) x 100/ Initial polysaccharides (g)
Calculations were based on dry weights.

5.3.4. Data for the Artificial Neural Network

Experimental data input for the artificial neural network was generated using various substrate loadings, enzyme loadings, initial pH, temperature and various combinations of these parameters. The conditions used and their range were based on the knowledge of enzymatic hydrolysis of apple pomace gained from the previous work. The bioreactors were operated in batch mode.

5.3.4.1. Determining the effect of substrate loading on the hydrolysis of apple pomace by Viscozyme L and Celluclast 1.5L

The effect of different apple pomace loadings on enzymatic hydrolysis was investigated. Final concentrations of 5%, 10%, 20% and 30% (wet, w/v) which corresponds to 0.5, 1, 2, and 3% dry weight were prepared in deionised water. Hydrolysis was performed using Viscozyme L - Celluclast 1.5L (50:50) (0.5 µL/mL, 0.019 mg/mL final for each) and Novozyme 188 (0.05 µL/mL, 0.0024 mg/mL) at room temperature in 1 L Schott bottle reactors mixed with compressed air as previously described (section 4.3.7) for 100 h. Aliquots of 5 mL each were collected at various time intervals and further enzyme reactions terminated by heating at 100°C for 5 min. pH was measured for each time interval. The yield of the liberated sugars was determined as described previously (section 5.3.3).

5.3.4.2. Determining the effect of various enzyme loadings on apple pomace hydrolysis

Various enzyme loadings were prepared: 0.12, 0.2, 0.4 and 0.6 mg protein/g apple pomace (wet w) which corresponded to 0.15, 0.25, 0.5 and 0.75 µL/mL (0.0114, 0.019, 0.038 and 0.057 mg/mL, respectively) for each of Viscozyme L and Celluclast 1.5L (50:50) and 10% volume of Novozyme 188 in deionised water. Apple pomace, 20% (wet, w/v) was used as substrate and the rest of the procedures were as described in section 5.3.4.1 above.

5.3.4.3. The effect of initial pH and hydrolysis temperature

The effect of initial hydrolysis pH was investigated using unbuffered deionised water, citrate buffer pH 4.0 and pH 5.0 (0.05 M). The rest of the procedures were as described in section 5.2.4.1 above. The effect of hydrolysis temperature was determined by using room temperature (22-26°C), 28°C and 37°C. The rest of the procedures were as described in section 5.3.4.1.

5.3.4.4. Interactive effects of various hydrolysis conditions

The effects of interaction of various hydrolysis conditions were investigated using various combinations of substrate concentration, enzyme loading, temperature and pH. The rest of the procedures were as described in section 5.3.4.1.

5.3.5. Building of the artificial neural network for the prediction of apple pomace hydrolysis

The experimental data generated following the hydrolysis of apple pomace using parameters (section 5.3.4) was used to develop the ANN model. Sugar concentrations were used in developing the ANN. There were 6 inputs patterns arranged in the form of a column vector;

$$\begin{bmatrix} p1 \\ p2 \\ p3 \\ p4 \\ p5 \\ p6 \end{bmatrix}$$

where:

- $p1$ is the lower temperature (22 for room temp, 28 and 37°C)
- $p2$ is the upper temperature (26 for room temperature, 28 and 37°C)
- $p3$ is the lower pH
- $p4$ is the upper pH
- $p5$ is the enzyme concentration
- $p6$ is the substrate concentration

The targets (release profiles) for the released sugars associated with each input were in the vector form;

$$\begin{bmatrix} t1 \\ t2 \\ t3 \\ t4 \\ t5 \\ t6 \end{bmatrix}$$

Where:

- $t1$ is the amount of glucose/reducing sugar after 1 hour

- $t2$ is the amount of glucose/reducing sugar after 6 hours
- $t3$ is the amount of glucose/reducing sugar after 24 hours
- $t4$ is the amount of glucose/reducing sugar after 50 hours
- $t5$ is the amount of glucose/reducing sugar after 72 hours
- $t6$ is the amount of glucose/reducing sugar after 100 hours

This resulted in 18x1 vectors which were taken as target vectors.

These readings were recorded in triplicate and there were 27 columns in the spread sheet with the readings concatenated into 18x1 vectors which were taken as target vectors. A typical part of the data is shown in Table 5.1.

Table 5.1: Part of the experimental data used to construct the ANN. Different hydrolysis conditions were employed, giving glucose and reducing sugars concentration as outputs. Room temperature was defined as 22-26°C and the unbuffered system as pH 3.0-5.0.

Input conditions	Temperature		22	22	22	22	28	22	22	22
	°C		26	26	26	26	28	26	26	26
	pH		3	3	3	3	3	3	3	3
			5	5	5	5	5	5	5	5
	mg/g substr	Enzyme	0.12	0.2	0.4	0.6	0.2	0.2	0.2	0.2
	%wet,w/v	Substrate	20	20	20	20	20	5	10	30
		Time (h)								
Glucose (g/L)		1	0.0625	0.2264925	0.4753731	0.630597	0.2168358	0.0943468	0.2032086	0.2819778
		6	0.7003106	0.6619403	1.1425373	1.1533582	0.7467118	0.6088617	0.7394958	0.7398782
		24	0.8408385	1.7089552	2.2649254	2.3067164	1.9579106	0.9702063	1.657754	1.9652454
		50	1.113354	3.0910448	3.5761194	4.461194	2.9778279	1.0847976	2.0290298	3.6162666
		72	1.7049689	3.7358209	4.4761194	4.4656716	3.8346486	1.0939649	2.118411	4.5363669
		100	1.8913043	4.0179104	4.3328358	4.3761194	3.906802	1.1375095	2.1298701	4.5148692
		1	0.0657997	0.2432836	0.511194	0.6552239	0.2292371	0.0897632	0.2043545	0.3034755
		6	0.7133152	0.7078358	1.1358209	1.1365672	0.7760241	0.6271963	0.7887701	0.9387316
		24	0.8175466	1.7962687	2.2895522	2.2776119	2.0706501	1.0252101	1.6325439	2.1221784
		50	1.2391304	3.2925373	3.661194	4.4343284	3.2078166	1.0847976	2.0725745	3.8054461
		72	1.5046584	3.6104478	4.480597	4.4656716	3.7399474	1.0847976	2.0725745	4.2611967
		100	1.7257764	4.2059701	4.2432836	4.4298507	4.0420894	1.1077158	2.0679908	4.8459334
		1	0.0638587	0.238806	0.5156716	0.6395522	0.2326193	0.0840336	0.1986249	0.3238982
		6	0.7111801	0.6652985	1.1369403	1.138806	0.7478392	0.6134454	0.7360581	0.8957363
		24	0.798913	1.8679104	2.3074627	2.2552239	2.0300639	0.9885409	1.7425516	2.3865998
		50	1.0574534	3.2253731	4.0507463	4.3985075	3.2980083	1.0962567	1.8456837	4.1150125
		72	1.5720497	3.6014925	3.9791045	4.519403	3.4332958	1.105424	2.0863254	4.3299893
		100	1.7350932	4.2283582	4.1895522	4.4522388	3.8797445	1.26356	2.0840336	4.5191688
Reducing sugars (g/L)		1	1.3371005	1.8014333	4.1056965	4.9273022	2.6548449	0.8349164	2.0621586	3.6431935
		6	5.9271498	5.2765744	8.1468127	9.7846424	6.6679762	2.438985	4.3885047	8.665393
		24	7.8042856	9.8671618	13.345532	14.838056	11.220354	3.3188924	6.1615047	13.944299
		50	9.9569644	14.011068	17.344133	16.344931	13.986546	3.6310309	7.3078062	20.732772
		72	12.518453	15.717066	18.95326	18.169326	16.281301	3.7844092	8.2372252	25.35834
		100	13.57777	16.104548	19.437613	18.712878	17.823157	3.9525873	8.7861583	28.256384
		1	1.3249917	1.8983039	4.2913651	5.0510813	2.6642628	0.8244221	2.0713075	3.7454457
		6	5.9271498	5.6263847	8.2544466	10.096781	6.7998278	2.29637	4.2485806	8.7111374
		24	7.9173013	9.9963225	13.673815	15.343935	11.753142	3.2960202	6.2153217	15.085219
		50	10.791127	14.188664	17.311842	17.884096	14.142615	3.5947044	7.3320238	21.144472
		72	12.770515	15.550234	19.066276	18.223143	17.072411	3.5973953	8.2695154	25.552081
		100	13.537407	16.728825	19.529102	18.599862	17.548691	3.8812798	8.7942308	28.821462
		1	1.2940469	1.9655751	4.6088852	5.1990779	2.7570971	0.8015499	2.0519334	3.8423163
		6	6.1854712	5.6263847	8.3836074	10.516553	6.7729193	2.4443667	4.0992385	8.0330436
		24	7.8527209	10.136247	13.59309	14.267596	11.629363	3.3310013	6.2207034	15.24667
		50	10.435935	14.274772	17.796195	18.336159	13.884294	3.593359	7.3131878	22.952722
		72	13.19836	16.287526	19.469903	18.546045	16.886742	3.8530259	8.5883809	26.480424

The triplicate targets were separated by perturbing the hydrolysis conditions/ inputs by 0.01 point. The first of the input values was retained and the remaining two values were perturbed by a small amount (0.01). This resulted in 81 different input patterns and associated targets, which were used to construct the ANN.

The ANN model developed in this study was a multiple layer neural network with interconnected neurons arranged in layers that are input, hidden and output layers (Figure 5.1). Each layer was interconnected by weights (w) and biases (b) which were adjustable, enabling it to model nonlinear functions. After experimentation with various architectures it was decided to use an ANN with 20 neurons each in the first and second hidden layers and 6 neurons in the third hidden layer. Transfer functions, *tansig*, *tansig* (tangent sigmoid function in the hidden layer) and *purelin* (pure linear function in the output layer) were used. Two ANNs were constructed each for glucose and reducing sugar.

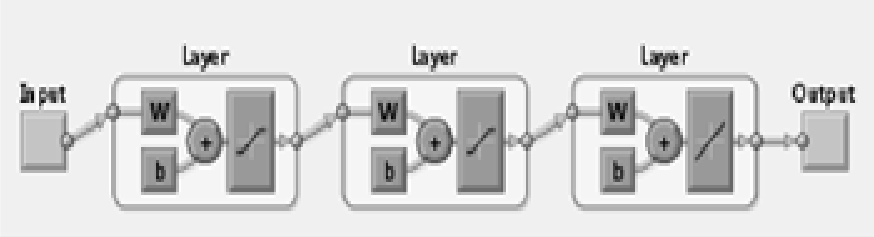


Figure 5.1: A generalised scheme of the ANN topography used for predicting glucose and reducing sugar concentration taken from Matlab. W – weight matrix, b – bias vector.

The fitting of the experimental data was performed in Matlab. When using MatLab, the data was scaled down before processing and then scaled up after processing using the scaled conjugate gradient (*trainscg*) training function. Feed-forward backpropagation learning algorithm (Levenberg-Marquardt), *trainlm* was used for ANN training using a 'leave one out' method, where training was performed on all the data except one for testing (randomly chosen). Two separate ANNs were constructed for the glucose and reducing sugar reactions. The weights and biases were adjusted using gradient descent with momentum as a learning method. The mean square error (MSE) was minimised by making adjustments to the network parameters and training over 500 epochs to avoid overtraining. After correct simulation on test points based on the MSE and correlation coefficient (R^2), training was then performed on all data. After training the ANN using the training data set, validation data was used to evaluate the performance of the training data set based on the ability to correctly predict/ simulate the validation data (new data). The simulation of ANN was performed on the input

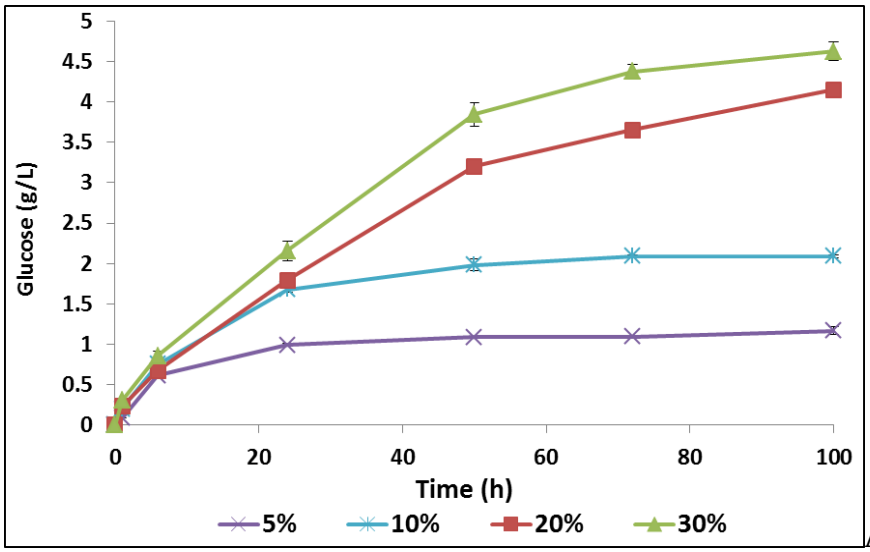
patterns to produce an activation, which is the sugar release profile predicted by the ANN for the input conditions, in order to find the optimum conditions.

The glucose and reducing sugar concentration release profiles using different input patterns were sorted according to the concentrations of the sugars in decreasing order. Surface plots were then generated for maximum glucose and reducing sugars concentrations released by varying the enzyme concentration and substrate concentrations while fixing the temperature at room temperature and pH as unbuffered.

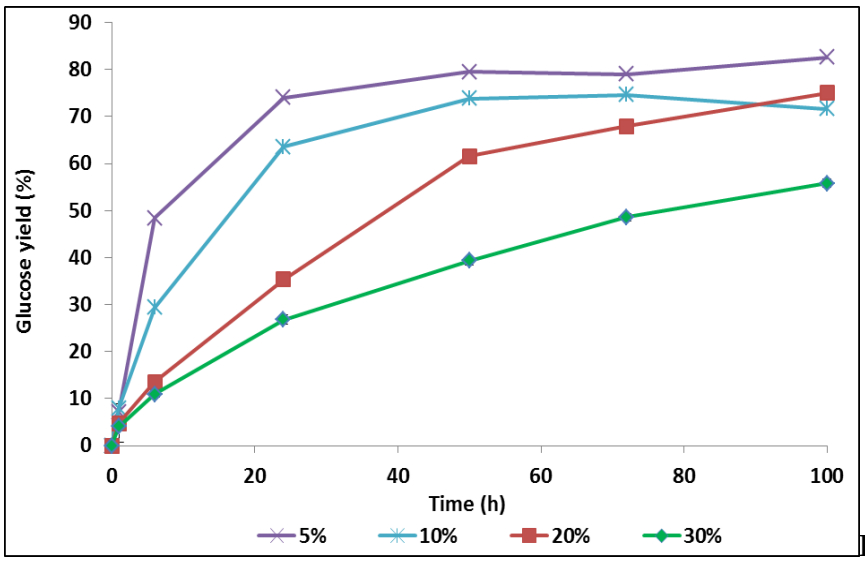
5.4. Results and Discussion

5.4.1. Effect of substrate loading

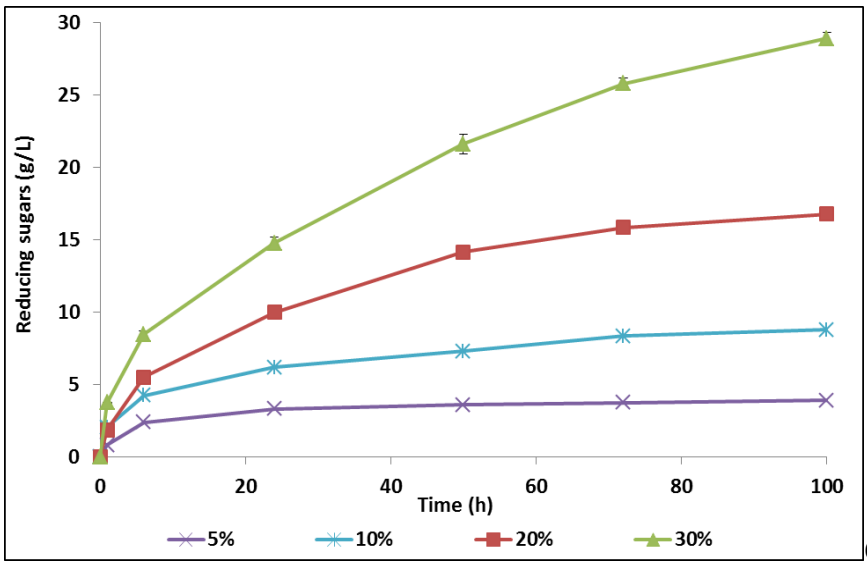
The effect of various substrate loadings on enzymatic hydrolysis of apple pomace was determined. The amount of glucose and reducing sugars released and their corresponding yields and pH are shown in Figure 5.2 (A-E). Table 5.2 shows the amount of enzyme (mg) that was utilised to produce glucose or reducing sugars.


Glucose (g/L)
Time (h)
5%
10%
20%
30%

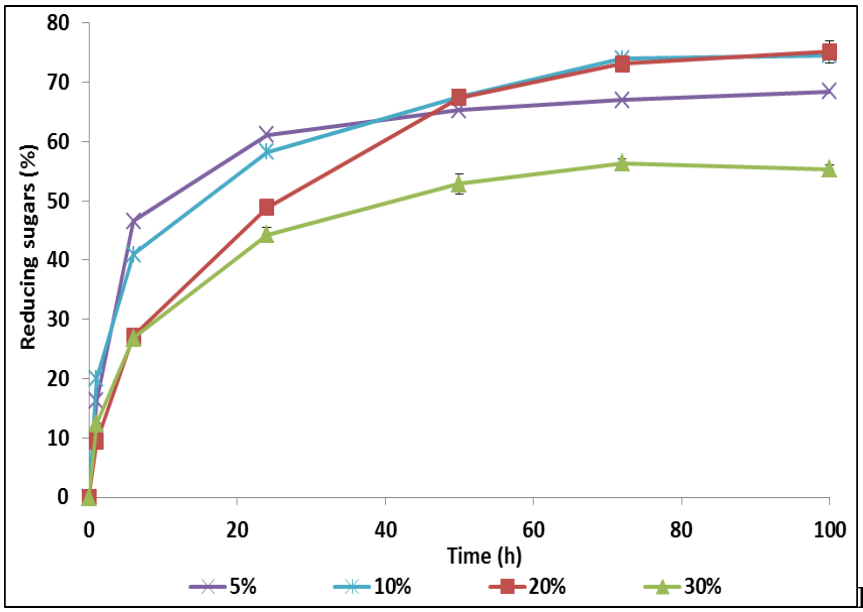
A


Glucose yield (%)
Time (h)
5%
10%
20%
30%

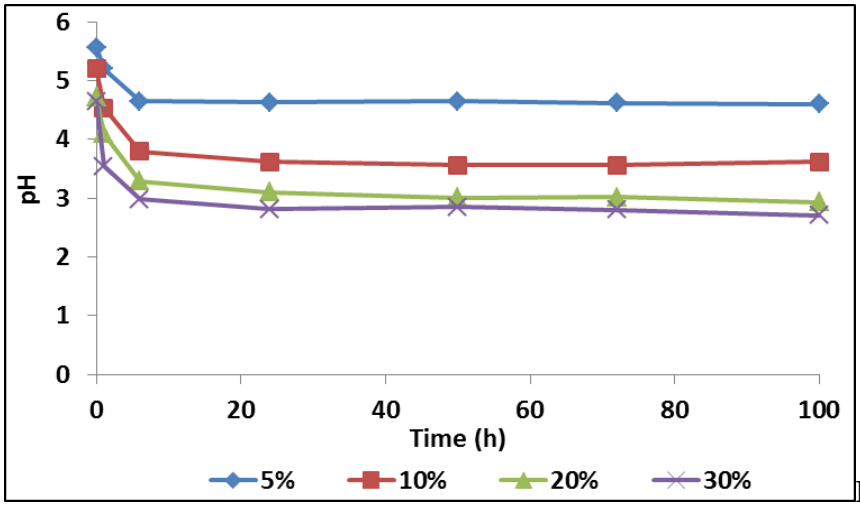
B


Reducing sugars (g/L)
Time (h)
5%
10%
20%
30%

C



D



E

Figure 5.2: Concentration of sugars released and pH at different time intervals for the batch reactor mixed with compressed air at room temperature with different starting substrate loadings. A (Glucose), B (glucose yield), C (reducing sugars), D (reducing sugar yield) and E (pH). Data points are presented as mean values ±SD (n=3).

Table 5.2: The amount of enzyme (mg) utilised to produce a g of reducing sugar or glucose from different substrate loadings at various time intervals.

Substrate (%)	5	10	20	30
Time (h)	**mg enzyme/ g reducing sugar**			
1	49.9(±0.6)	21.2(±0.05)	20.5(±0.5)	10.9(±0.2)
6	17.4(±0.4)	9.9(±0.2)	7.4(±0.2)	5.0(±0.1)
24	13.2(±0.04)	6.9(±0.02)	4.1(±0.03)	3.0(±0.1)
50	12.4(±0.04)	6.0(±0.01)	3.0(±0.02)	2.6(±0.1)
72	12.1(±0.3)	5.5(±0.1)	2.8(±0.04)	2.5(±0.1)
100	11.8(±0.06)	5.4(±0.01)	2.7(±0.07)	2.4(±0.1)
	mg enzyme/g glucose			
1	459.0(±15.4)	206.2(±1.8)	191.2(±4.1)	135.1(±5.4)
6	67.4(±0.6)	55.6(±1.2)	67.1(±1.4)	50.1(±3.7)
24	44.1(±0.7)	25.7(±0.5)	25.7(±0.7)	20.9(±1.2)
50	41.0(±0.1)	22.1(±0.8)	14.7(±0.3)	14.4(±0.5)
72	41.0(±0.2)	21.8(±0.1)	13.3(±0.2)	14.1(±0.3)
100	39.6(±1.6)	21.7(±0.2)	12.1(±0.2)	13.3(±0.4)

It was apparent that an increase in substrate concentration resulted in an increase in the amount of sugars released. The release of sugars increased linearly at the beginning of the reaction, but the rate decreased with time (Andric *et al*., 2010c; Bansal *et al*., 2009; Zhang *et al*., 1999). The initial rapid release of sugars can be attributed to high synergy between enzymes as the substrate was modified, as well as the availability of easily hydrolysable sites of the substrate. The fast initial rate of sugar release conforms to the Michaelis-Menten model. However, it cannot explain what happens over extended periods of time as a result of changes in substrate structure, changes in the interaction between the enzyme and substrate interface, depletion of hydrolysable substrate, enzyme adsorption on residual substrate and lignin, enzyme deactivation, end-product inhibition, inhibition from high concentrations of inhibitory compounds and mass transfer limitations at high substrate loadings (Bommarius *et al*., 2008; Movagarnejad *et al*., 2000; Sarkar and Etters, 2004; Zhang *et al*., 1999; Zhang *et al*., 2010). Glucose was released rapidly at the beginning of hydrolysis at low substrate concentration (Lu *et al*., 2010). The fast rate of release of glucose may be due to the removal/hydrolysis of the first layer of cellulose (Gan *et al*., 2003). The glucose production rate for both 5 and 10% substrate loadings began to decrease after 24 h, giving final glucose concentrations of 1.2 and 2.1 g/L, respectively at 100 h (Figure 5.2A). At 20 and 30% substrate loadings, the rate of glucose production started to decrease after 50 h with final

glucose concentrations of 4.2 and 4.6 g/L, respectively, after 100 h. For the reducing sugars, 20 and 30% substrate loadings followed the same pattern as glucose production, with final reducing sugars of 16.8 and 28.9 g/L, respectively (Figure 5.2C). The rate of reducing sugar release for 5 and 10% substrate loadings started to decrease after 6 h of hydrolysis and resulted in 3.9 and 8.8 g/L of reducing sugars, respectively, after 100 h. The results indicate that low substrate concentrations required shorter hydrolysis times as compared to high substrate concentration. At high substrate concentrations viscosity was also high initially which posed mass transfer limitations.

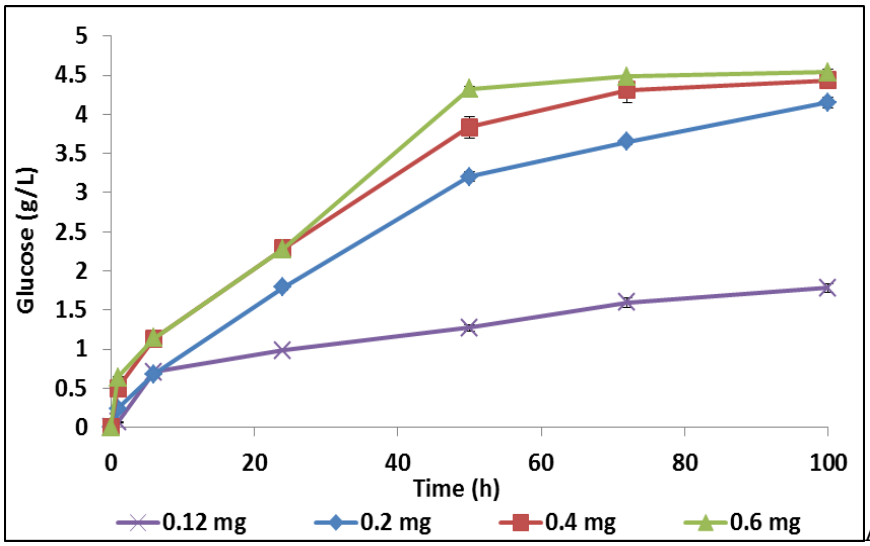
At higher substrate loadings, conversion efficiency decreased. The yields decreased with an increase in substrate concentration, in agreement to what was reported by Ioelovich and Morag (2012) and Zhang *et al*. (2009). Glucose and reducing sugar yields were higher at 5 and 10% substrate during the first 24 h and were maintained until the end of the reaction (Figure 5.2B and D). The maximum yields for 20 and 30% substrate were reached after 50 h of hydrolysis for reducing sugars, but were not attained for glucose, even after 100 h. The initial enzyme: substrate ratio was low for high substrate loadings, resulting in lower conversion efficiency than at low substrate loadings (Rosgaard *et al*., 2007). The final glucose yields at 100 h were 80, 75, 75 and 55.8% for 5, 10, 20 and 30% substrate loadings, respectively, and for reducing sugars 68.4, 74.6, 75 and 55.4%, respectively. Higher initial substrate concentrations required longer hydrolysis times for the reactions to be completed, which was in agreement with a reported study on CMC and corn stover using cellulases (Al-Zuhair, 2008; Imai *et al*., 2003; Lu *et al*., 2010). The fact that 100% yield was not attained, even at low substrate loadings, may be as a result of end-product inhibition, depletion of hydrolysable sites and adsorption of enzyme on the residual substrate which may contain mainly lignin. Although Novozyme 188 was used to supplement the β-glucosidase activity of Viscozyme L and Celluclast 1.5L, and hence reduce the inhibition of cellulases by cellobiose, accumulation of glucose may also be inhibitory to β-glucosidase (Sarkar and Etters, 2004; Teeri, 1997). The low glucose yields at 20 and 30% may be attributed to low reaction rates due to high viscosity and mass transfer limitations, and end-product inhibition due to higher concentrations of glucose. The enzymes may also have been limiting or adsorbed onto the substrate. Viscosity will be high at the beginning of hydrolysis, thereby posing mass transfer limitations, leading to low rates of glucose release at high substrate loadings (Lu *et al*., 2010; Mussatto *et al*., 2008; Sarkar and Etters, 2004). Operation at high solid concentrations has the advantage of increasing sugar concentration, which will lower capital operational costs

associated with reduced reactor volume, reduced energy costs and ethanol separation (Hodge *et al*., 2009; Lu *et al*., 2010). It has been reported that the tank volume and operational costs can be reduced by using high substrate concentrations (Hodge *et al*., 2009; Zhang *et al*., 2009; Zhang *et al*., 2010). However, high solids require more energy for mixing and result in the build-up of more residual solids.

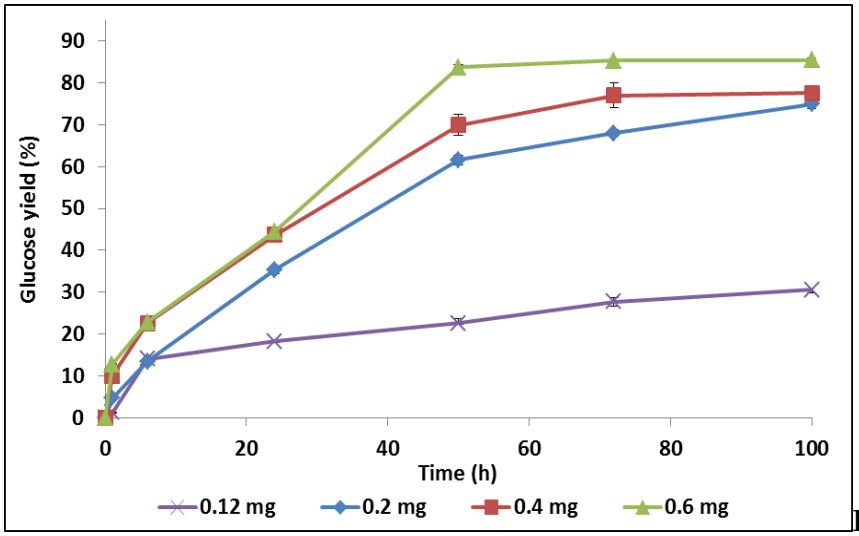
There was no clear difference between glucose released by different substrate concentrations within the first 6 h, but there was an increase in reducing sugars released with an increase in substrate concentrations. This may be due to the fact that the pectin or hemicellulose component of apple pomace is degraded first thereby exposing cellulose and the fact that there are low levels of glucose (22.3%) in apple pomace (Parmar and Rupasinghe, 2013). Other sugars like arabinose, galactose as well as galacturonic acid were released first before glucose, contributing to the differences in the observed trends between reducing sugars and glucose. The results for reducing sugars show that, at higher substrate loadings, the initial rate of reaction was faster, hence the higher production of sugars in agreement to what was obtained by Gan *et al*. (2003). The decrease in pH over time was related to the concentration of substrate and hence product concentration, with high substrate concentrations recording lower pH values (Figure 5.2E). Large amounts of enzyme were required during the initial stages of hydrolysis (Table 5.2). This may have been due to high enzyme synergy occurring during initial stages of hydrolysis (section 3.4.4).

5.4.2. Effect of enzyme loading

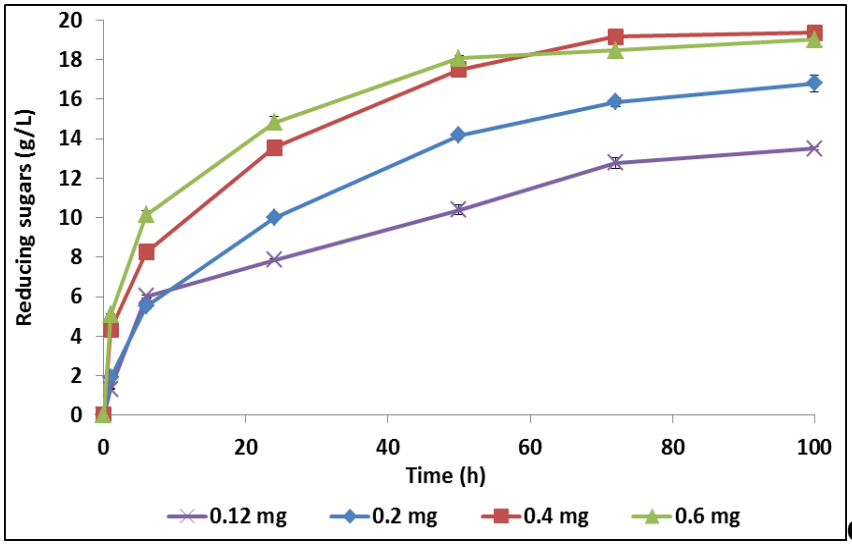
The effect of enzyme loadings on enzymatic hydrolysis of apple pomace was established. The amount of glucose and reducing sugars released and their corresponding yields and pH are shown in Figure 5.3 and the amount of enzyme (mg/g of substrate) that was utilised to produce glucose and reducing sugars is shown in Table 5.3.


Glucose (g/L)
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
0
20
40
60
80
100
Time (h)
0.12 mg
0.2 mg
0.4 mg
0.6 mg

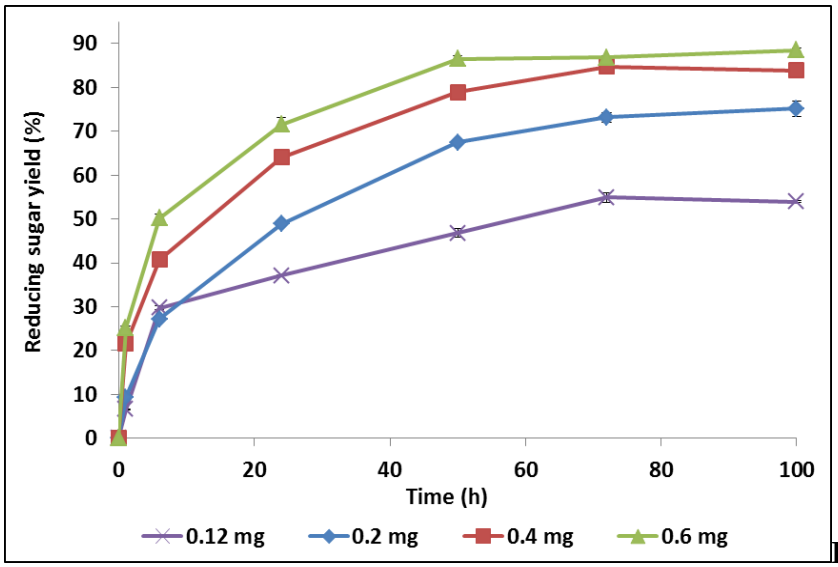
A


Glucose yield (%)
90
80
70
60
50
40
30
20
10
0
0
20
40
60
80
100
Time (h)
0.12 mg
0.2 mg
0.4 mg
0.6 mg

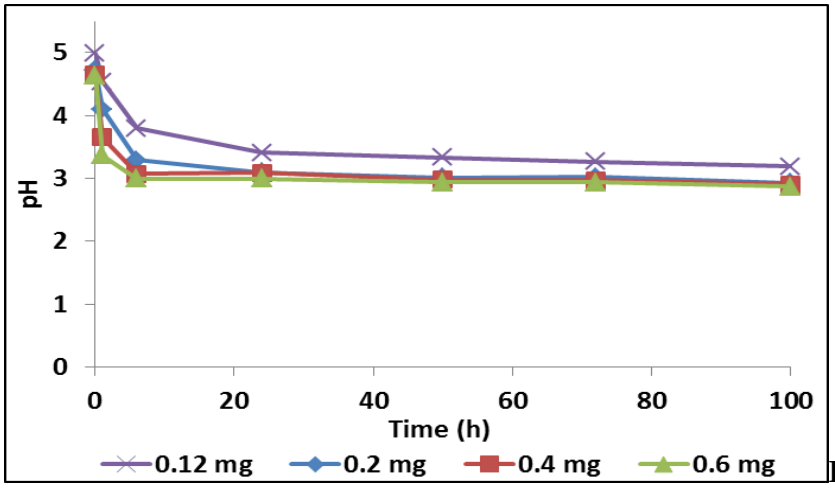
B


Reducing sugars (g/L)
20
18
16
14
12
10
8
6
4
2
0
0
20
40
60
80
100
Time (h)
0.12 mg
0.2 mg
0.4 mg
0.6 mg

C



D



E

Figure 5.3: Concentration of sugars released and pH at different time intervals for the batch reactor mixed with compressed air at room temperature with 20% (wet, w/v) apple pomace. A (Glucose), B (glucose yield), C (reducing sugars), D (reducing sugar yield) and E (pH). Data points are presented as mean values ±SD (n=3).

Table 5.3: The amount of enzyme (mg) utilised to produce a g of reducing sugar or glucose using different enzyme loadings at various time intervals.

Enzyme loading (mg/g substrate)	**0.12**	**0.2**	**0.4**	**0.6**
Time (h)	**mg enzyme/g glucose**			
1	180.6(5.7)	191.2(4.1)	200.4(4.7)	210.9(2.4)
6	34.6(0.2)	67.1(1.4)	80.1(0.1)	119.0(0.5)
24	31.3(0.5)	25.7(0.7)	41.5(0.2)	61.2(0.4)
50	23.8(1.1)	14.7(0.3)	25.5(1.9)	31.7(0.1)
72	18.1(0.8)	13.3(0.2)	23.6(0.9)	31.9(0.1)
100	17.5(1.1)	12.1(0.2)	24.7(0.6)	32.7(0.2)
	mg enzyme/g reducing sugar			
1	18.5(0.2)	21.5(0.5)	23.8(0.6)	24.1(0.4)
6	4.1(0.1)	7.4(0.2)	9.9(0.1)	12.1(0.3)
24	3.3(0.01)	4.1(0.03)	6.3(0.05)	8.5(0.2)
50	2.6(0.1)	3.0(0.02)	5.1(0.05)	7.2(0.3)
72	2.2(0.04)	2.8(0.04)	4.8(0.04)	7.0(0.04)
100	2.2(0.01)	2.7(0.1)	4.9(0.1)	6.9(0.03)

The rate of sugar release was high during the first 6 h and began to level off after 50 h. At high enzyme loadings, the initial reaction rates were faster and higher, leading to higher amounts of sugars being released as compared to low enzyme loadings. At high enzyme loadings, the enzyme to substrate ratio was high, resulting in high conversion efficiency (Rosgaard *et al*., 2007; Zhang *et al*., 2009). The reaction reached its optimum after about 60 h, regardless of the concentration of enzyme used. It was expected that an increase in the enzyme concentration would reduce the time of hydrolysis (Zhang *et al*., 2010). The amount of glucose released after 100 h from 0.12, 0.2, 0.4 and 0.6 mg enzyme/g of substrate were 1.8, 4.2, 4.4 and 4.5 g/L (Figure 5.3A), respectively, which corresponded to a 30.6, 75, 77.6 and 85.4% yield (Figure 5.3B), respectively. The concentration of reducing sugars produced after 100 h from 0.12, 0.2, 0.4 and 0.6 mg enzyme/g of substrate were 13.5, 16.8, 19.3 and 19 g/L (Figure 5.3C), respectively, which corresponded to a 54, 75, 83.9 and 88.5% yield (Figure 5.3D), respectively. At low enzyme concentrations, all hydrolysis sites on the substrate were not yet saturated, and the degree of synergy was normally low. An increase in enzyme concentration resulted in an increase in the release of sugars, however a concentration of 0.4 and 0.6 mg enzyme/g substrate resulted in almost the same amount of sugars being released. This may be due to the enzymes reaching saturation point, or may be

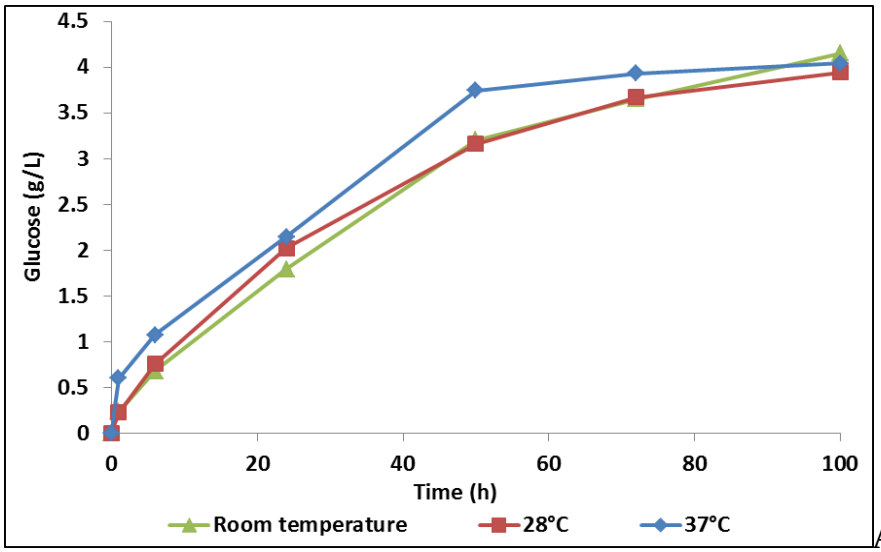
due to end-product inhibition due to the build-up of sugars. Enzyme jamming can also take place as enzyme molecules interfere with each other (Bommarius *et al*., 2008). The relative number of binding sites may be reduced at high protein loadings, leading to enzymes competing for the same binding sites on the substrate and a reduction in hydrolysis rate (Andric *et al*., 2010b; Banerjee *et al*., 2010b; Converse and Optekar, 1993; Van Dyk and Pletschke, 2012). Beyond the optimum enzyme loading, there will be limited surface area on the substrate for the enzymes to bind and enzymes may unproductively bind to lignin (Rosgaard *et al.*, 2007). As a result, excess enzyme molecules are adsorbed on the substrate to form multiple layers, but only the substrate molecules adsorbed onto the active sites will take part in hydrolysis (Kristensen *et al.*, 2009; Lin *et al*., 2010). During the initial stages of hydrolysis, there was a rapid decrease in pH with an increase in enzyme concentration (Figure 5.3E). The results indicated that there was increased hydrolysis with increased enzyme concentration. The decrease in pH was higher with high enzyme loadings, which corresponded to large amounts of sugars being released following the same pattern as was observed with varying substrate concentrations (Figure 5.2E). A larger amount of enzyme was utilised to release a g of product at high enzyme loadings and during the initial stages of hydrolysis (Table 5.3). The efficiency of the enzymes in terms of the amount of enzyme utilised per amount of sugar released shows that, at high enzyme loading, the efficiency of the enzyme decreases (Gan *et al*., 2003).

Comparing the results for substrate concentration (Figure 5.2) and enzyme loading (Figure 5.3), the results indicated that enzyme concentration has a more significant effect on the yield of sugars than substrate concentration. This is in agreement with Mussatto *et al*. (2008) in a study on the hydrolysis of brewer's spent grain by Celluclast 1.5L. Yield increases with an increase in enzyme concentration but decreases with an increase in substrate concentration. Rosgaard *et al*. (2007) also reported that enzyme concentration was more important than substrate concentration in the hydrolysis of pre-treated barley straw by Celluclast 1.5L and Novozyme 188. Enzyme loading has also been reported to be more significant than substrate loading in sugarcane bagasse hydrolysis (Vasquez *et al*., 2007).

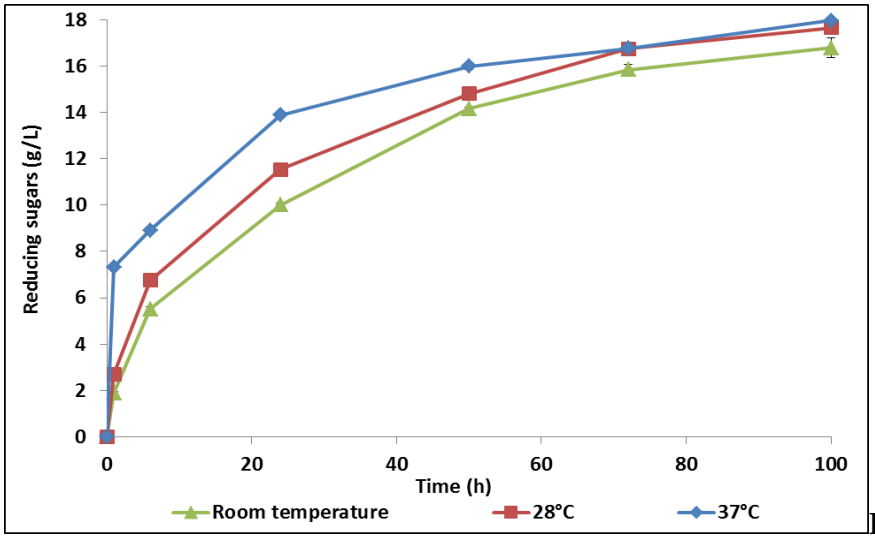
5.4.3. Influence of temperature

The effect of temperature on the hydrolysis of apple pomace by Viscozyme L and Celluclast 1.5L was determined following incubation at room temperature, 28°C and 37°C. The amounts of glucose and reducing sugars released by each treatment were as shown in Figure

5.4A and B, respectively. The temperatures were chosen based on the previous data obtained (section 4.4.1.) and the fact that pomace from the juice industry can exit the factory at high temperature. A temperature of 37°C could be a good compromise between saccharification and fermentation if SSF is considered.



A



B

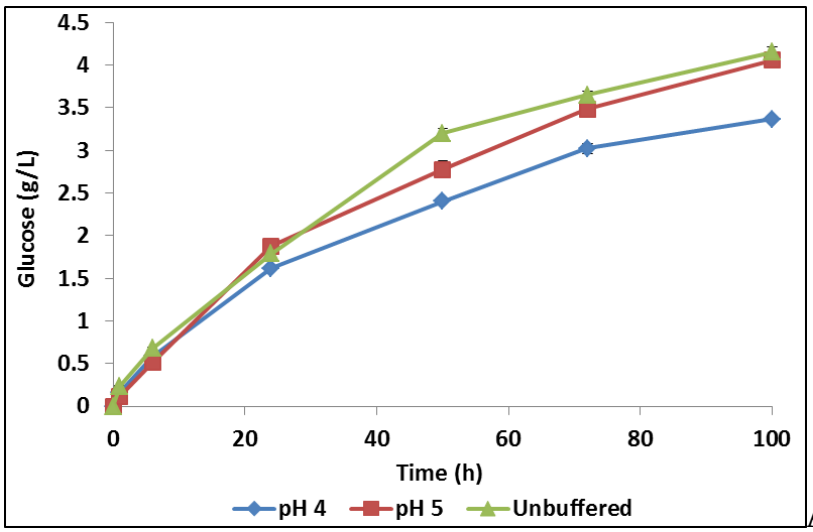
Figure 5.4: The amount of sugars released at different time intervals for the batch reactor mixed with compressed air at different temperatures with 20% (wet, w/v) apple pomace and an enzyme loading of 0.2 mg/ g substrate. Unbuffered deionised water was used in the reactions. A (Glucose) and B (reducing sugars). Data points are presented as mean values ±SD (n=3).

At 37°C, the initial reaction rate was high as compared to 28°C and room temperature, but there was no significant difference in the amount of sugar release after 100 h. The amount of glucose released was 4, 3.9 and 4.2 g/L and reducing sugars, 18, 17.7 and 16.8 g/L at 37°C, 28°C and room temperature, respectively, after 100 h hydrolysis. The results were in

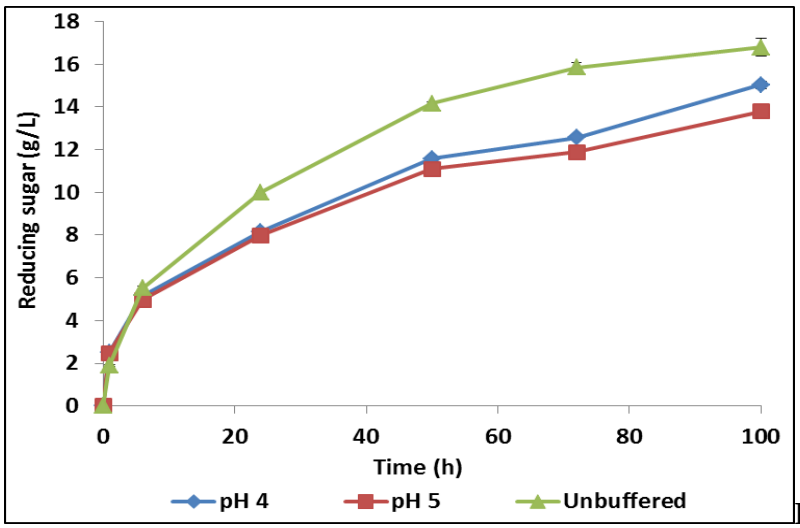
agreement with results obtained in the previous section that showed a small difference in reducing sugar release at these temperatures (section 4.4.1).

5.4.4. Influence of pH

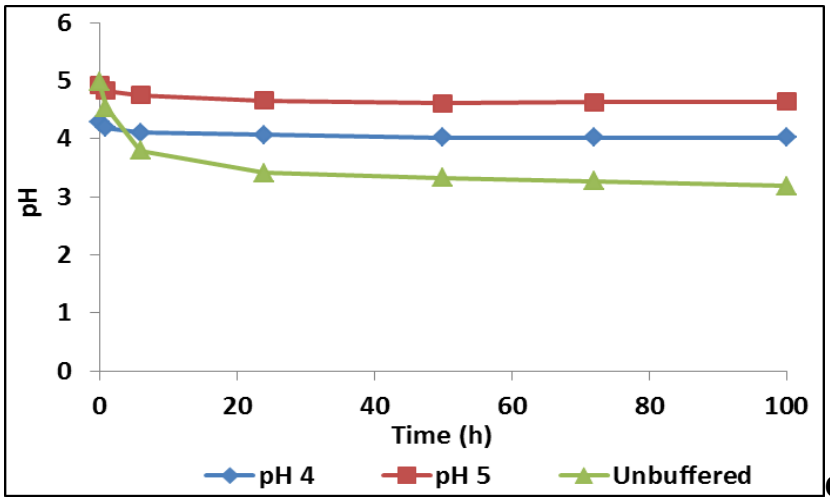
The effect of initial pH on the the hydrolysis of Viscozyme L and Celluclast 1.5L was investigated using an unbuffered system, pH 4.0 and pH 5.0. Figures 5.5A and B, show the amounts of glucose and reducing sugars released over time. The change in pH over time was as shown in Figure 5.5C. The conditions were chosen based on earlier results (section 2.4.2.2 and 4.4.1) and the fact that the cost of using a buffered system can be reduced by producing value-added products.



A



B



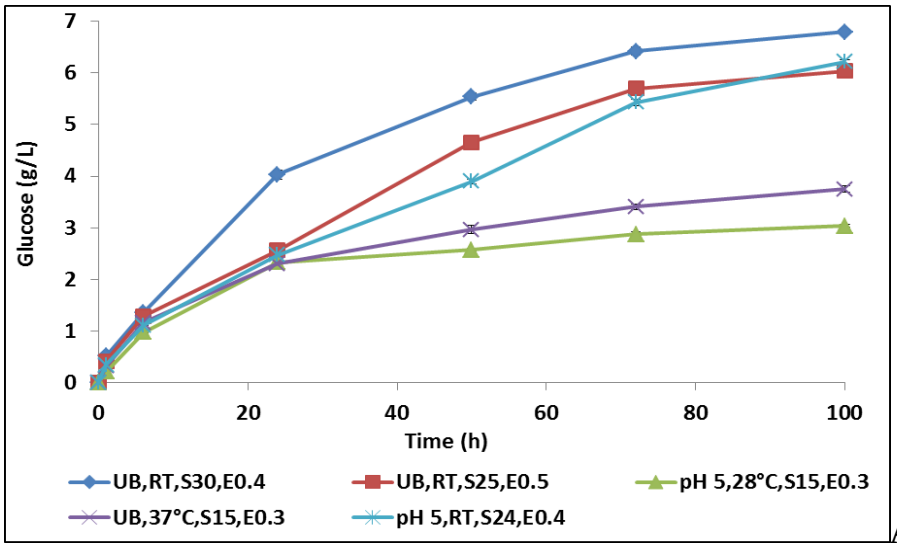
C

Figure 5.5: The amount of sugars released and pH at different time intervals for the batch reactor mixed with compressed air at different initial pH conditions with 20% (wet, w/v) apple pomace and an enzyme loading of 0.2 mg/g substrate. Hydrolysis was performed at room temperature. A (Glucose), B (reducing sugars) and C (pH). Data points are presented as mean values ±SD (n=3).

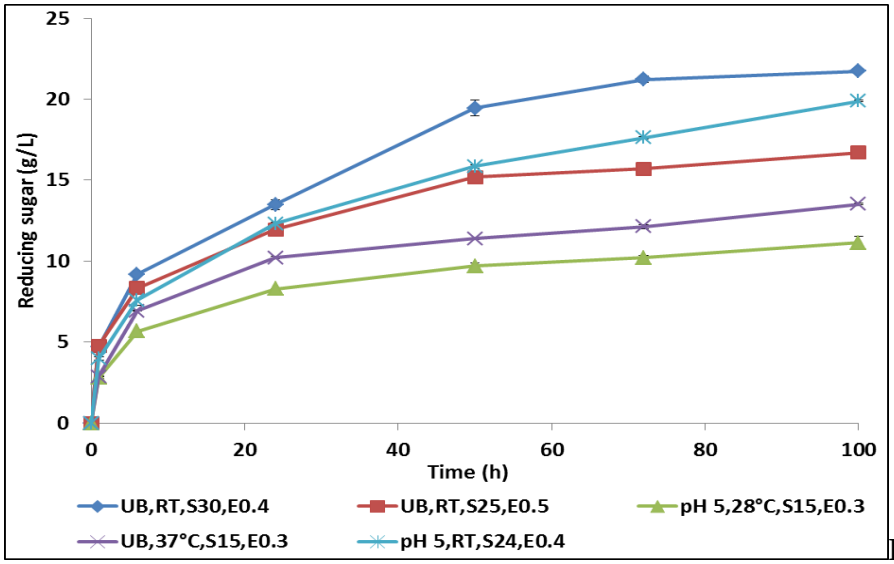
Higher amounts of sugars were released using the unbuffered system compared to the buffered system. A pH of 5.0 resulted in the release of slightly more glucose than pH of 4.0, but vice versa for reducing sugars. The amount of glucose released was 3.4, 4.1 and 4.2 g/L and reducing sugars, 15, 13.8 and 16.8 g/L using a buffer at pH 4.0, pH 5.0 and an unbuffered system, respectively, after 100 h hydrolysis. In the unbuffered system, the initial pH was around pH of 5.0, but decreased to around pH 3.2 after 100 h. The enzymes present in the enzyme cocktails have different pH optimum values (section 2.4.2.2 and 4.4.1). The results indicated that the initial pH as well as the change in pH as the reaction progressed was important for the hydrolysis of apple pomace. The results were in agreement with previous results that indicated that the unbuffered system yielded more reducing sugars than the buffered system at pH 5.0 (section 4.4.1). Parmar and Rupasinghe (2013) compared the effect of pH 4.0, 4.5 and 5.0 on the hydrolysis of apple pomace and reported that a pH of 4.0 was optimum for cellulose conversion to glucose.

5.4.5. Interactive effect of different hydrolysis parameters

The effect of a combination of different parameters, including some conditions which were not investigated in the experiments above, on the enzymatic hydrolysis of apple pomace, was investigated. The amount of sugars released over time was as shown in Figure 5.6.



A



B

Figure 5.6: The amount of sugars released at different time intervals for the batch reactor mixed with compressed air a combination of parameters. Apple pomace was used as the substrate. A (Glucose) and B (reducing sugars). UB – unbuffered, RT – room temperature, S – substrate (%, wet w/v), E – enzyme (mg/g substrate). Data points are presented as mean values ±SD (n=3).

The results indicated that substrate and enzyme loading were the main factors that influenced apple pomace hydrolysis by Viscozyme L and Celluclast 1.5L. Larger amounts of sugars were released (6.8 g/L glucose and 21.7 g/L reducing sugars) when the reactions were performed using high substrate and enzyme concentrations at room temperature in an unbuffered system. Reactions operated using low enzyme and substrate concentration resulted in a low release of sugars, 3 g/L for glucose and 11.1 g/L for reducing sugars. The results indicated that there was a significant effect as a result of the interaction taking place

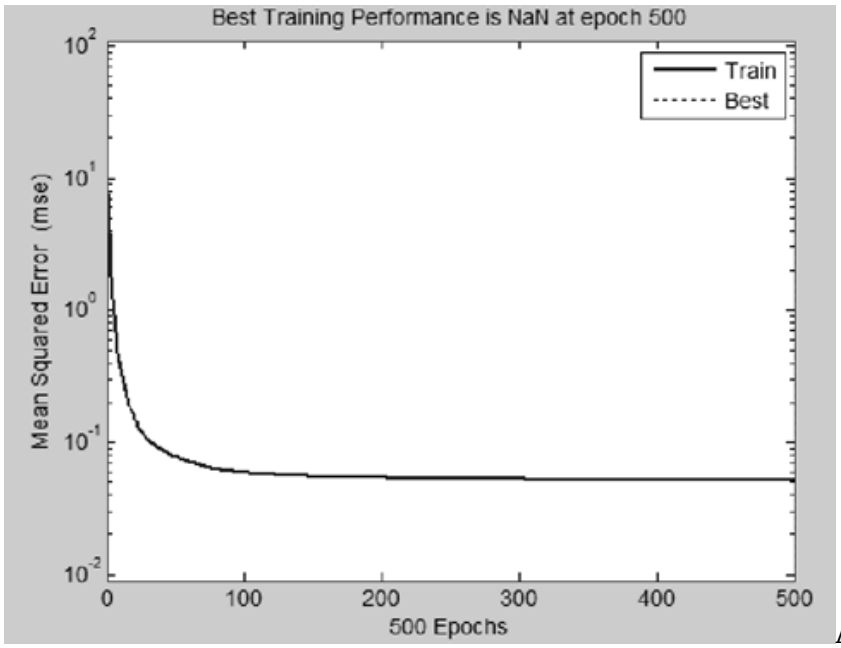
between the hydrolysis conditions. The work by Vasquez *et al*. (2007) considered pH, percentage solids, enzyme loading and temperature for sugarcane bagasse hydrolysis and they reported that enzyme loading had the highest effect, followed by temperature. There was a less significant effect on hydrolysis as a result of interaction of factors. pH had less an effect than other variables in the range considered. Parmar and Rupasinghe (2013) reported that pH, temperature, time and enzyme dosage were significant for the release of sugars from apple pomace using a combination of Celluclast 1.5L, Pectinex 3XL and Novozyme 188.

Most authors prefer models using constant temperature and pH (Sousa *et al*., 2011). Vasquez *et al*. (2007) considered pH, percentage solids, enzyme loading and temperature for sugarcane bagasse hydrolysis, using a factorial design and developing a quadratic model. Enzyme loading had the largest effect, followed by temperature. There was a less significant effect on hydrolysis as a result of interaction of factors. pH had less an effect than other variables in the range considered. pH 5.0 was selected because this is the main pH used in literature for cellulose hydrolysis. At this pH there is less contamination by bacteria, while *S. cerevisiae* performs well in the acidic pH range. Optimal conditions for sugar concentration and yield may be different. Gan *et al*. (2003) developed a model that factored in enzyme deactivation, in addition to the other parameters mentioned above.

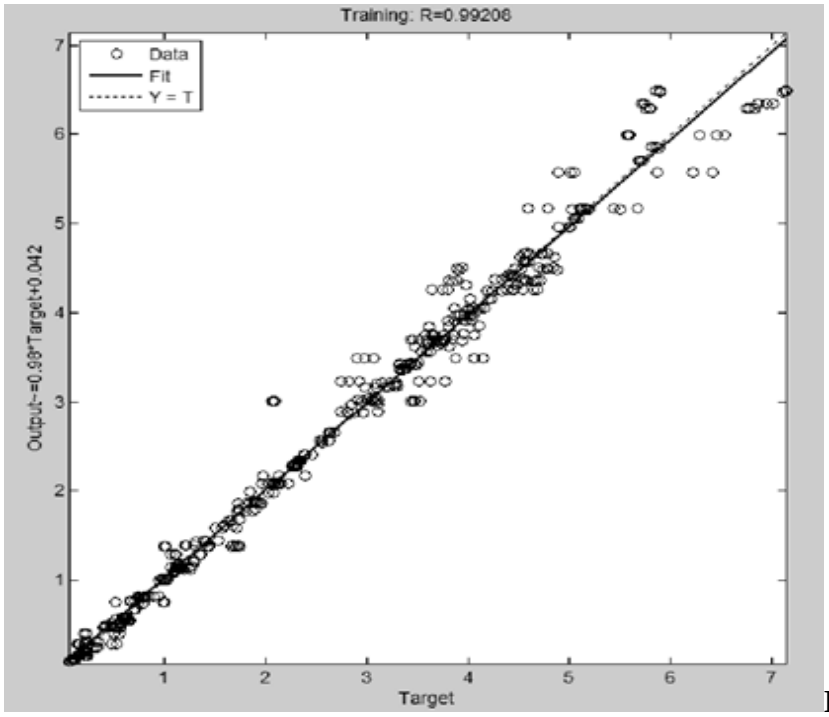
5.4.6. ANN model for prediction of apple pomace hydrolysis

The performance of the ANNs is influenced by the choice of design parameters, which include training algorithm, activation function, number of hidden layers and neurons, training duration and learning rate (O'Dwyer *et al*., 2008; Sasikumar and Viruthagiri, 2010). Careful selection of input variables is also important for the performance of the ANN model (Puig-Arnavat *et al*., 2013). Since all conditions that influence enzymatic hydrolysis cannot be investigated all at once, the most influential conditions for lignocellulose hydrolysis were therefore investigated as guided by literature (Bansal *et al*., 2009; Holtzapple *et al*., 1984; Vasquez *et al*. 2007). In this study, results of sugar concentration were used and a number of training methods were employed on different data arrangements, for example using an input vector of 6x1 and target of 18x1 and also using an input vector of 6x1 and target of the average of the triplicate, however these approaches didn't work due to the limitation of the data size and the fact that training in ANN is difficult when there are different targets associated with the same input. It was finally decided to keep the first of each input value and perturb each of the remaining two input values by a small amount (0.01). This procedure was

justified since a small amount was used. A total of 81 different inputs and associated targets were therefore obtained, giving enough data for ANN training. A number of ANNs were constructed and the best network architecture was selected. Training was done on all data leaving one for testing, a 'leave one out' method. The generation ability of the ANNs developed was assessed using the validation data set (Nodeh, 2012). Initially, the ANN was trained over 1000 epochs (number of runs) and the performance levelled out after about 500 epochs, therefore the ANN was trained over 500 epochs to avoid overtraining. The error between the experimental data output and the ANN outputs was reduced by adjusting weights and biases of the ANN (O'Dwyer *et al*., 2008). The average R^2 values and mean square error (MSE) of the outputs over all the runs were determined to assess the simulation performance of the model on the experimental data. The trained ANN accurately fitted the experimental data as indicated by the R^2 of 0.99 and small MSE value (Figure 5.7). This model was successful in predicting sugar release. Normally the experimental data is randomly split into three data sets, training, test and validation set depending on the size of the data (Puig-Arnavat *et al*., 2013; Sasikumar and Viruthagiri, 2010). Due to the size of the data set used, the test set and validation set were the same. During training the data was loaded and organised to construct the neural network and to train it. During testing, the performance of the neural network was assessed by simulating the network on the training and test data sets. Validation data was used to simulate the neural network on new data.



A



B

Figure 5.7: Correlation between experimental data and ANN simulated/ predicted slopes and intercepts for sugar concentrations performed on all the data. A- correlation coefficient, B- MSE.

After correct simulation of the ANN model on the test data points, training was performed on all the data. Validation data was used to simulate the neural network on new data (Nodeh, 2012). The performance of the ANN model for glucose and reducing sugar concentration using the training and validation data set is shown in Figure 5.8. The developed ANN was successfully able to predict the sugar concentrations on new data as the experimental values

fitted closely to the predicted values by ANN. This was a further confirmation that the training of the ANN was successful. The results agree with what is reported in literature, that ANN has an excellent data fitting and prediction ability, without depending on mathematical equations like traditional kinetic models (Bhotmange and Shastri, 2011; O'Dwyer, 2008; Sousa *et al*., 2011; Wang *et al*., 2011). ANN has been reported to be flexible so that if any new data is added, the system can relearn and new observations can be added anytime (Bhotmange and Shastri, 2011; Rivera *et al*., 2010; Wang *et al*., 2011).

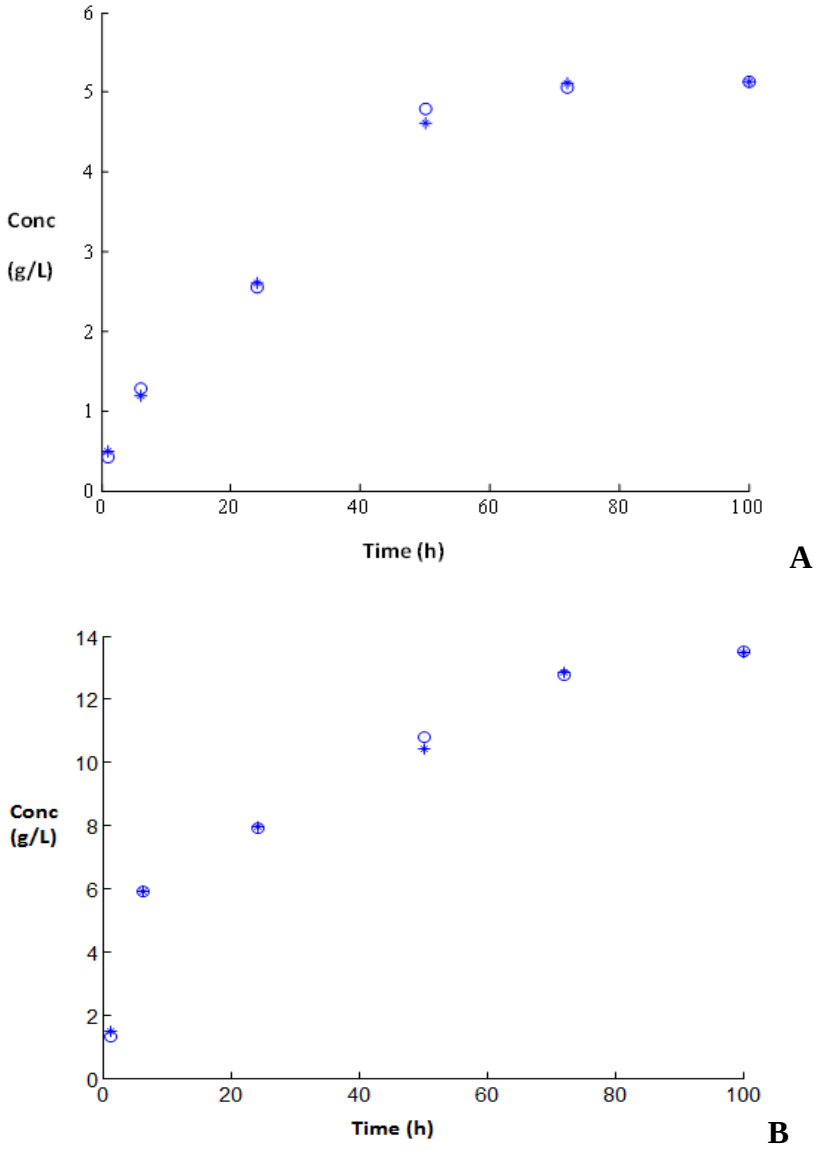


Figure 5.8: Experimental data and performance of ANN model for glucose (A) and reducing sugars (B) using training dataset (o) and validation dataset (ж). Conc – concentration.

There was poor simulation at 37°C and pH 4.0, which may be attributed to insufficient data at these conditions.

After successful development and training of the ANN model, simulation (outputs for given inputs patterns) was performed in order to find optimum conditions for apple pomace hydrolysis. The ANN was simulated on the input patterns to produce an activation vector which is a sugar release profile of the inputs conditions. A Matlab program was used to sort sugar profiles according to maximum sugar concentration for each input condition. The maximum glucose and reducing sugar concentrations produced when substrate and enzyme concentrations were varied and temperature was fixed at room temperature and pH fixed as unbuffered were surface plotted as shown in Figure 5.9. Room temperature and an unbuffered system were shown to be optimal conditions for apple pomace hydrolysis (section 4.4.1, 5.4.3 and 5.4.4) and a lot of experimental data was generated using these conditions. The optimal glucose concentration of 6.5 g/L was released using a substrate concentration of 30% (wet, w/v) and enzyme concentration of 0.5 mg/g of substrate. However, the concentrations for reducing sugars were different, a substrate concentration of 30% (wet, w/v) and enzyme concentration of 0.2 mg/g of substrate gave a maximum of 28.9 g/L reducing sugars.

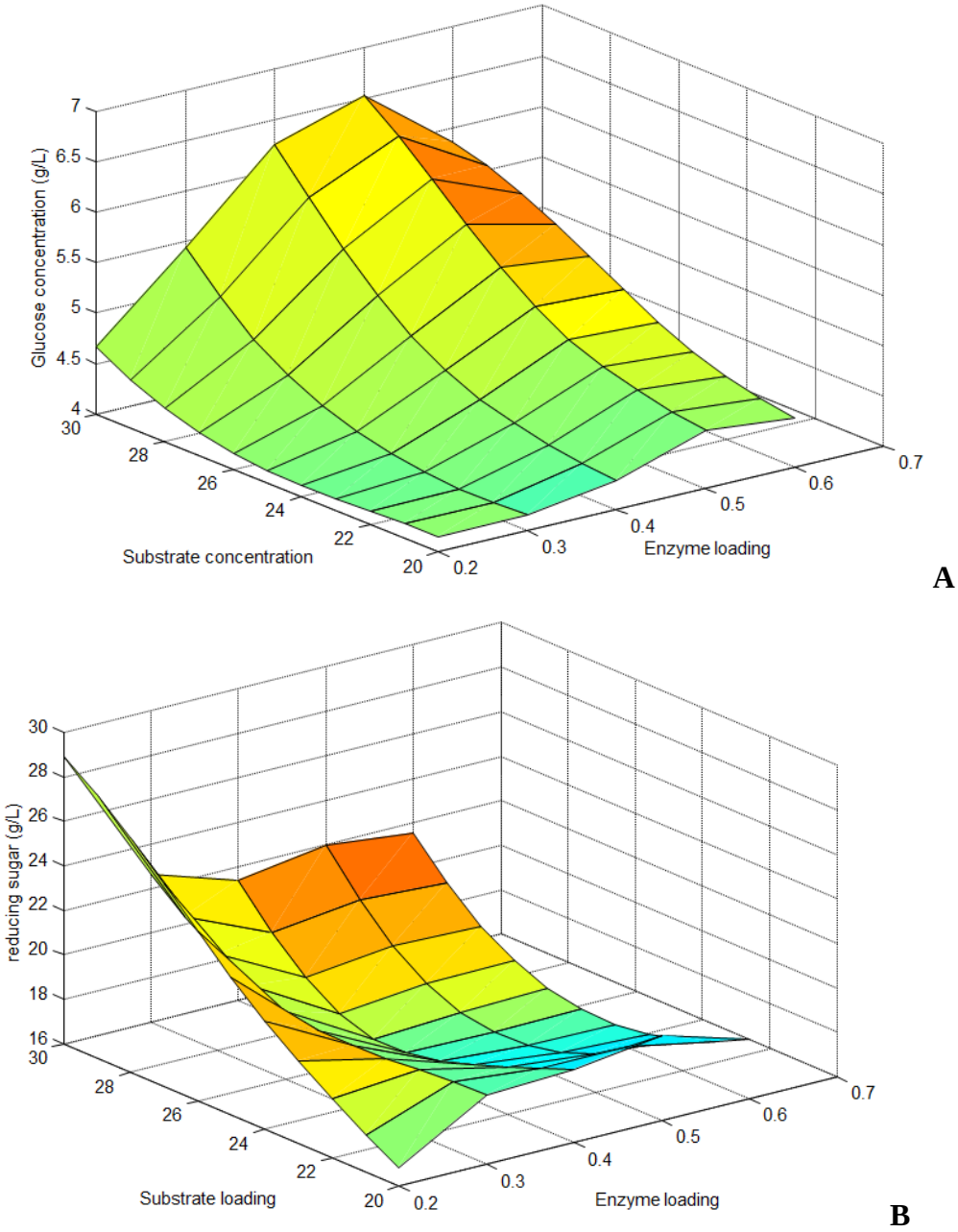


Figure 5.9: Response surface plot generated by the ANN model showing maximum glucose (A) and reducing sugars (B) released under different substrate and enzyme concentrations. Substrate concentration (%, wet w/v), enzyme loading (mg/g substrate), gluc (glucose) and RS (reducing sugar).

The results indicated that substrate and enzyme concentrations had an influence on each other and were the two most important parameters for apple pomace hydrolysis. For both products, increasing enzyme concentration beyond 0.6 mg/g of substrate did not increase sugar release. The results indicated that high enzyme loadings did not result in high sugar release was in agreement with what was reported by Rivera *et al*. (2010). However, an increase in substrate

concentration resulted in an increase in sugar release. These results agreed with results in Figure 5.2 and 5.3 for varying substrate and enzyme concentrations, respectively. An increase in substrate concentration from 20-30% resulted in a large difference in reducing sugar release, but a small difference in glucose release. Enzyme concentration appeared to play a larger role in glucose release than substrate concentration. However, for reducing sugars, substrate concentration seemed to have a greater influence than enzyme concentration. Since the ANN predicted optimal conditions for reducing sugar and glucose release were different, the conditions chosen for an industrial application will depend on the targeted product. It's important to use the minimal enzyme loadings for biofuel production and glucose will be a targeted product. However, if it will be ideal to operate under similar hydrolysis conditions, a compromise has to be reached. In this case enzyme loading of 0.4 mg/g substrate and substrate concentration of 30% will be ideal for both reducing sugar and glucose, considering enzyme costs and product concentration.

The results indicated that an ANN, although a simple model, can be a powerful method to simulate complex processes like apple pomace hydrolysis. It has been reported that enzymatic reactions involving different enzyme concentrations are difficult to understand and describe statistically (e.g. multivariate regression models), but ANN can be used very successfully (Rivera *et al*., 2010). The advantage of ANN over kinetic and regression models is that it can work efficiently using less data and it is not limited by experimental design (Bhotmange and Shastri, 2011; Sousa *et al*., 2011; Zhang *et al*., 2012). New data can be added and the system can relearn and new observations can be added at any point (Bhotmange and Shastri, 2011; Rivera *et al*., 2010; Wang *et al*., 2011). ANN has been successfully used to model biomass gasification processes in bioreactors (Puig-Arnavat *et al*., 2013). O'Dwyer *et al*. (2008) successfully predicted the digestibility of poplar wood based on structural features using ANN. The disadvantage of ANN is that it doesn't explain the behaviour of the enzyme-substrate interactions in a mechanistic way (Brown *et al*., 2010; Sousa *et al*., 2011; Wang *et al*., 2011).

5.5. Conclusions

Enzymatic hydrolysis of apple pomace is influenced by a number of factors, which include temperature, initial pH, enzyme concentration, substrate concentration and time of hydrolysis. Investigating the interaction of these factors was important for the purpose of this study.

Enzyme concentration and substrate concentration were the most significant factors in apple pomace hydrolysis. An ANN model was successfully developed using experimental data involving temperature, initial pH, enzyme concentration, substrate concentration as inputs and glucose and reducing sugars as outputs. The ANN was an effective tool in approximating data from apple pomace hydrolysis which is non-linear as indicated by the R^2 value of 0.99 and a small MSE value. Enzyme loadings of 0.5 and 0.2 mg/g substrate and a substrate concentration of 30% were optimal for glucose and reducing sugar release from apple pomace, respectively. The ability of the ANN to simulate and predict the amount of sugars released from apple pomace under different hydrolysis condition is important for the design of a cost-effective process for apple pomace hydrolysis. Application of the ANN model for apple pomace hydrolysis optimisation was successful and therefore can be used to find optimal sugar concentrations and their corresponding hydrolysis conditions without any experiments being performed. Other hydrolysis conditions can be successfully modelled using the ANN procedure employed in this study. ANN can therefore be successfully applied in complex systems like lignocellulose hydrolysis.

The next and final chapter of this study, Chapter 6, deals with the general conclusions and recommendations for future studies.

CHAPTER 6 - GENERAL CONCLUSIONS AND FUTURE WORK

The use of commercial enzyme cocktails to degrade complex lignocellulosic biomass like apple pomace was investigated in this study. Large quantities of fruit waste are produced in South Africa which can utilised to produce value-added products, as well as avoiding environmental pollution.

The use of abundant and renewable lignocellulosic wastes such as corn stover, wheat straw, sugarcane bagasse and rice straw have received global attention as an alternative source for biofuel production (Balat, 2011; Ge *et al*., 2011; Gomez *et al*., 2008; Hu *et al*., 2008; Wyman, 2007). Traditionally, liquid transport fuels are obtained from fossil fuels, however because they are fast depleted and are non-renewable (Merino and Cherry, 2007). Fossil fuels also cause global conflicts, climate change and global warming as has been reviewed in section 1.1.1.2 (Dashtban *et al*., 2009; Garcia-Aparicio *et al*., 2011; Himmel *et al*., 2007; Merino and Cherry, 2007; Solomon *et al*., 2007). Bioethanol production has been commercialised in countries like USA, Brazil and some EU countries from starch crops such as corn, sweet sorghum, sugar cane, wheat barley and sugar beet (Balat, 2011; Dashtban *et al*., 2009; Ge *et al*., 2011). However, these first generation biofuels have their own problems as a result of competition with humans and animals for food, hence attention has been shifted toward lignocellulosic biomass. Production of biofuels from lignocellulose involves four stages: pretreatment, saccharification, fermentation and finally distillation. The first two stages are the most limiting stages towards biofuel production.

South Africa produces a lot of fruit waste from fruit and juice industries, e.g. apple pomace, which is lignocellulosic in nature. Apple pomace has a high moisture content, high COD and BOD and is highly nutritious, making it a target for decomposition by microorganisms. This leads to environmental pollution and health hazards. Fruit juice industries sell the pomace to farmers for animal feeding at a lower price, dump it on the land or discharge it into water systems (Van Dyk *et al*., 2013). Utilisation of apple pomace for value addition will alleviate these problems. Lignocellulosic biomass comprises cellulose, hemicellulose, pectin and lignin. The composition for apple pomace and its abundance makes it a feasible target for value addition through production of biofuels and biorefinery chemicals like sorbitol, xylitol, pectin, acetic acid, phenolics and vanillin (Bhushan *et al.*, 2008; Foyle *et al*., 2007; Joshi and

Attri, 2006). In order to utilise apple pomace to produce value-added products, it has to first be hydrolysed into sugar monomers. Hydrolysis can be done using enzymes or chemicals. However, enzymatic processes are favoured because they are environmentally friendly and specific in action. Lignocellulosic material is recalcitrant in nature due to the presence of crystalline cellulose and lignin. As a result, individual enzymes are not able to access and degrade their respective substrate components. The complete degradation of lignocellulose requires a combination of cellulases, hemicellulases, pectinases and ligninases working together synergistically, which results in increased activity as compared to the activity of enzymes working individually. Synergy experiments previously published have been performed between cellulases only and hemicellulases only and mainly on pre-treated substrates. These enzymes are produced naturally by bacteria and fungi, however a number of these enzymes have been extracted and are commercially available (Banerjee *et al.*, 2010a; Merino and Cherry, 2007). Isolated enzymes are preferred to whole cell organisms because they have greater specificity, are easier to handle and store, and the enzyme concentration used in the process is not dependent on microbial growth. However, the commercial enzyme mixtures have not been fully characterized and enzyme costs are very high, which hinders commercial production of biofuels. In order to reduce the enzyme costs, proper combinations and ratios have to be formulated. Enzymes can also be immobilized for recycling and increased stability.

A number of bioreactor systems can be used for industrial production of biofuels, namely batch, fed-batch or semi/continuous systems. The bioreactor system should enable high productivity at reduced costs, consistently produce high quality product and be easy to monitor and control process parameters (e.g. temperature, pH and mixing). New substrate should be easily added and product removed to avoid end-product inhibition. The design, simulation and control of a bioreactor system will depend on a good kinetic model that aids in the understanding of hydrolytic process and its optimisation (Hodge *et al*., 2009). A kinetic model will also provide an insight into the feasibility of commercial biofuel production.

The aim of this study was to degrade apple pomace using a combination of commercial enzyme preparations in a sustainable and cost effective manner and process. Obtaining sugar monomers that can be utilized to produce value-added products from the breakdown of the lignocellulose components was also important. This study considered a bioreactor system with rationally selected key commercial enzyme mixtures, focusing on integration of their

synergistic action to depolymerise lignocellulosic residues from apple pomace. In order to meet the objectives of this study the following questions had to be addressed;

a) What are the most feasible commercial enzyme mixtures required for complete degradation of apple waste based on the waste composition?
b) What are the optimal ratios and combinations of enzymes and the optimal conditions required to degrade apple pomace biomass effectively?
c) What are the sugar yields as a result of the synergistic cooperation between commercial enzyme mixtures for apple pomace degradation?
d) How can a simple and cheap bioreactor for juice industrial application and model apple pomace hydrolysis be designed?

Determination of the chemical composition of apple pomace was important, as the chemical composition assisted in the selection of appropriate enzymes and calculation of yield. The composition of apple pomace, as published in the literature, is not clear as composition depends on the analytical method used, as well as the method used in juice extraction and the seasonal variations in the apple substrate. The main sugars detected in apple pomace were glucose (22.3%), arabinose (12.5%) and galactose (5%) and low amounts of xylose (1.1%), which can be utilised for biofuel production. It was suspected that the major remaining component was galacturonic which can also be utilised for value addition. Although there are substantial amounts of lignin (19.8%) in apple pomace (which poses a challenge for its complete degradation), results from this study (inhibition studies) indicated that a 2 g/L lignin content had no significant effect on the activity of Viscozyme L and Celluclast 1.5L. The presence of these sugars and galacturonic acid in apple pomace was an indication that apple pomace is a suitable target for value-addition in South Africa. The yield of glucose and reducing sugars after enzymatic hydrolysis was calculated based on these compositions.

Based on the apple pomace composition, three commercial enzyme mixtures, Biocip membrane, Viscozyme L and Celluclast 1.5L were selected considering product information from suppliers, local availability and cost. The enzymes were screened based on their synergistic action to efficiently degrade apple pomace. Viscozyme L and Celluclast 1.5L in combination displayed a higher degree of synergy (1.6) and release of sugars than any other combination, and hence this enzyme combination was used in subsequent experiments in this study. The high degree of synergy was an indication that there was cooperation between the

enzymes of the two mixtures for apple pomace degradation. Biocip Membrane and Viscozyme L and the combination of the three enzymes showed a low degree of synergy due to possible competition between enzymes in these mixtures. Following the selection of best enzymes, an optimal enzyme ratio was determined. A ratio of 50:50, Viscozyme L: Celluclast 1.5L, was the best in terms of synergy and sugar release. Using the two enzyme mixtures at their optimal ratio means that fewer enzymes can be utilised in apple pomace hydrolysis, leading to a reduction in enzyme costs which is one of the major bottle-necks to commercial biofuel production.

To understand the behaviour of the enzyme mixtures, the enzymes present in the mixtures were identified through measuring their activities on specific substrates. The study demonstrated that there were many enzymes present in both Viscozyme L and Celluclast 1.5L. Viscozyme L contained mainly hemicellulases and pectinases, while Celluclast 1.5L contained mainly cellulases and xylanases, with both enzyme mixtures containing very little β-glucosidase. It was clear that the enzyme mixtures contained the main enzymes required and complemented each other for the degradation of cellulose, hemicellulose and pectin components of apple pomace.

Hydrolysis conditions (temperature and pH optima) for Viscozyme L and Celluclast 1.5L were also similar and the enzymes were very stable for a long period of time (15 days), which is an advantage as the enzymes can be applied together in industrial bioreactors with minimum operational modification and enzyme costs. The stability of the enzymes enabled them to be used in their free form for optimal hydrolysis of apple pomace. The temperature optimum was 50°C for both enzymes and pH optima were observed at pH 5.0 and pH 3.0 for Viscozyme L and Celluclast 1.5L, respectively.

Enzyme activity can be influenced by some metabolites or compounds present or produced in the reaction mixture. It was therefore important to determine the effect of some of these compounds on enzyme activity. Viscozyme L and Celluclast 1.5L activity was affected by the presence of alcohols, sugars, organic acids, lignin, phenolic compounds and metal ions to varying degrees, depending on their concentrations. Oligosaccharides like cellobiose and xylobiose were more inhibitory than monomers, indicating that there is need to hydrolyse the apple pomace into sugar monomers to reduce end-product inhibition, which can be achieved by using the right proportions of enzymes and supplementation with β-glucosidase. Ethanol

was less inhibitory to the enzymes than other alcohols, making it feasible to use a SSF bioreactor system for bio-ethanol production. However, when considering the inhibitory effect of organic acids which may be generated during fermentation on the hydrolytic enzymes, a SHF bioreactor system is recommended. The effect of various inhibitors on Viscozyme L and Celluclast 1.5L can be reduced by using enzymes for pre-treatment, using a SHF bioreactor system, and using water that is not contaminated with metal ions e.g. deionised water.

The optimal time of apple pomace hydrolysis by the combination of Viscozyme L and Celluclast 1.5L was 24 h, using simultaneous synergy. It was noted that the degree of synergy was highest at the initial hydrolysis stages, but decreased with increased hydrolysis time. Greater cooperation of enzymes in the hydrolysis of apple pomace was required during initial stages as the substrate will be opening up (Van Dyk *et al*., 2012). The results of this study indicated that the degree of synergy does not always correspond to yield. Pre-treatment of apple pomace with ligninases did not improve sugar release by Viscozyme L and Celluclast 1.5L and therefore addition of ligninases was not required as they would only contribute to higher enzyme and production cost. The operation of bioreactors for a shorter time with less enzymes will greatly reduce the cost of biofuel production.

To improve the stability of the enzymes and enable their recycling for bioreactor application, different immobilisation methods, which included immobilisation on nylon mesh, nylon beads, chitin, sodium alginate beads, silica gel beads and CLEAs were attempted without much success. The major challenge with immobilisation was the low protein loadings and loss of activity of the enzymes. The failure of the immobilisation strategies were attributed to the presence of many enzymes in the mixture with different chemical properties and the presence of additives in the enzyme cocktails. It can be concluded that free enzymes can be successfully used for apple pomace hydrolysis due to their good stability. This will also save costs associated with immobilising the enzymes.

Having characterised the substrate and the enzyme mixtures, the study then focused on: optimising the bioreactor conditions in terms of temperature, pH, type of water, reactor volume and mixing; designing a simple and cheap bioreactor for industrial application and scaling it up to bigger volumes; investigating the effect and efficiency of different substrate feeding regimes; and determining the effect of using high substrate concentration on

enzymatic hydrolysis. A simple and cheap bioreactor using compressed air for mixing was successfully designed for 1 L reactor volumes. Operating the bioreactors at room temperature using an unbuffered system was found to be the best for optimal hydrolysis of apple pomace. Using these hydrolysis conditions could make the process cost effective. The study demonstrated that increasing substrate loadings resulted in an increase in sugar release, but with a decrease in yield. The addition of new substrate resulted in a subsequent increase in the amount of sugars released, which further proved that Viscozyme L and Celluclast 1.5L were stable for longer periods of time under the assay conditions used. Supplementing β-glucosidase to Viscozyme L and Celluclast 1.5L resulted in the doubling of glucose released after 200 h. Novozyme 188 addition became more significant at high substrate loadings and after 50 h hydrolysis. The study showed that it was best to operate the bioreactors in batch mode, rather than in fed-batch mode. This offers the advantage of easy application. Using 20% (wet w/v) apple pomace concentration in batch mode resulted in the release of 4.2 g/L glucose and 16.8 g/L reducing sugars with a glucose yield of 75% after 100 h. The obtained yield was quite high and demonstrated that the enzymes were efficient considering that the reaction was running for 100 h, which was short as far as lignocellulose hydrolysis is concerned. Besides glucose, other products such as galacturonic acid, xylose and arabinose were released from the hydrolysis of apple pomace. These products can be utilised for value-addition, making the apple pomace degradation process cost-effective. The produced sugars can be converted to bioethanol through fermentation.

To understand how the enzymes work and interact with the substrate and to predict optimal sugar release from apple pomace, the effect of different hydrolysis conditions were investigated, e.g. enzyme loading, substrate loading, temperature, initial pH and interaction of these factors. Enzyme loading was found to be the most important factor for apple pomace hydrolysis. The data was used to develop an ANN model for predicting apple pomace hydrolysis. The best model had 20, 20 and 6 neurons in the first, second and last hidden layer, respectively. The developed model successfully predicted sugar release from apple pomace. Optimal conditions obtained from ANN are important for the design of a cost-effective bioreactor system. The fact that the ANN was able to correctly predict sugar release from apple pomace, which is a lignocellulose substrate, means that it can also be applied to other complex lignocellulose substrates.

Recommendations and future work

Based on the insight and understanding gained from this work some of the recommendations for future work include:

a) Fermentation of sugar monomers to ethanol and other products such as acetic acid is recommended (Parmar and Rupasinghe, 2012).
b) Phenolic compounds present before and after enzyme hydrolysis should be determined as these can be used for value addition and also aid in understanding the behaviour of the enzymes. Some of the phenolics and organic acids such acetic acid have a potential to inhibit fermentation microorganisms (Hogde *et al*., 2009, Parmar and Rupasinghe, 2012, Van Dyk *et al*, 2013; Zhang *et al*., 2009). In the review by Van Dyk *et al*. (2013) it was reported that phenolics were released from fruit waste by using a combination of pectinases and cellulases.
c) It will be important to measure the release of oligomers in the enzyme reactions in future studies as this will give an idea of the extent of hydrolysis and whether additional enzymes were required to release monomers and at what stage additional enzymes should be added. A method for identifying oligomers, e.g. High Performance Size Exclusion (HPSEC) and High Performance Anion Exchange Chromatography (HPAEC) could be used. The disadvantage with TLC is that it would require a number of standards for each run and that it is not quantitative.
d) A fast and specific method for identification of hydrolysis products is required, e.g. HPLC or GC by first derivatising the carbohydrates (Vanderghem *et al*., 2010; Parmar and Rupasinghe, 2012). These methods will allow all sugars present to be determined in one run. Megazyme kits are expensive, laborious and time-consuming as different analyses have to be performed for each sugar separately.
e) An in-depth synergy study involving other auxillary enzymes, such as ferulic and acetyl esterases, can be performed to improve sugar yield.
f) Use of enzyme surfactants, e.g. Tween 20, Tween 80, polyoxyethylene glycol or BSA, can improve yields due to stabilisation of cellulases and reduction of irreversible binding of cellulases to the substrate, especially lignin, by modifying the cellulose surface (Rodhe *et al*., 2011, Sun and Cheng, 2002). The surfactants can bind to lignin and prevent unproductive binding of enzymes (Sipos, 2010).
g) Performing enzyme adsorption studies by detecting the amount of enzyme in the supernatant after hydrolysis will indicate whether recycling of enzymes was possible. The

studies will also provide more insight into the interaction between the enzymes and the substrate.

h) There was a problem with mixing at high substrate concentrations using both shake flasks and compressed air due to the high viscosity of the medium. High substrate concentrations are required for the production of high sugar concentration and lowering the costs for downstream processing. Therefore, applying mixing methods that are closer to industrial application/ implementation, such as impellers, should be implemented (Zhang *et al*., 2009). This will be important for scale-up to bigger bioreactors/ pilot bioreactors to sizes suitable for industrial application. It will also be important to use the actual apple pomace from the fruit juice industry in the pilot scale bioreactor.

i) The bioreactors used in this study were difficult to set up, sample and control, therefore for pilot bioreactors an automated system should be employed for easy monitoring of parameters.

j) In this study, enzyme immobilisation did not yield positive results, therefore the use of membrane reactors (ultrafiltration) to remove glucose and other sugars while recycling the enzymes should be considered (Andric *et al*., 2010c). This approach will not only prevent product inhibition, but will also enable the reuse of the enzymes. Another way to recycle the enzyme is by resuspending residuals (Vanderghem *et al*., 2010; Zhang *et al*., 2010). Enzymes bind to the substrate through CBDs, so the enzyme can be recovered by reusing solid residues. The residual solids can be used in the next batch reactor without the need to add more enzymes. However, there is need to investigate if the enzymes will remain bound to the substrate. This will aid in reducing enzyme costs. Recycling unhydrolysed substrate can also aid in improving the overall sugar yield (Zhang *et al*., 2010). The use of CLEAs for enzyme immobilisation appeared more promising than other methods tested. CLEAs can be immobilised on sodium alginate beads, silica gel or magnetic metal particles, to avoid disintegration of the beads and to aid in recovery and reuse.

h) Enzymatic degradation of other fruit wastes such as grape, pine and citrus wastes should be investigated since these are also produced in large quantities in South Africa. This will give an idea on enzyme ‘tunability’ on different fruit substrates as well as whether additional enzymes will be required.

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APPENDICES

APPENDIX 1- STANDARD CURVES

APPENDIX 1A- GLUCOSE, XYLOSE AND GALACTURONIC ACID STANDARD CURVES

Enzyme activity was measured by the reducing sugars formed in a modified dinitrosalicylic acid (DNS) method (Miller, 1959) using glucose as the standard. The composition of the DNS reagent was as follows: 2 g sodium hydroxide, 2 g 3,5 dinitrosalicylic acid, 40 g potassium sodium tartrate, 0.4 g phenol and 0.1 g sodium metabisulfite, made up to a total volume of 200 mL using Millipore water.

A glucose standard curve was prepared using glucose concentrations of 0-1 mg/mL (Figure A.1). A xylose standard curve was generated with concentrations of xylose between 0–1 mg/mL (Figure A.2) and a galacturonic acid standard curve was generated using galacturonic acid concentration ranging from 0–1 mg/mL (Figure A.3). A volume of 150 µL of each concentration of the sugar standard was added to 300 µL of DNS reagent. The mixture was then heated at 100°C for 5 min, cooled on ice for 5 min and readings taken at 540 nm on a Powerwave$_X$ microplate reader from Bio-Tek Instruments using KC Junior software®. The standard curve was generated in Microsoft Excel®.

The standard curves show that the DNS assay does not display a linear response at low (below 0.2 mg/mL) concentrations of glucose, xylose and galacturonic acid. Interpolations were therefore performed for concentrations of 0.2 mg/mL and above.

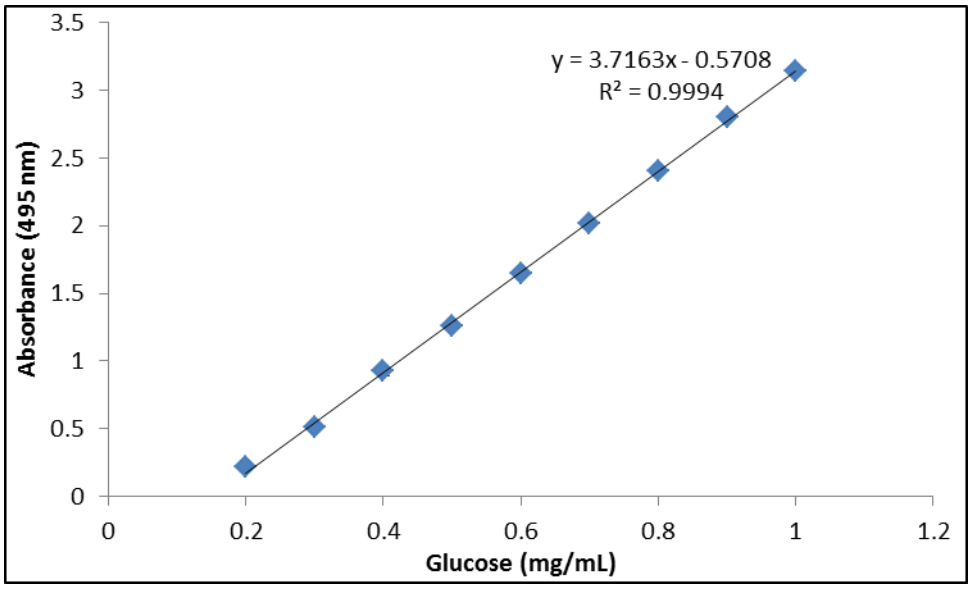

Figure A.1. Glucose standard curve. Data points are presented as mean values ±SD (n=3).

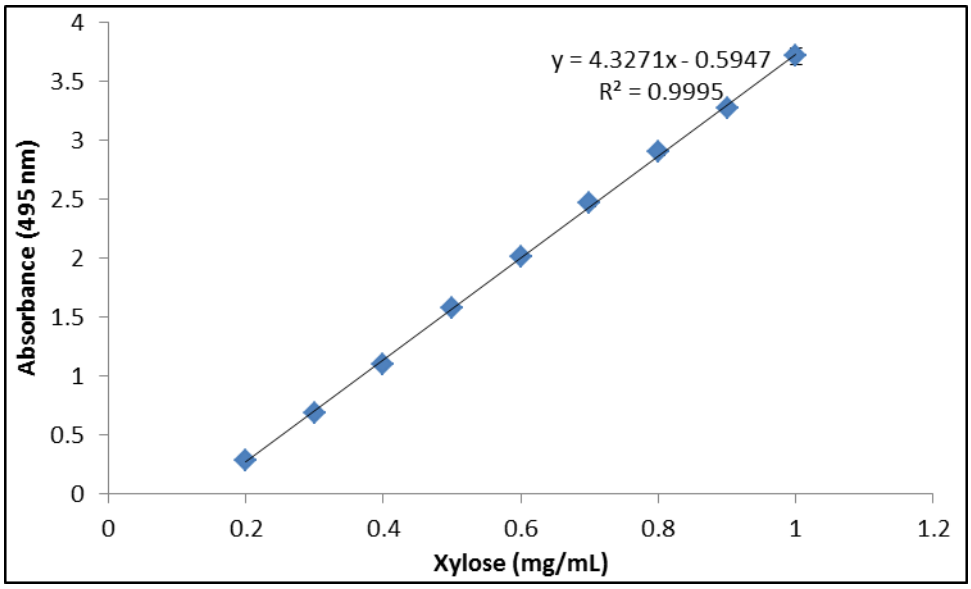

Figure A.2. Xylose standard curve. Data points are presented as mean values ±SD (n=3).

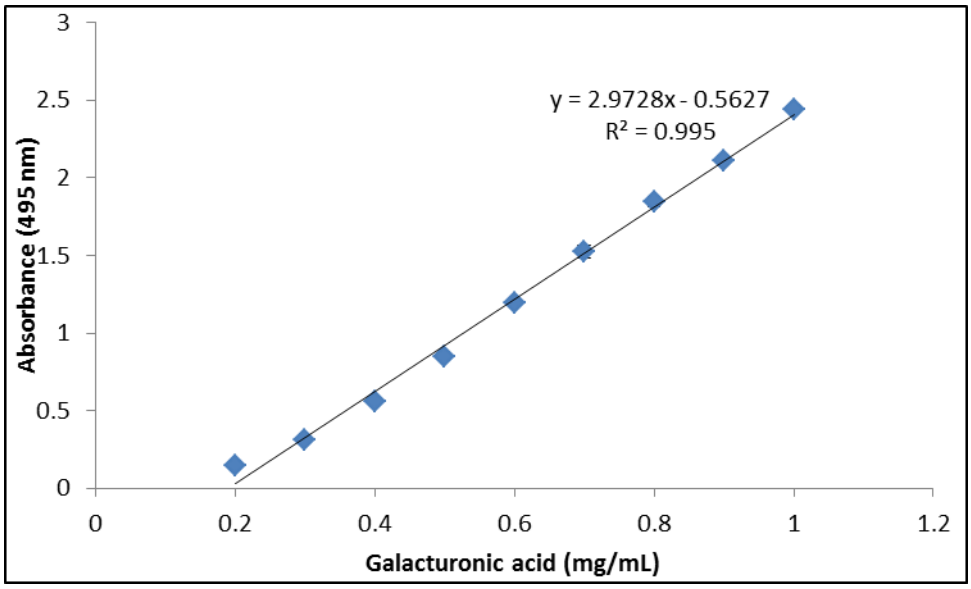

Figure A.3. Galacturonic acid standard curve. Data points are presented as mean values ±SD (n=3).

APPENDIX 1B - NITROPHENOL STANDARD CURVE

A 4-Nitrophenol standard curve was generated using a modified 4-nitrophenol assay method (Berghem and Pettersson, 1974), using 4-nitrophenol in the range of 0.001–0.08 µmol/mL (Figure A.4). A volume of 500 µL 2 M Na_2CO_3 was added to a volume of 500 µL of each 4-nitrophenol concentration and the absorbance of the resulting colour developed was measured at 405 nm using a Powerwave$_x$ microplate reader (from Bio-Tek instruments with KC Junior software®).

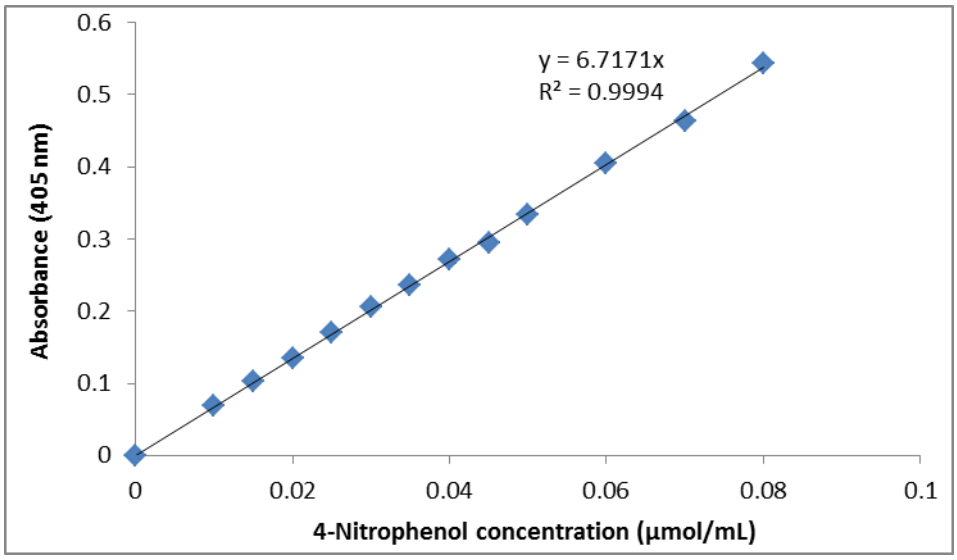

Figure A.4. 4-Nitrophenol standard curve. Data points are presented as mean values ±SD (n=3).

APPENDIX 1C - PROTEIN STANDARD CURVE

The protein standard curve was generated using a modification of the Bradford protein assay (Bradford, 1976). Bovine serum albumin (BSA) was used as a standard with commercial Bradford's reagent. Various concentrations of BSA were prepared ranging from 0.1–1.4 mg/mL. A volume of 5 µL sample was mixed with a volume of 250 µL Bradford's reagent and allowed to stand at room temperature for 5 min before readings were taken. Absorbance readings were taken at 595 nm using a Powerwave$_X$ microplate reader (Bio-Tek instruments with KC Junior software®). A standard curve was generated in Microsoft Excel® (Figure A.5).

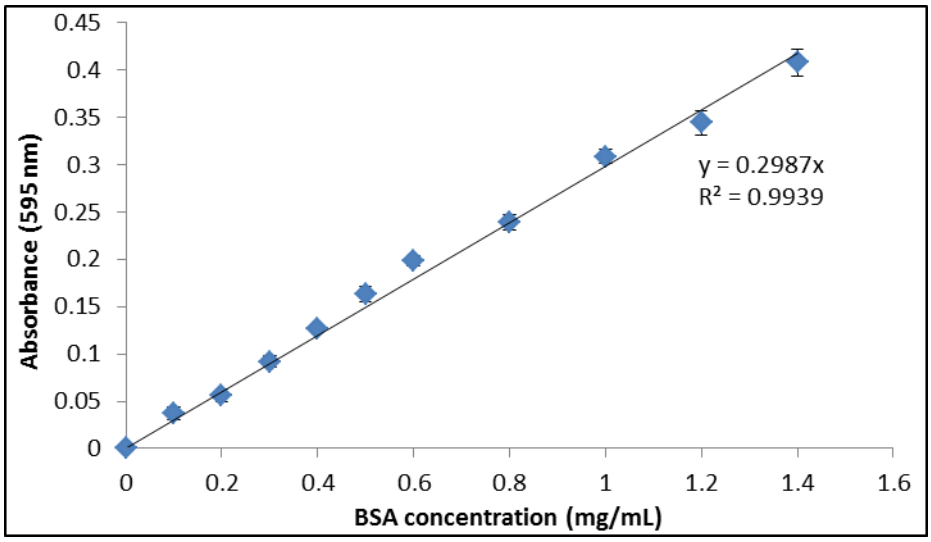

Figure A.5. BSA standard curve using 5 µL sample and 250 µL Bradford's reagent. Data points are presented as mean values ±SD (n=3).

APPENDIX 1D - PHENOL SULPHURIC ACID STANDARD CURVE

The phenol sulphuric acid standard curve was generated using a modified method of Dubois *et al.* (1956). Various glucose concentrations were prepared in the range of 0.0125–0.4 mg/mL. A volume of of 300 µL concentrated sulphuric was added to 100 µL of each standard and then 60 µL of 5% phenol, this was then mixed by vortexing. The mixture was heated at 90ºC for 10 min and then cooled to room temperature. Absorbance readings were taken at 490 nm using a Powerwave$_X$ microplate reader (Bio-tek Instruments with KC Junior software®). A standard curve was generated in Microsoft Excel® (Fig A.6).

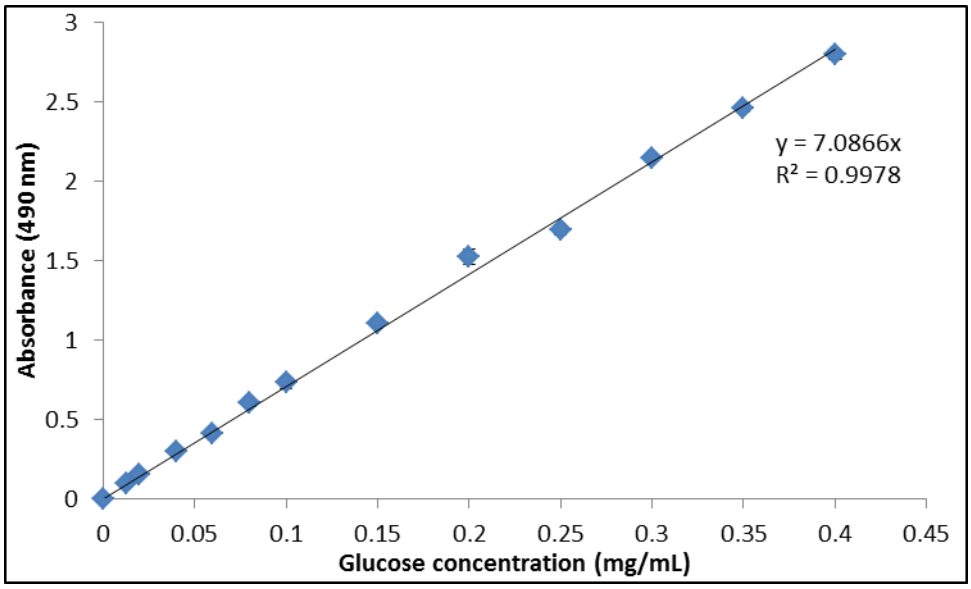

Figure A.6. Phenol sulphuric acid standard curve. Data points are presented as mean values ±SD (n=3).

APPENDIX 1E – MEGAZYME KIT ASSAY STANDARD CURVE

Megazyme kit assay procedures were used to generate standard curves for glucose, galacturonic acid, arabinose, xylose and galactose (Megazyme, Ireland). Assays were carried out on microtire plates. Standard curves were prepared using different concentrations ranges for each sugar; glucose 0-1 mg/mL (Figure A.7), galacturonic acid, 0–4 mg/mL (Figure A.8), arabinose 0-2 mg/mL (Figure A.9), xylose, 0–0.25 mg/mL (Figure A.10) and galactose, 0-0.4 mg/mL (Figure A.11) Absorbance readings were taken at 510 nm for glucose and 340 nm for galacturonic acid, arabinose, xylose and galactose on a Powerwave$_X$ microplate reader from Bio-Tek Instruments using KC Junior software®. The standard curve was generated in Microsoft Excel®.

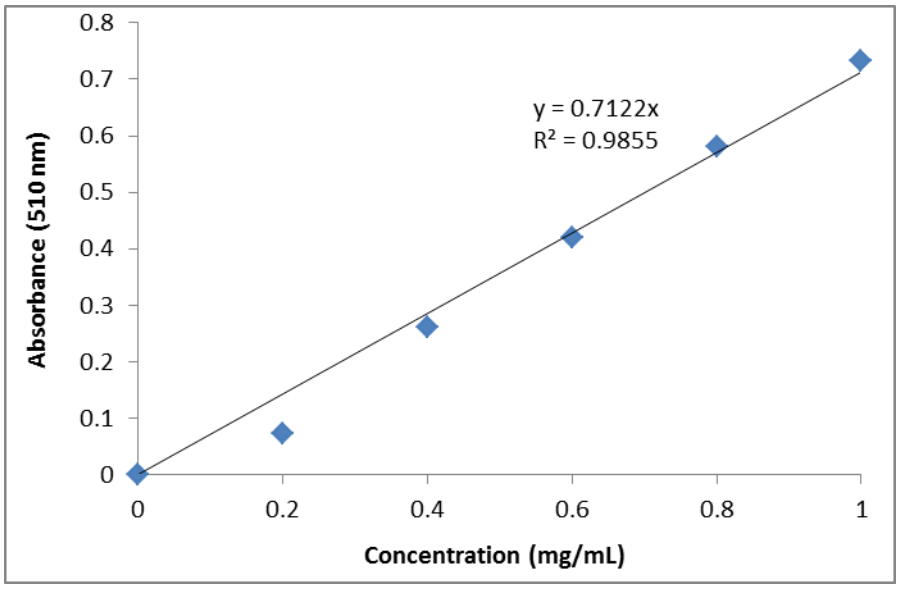

Figure A.7. Glucose standard curve. Data points are presented as mean values ±SD (n=3).

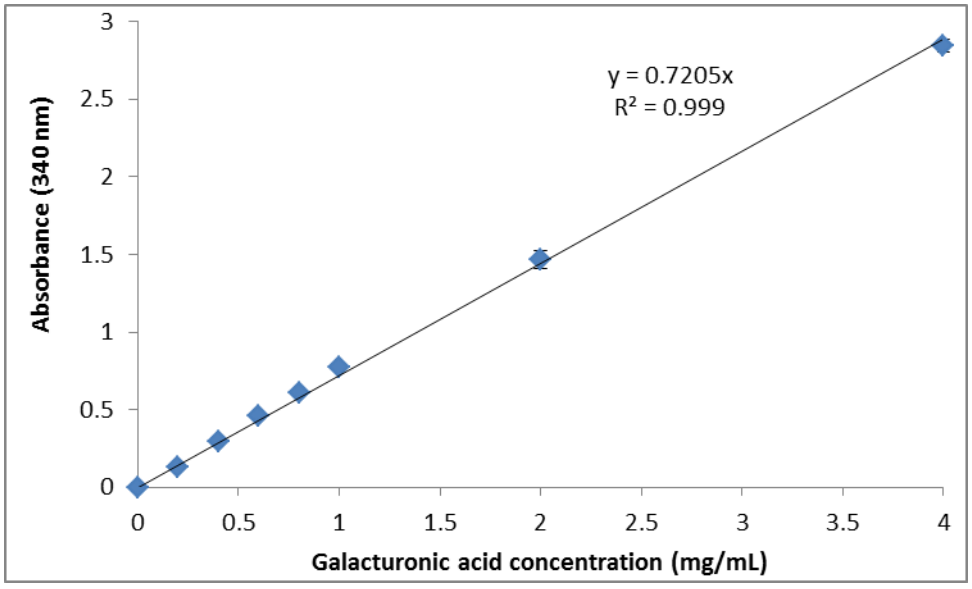

Figure A.8. Galacturonic acid standard curve. Data points are presented as mean values ±SD (n=3).

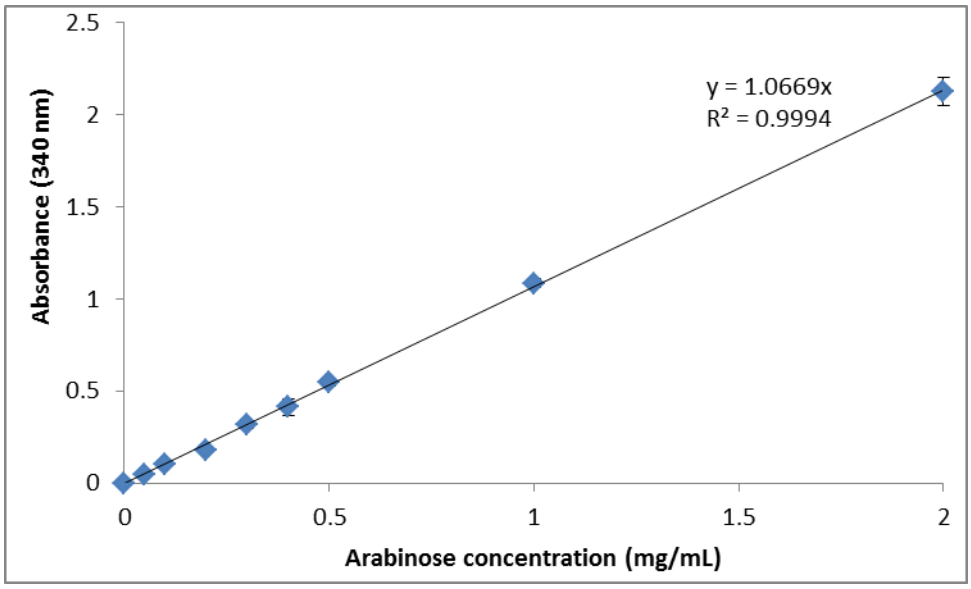

Figure A.9. Arabinose standard curve. Data points are presented as mean values ±SD (n=3).

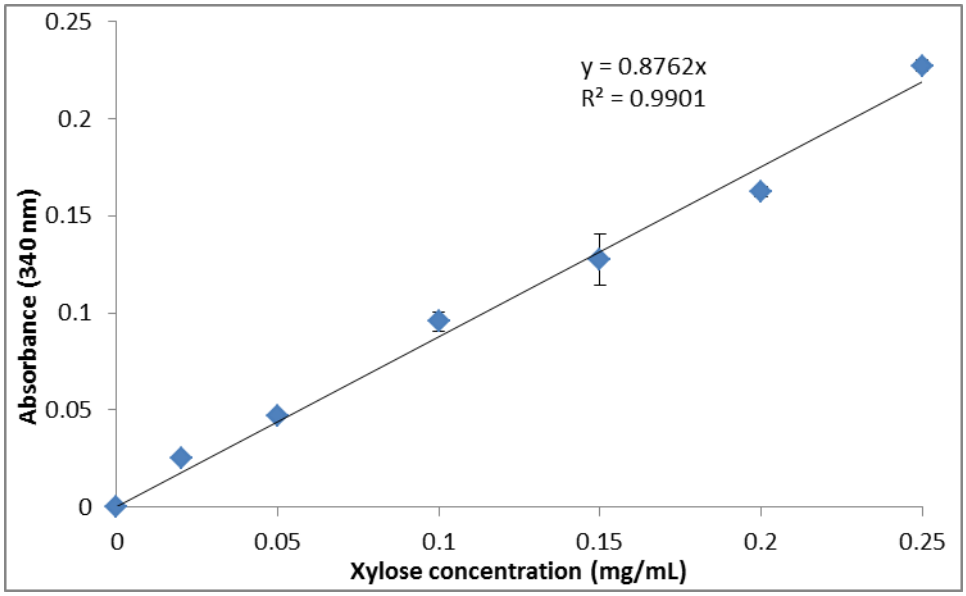

Figure A.10. Xylose standard curve. Data points are presented as mean values ±SD (n=3).

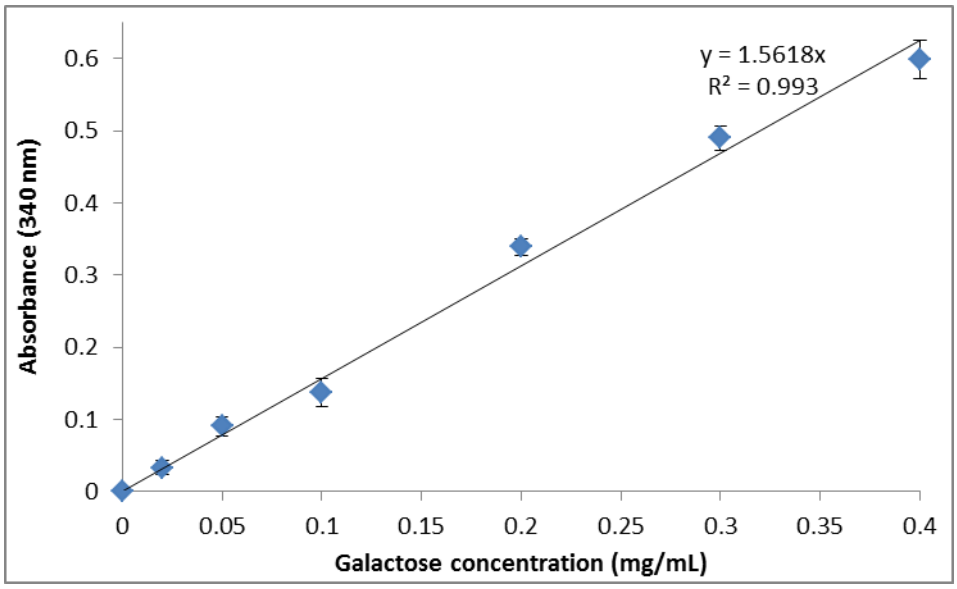

Figure A.11. Galactose standard curve. Data points are presented as mean values ±SD (n=3).

APPENDIX 2 - LIST OF REAGENTS

Table A1: The name of reagents used and the suppliers

Name of reagent	Name of supplier (Catalogue number)
1,4- β-D-Xylotetrose	Megazyme (O-XTE)
1,4- β-D-Xylotriose	Megazyme (O-XTR)
1,4-β-D-Xylobiose	Megazyme (O-XBI)
1-Butanol	Sigma-Aldrich (360465)
1-Propanol	Sigma-Aldrich (402893)
2-mercaptoethanol	Fluka (63700)
3,5-Dinitrosalicylic acid	Sigma (D0550)
4-Nitrophenol	Aldrich (42,575-3)
4-Nitrophenyl-β-D-cellobioside	Sigma-Aldrich (N5759)
4-Nitrophenyl-β-D-glucopyranoside	Sigma (N1377)
4-Nitrophenyl-β-D-mannopyranoside	Sigma (N1268)
4-Nitrophenyl-β-D-xylopyranoside	Sigma (N2132)
4-Nitrophenyl-β-L-arabinofuranoside	Sigma (N3641)
Acetone	Merck (8.22251.2500)
Acrylamide	Sigma (A8887)
Ammonium chloride	Saarchem (112 27 20 EM)
Ammonium persulphate	Sigma (A3678)
Ammonium sulphate	Merck (1.01217.1000)
Pectin apple	Sigma (76282)
(3-aminopropyl) triethoxysilane (APTES)	Sigma-Aldrich (A3648)
Avicel PH101	Fluka (11365)
Birchwood xylan	Fluka (95588)
Bis-Tris	Fluka (14880)
Boric acid	Merck (1404020EM)
Bovine serum albumin (BSA)	Sigma (A4503)

Bradford's reagent	Sigma (B6916)
Bromophenol blue	Sigma (B8026)
Butyric acid	Merck (8.00457.0100)
Calcium carbonate	Saarchem (1524500)
Calcium chloride	Merck (1524900EM)
Carboxymethyl cellulose	Sigma (C-5678)
Citric acid	Merck (1.00244.0500)
Coomassie Brilliant blue R250	Merck (1.12553)
Copper Sulfate	Sigma (C3036)
ρ-Coumaric acid	Sigma (C9008)
D-(+)-Cellobiose	Sigma-Aldrich (C7252)
D-(+)-Glucose monohydrate	Merck (1.04074.0500)
D-(+)-Xylose	Sigma (X-3877)
Di-potassium hydrogen phosphate	Merck (1.05104.1000)
Di-sodium carbonate	Merck (1.06392,0500)
Ethanol	Merck (1.00980.0500)
Ferrous Sulfate	Merck (2346000)
Formic acid	Merck (SAAR2437080LC)
Fructose	Sigma (F-0127)
Galactose	Sigma (G-6404)
Galacturonic sodium salt acid	Fluka (73960)
Gallic acid	Sigma (G7384)
Glacial acetic acid	Merck (SAAR102 1000LC)
Glycine	Merck (1.04169)
Guaiacol	Sigma (G 5502)
Hydrochloric acid	Merck (1.00319.2500)
HPTLC plates (Silica gel 60 F254)	Merck, Darmstadt
Isopropanol	Sigma (278475)
L-(+)-arabinose	Sigma (A-3256)
Lactic acid	Sigma-Aldrich (W261106)
Lignin	Aldrich (47 1003)
Locust bean gum	Sigma (G0753)
Magnesium chloride	ACE (9607318107)
Magnesium sulphate	Saarchem (412 39 20 EM)
Malic acid	Sigma (94916)
Mannose	Sigma (M2069)
Methanol	Merck (8.22283.2500)
N,N-methylene bisacrylamide	Sigma (M7279)
PageBlue^{TM}Protein staining solution	Fermentas (R0571)
PegGold protein maker II	pegLab (27-2010)
Phenol	Merck (8.22296.0100)
Phosphoric acid	Merck/ Fluka (4818000LC)
Polygalacturonic acid	Sigma (P3850)
Potassium chloride	Saarchem (5042020)
Potassium di-hydrogen phosphate	Merck (1.04877.1000)
Di-potassium hydrogen phosphate	Merck (1.05104.1000)
Potassium hydroxide	Saarchem (5042020)
Potassium sodium tartarate	Merck (1.08087.1000)

Sodium azide	Merck (8.22335)
Sodium borohydride	Fluka (71321)
Sodium chloride	Saarchem (5822320)
Sodium di-hydrogen orthophosphate dihydrate	Merck (SAAR5822680 EM)
Sodium dodecyl sulphate (SDS)	BDH biochemical (301754)
Di-sodium hydrogen orthophosphate	Sigma (5136)
Sodium hydroxide	Saarchem (5823200)
Sodium metabisulfite	Sigma-Aldrich (255556)
Sodium potassium tartrate	Merck (1.08087)
Sulfuric acid	Merck (1.01833.2500)
Syringic acid	Sigma (S6881)
N,N,N,N-Tetramethylethylenediamine(TEMED)	Sigma (T9281)
Tris (hydroxymethyl) aminomethane	Merck (1.08382)
Tris base	Merck (1.08382.1000)
Vanillin	Sigma-Aldrich (V110-4)
Zinc sulphate	Merck (7582860 EM)

APPENDIX 3 - SDS – PAGE

Enzymes/ proteins in the commercial cocktails were analysed using sodium dodecyl sulphate polyacrylamide gel electrophoresis using the modified method by Laemmli (1970) and the BioRad Mini-Protean® 3 Cell instruction manual. The solutions were prepared as follows:

1. Acrylamide/Bis (30%)

87.6 g acrylamide

2.4 g N'N'-bis-methylene-acrylamide

The chemicals were dissolved in DH_2O and volume made up to 300 mL with DH_2O. The solution was wrapped in aluminium foil and stored at 4°C in the dark.

2. 10% (w/v) SDS

10 g SDS was dissolved in 90 mL DH_2O with gentle stirring and brought to 100 mL with DH_2O.

3. Resolving buffer gel (1.5M Tris-HCl, pH 8.8)

27.23 g Tris base was dissolved in 80 mL DH_2O. After adjusting the pH to pH 8.8 with 6 N HCl, the volume was brought to a total volume to 150 mL with DH_2O and stored at 4°C.

4. Stacking buffer gel (0.5 M Tris-HCl, pH 6.8)

6 g Tris base was dissolved in 60 mL DH_2O. pH was adjusted to pH 6.8 with 6 N HCl and total volume brought to 100 mL with DH_2O. The solution was stored at 4°C.

5. 10x Running buffer, pH 8.3

30.3 g Tris base

144.0 g Glycine

10.0 g SDS

The chemicals were dissolved in DH_2O and total volume brought to 1 L with DH_2O. A 1x running buffer was prepared by diluting the 10x buffer with DH_2O.

6. 10% APS (prepared freshly prior to use)

100 mg (0.1 g) ammonium persulfate was dissolved in 1 mL DH_20.

7. Sample buffer (SDS Reducing buffer)

3.55 mL DH_2O

1.25 mL 0.5 M Tris-HCl, pH 6.8

2.5 mL glycerol

2.0 mL 10% (w/v) SDS

0.2 mL 0.5% (w/v) bromophenol blue

The sample buffer was stored at room temperature.

USE: 50 µL β-Mercaptoethanol (reducing agent) was added to 950 µL sample buffer prior to use. The sample was diluted at least 1:2 with sample buffer and heated at 95°C for 4 min.

8. Coomassie Brilliant Blue staining solution

1 g Coomassie brilliant blue R250

450 mL methanol

450 mL DH_2O

100 mL Glacial acetic acid

9. Coomassie destain solution

45% methanol, 45% distilled water and 10% glacial acetic acid

10. Preparation of SDS-PAGE gels

Resolving gel

The 10% resolving gel was prepared by adding the following solutions in sequence;

4.1 mL distilled MilliQ water

3.3 mL 30% acrylamide stock solution

2.5 mL 1.5 M Tris-HCl buffer (pH 8.8)

0.1 mL 10% SDS stock solution

0.1 mL 10% ammonium persulfate (APS) solution

0.05 mL N,N,N,N-Tetramethylethylenediamine (TEMED)

APS and TEMED were added immediately prior to pouring of the gel.

Stacking gel

The 4% stacking gel was prepared by adding the following solutions in sequence:

6.1 mL distilled MilliQ water

1.3 mL 30% acrylamide stock solution

2.5 mL 0.5 M Tris-HCl buffer (pH 6.8)

0.1 mL 10% ammonium persulfate (APS) solution

0.05 mL N,N,N,N-Tetramethylethylenediamine (TEMED)

The resolving gel solution was poured into the gel apparatus and immediately overlayed with 300 µL isopropanol, and allowed to set. The isopropanol was removed using filter paper prior to pouring of the stacking gel on top of the resolving gel. Plastic combs were immediately inserted into the gel and the gel was allowed to set.

Gels were visualised under UV light with a Uvitec gel documentation machine (Cambridge, UK) with Uvipro chem program.