Effect of alkaline pre-treatments on the synergistic enzymatic hydrolysis of sugarcane (*Saccharum officinarum*) bagasse by *Clostridium cellulovorans* XynA, ManA and ArfA

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Abstract

The continual increase in industrialization and global population has increased the dependency and demand on traditional fossil fuels for energy; however, there are limited amounts of fossil fuels available. The slow depletion of fossil fuels has sparked a fresh interest in renewable sources such as lignocellulose to produce a variety of biofuels, such as biogases (e.g. methane), bioethanol, biodiesel and a variety of other solvents and economically valuable by-products. Agricultural crop wastes produced in surplus are typically lignocellulosic in composition and thus partially recalcitrant to enzymatic degradation. The recalcitrant nature of plant biomass and the inability to obtain complete enzymatic hydrolysis has led to the establishment of various pre-treatment strategies. Alkaline pre-treatments increase the accessibility of the exposed surface to enzymatic hydrolysis through the removal of acetyl and uronic acid substituents on hemicellulose. Unlike the use of steam and acid pre-treatments, alkaline pre-treatments solubilize lignin and a small percentage of the hemicellulose, increasing enzyme accessibility and thus the hydrolysis of lignocellulose. The majority of *Clostridium cellulovorans* associated enzyme synergy studies have been devoted to an understanding of the cellulolytic and hemi-cellulolytic degradation of plant cell walls. However, little is known about the effect of various physical and chemical pre-treatments on the synergistic enzymatic degradation of plant biomass and possible depolymerization of plant cell walls. This study investigates the use of slake lime, sodium hydroxide and ammonium hydroxide to pre-treat sugarcane bagasse under mild conditions and elucidates potentially important synergistic associations between the *C. cellulovorans* enzymes for the enhanced degradation of lignocellulose.

The primary aims of the study were addressed using of a variety of techniques. This included suitable vector constructs for the expression and purification of recombinant *C. cellulovorans* enzymes, identification of the effects of various pre-treatments on enzyme synergy, and identification of the resultant reducing sugars and phenolic compounds (released during the pre-treatment of the bagasse). This study also made use of physical and chemical pre-treatment methods, protein purification using affinity, high performance liquid and thin layer chromatography, mass spectrometry, sodium dodecyl sulphate and fluorophore-assisted

polyacrylamide gel electrophoresis (FACE) , enzymatic degradation and synergy studies with various substrates indirectly using the 3, 4-dinitrosalicylic acid (DNS) reducing sugar assay.

From this investigation, the following conclusions were made: alkaline pre-treatment successfully solublised, redistributed and removed lignin from the bagasse, increasing the digestibility of the substrates. In summary, the most effective pre-treatment employed 0.114 M ammonium hydroxide / gram bagasse at 70°C for 36 hours, followed by hydrolysis with an enzyme cocktail containing 25% ManA and 75% XynA. This increased the production of sugars approximately 13-fold. Analysis of the sugars produced by the synergistic hydrolysis of sugarcane bagasse (SCB) indicated the presence of xylose, indicating that the enzymes are potentially bifunctional under certain conditions.

This study indicated that the use of mild pre-treatment conditions sufficiently removed a large portion of lignin without affecting the hemicellulose moiety of the SCB. This facilitated the potential use of the hemicellulose component for the production of valuable products (e.g. xylitol) in addition to the production of bioethanol. Thus, the potential use of additional components of holocellulose may generate an additional biotechnological benefit and allow a certain degree of flexibility in the biofuel industry, depending on consumer and industrial needs.

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List of Abbreviations

µl	Microlitre
2 × YT	2 × Yeast-Tryptone
2FI	2 Factorial
3D	Three dimensional
AFEX	Ammonia fibre/freeze explosion
$(Al)_2SO_4$	Aluminium sulfate
ANOVA	One way analysis of variance
ANTS	8-aminonaphthalene-1,3,6-trisulphonate
APS	Ammonium persulphate
APS	Adenosine phosphosulphate
ArfA	α-Arabinofuranosidase A
ARP	Ammonia recycle percolation
BSA	Bovine serum albumin
$Ca(OH)_2$	Lime
$CaCl_2$	Calcium chloride
CBM	Carbohydrate binding module
CMC	Carboxymethylcellulose
CMX	Carboxymethylated derivative of xylan
CO_2	Carbon dioxide
Da	Dalton
DF	Degrees of freedom
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNS	3, 4-dinitrosalicylic acid
DP	Degree of polymerization
E. coli	*Escherichia coli*
EIA	Energy Information Administration
ESI	Electron spray ionization
FACE	Fluorophore-assisted carbohydrate electrophoresis
FAO	Food and Agriculture Organization of the United Nations
$FeCl_2$	Ferric chloride
F-value	Factor value
g/g	Gram per gram
g/M	Gram per molar
GH	Glycosyl hydrolase
h	Hours
H_2S	Hydrogen sulfide
HCl	Hydrochloric acid
HMF	Hydroxymethylfurfural or 5-(Hydroxymethyl)furfural
HPLC	High performance chromatography
IEA	International Energy Agency
ILS	Ionic liquids
IPTG	Isopropylthiogalactoside
kDa	Kilodalton

LBG	Locust Bean gum
LWH	Liquid hot water
ManA	Mannanase A
MES	morpholineethanesulfonic acid
mg	Milligrams
min	Minutes
ml	Millilitre
mM	Millimolar
MM	Molecular marker
mRNA	Messanger ribonucleic acid
MS	Mass spectroscopy
Na_2S	Disodium sulfide
NaCl	Sodium chloride
$NaCNBH_3$	Sodium cyanoborohydride
NaH_2PO_4	Sodium dihydrogen phosphate
NaOH	Sodium hydroxide
NH_4OH	Ammonium hydroxide
Ni-NTA	Nickel nitrilotriacetic
NMR	Nuclear magnetic resonance
NREL	National Renewable Energy Laboratory
O/N	Overnight
OD	Optical density
OSX	Oat spelt xylan
PAGE	Polyacrylamide gel electrophoresis
PAPS	Phosphoadenosine 5’ phosphosulphate
pI	Isoelectric Point
PIPES	piperazine-N,N’-bis(2-ethanesulfonic acid)
RNA	Ribonucleic acid
RSM	Response Surface Methods
SCB	Sugarcane bagasse
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Scanning electron microscopy
TEMED	N,N,N’,N’-tetramethylethylenediamine
TFA	Trifluoroacetic acid
TLC	Thin layer chromatography
Tris	Tris(Hydroxymethyl)Aminomethane
TRS	Total reducing sugars
UV	Ultraviolet
Vis	Visible
WEO	World Energy Outlook
XynA	Xylanase A

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Research outputs emanating directly from this study

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2) Beukes, N. and Pletschke, B.I. (2011) Effect of alkaline pre-treatment on enzyme synergy for efficient lignocellulose hydrolysis. *Bioresource Technology*, **102**:5207-5213.

International conference proceedings:

1) Beukes, N. and Pletschke, B.I. The effects of lime pre-treatment on the hemicellulolytic degradation of sugarcane bagasse. Third SMBBM International Congress of Biochemistry /IUBMB Special Meeting on Plant Stresses /6th FASBMB Congress. Hotel Mogador Agdal, Marrakesh, Morocco, 20-25 April 2009.

2) Beukes N., Chan H., Doy R.H. and Pletschke B.I. Synergistic degradation on untreated and lime pre-treated sugarcane bagasse using cellulosomal and non-cellulosomal enzymes. Gordon Research Conference on Cellulosomes, Cellulases and Other Carbohydrate Modifying Enzymes. Proctor Academy, Andover, New Hampshire, U.S.A. July 26 – 31, 2009.

3) Pletschke B.I., Beukes N., Dredge R., Waithaka C., Van Dyk J.S., Jones S. and Abboo S. Enzyme synergy for efficient lignocelluloses degradation: Fundamentals and application. 51st Annual Conference of Association of Microbiologists of India (AMI). AMI-2010. Ranchi, India. December 14-17, 2010.

National conference proceedings:

1) Beukes, N. And Pletschke, B. The effect of alkaline pre-treatment on the degree of synergy with sugarcane bagasse. 22nd South African Society for Biochemistry and Molecular Biology (SASBMB) Congress. Ilanga Estate. Bloemfontein, 18-20 January 2010.

Chapter 1: Literature review

1.1 Traditional versus renewable energy sources

The traditional energy sources that have been exploited since the industrialisation of different countries are commonly referred to as fossil/non-renewable fuels; however, the term non-renewable is debatable since fossil fuels are renewable over a very long period of time. With increased industrialisation and increasing populations, the demand on the fossil fuels for energy has increased exponentially (Sun and Cheng, 2002) and the increase is predicted to continue (Figure 1). In general, the consumption of energy can be divided into three groups, heat, grid electricity and transportation fuels (Pehnt *et al.,* 2006). The use of lignocellulosic biomass makes a small contribution to all three energy groups to varying degrees (Gomez *et al.,* 2008).

The growth of the world energy consumption is currently at 2% per annum (Mason, 2007); however, the Energy Information Administration (EIA) has predicted that the energy consumption would only grow at an average rate of 1.1% per annum (Energy Information Administration, 2007). With the increase in the global energy consumption the International Energy Agency (IEA) predicted that the demand for oil would increase by approximately 1.3% per annum between 2005 and 2030 (International Energy Agency, 2006). In 2006, the World Energy Outlook (WEO) claimed that fossil fuels will remain the major source of energy, where oil is the main fossil fuel used in a modern society; however, the dependency on coal (currently the second largest primary fuel source) is expected to increase (International Energy Agency, 2006).

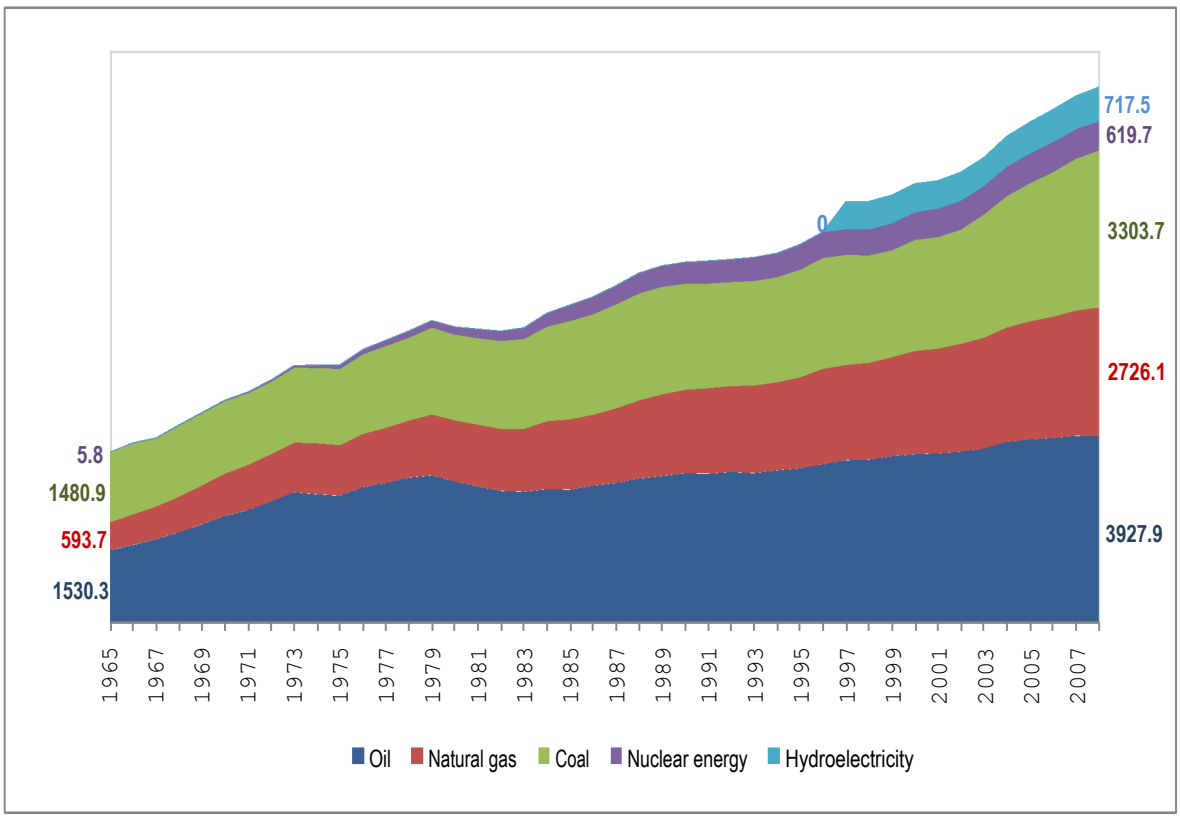


Figure 1: Comparison of global energy consumption between 1965 and 2008. The data for natural gas, coal, nuclear energy and hydroelectricity are given (colour coded) as million tonnes oil equivalents to allow comparison to the global oil consumption (BP Statistical 2010 review of World Energy).

During the period of 1950 – 1972, the fossil fuel prices were constant and comparable; however, during the period of 1972 – 1998 the fossil fuel prices were relatively constant, with occasional fluctuation. The last decade has seen an increase in volatility in fossil fuel prices (Figure 2) (Shafiee and Topal, 2010).

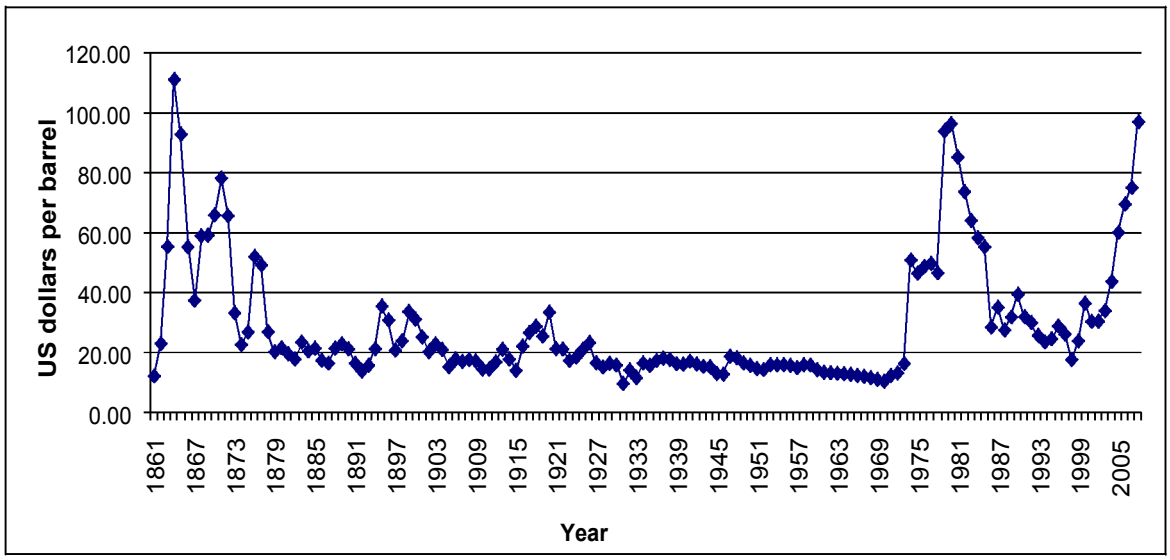


Figure 2: An instability in the global crude oil market has been observed since the 1960s and can be illustrated as the fluctuation in crude oil prices between 1861 and 2008 (BP Statistical Review of World Energy, 2010).

Since the explosion of industries in first world and developing countries, there has been a rapid, steady decrease in fossil fuel resources. The depletion of the fossil fuels has sparked a renewed interest in the potential use of renewable sources such as lignocellulose to produce a variety of fuels, such as biogases, bioethanol, biodiesel and a variety of other solvents (Sun and Cheng, 2002). Another factor responsible for the renewed interest in the production of biofuels, specifically bioethanol, is the continual fluctuation in oil prices (Figure 2).

The fluctuation of oil prices, the impending depletion of traditional fossil fuels, as well as several political reasons, have forced several countries to invest a great deal of research and development into finding an alternative energy source. Alternative energy sources that have been investigated include bituminous shales and sand, as well as the conventional energy alternatives, including geothermal and nuclear energy, wind power, the conversion of coal and, more recently, plant biomass for the production of bioethanol. Plant biomass has appeared to emerge as the most promising source of alternative energy as it can be utilised in both physico-chemical and biological conversion into several valuable products (Figure 3) (Demirbas, 2008; Howard *et al.*, 2003; Szczodrak and Fiedurek, 1996; Yang and Wyman, 2008).

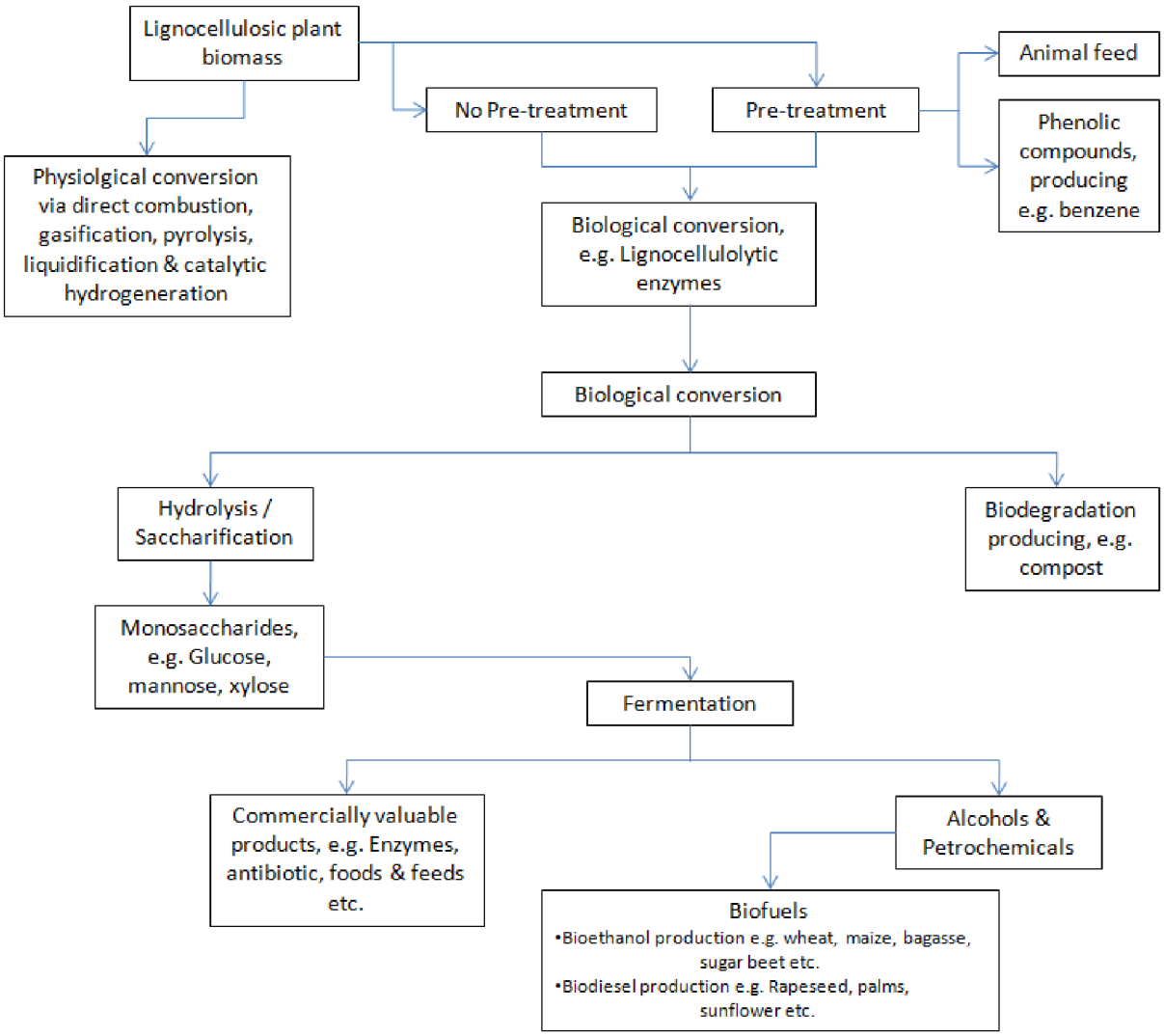


Figure 3: General utilisation of biomass in industry (modified from Demirbas, 2008; Howard *et al.*, 2003; Szczodrak and Fiedurek, 1996; Yang and Wyman, 2008).

The production of ethanol from lignocellulose is widely considered to be an important biofuel due to its impact in the petrochemical industry. It is believed that bioethanol has the potential to replace petrol, and is at presently blended into petrol in some countries such as the US and Brazil (Sun and Cheng, 2002). To date, the most important biofuels produced are biodiesel and bioethanol. Biodiesel and bioethanol are often referred to as first generation biofuels as the production of these products from readily available sucrose (extracted from sugarcane) and glucose (extracted from starch rich crops) sources. Biodiesel production is dependent on oil crops such as rapeseed, sunflower or soy crops, whereas

bioethanol is dependent on starch crops such as maize, wheat and sugarcane. Second generation biofuel, on the other hand, is obtained from deconstruction of complex cell wall carbohydrates, thus making use of the entire crop as a fuel source (Peters and Thielmann, 2008).

Biofuels have several advantages:

1) Biofuels can be obtained from a variety of readily available biomass,
2) they represent the carbon dioxide cycle in general combustion,
3) they are environmentally friendly as they are considered to be carbon neutral or commonly referred to as having a net carbon emission,
4) biofuels are biodegradable,
5) their chemical and physical properties allow for possible mixing with petrol and diesel, and
6) biofuels can be produced by most countries, thus they can potentially reduce the demand of oil importation (Puppan, 2002; Rajagopal *et al.*, 2007).

Despite the potential advantages of biofuel production, biofuels have distinctive drawbacks. The largest disadvantage to biofuel production is that biofuels are considered land and water intensive, which have subsequently (and partly) given rise to the fuel vs. food debate (Rajagopal *et al.,* 2007).

The main concern with an increase in the production of biofuels, primarily from food crops, is the impact of crop divergence not only on the availability of food, but on the market value of the crops, for example the maize price in the US. The food vs fuel debate is driven by the increasing percentage of arable land that is being diverted to the growth of crops for the production of fuel instead of food stocks. The US is responsible for the production and export of approximately 70% of the worlds maize requirements (Westcott, 2007). For example, in 2004 the US dedicated 32 million tons of maize to the production of ethanol. Even though this quantity only constituted approximately 12% of the total maize production in the US, it would have fed 100 million people at the average world consumption level (Brown, 2006). On a smaller scale, the quantity of grain required for the production of fuel to fill a 25 gallon utility vehicle, would provide one person with sufficient calories for a year (Brown, 2006; Runge and Senauer, 2007).

Although the effect of crop divergence from food to fuel at a global scale is largely unknown, these effects have been observed in Mexico. Mexico imports approximately 80% of the country's maize requirement form the US. With increasing US maize prices, the price for tortillas per kilogram has doubled. Tortillas are made from white maize that is predominantly grown locally. The rise in the tortilla prices resulted from the food processing and animal feed manufacturers buying up the locally grown white maize for a cheaper rate than the imported yellow maize that was traditionally used (Brown, 2006).

There are several individuals and groups that constantly call for the global rethinking of how the production of biofuels should proceed, especially in light of the effect of biofuels production have on the world's food supply. On the other hand, there are several groups that believe that food and fuel crops can co-exist without one being compromised or favoured. The problem with this scenario is that it is only attainable with a suitable international policy that can propose a biofuel target and, in particular, bioethanol production goal, and the production of an affordable, abundant food source. In 2006, J. von Braun (Director General of the Food Policy Research Institute) and R.K. Pachauri (Director General of the Energy and Resources Institute, New Deli, India) presented the argument that the effective maintenance and utilisation of arable land would provide a means for promoting and establishing a sustainable balance between food and fuel crops. They mainly proposed that the arable land should be rotated, reserving the favourable land for food crops, and increasing biofuel production in rural, developing countries. However, J. Diouf (Director General of the Food and Agriculture Organisation of the United Nations) argues that international policies should first address the barriers to ethanol production and import in developing countries. Diouf also argues, that developing countries that have the ecosystems and climate suited for biomass production, should rather use their arable land for crop production. This would subsequently give the developing countries the correct competitive advantage and potentially aid in the reduction of hunger and poverty (Blas, 2007). The increase in the biofuel industry, in particular bioethanol, has resulted in a thriving US maize industry; however, this industry's success could wane, especially if the petroleum oil prices drop and stabilise at a low cost. A decreasing oil price would result in the production of ethanol becoming no longer viable due to the production of ethanol being relatively expensive (Runge and Senauer, 2007).

With the continuation of the food vs fuel debate, an alternative fuel source that addresses the concerns of the availability and cost of viable food crops has emerged. The use of lignocellulosic materials, such as trees, grasses, wood chips, crop residues from wheat, rice straw and maize stalks have shown to be efficient for the production of biofuels (Hill *et al.*, 2006; Brown, 2007; Runge and Senauer, 2007; Westcott, 2007). Fast growing trees, switchgrass and sugarcane have been identified as the most promising alternatives to food crops for the production of bioethanol. There is also ongoing research to identify possible oil rich plants that may be used for the production of biodiesel Switchgrass and sugarcane are favoured due to the ability of these crops to grow on land that is generally unsuitable for annual crops and in an unfavourable climate for maize or soybean crops (Hill *et al.,* 2006; Brown, 2007; Runge and Senauer, 2007).

1.2 Biofuel Production

As mentioned previously, the general human consumption of energy can be classified into under three groups: heat, grid electricity and transport fuels. Over the years it has been observed that plant biomass can make a contribution to all three groups of energy consumption; however, the greatest contribution can be made in the context of the production of transport fuels (Gomez *et al.,* 2008). The production of biofuels has been characterised into two phases, first and second generation technology. The defining feature between first and second generation biofuel is that first generation biofuel affects short term goals, whereas second generation biofuels is expected to provide both the short term benefits of first generation biofuel as well as provide long term benefits (Rajagopal *et al.,* 2007).

First generation biofuels are produced primarily by food crops and the main benefits are low carbon dioxide (CO_2) emmissions. The main concern regarding first generation biofuels is the impact biofuel production may have on the land biodiversity and the competition with food crops (Pimentel and Patzek, 2005).

Second generation biofuel is largely dependent on cellulosic feedstocks, which have a higher cellulose yield per hectare in comparison to sugar or starch feedstocks (Somerville, 2007). Second generation biofuels are produced from potentially cheaper and more abundant crops that are typically lignocellulosic in nature. These lignocellulosic crops, like food crops

used in first generation biofuels, produce low CO_2 emissions, but unlike first generation biofuels crops, the lignocellulosic crops will not compete with food crops (Petersson *et al.*, 2007).

As mentioned previously the production of biofuels is becoming increasingly popular due to the slow depletion of fossil fuels and the recent instability in the Arab world may result in the increase in the global petroleum price. The most common renewable biofuel source is bioethanol, which is traditionally derived from starch obtained from maize and sucrose from sugarcane bagasse (SCB) (Gray *et al.,* 2006). The production of biofuel e.g. ethanol may occur once the biomass has been cultivated and harvested. The biomass is subsequently pre-treated to remove lignin and other undesired plant components in order to facilitate an effective means to hydrolyse the biomass into fermentable sugars. In the down-stream processes, the reduced sugars are fermented (to ethanol) and the residual waste is disposed of.

A recent development in the field of biofuels is the production of third generation biofuel, which is essentially the production of biohydrogen from the direct fermentation of lignocellulosic feedstocks via "consolidated bioprocessing" (Levin *et al.,* 2009). The term consolidated processing refers to a single step process during which hydrolytic enzymes are produced and the hydrolysis of lignocellulosic biomass and the fermentation of reduced sugars occurs through the action of a single microorganism (Demain *et al.,* 2005; Lynd *et al.*, 1989, 2002; Lynd, 1996).

1.3 Plant cell wall composition

An important feature of plant cell walls or plant biomass is the heterogeneous composition which varies depending on the plant species (Ding and Himmel, 2006; Zhang and Lynd, 2004). The important plant biomass components that require consideration if the plant biomass may be considered as a potential source of renewable energy, are essentially cellulose, hemicellulose and lignin (Ding and Himmel, 2006; Zhang and Lynd, 2004). Lignocellulose recalcitrance is partially due to the low degree of accessibility of the cellulose fibres to enzyme hydrolysis due to the crystalline conformation of the cellulose moiety and the presence of hemicellulose and lignin in lignocellulose.

1.3.1 Cellulose

In general, lignocellulosic biomass consists of three polymers: cellulose, hemicellulose and lignin. Cellulose is considered the most abundant plant polymer as it generally constitutes a third to half of the plant material and is constantly being replenished through photosynthesis (Goodger, 1976). Cellulose is chemically characterised as a liner polymer consisting of anhydroglucose linked between the one and four carbon units via β-1,4-glycosidic bonds (Figure 4) (Fengel and Wegener, 1984; Kadla and Gilbert, 2000).

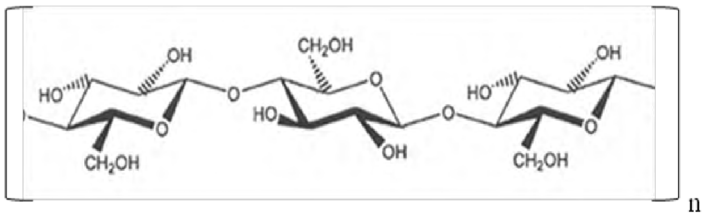


Figure 4: Chemical structure of cellulose (modified from Desvaux, 2004).

Cellulose consists of crystalline and amorphous regions in its structure. The chemical uniformity of the cellulose chains allows for the spontaneous crystalisation between the parallel cellulose chains via hydrogen bonding to produce microfibrils (Schwarz, 2001; Laureano-Perez *et al.,* 2005).

In general, the hydrolysis of cellulose occurs through the synergistic action of both endo- and exoglucanases and β-glucosidases, resulting in the formation of cellobiose and glucose as the hydrolysis products (Szczodrak and Fiedurek, 1996).

1.3.2 Hemicellulose

Hemicellulose is the second most abundant plant polymer. Hemicellulose generally constitutes 20-35% of plant biomass composition. Unlike cellulose that has a homogeneous composition, hemicellulose is a heterogeneous mixture of predominantly pentose sugars (xylose and arabinose) and hexose sugars (mannose, glucose, galactose) and sugar acids. The mixture of the sugar chains varies according to the source of hemicelluloses, e.g. hardwoods consist mostly of xylans, whereas softwoods consist mostly of glucomannans (McMillian, 1994). In general, hemicellulose has a lower molecular weight in comparison to cellulose,

and as previously mentioned, consists of a variety of sugars that form short lateral chains which branch off from the hemicelluloses backbone (Fengel and Wegener, 1984). The lateral side chains may consist of varying quantities of mannose, arabinose, galactose and glucuronic acid depending on the plant source (Gray *et al.,* 2006). Hemicellulose serves to increase the structural rigidity of the plant cell walls by linking the cellulose and lignin components of the plant cell walls (Laureano-Perez *et al.,* 2005). The hemicelluloses interact with the outer surface of the cellulose microfibrils via hydrogen bonding, preventing the microfibrils from interacting with each other (Gomez *et al.,* 2008). Even though the general structure of xylan is more complex than the structure of cellulose and requires a larger variety of enzymes (Table 1) to facilitate the hydrolysis of the heteropolysaccharide, it does not form a tightly packed crystalline structure observed in cellulose and is thus more accessible to enzymatic degradation (Gilbert and Hazlewood, 1993).

Table 1: A summary of the enzymes generally associated with the degradation of hemicellulose (Saha, 2000).

Enzyme	**Activity**
Endo-xylanase	– Hydrolyses the interior β-1,4-xylose linkages of the xylan backbone
Exo-xylanase	– Hydrolyses the β-1,4-xylose linkages releasing xylobiose
β-Xylosidase	– Releases xylose from the xylobiose and short chain xylooligosaccharides
α-Arabinofuranosidase	– Hydrolyses the terminal nonreducing α-arabinofuranose from arabinoxylans
α-Glucuronidase	– Releases glucuronic acid from glucuronoxylans
Acetylxylan esterase	– Hydrolyses the acetylester bonds in acetyl xylans
Ferulic acid esterase	– Hydrolyses the feruloylester bonds in xylans
ρ-Coumaric acid esterase	– Hydrolyses the *ρ*-coumaryl ester bonds in xylans

Xylanases are responsible for the depolymerisation of xylan, through the random hydrolysis of the xylanolytic backbone. Xylosidases cleave off the smaller oligosaccharides; and arabinofuranosidases, glucoronidases, galactosidases and acetyl xylan esterases are responsible for the liberation of side chains from the xylanolytic backbone (Subramaniyan and Prema, 2002). Xylan chains have been reported to be bound to rhamnose and galacturonic acid on the reducing ends of the xylan chain backbone, rendering the end groups of xylan chains alkaline resistant (Kulkarni *et al.,* 1999). The acetyl groups on the xylan chains are responsible for xylan‟s partial solubility in water (Whistler and Richards, 1970);

however, the acetylated substituents are readily removed when the xylan is subjected to alkaline extraction (Dekker, 1989).

Xylans are heteropolysaccharides that have a homopolymeric backbone consisting of 1,4-β-D-xylose units; however, xylans may also contain arabinose, glucuronic acid, acetic acid, ferulic and *ρ*-coumaric acid. The combination and the frequency of which these different components occur in hemicellulose vary according to the source of the hemicellulose (Aspinall, 1980). For example, birch wood xylan contains approximately 89.3% xylose, 1% arabinose, 1.4% glucose, and 8.3% anhydrouronic acid (Kormelink and Voragen, 1993), whereas rice bran xylan contains approximately 46% xylose, 44.9% arabinose, 6.1% galactose, 1.9% glucose, and 1.1% anhydrouronic acid (Shibuya and Iwasaki, 1985).

Xylan forms covalent and non-covalent interactions with various other components, such as lignin and cellulose, which increases the recalcitrance of biomass (Subramaniyan and Prema, 2002). Xylans have been described as alkaline-soluble, thus these compounds can be precipitated from aqueous solution via alcohols. These have a higher solubility in mineral acids than cellulose (Wilkie, 1983). Xylans are generally situated between lignin and cellulose fibres; however, xylans are intertwined and covalently linked at various points within the overlying sheaths of lignin, resulting in a coat around the underlying cellulose strands (Biely, 1985; Jeffries, 1990; Joseleau *et al.,* 1992).

Arabinoxylans contain ferulic and *p*-coumaric acids that are bound to the arabinofuranosyl substituent‟s C-5 position. Approximately 80% of the arabinoxylan backbone is substituted with side chains of either arabinose or glucuronic acid at the O-2 and / or O-3 position on the xylose backbone (Chanliaud *et al.,* 1995, Saulinier *et al.,* 1995).

Mannan forms a significant part of the hemicellulolytic component of softwoods, legumes, nuts and beans (Puls and Schuseil, 1996). Galactomannan is a mannopyranose chain with galactopyranose side chains (Figure 5) (Kusakabe *et al.,* 1986).

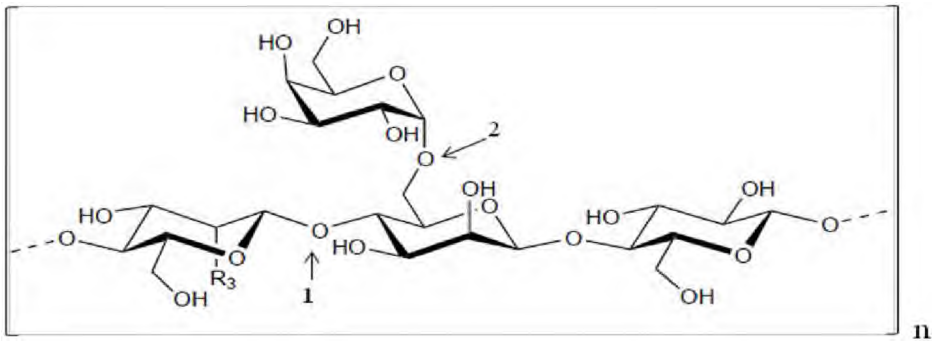


Figure 5: The structure of galactomannan and some of the enzymes required for the hydrolysis of galactomannan. 1) Endomannanase and 2) α-galactosidase (modified from Shallom and Shoham, 2003).

1.3.3 Lignin

Lignin is often found intertwined with the cellulose and hemicellulose components of plant biomass and forms a protective layer around the cellulose and hemicellulose fibres against microbial attack and oxidative stresses, as well as providing structural support and impermeability (Figure 6) (Boudet *et al.,* 2003; Chang and Holtzapple, 2000; Fengel and Wegener, 1984; Hsu *et al.,* 1980; Hsu, 1996; Mosier *et al.,* 2005; Rydholm, 1965; Wenzel, 1970; Zhang *et al.,* 2007).

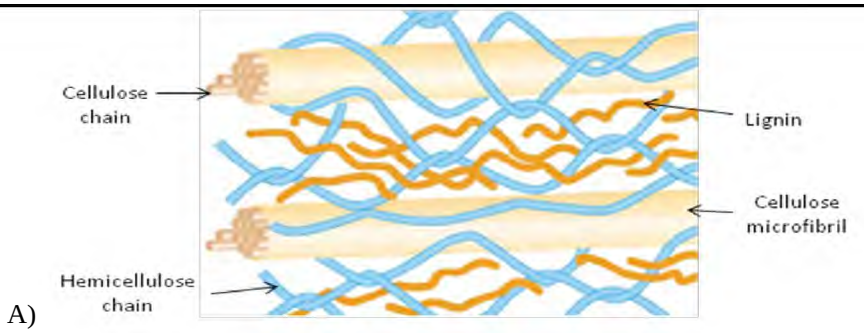


Figure 6: Schematic representation of lignin reinforced plant cell walls. Illustrating the internal association between lignin, cellulose and hemicellulose fibres. (modified from Boudet *et al.*, 2003; Gomez *et al.*, 2008).

Lignin is characterised as a heterogeneous plant polymer (Figure 7) that is produced through the dehydrogenative polymerization of *ρ*-coumaryl, coniferyl and sinapyl alcohols; however, once the above mentioned cinnamyl alcohols are incorporated into lignin they are referred to as the *ρ*-hydroxyphenyl, gauiacyl and syringyl lignin units (Sederoff *et al.,* 1999; Boerjan *et al.,* 2003). The various hydroxyphenylpropanoid components are cross linked with various polysaccharides (Figure 7). Lignins are essentially composed of three types of phenolic compounds that can generate a wide variety of bonding motifs, namely: *β*-aryl ethers, phenylcoumarans, resinols, biphenyls and biphenyl ethers (Figure 7). The variety of potential bonding motifs causes the structural heterogeneity and aromaticity while the extensive carbon-crosslinking increases the recalcitrance of lignin (Chang, 2007; Ralph *et al.,* 2004). Lignin can be covalently bound to the hemicellulose moiety of plant biomass via ferulic acid ester linkages (Gray *et al.,* 2006).

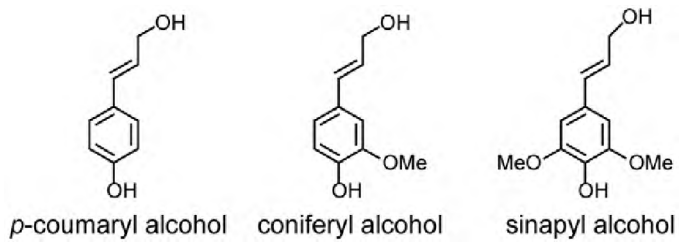


Figure 7: Monomeric components that can be cross linked to form lignin via a variety of different binding motifs (modified from Chang, 2007).

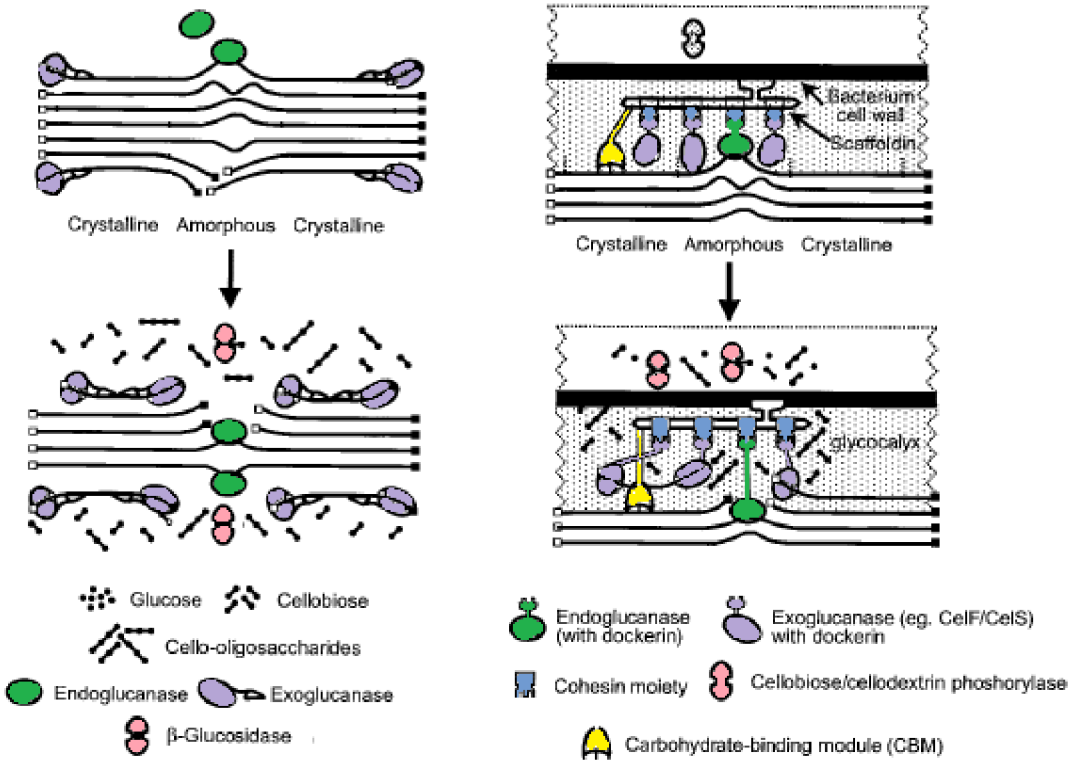
1.4 Sugarcane (Saccharum officinarum) bagasse

The potential use of agricultural wastes has sparked a renewed interest in both the production of biofuels and as a source of raw materials that may have industrial applications, e.g. sugarcane bagasse (SCB). SCB is well established as an important agricultural waste product. Sugarcane is a perennial grass that is cultivated and harvested primarily for its sucrose content (Lee *et al.,* 2009). The sugarcane stalk consists of an inner sucrose containing pith surrounded by an outer layer of lignocellulosic fibres. During sugarcane processing, the entire stalk is crushed to extract the sucrose. The remaining pith fibres and lignocellulosic rind is then referred to as bagasse (Han and Wu, 2004; Rowell and Keany, 1991). The chemical composition of SCB may vary depending on growth conditions with regard to

climate and location; however, bagasse is composed approximately of 40% cellulose, 24% hemicellulose and 25% lignin (Saha, 2003). SCB is the sole lignocellulosic residue that remains after the juice has been extracted and is commonly burnt as a fuel source. However, SCB may serve as a valuable renewable source of biomass for the production of biofuel, as SCB is produced in large quantities in sugar mills (Frollini *et al.,* 2004; Monteiro *et al.,* 1998; Paiva and Frollini, 2002; Simkovic *et al.*, 1990; Stael *et al.,* 2001; Zarate *et al.,* 2000). Besides from being used as an energy source in sugar mills, SCB has also been used as the raw material to generate fuel to power the sugar mills, the paper and pulp industry, and the hydrolysed SCB products have been used in different fermentation processes (Pandey *et al.,* 2000).

1.5 Cellulosomes versus free cellulase systems

The degradation and subsequent utilisation of degradation products of cellulosic biomass by microorganisms varies according to the niche the particular organism colonises. As a result, the enzyme systems that are employed by the micro-organisms are either in the form of free enzymes or complex enzymes that are closely associated with the micro-organism (Henrissat *et al.,* 1998). The cellulase systems employed by aerobic micro-organisms are typically non-complex, whereas anaerobic micro-organisms employ complex cellulase systems that are commonly known as cellulosomes (Figure 8).



(A) (B)

Figure 8: Comparison between (A) non-complexed and (B) complexed cellulase systems employed by aerobic and anaerobic microorganisms respectively, in order to hydrolyse complex plant biomass (modified from Lynd *et al.*, 2002).

Aerobic micro-organisms secrete a large battery of free enzymes that have the ability to hydrolyse the cellulose that is embedded between hemicellulose and lignin, ensuring the production of large quantities of saccharides that are utilised by the micro-organisms. Anaerobic micro-organisms typically lack the ability to penetrate lignin and other cellulosic materials that limit the accessibility to the cellulose fibres. This may have led to the secretion of cellulases and other hydrolytic enzymes that aggregate to produce cellulosomes (Lynd *et al.*, 2002). It has previously been noted that the most effective form of a cellulolytic system is not in the form of free enzymes as previously thought. Cellulosomes that are bound to the surface of anaerobic bacteria are potentially the most effective forms of cellulase systems (Chang, 2007). Cellulosomes were identified and defined as a cellulose binding multi-enzyme complex in 1983 by Edward Lamed. Thus cellulosomes are essentially large multi-

enzyme complexes that may comprise of a variety of different enzymes that are required for the degradation of complex plant biomass.

1.5.1 Cellulosomes

Cellulosomes were originally identified as the multi-enzyme complex first isolated from *Clostridium thermocellum*, from which the cellulosome structure was studied thus giving the cellulosome a distinct set of characteristics (Figure 9). Subsequently, cellulosomes were isolated from *C. cellulovorans, C. cellulolyticum, Acidothermus cellulolyticus, Bacteroides cellulosolvens and Ruminococcus flavefaciens*, which provided greater insight into the cellulosome architecture (Nordon *et al.*, 2009).

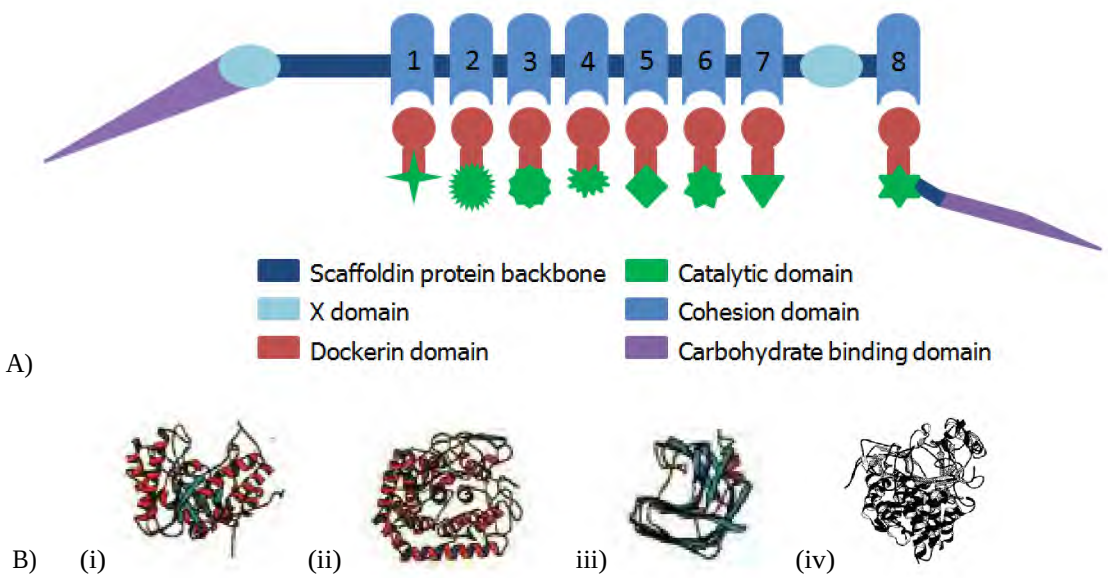


Figure 9: Cellulosome structure. A) The basic cellulosome structure is composed of a variety of fifferent components that may vary according to different species. B) The crystal structures for the glycosyl hydrolase family 5 (i) is an example of mannanases and some endoglucanases, which has the general $(\beta/\alpha)_8$ barrel structure; for the glycosyl hydrolase family 9 (ii) is an example of endoglucanases E has the general $(\alpha/\alpha)_6$ barrel; for the glycosyl hydrolase family 11 (iii) is an example of xylanases has the general β-jelly roll structure; and family 48 (iv) is an example of exoglucanases. In this case, ExgS has the general $(\alpha/\alpha)_6$ barrel structure (modified from Bayer *et al.*, 1998a; Davies and Henrissat, 1995; Desvaux, 2005; Doi and Tamaru, 2000; Henrissat and Bairoch, 1993; Kosugi *et al* 2002a; Tamaru and Doi, 1999).

1.5.1.1 Scaffoldin proteins

Scaffoldin proteins vary depending on the bacterial source; however, the functions of the scaffoldin proteins remain the same (Figure 10) (Schwarz, 2001).

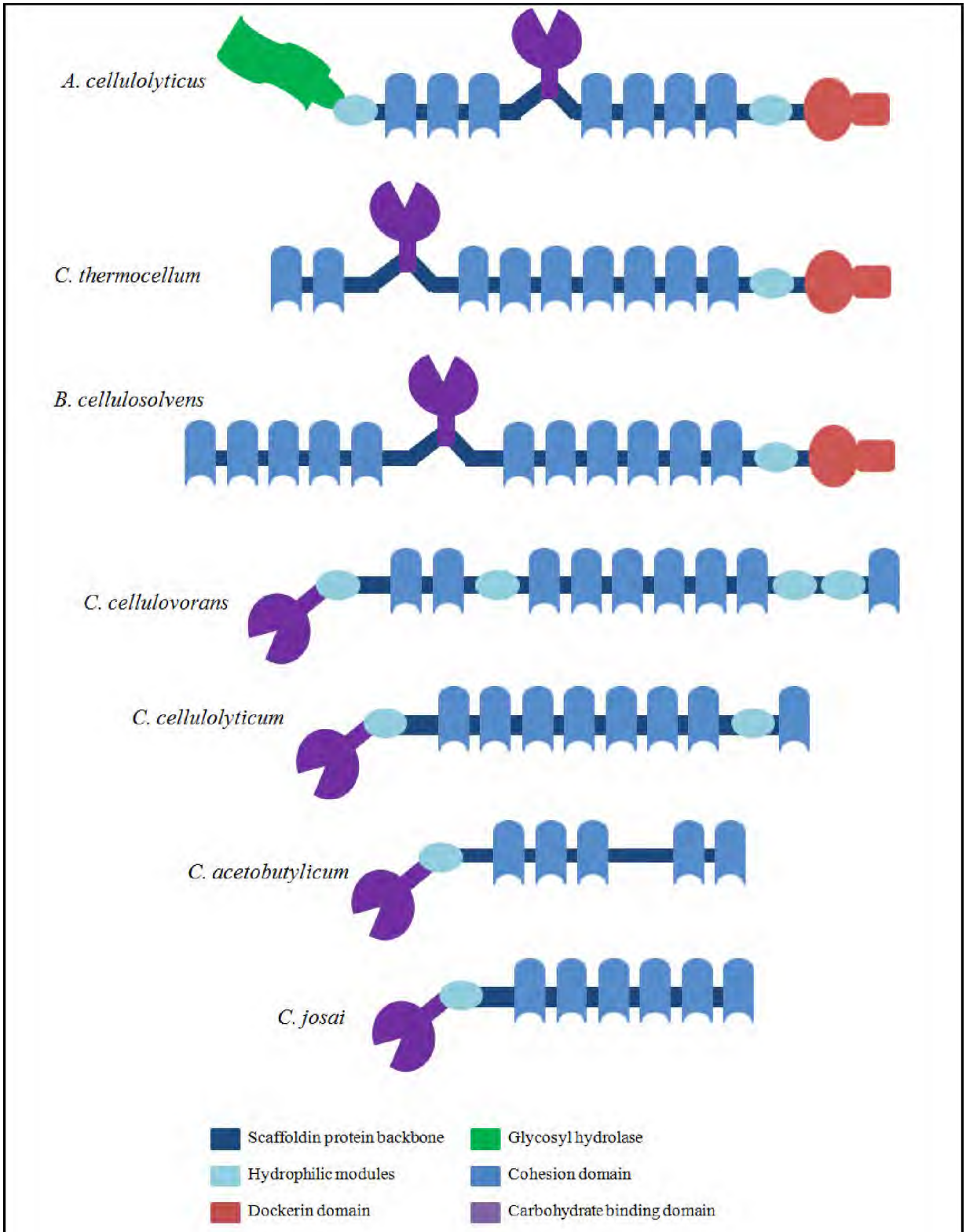


Figure 10: Schematic representations of the different types of scaffolding proteins that has been identified. The scaffolding protein consists of different components: the carbohydrate-binding (CBM), cohesion domains, type II dockerin domains, glycosyl hydrolase family 9 (in the case of *A. cellulolyticus*), and hydrophilic modules (modified from Schwarz, 2001).

1.5.1.2 Cohesion and dockerin domains

In general, the cellulosome is comprised of a variety of enzymatic subunits that are bound to the inert scaffolding protein via calcium dependent interactions between cohesion and dockerin domains (Nordon *et al.,* 2009). Cohesion domains have generally been classified into two types depending on the cohesion domains‟ respective homology to the cohesion domains found on the *C. theromocellum* scaffoldin. In 2001, Ding *et al.* described cohesions with a homology with ScA and ScB cohesions described in *R. flavefaciens* as type III cohesion domains. The interactions between the cohesion and dockerin domains are of a highly specific "lock-and key" based mechanism (Nordon *et al.,* 2009). As mentioned previously, the interaction between the cohesion and dockerin domains are calcium dependent. Type I dockerin domains have two calcium binding sites which are required for the dockerin domains to maintain the correct conformations as to allow cohesion recognition and specificity (Schaeffer *et al.,* 2002) after which the dockerin domains undergo a conformational change to maintain the bound state (Carvalho *et al.,* 2007). Carvalho *et al.* (2007) indicated that the recognition between the dockerin and cohesion domain occurs through helix III on the dockerin domain (Figure 11); however, Carvalho *et al.,* (2007) also indicated that the 22 amino acid duplication in the dockerin helix I potentially allows a reversible binding between the dockerin and cohesion domains. The binding of the type I cohesion domains to the dockerin domain does not display any conformational flexibility unlike that of the type II dockerin domains (Adams *et al.,* 2005, Carvalho *et al.,* 2003).

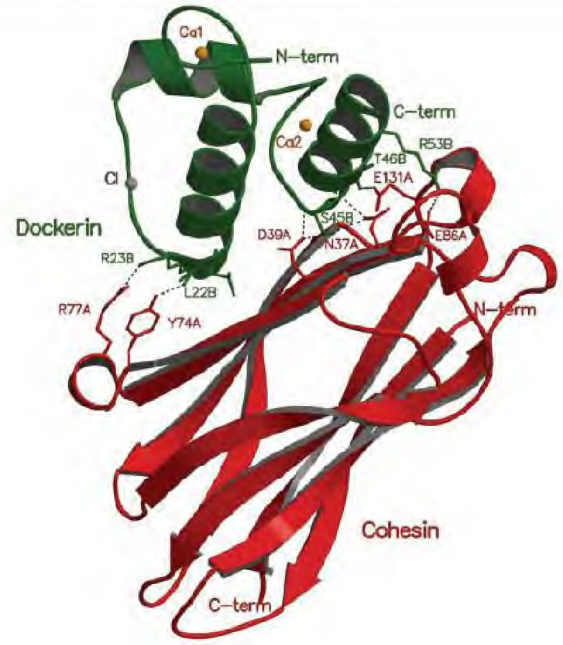


Figure 11: Cohesion and dockerin domain interactions (Carvalho *et al.*, 2003).

1.5.1.3 Carbohydrate binding modules

A carbohydrate binding module is defined as a contiguous amino acid sequence found in a carbohydrate active enzyme that has the ability to bind to carbohydrates (Boraston *et al.*, 1998, 1999; Coutinho and Henrissat, 1999). To date 23 CBM families have been identified. The CBM compositions may range between 30 and 200 amino acids, and may exist as up to three domains in a single protein (Shoseyov *et al.*, 2006). All the CBM families have structural similarities as it is thought that the binding capacities are due to the presence of a hydrophobic surface consisting of bulky aromatic amino acids (Shoseyov *et al.*, 2006).

1.5.1.4 Cellulosome attachment to substrates

Once the cellulosome has been assembled, the complex binds to the cellulosic or lignocellulosic biomass via the cellulose binding module (CBM) present on the scaffolding protein in such a way so as to allow for the hydrolytic components of the cellulosome to come into contact with the substrate. This provides the bacterial cell attached to the complex with a suitable proximity to allow the absorption of nutrients/ sugars released from the degradation of the substrate (Figure 12) (Nordon *et al.*, 2009).

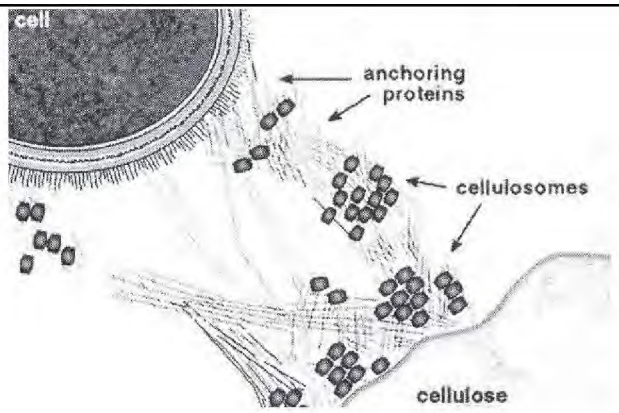


Figure 12: Schematic diagram illustratings the attachement between the cellulosomes, the bacterial cell wall and the cellulosic substrate (modified from Bayer *et al.*, 1998b).

Cellulosomes are believed to promote synergy between enzymes due to the close proximity between juxtaposed enzymes, and to minimise the diffusion distance of the oligosaccharides to the micro-organism (Bayer *et al.*, 1994; Lamed *et al.*, 1983; Schwarz 2001).

1.5.2 Free enzymes

1.5.2.1 α-L-Arabinofuranosidases

α-L-Arabinofuranosidases are considered ancillary enzyme, that are involved in the removal of arabinose from the hemicellulose moiety of lignocellulose (Saha, 2000). Arabinofuranosidases hydrolyse the (1--+3)- and the (1--+5)-α-arabinosyl linkages of the arabinoxylan generally from the terminal non-reducing residues and form part of the xylanolytic system employed by microbes for the complete degradation of arabinoxylans (Bachmann and McCarthy, 1991; Greve *et al.*, 1984; Lee and Forsberg, 1987; Poutanen, 1998; Saha and Bothast, 1999). α-L-Arabinofuranosidases are classified into glycanase families 43, 51, 54 and 62 on the basis of amino acid sequences (Saha, 2000).

Arabinofuranosidases have a wide variety of physico-chemical characteristics and have been isolated from bacterial, fungal and plant sources (Table 2).

Table 2: Summary of arabinofuranosidases isolated from different sources modified from Saha (2000).

Comparative properties of some fungal α-arabinofuranosidases							
Organism	**No. of Isomers**	**kDa**	**pI**	**°C**	**pH**	**Substrates**	**References**
Aspergillus awamori	2	62 - 81	3.3 - 3.6	60	4	BA	Kaneko *et al.*, 1998a
Aspergillus nidulans	1	65	3.3	65	4		Ramon *et al.*, 1993
Aspergillus niger	2	53 - 67	3.5 - 3.6	60	4	BA, AX	Kaneko *et al.*, 1993; Kaji and Tagawa, 1970
Aspergillus sojae	1	34.3	3.9	50	5		Kimura *et al.*, 1995
Aspergillus terreus	3	39 - 59	7.5 - 8.5		3.5 - 4.5	OSX, RAX, AGX, BA	Luonteri *et al.*, 1995
Aureobasidium pullulans	1	210		75	4.0 - 4.5	BA, AX, OSX	Saha and Bothast, 1998b
Cochliobolus carbonum	1	63		50	3.5 - 4.0	BA, AX	Ransom and Walton, 1997
Cytophaga xylanolytica	1	160 - 240	6.1	45	5.8	BA, AX	Renner and Breznak, 1998
Dichomitus squalens	1	60	5.1	60	3.5	BA, AX	Brillouet et al., 1985
Penicillium capsulatum	2	62.7 - 64.5	4.1 - 4.4	55 - 60	4	BA. AX, AXO	Filho *et al.*, 1996
Sclerotinia sclerotiorum	1	63	7.5		4.0 - 4.5	BA, AX	Baker *et al.*, 1979
Penicillium purpurogenum	1	58	6.5	50	4	AX	De loannes *et al.*, 2000
Trichoderma reesei	1	53	7.5		4	AX, AXO	Poutanen, 1998
Comparative properties of some bacterial α-arabinofuranosidases							
Bacillus polymyxa	1	166	4.7			AXO	Morales *et al.*, 1995a
Bacillus stearothermophilus	1	110		70		Delignification	Bezalel *et al.*, 1993
Bacillus subtilis	1	65	5.3		6.5	BA	Weinstein and Albershein, 1979
Bacteroides xylanolyticus	1	364 (61)		50	5.5 - 6.0	AXO	Schyns *et al.*, 1994
Bifidobacterium adolescents	1			30 - 40	6	AX	
Butyrivibrio fibrisolvens	1	240	6	55	6.0 - 6.5	BA, AX, OSX	Hespell and O″Bryan, 1992
Clostridium acetobutylicum	1	94	8.2		5.0 - 5.5	BA	Lee and Forsberg, 1987
Ruminococcus albus	1	310	3.8		6.9	AH	Greve *et al.*, 1984
Streptomyces sp. 17–1	1	92	4.4		6	BA, AX, AG	Kaji *et al.*, 1981
Streptomyces diastaticus	2	38 - 60	8.3 - 8.8		5.0 - 6.5	AX	Tajana *et al.*, 1992
Streptomyces diastaatochromogenes	1	73			6	BA	Higashi *et al.*, 1983
Streptomyces purpurascens	1	495	3.9		6.5	A2, A3	Komae *et al.*, 1982
Comparative properties of some plant α-arabinofuranosidases							
Spinach leaf	2	68 - 118	4.2 - 4.8		4.8		Hirano *et al.*, 1994
Carrot cell culture	1	84	5.5	55	3.8	BA	Konno *et al.*, 1994
Radish seeds	1	64	4.7			BA, AG	Hata *et al.*, 1992
Soybean seeds	1	87	4.8				Hatanaka *et al.*, 1991
Japanese pear fruit	1	42			5		Tateishi *et al.*, 1996

*BA- Beet arabinan, AX – arabinan, OSX – oat spelt xylan, RAX – rye arabinoxylan, AGX - arabinoglucan, AXO – arabinoxylan oligosaccharide, A2 –arabinobiose, A3 – arabinotriose, CX – corn endosperm xylan, AG – arabinogalactan.

These enzymes may not only vary in size depending on the source, but also on structural complexity e.g. monomeric and dimeric forms have been isolated and different isomers may be isolated from the same source (Saha, 2000). The different isomers may also vary in substrate specificity depending on the source of the isolated enzyme (Saha, 2000).

1.5.2.2 Mannanases

Mannanase are members of family five of the glycosyl hydrolases (GH5) (Tamaru and Doi, 2000). Like other hemicellulases, mannanases are secreted by microorganisms as multiple enzyme isomers (Johnson *et al.,* 1990). Endo-β-mannanases depolymerise the mannan backbone through random cleavage to release mannobiose units as well as a variety of other manno-oligosaccharides. The cleavage by exo-β-mannosidases releases mannose (Halstead *et al.,* 1999). Random hydrolysis of the 1,4-β-D-mannopyranosyl linkages in 1,4-β-mannans, glucomannans, and galactomannans (McCleary, 1988), via a retaining mechanism the glycosyl hydrolase (GH) family 5 releases mannose, glucose and galactose (Davies and Henrissat, 1995). The GH 5 mannanases have eight amino acids, which are strictly conserved within the sixty GH 5 cellulases (Sakon *et al.,* 1996; Wang *et al.,* 1993). The eight highly conserved amino acids are located in or near the active site. They are therefore believed to either aid in substrate binding (Davies *et al.,* 1997) or stabilise and protonate the catalytic glutamate residue (Dominguez *et al.,* 1995, 1996; Ducros *et al.,* 1995; Sakon *et al.,* 1996).

β-mannanases liberate β-1, 4-manno-oligosaccharides through the hydrolysis of mannan. The manno-oligosaccharides are subsequently hydrolysed by β-mannosidases. Approximately 50 mannanses are found within the GH families 5 and 26 (Howard *et al,* 2003). Endo-1,4-β-mannanases are used in combination with 1,4-β-endoxylanases in the paper and pulp industry to bio-bleach softwood pulps (Viikari *et al.,* 1994).

The ManA dockerin (duplicated sequence/DS) is present at the *N*–terminal of the *Clostridium cellulovorans manA* gene (Tamaru and Doi, 2000). The ManA catalytic domain shows a high degree of similarity to fungal mannanases, whereas its dockerin domain shows a high degree of similarity to other dockerin domains within the *Clostridium* genus (Tamaru and Doi, 2000).

The cellulosomal mannanase (ManA) from *C. cellulovorans*, which is encoded by a 1,275 base pairs (bp) gene, is approximately 45 kDa in size and consists of 425 amino acids. The predicted molecular weight of ManA is 47 kDa (Tamaru and Doi, 2000). The predicted molecular weight of 47 kDa includes a signal sequence (Ala-X-Ala-Ala), the removal of which produces a protein with a molecular weight of approximately 44.5 kDa (von Heijne, 1985). ManA is a member of family five of the glycosyl hydrolases, which includes some endoglucanases (Ethier and Doi., 1998). ManA has a pH and temperature optimum of 7.0 and 45°C, respectively, using a piperazine-*N*,*N*'-bis(2-ethanesulfonic acid) (PIPES) buffer at pH 7.0 (Tamaru and Doi, 2000). ManA was able to degrade glucomannan and locust bean gum effectively and was unable to degrade carboxymethylcellulose (CMC), thus indicating that ManA effectively hydrolyzes β-(1-4)-mannosidic linkages (Tamaru and Doi, 2000).

1.5.2.3 Xylanases

Hemicellulases are multi-domain hydrolytic proteins (Henrissat and Davies, 2000; Prates *et al.,* 2001). The multi-domain components consist of catalytic and non-catalytic components. The non-catalytic domain consists of a CBM that facilitates the binding of the hydrolytic enzymes to the substrate through the binding of dockerin and cohesion domains either to the cell surface or to enzyme complexes, and in this case the cellulosome (Prates *et al.,* 2001; Shallom and Shoham, 2003). In general, hemicellulases are either glycosyl hydrolases, which catalyse the hydrolysis of glycosidic bonds, or carbohydrate esterases, which catalyse the hydrolysis of the ester linkage of acetate or ferulic acid side chains (Henrissat and Bairoch, 1993; Rabinovich *et al.,* 2002a and b).

Xylanases belong to the glycoside hydrolases families 10 and 11 (Howard *et al.*, 2003). Xylanases are economically important due to the wide range of industrial processes that the enzymes are associated with. Industrially, xylanases are utilised for a wide range of processes, for example the bleaching of pulp in the paper and pulp industry (Bocchini *et al.*, 2003; Damiano *et al.,* 2003; Shoham *et al.*, 2003), bioconversion of lignocellulosic material and agro-wastes to fermentative products for the production of fuel (Linko etal., 1989; Wong *et al.,* 1988), increasing the digestibility of animal feed (Bedford and Classen, 1992), the extraction of coffee, plant oils and starch (Wong and Sadler, 1993), clarification of fruit juices and the degumming of vegetable fibres (Kapoor *et al.,* 2001; Wong *et al.*, 1988). The

molecular weight of xylanase isomers range between 12 to 145 kDa, some isomers may form heterodimers depending on the conditions under which they are expressed (Petrosyan *et al.*, 2002). High molecular weight endoxylanases with low pI values belong to glycanase family 10, while the low molecular weight endoxylanases with high pI values belong to glycanase family 11 (Kuno *et al.*, 2000).

Due to the complexity and variability of xylan, the degradation requires synergy between several enzymes that are required for the complex hydrolysis of xylan (Figure 13), namely: 1,4-β-endoxylanases (E.C. 3.2.1.8), which are responsible for the random cleavage of the backbone, and 1,4-β-xylosidases (E.C. 3.2.1.37), which are responsible for the hydrolysis of the smaller oligosaccharides (Subramaniyan and Prema, 2002), α-L-arabinofuranosidase, α-glucuronidase, acetylxylan esterases and phenolic acid esterases (Figure 13) (Coughlan and Hazlewood, 1993).

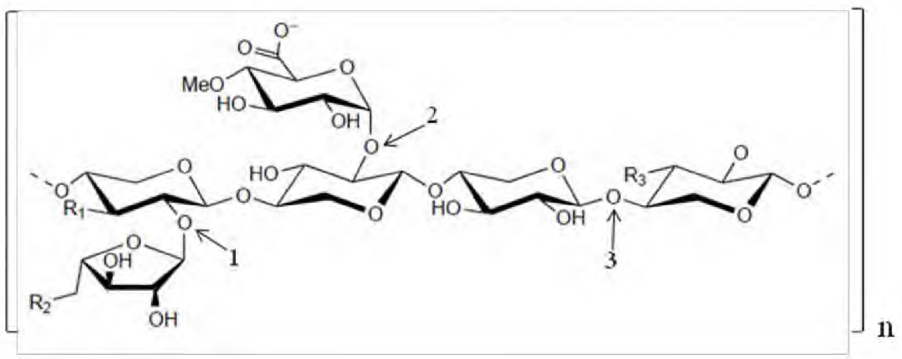


Figure 13: Some of the main enzymes involved in the hydrolysis of xylan. 1) α-L-Arabinofuranosidase, α-D-glucuronidase and endo-xylanase (modified from Shallom and Shoham, 2003).

Due to the heterogeneity of xylan, not all of the xylanosidic linkages are accessible to hydrolysis. The degradation of xylan not only requires various enzymes, but also the hydrolytic activity of xylanase isomers, which possess overlapping but different specificities (Wong *et al.*, 1988). Thus, the expression of multiple xylanolytic enzymes by the microorganism ensures cooperative action through xylanase synergy (Kelett *et al.*, 1990).

Many xylanases are multi-domain enzymes, i.e. they comprise several discrete domains such as the cellulose binding domain in addition to a catalytic domain (Tomme *et al.*, 1995). They generally have acidic or neutral pH optima. Those xylanases that have

alkaline pH optima are of great interest to the paper and pulp industry as xylans are more readily soluble in alkaline solutions (Onysko, 1993; Tull *et al.*, 1991). Similarly to cellulases, xylanases may also exist as endo- and exoxylanases. Endoxylanase activity was differentiated from general xylanolytic activity by using a caboxymethylated derivative of xylan (CMX) which is specific for endoxylanases (Khasin *et al.*, 1988), as they are responsible for the cleavage of the internal glycosidic linkages of the backbone (Reilly, 1981). The depolymerising activity of endoxylanases results in the conversion of complex polymeric substances into xylooligosaccharides and xylose (Shoham *et al.*, 1992).

The multiplicity of xylanases is a result of a variety of factors such as differential mRNA processing, post-secretion modification through proteolytic cleavage and various post-translational modifications such as glycosylation and auto-aggregation (Biely, 1985, Coughlan *et al.*, 1993).

The expression of xylanases is constitutive and is controlled by catabolite repression. Products of xylan hydrolysis are small molecular weight sugars, e.g. xylose, xylobiose, xylotriose and other oligosaccharides (Wang *et al.*, 1993; Zhao *et al.*, 1997). These molecules enter cells and sustain growth by acting as energy and carbon sources. Xylose also induces the production of xylanases (Biely, 1985; Zhao *et al.*, 1997).

The cellulosomal xylanases in *C. cellulovorans* have not been well studied; however, Kosugi *et al.* (2001) have indicated that, despite the fact that the majority of xylanases are produced independently of the cellulosome, some possess a dockerin domain, which is essential for binding to the cohesion domains on the scaffoldin protein. Cellulosomal xylanase activity has also been reported in *C. acetobutylicum, C. stercorarium and C. papyrosolvens* C7 (Nielsen *et al.*, 1993; Wong *et al.*, 1988).

Hydrolysis of xylan-based substrates may result in either retention or inversion of the anomeric centre of the reducing sugar monomer of the carbohydrate, suggesting one or two chemical transitional states (Clarke *et al.*, 1993). The glycosyl transfer that occurs results in the nucleophilic substitution at the saturated carbon of the anomeric centre and takes place with either retention or inversion of the anomeric configuration (Figure 14) (Clarke *et al.*,

1993). The general double displacement mechanism for the anomeric-retention of the products occurs as follows (Figure 14):

1) acid catalysis results in the protonation of the substrate (Clarke *et al.,* 1993),
2) the carboxyl group of the enzyme is positioned on a covalent glycosyl enzyme intermediate with the carboxylate in which the anomeric configuration of the sugar is opposite that of the substrate (Clarke *et al.,* 1993),
3) the covalent intermediate is reached from both directions through transition states involving oxo-carbonium ions (Clarke *et al.,* 1993), and
4) various non-covalent interactions provide most of the rate enhancement (Clarke *et al.,* 1993).

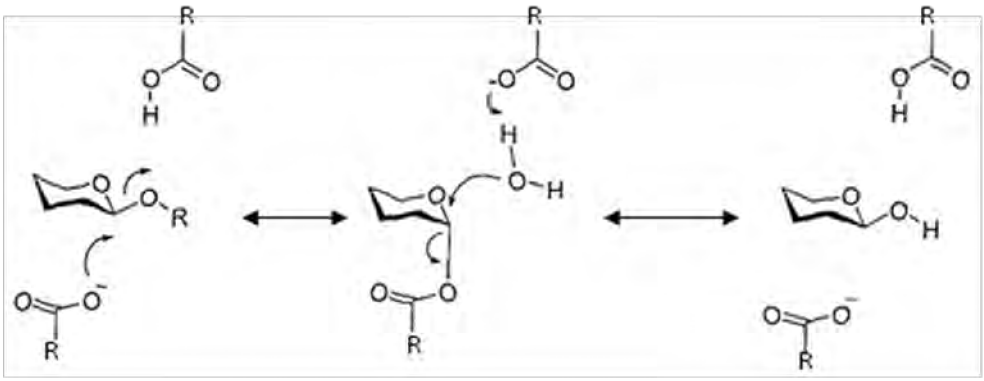


Figure 14: The general catalytic mechanisms of glycosyl hydrolases, illustrating the general retention mechanism or double displacement mechanism (modified from Schlülein, 2000).

Thus, the proposed mechanism for ArfA/ManA/XynA activity occurs in a similar fashion, where:

1) substrate is recognized and bound by the respective enzymes,
2) the glycosyl residue becomes distorted and is pulled down toward the catalytic residues. This results in the glycosidic bonds becoming strained, and the bonds break to form the substrate-enzyme covalent intermediate, and
3) an activated water molecule, following the classic retaining glycosyl hydrolase mechanism, attacks the intermediate. The products are released (Leggio *et al.,* 2000).

C. cellulovorans constitutively expresses several xylanases ranging from 28 – 57 kDa (Han *et al.,* 2003); however, the major xylanases are 47 and 57 kDa (Kosugi *et al.,* 2001). Kosugi *et al.* (2001) reported that the expression of xylanases varied when *C. cellulovorans* was grown on different carbon sources, namely glucose, cellobiose, cellulose, Avicel, birchwood and oat spelt xylan. However, the highest xylanase activity observed when the bacterium was grown on birchwood xylan, using a sodium phosphate buffer (pH 7.0) at 37°C. The 2.7kb *xynA* (Han *et al.,* 2003) encodes a 57 kDa protein with the signal sequence ATKTITXNETGNF (Kosugi *et al.,* 2001).

1.6 Enzyme synergism

Synergism has been observed in a variety of enzyme systems; however, the synergism that exists in cellulase systems is the most established. Synergism facilitates an enhanced hydrolytic activity that is greater than the collective sum of the individual enzymes activities, using complex lignocellulose biomass. To date, four forms of synergism in cellulase systems have been described: (i) between endo- and exoglucanases, (ii) between exoglucanases, (iii) between exoglucanases and β-glucosidases, and (iv) between catalytic domains and CBMs (Din *et al.,* 1994; Teeri, 1997). The majority of the synergistic relationships that have been elucidated occurred between cellulases predominantly secreted from aerobic micro-organisms. In recent years, the synergism between cellulases secreted by anaerobic micro-organism has been studied (Beldman *et al.,* 1988; Gaudin *et al.,* 2000; Henrissat *et al.,* 1985; Irwin *et al.,* 1993, 2000; Jeoh *et al.,* 2006; Murashima *et al.,* 2002b; Nidetzky *et al.,* 1994; Watson *et al.,* 2002; Wood and McCrae, 1972, 1979; Wood *et al.,* 1989; Woodward *et al.,* 1988). The enzyme composition of cellulosomes is an important feature for consideration, as these multi-enzyme complexes do not consist solely of cellulases. Unfortunately, the synergistic relationships between cellulosomal enzymes and other cellulosomal and/or non-cellulosomal enzymes are not well established. Previous synergism studies occurred between a variety of proteins/ enzymes such as: scaffolding proteins, xylanasases, acetyl xylan esterase, α-arabinofuranosidase, β-galactosidase and mannanases (Beukes *et al.,* 2008; Blum *et al.,* 2000; Ciruela *et al.,* 1998; Kosugi *et al.,* 2002a,b; Koukiekolo *et al.,* 2005; Murishima *et al.,* 2003).

1.7 Designer cellulosomes

Despite the short period of time over which designer cellulosomes have been studied, it has been established that designer cellulosomes can be created and be functional *in vivo* (Perret *et al.,* 2004; Sabathé and Soucaile, 2003) and *in vitro* (Doi *et al.,* 1998; Murashima *et al.,* 2002a). The concept of designer cellulosomes was derived from two characteristics, namely the presence of synergistic relationships and the modular nature of cellulosomal enzymes (Bayer *et al.,* 1994).

Since establishing of the concept that free enzymes act synergistically to enhance the rate of lignocellulose degradation, there has been an increasing interest in how complexed enzymes act synergistically. As mentioned previously, the synergistic associations that occur between enzymes in cellulolytic/lignocellulolytic systems have been enhanced through the construction of designer cellulosomes. This interest led to the development of artificial enzyme complexes (designer cellulosomes). The production of designer cellulosomes with specific hydrolytic properties provides a means to understand the hydrolytic properties of cellulosomes (Cho *et al.,* 2004). The construction of designer cellulosomes appeared to improve the efficiency of the enzymes that displayed a synergistic association and led to an improvement in the subsequent degradation of plant biomass. The enhanced synergy associated with the construction of designer cellulosomes therefore sparked an enhanced interest in the potential use of designer cellulosomes for the degradation of lignocellulosic biomass. The construction of designer cellulosomes has occurred over the last decade. Designer cellulosomes are ascribed two important features: they encourage an enhanced synergistic association between cellulosomal enzymes by keeping the enzymes in close proximity and increase substrate targeting and binding though CBMs (Fierobe *et al.,* 2005).

The modular nature of cellulosomal enzymes led to the proposal that cellulosomal enzymes may be linked together in a variety of combinations to produce chimeric miniature cellulosomes. These chimeric miniature cellulosomes became known as designer/recombinant cellulosomes (Fierobe *et al.,* 2005).

Fierobe *et al.* (2002) produced chimaeric mini-cellulosomes consisting of a mini-scaffoldin protein with a functional CBM and two cohesion domains with divergent binding

specificities for cellulase dockerin domains from *C. cellulolyticum*. In 2005, Fierobe *et al.* went a step further and produced a trifunctional chimaeric cellulosome by adding an additional cohesion domain from *C. thermocellum*, which produced a 7-fold increase in the enzymatic activity with microcrystalline cellulose, in comparison to the hydrolytic activity of the free enzyme.

In 2007, Cha and co-workers made use of a scaffoldin containing a range of cohesion domains, for either an endoglucanase or a xylanase from *C. cellulovorans.* After producing the mini-cellulosomes they demonstrated that even though the complex had a moderate degree of synergy there was an 11 to 18 fold increase in the enzymatic activity in comparison to the free enzymes.

In contrast to only using bacterial enzymes for the production of mini-cellulosomes, Mingardon *et al.* (2007) included a fungal cellulase from *Neocallimastix patriciarum* in a *C. cellulolyticum* based cellulosome. This mini-cellulome produced a 26 fold increase in enzymatic activity in comparison to the hydrolytic activity of the individual enzymes (Mingardon *et al.*, 2007).

The potential to include enzymes from a variety of sources as reported by Mingardon *et al.* (2007) indicated the flexibility in chimeric cellulosome design in order to maximise the hydrolytic activity as a significant advantage over the use of free enzymes. However, the cost of production of chimeric cellulosomes *in vitro* is high, thus limiting the use of this technology in large-scale applications. A new approach into the production of chimeric cellulosomes employing an *in vivo* system has been used, and in comparison to the *in vitro* synthesis of chimeric cellulosomes the *in vivo* system is a more cost effective approach. Murashima *et al.* (2002b) made use of *Bacillus subtilis* as the *in vivo* expression system for the production of chimeric cellulosome subunits based on *C. cellulovorans*.

1.8 Pre-treatments

Despite the enhanced enzymatic hydrolysis of lignocellulose resulting from synergistic relationships between the different enzymes in non-complexed and complexed systems, the complete hydrolysis of lignocellulose has never been achieved due to the recalcitrant nature of plant biomass. The recalcitrant nature of biomass is due to the

complexity of the biomass structure. Cellulose fibrils are embedded between a mixture of amorphous hemicelluloses and lignin, and as a result, the conversion of cellulosic and lignocellulosic biomass at an industrial scale, requires the use of pre-treatment technologies (Mitchell *et al.,* 1992). The recalcitrant nature of plant biomass and the inability to obtain complete enzymatic hydrolysis has led to the establishment of various pre-treatment strategies.

Since the 1970"s there has been a steady increase in supporting evidence, that at present non-renewable fossil fuels may be partially replaced with renewable fuel sources. The main rationale behind the increased research into the use of renewable fuel sources are the slow depletion of fossil fuel reserves and the increased accumulation of greenhouse gases (Caldeira *et al.,* 2003; Demain *et al.,* 2005; Farrell *et al.,* 2006; Ragauskas *et al.,* 2006).

In literature, the term "pre-treatment" is defined as a process step that facilitates the conversion of the recalcitrant, native conformation of lignocellulosic biomass, which is predominantly amorphous in nature, thus enhancing the efficiency of enzymatic hydrolysis by increasing the surface area of the substrate accessible to degradation (Lynd *et al.,* 2002). Thus the term pre-treatment refers to the disruption of the carbohydrate-lignin shield surrounding the holocellulose; which will increase the surface area of the cellulose and hemicellulose accessible to enzyme degradation (Holtzapple, 1993; McMillian, 1994; Wyman *et al.,* 2005).

The pre-treatment strategies that are usually employed are either physical, chemical, biological and thermal in nature; however, a combination of chemical and physical processes has been previously used (Hsu, 1996; McMillan, 1994). Physical and thermal pre-treatments generally include a mechanical reduction in the particulate size, steam explosion and hydrothermolysis (Millett *et al.,* 1976; Rivers and Emert, 1987; Sidiras and Koukios, 1989; Tassinari *et al.,* 1980, 1982).

Between cellulose, hemicellulose and lignin, it has been noted that the different types of hemicellulose are the most susceptible to being altered or removed by various thermal-chemical pre-treatments, during which the hemicellulose side chains react first, followed by the backbone (Levan *et al.,* 1990; Sweet and Winandy, 1999; Winandy, 1995).

1.8.1 Physical or mechanical pre-treatments

Physical or mechanical pre-treatment is essentially the process of milling, whereby lignocellulosic biomass is cut into smaller pieces. By decreasing the particle size, this type of pre-treatment increases the surface area and subsequently decreases the degree of holocellulose polymerization (Palmowski and Muller, 1992). Using physical pre-treatments such as milling has been shown to increase the hydrolysis of lignocellulose between 5 and 25% (Delgenés *et al.,* 2002; Hartmann *et al.,* 1999).

1.8.2 Thermal pre-treatments

In order to reach the cellulosic component of lignocellulose using only thermal pre-treatment, temperatures of above 150 - 180°C are required to start solubilising the hemicellulose and lignin fractions of the lignocellulose (Bobleter, 1994; Garrote *et al.,* 1999). One of problems associated with the partial solubilisation of lignin is the production of highly reactive, soluble phenolic compounds that will recondense on the lignocellulose if not removed quickly, thus when using thermal pre-treatment, a short exposure time is required (Gossett *et al.,* 1982; Liu and Wyman, 2003). However, if the pre-treatment conditions are too harsh it will not only cause the condensation and precipitation of soluble lignin components, but that of soluble hemicellulosic compounds as well, such as furfural and hydroxymethylfurfural (HMF) (Bobleter and Concin, 1979; Lora and Wyman, 1978; Negro *et al.,* 2003). The two thermal pre-treatments that are employed are steam explosion and liquid hot water (LHW)

Steam explosion pre-treatment occurs when lignocellulose biomass is rapidly heated under high pressure steam in order to hydrolyse the hemicellulosic component of biomass. The pre-treatment is terminated by a rapid decrease in the pressure (Abatzoglou *et al.,* 1992; Avellar and Glasser, 1998; Brownell and Saddler, 1984; Glasser and Wright, 1998; Heitz *et al.,* 1991; Ramos *et al.,* 1992). Using steam explosion, the hemicellulose moiety is thought to be hydrolysed by the acetic acid that is produced by the hydrolysis of the acetyl groups associated with the hemicellulose and is released during the steam pre-treatment (Baugh *et al.,* 1988a,b; Weil *et al.,* 1997a,b). The process by which the steam pre-treatment formed acids catalyze the hydrolysis of the lignocellulose is also termed auto-cleave steam explosion

(Bobleter *et al.,* 1991; Mok and Antal, 1992). Steam explosion will solubilise between 80-100% of the hemicellulosic component of lignocellulose, and will increase the accessibility of the cellulose surface area to enzyme attack; however, steam explosion is ineffective with regard to the solubilisation of lignin, which is redistributed (Brownell and Saddler, 1987; Heitz *et al.,* 1991; Puls *et al.,* 1985).

The treatment of lignocellulose with liquid hot water instead of steam explosion decreases the possibility of the formation of inhibitors that are mostly associated with the solubilisation of lignin. LHW is generally used to solubilise hemicellulose in order to increase the accessibility of the cellulose (Kohlmann *et al.,* 1995; Mosier *et al.,* 2005; Weil *et al.,* 1997a,b).

1.8.3 Chemical pre-treatments

Chemical pre-treatments generally make use of acids, bases and organosolv processes (Mosier *et al.,* 2005). The use of chemical pre-treatments is commonly seen in the paper and pulping industry. The main aim of chemical pulping in industry is to remove lignin from the fibrous lignocellulosic plant cell walls, and to separate the fibres at the middle lamella without causing mechanical damage to the plant cell wall. The main obstacle with regard to the delignification of plant cell walls is primarily due to the insoluble, cross-linked, heterogeneous nature of lignin. As a result of the recalcitrant nature of lignin, delignification requires harsh pre-treatment conditions, such as high temperatures and a combination of chemicals over a long period of time (Baucher *et al.,* 2003).

1.8.3.1 Organic solvents pre-treatments (Organosolv processes)

Organosolv processes essentially make use of Lewis acids ($FeCl_3$ and $(Al)_2SO_4$) that are dissolved in aqueous alcohols (glycerol, phenol and ethylene glycol). These solvents are believed to disrupt the cellulose structure to promote hydrolysis (Mosier *et al.,* 2005; Wood and Saddler, 1988).

The use of ionic liquids (ILS) as a pre-treatment strategy for treating biomass is potentially an effective, environmentally friendly route. ILS form hydrogen bonds with the protons present on the sugar hydroxyl groups resulting in the formation of a non-covalent

bonds between the biomass polymers (cellulose and hemicelluloses), disrupting the bonds between the lignin and polysaccharide chains (Dadi *et al.,* 2006; Reichert *et al.,* 2001).

1.8.3.2 Alkaline pre-treatments

Alkaline pre-treatments proceed at ambient temperatures and at low pressures, which is advantageous, as it eliminates the cost of maintaining the high temperatures and pressures that are usually required in other pre-treatments. The disadvantage of using alkaline pre-treatments is the time required. Most pre-treatments, such as the use of acid and steam, proceed over minutes rather than hours or days which is typically required for alkaline pre-treatments (Mosier *et al.,* 2005). Alkaline pre-treatments increase the accessibility of the surface exposed to enzymatic hydrolysis through the removal of acetyl and uronic acid substituents on hemicellulose (Chang and Holtzapple, 2000). Unlike the use of steam and acid pre-treatments, alkaline pre-treatments e.g. lime ($Ca(OH)_2$) solubilise lignin and a small percentage of the hemicellulose (Chang *et al.,* 1997; Kaar and Holtzapple, 2000). During the alkaline pre-treatment, the lignocellulose undergoes two reactions, namely solvation and saponification, which cause the structure of the lignocellulose to swell, decreasing the degree of polymerisation, thus making the lignocellulose components more accessible to enzymatic and microbial degradation (Hendriks and Zeeman, 2009). It has also been found that alkaline solutions can be used in the solubilisation, redistribution and condensation of lignin, which also leads to the modification of the crystalline cellulose (Gregg and Saddler, 1996). The most common alkaline pre-treatments that are employed make use of sodium hydroxide (NaOH) and $Ca(OH)_2$.

The cheapest alkaline chemical commonly used as pre-treatment agent is $Ca(OH)_2$ which has been documented to remove lignin and acetyl groups, thus increasing the rate at which lignocellulose is hydrolysed (Chang, 2007). The degree at which $Ca(OH)_2$ removes lignin from plant biomass is dependent on the type of biomass that is being treated and thus the quantity of lignin present in the sample, e.g. $Ca(OH)_2$ is less effective against woody than herbaceous and agricultural plants, primarily due to the higher quantity of lignin present in woody plants (Yang and Wyman, 2008). The use of $Ca(OH)_2$ as a pre-treatment step has its advantages: 1) $Ca(OH)_2$ is a relatively low in cost; 2) it is non-toxic; 3) it is ubiquitous since it is available worldwide in lime stone deposits; and 4) it can be recovered from solution for

recycling by saturating the water used to wash pre-treated biomass with carbon dioxide to produce calcium carbonate that precipitates out of solution. The calcium carbonate can subsequently be fed into a $Ca(OH)_2$ kiln to regenerate $Ca(OH)_2$ (Yang and Wyman, 2008).

A well-known example of alkaline chemical pulping of lignocellulosic biomass is known as the Kraft process. The delignification of biomass with the Kraft process is based on the use of NaOH and sodium sulphide (Na_2S) as delignifying agents (Baucher *et al.,* 2003). NaOH is commonly used in the chemical pre-treatment of lignocellulose due to its ability to delignify biomass; however, in the large scale production of biofuels the use of NaOH as a pre-treatment may not be cost effective (Fan *et al.,* 1981; Koullas *et al.,* 1993; Lin *et al.,* 1981; Millett *et al.,* 1976; Playne, 1984; Wyman *et al.,* 2005). During the Kraft process the lignocellulose is cooked with NaOH and Na_2S, during which point the Na_2S reacts with NaOH to produce NaHS and hydrogen sulphide (H_2S). The resulting sulphur derivatives subsequently react with the lignin components producing soluble thiolignins (Baucher *et al.,* 2003).

Other pre-treatments that are commonly employed make use of ammonia, which is responsible for improving the digestibility of cellulose by decreasing the crystallinity of the fibrils, or at high temperatures the ammonia can depolymerise the lignin to release the polysaccharide matrix (Teymouri *et al.,* 2005). Ammonia fibre/freeze explosion (AFEX) solubilises some of the lignin and hemicellulose present in the plant biomass, while decrystallising the structure of the cellulose fibres. The AFEX process pre-treats lignocellulosic biomass with liquid ammonia under pressure. The rapid release of pressure (that completes the pre-treatment) results in the decrystallisation of the cellulosic component, hydrolyses small quantities of hemicellulose and alters the lignin structure so that the structure of the lignocellulose is disrupted (Dale and Moreira, 1982; Dale *et al.,* 1985). Even though AFEX does not remove a substantial quantity of hemicellulose and lignin, enzyme activity against the substrate has been reported to increase. The decrystalisation of the cellulose fibres causes the crystalline regions of the cellulose to swell, increasing the accessibility of the β-glucosidic bonds to enzymatic attack (Dale and Moriera, 1982; Lin *et al.,* 1981).

Ammonia recycle percolation (ARP) pre-treatment is another process that makes use of ammonia. During ARP, ammonia solution is passed though a reactor packed with lignocellulose at temperatures of between 80°C and 180°C, and the ammonia present in the effluent is recycled.

1.8.3.3 Oxidative pre-treatment

The oxidative pre-treatment of lignocellulose makes use of various oxidising chemicals, such as hydrogen peroxide, peracetic acid and sodium hypochlorite, facilitating the removal of lignin and hemicellulose from the biomass (Hon and Shiraishi, 2001). The problem with using an oxidative pre-treatment strategy is that it is a non-selective pre-treatment and the loss of both hemicellulose and cellulose components of lignocellulose is often observed if the pre-treatment is left to proceed (Hon and Shiraishi, 2001). When the lignin is oxidised, soluble aromatic compounds are produced and are often toxic and act as inhibitors. These inhibitors often affect the conversion of the cellulose moiety of the lignocellulose to ethanol (Hendriks and Zeeman, 2009). Examples of oxidative pre-treatment include the use of peracetic acid and hydrogen peroxide. Teixeria *et al.* (1999) made use of peracetic acid to pre-treat SCB for the removal of lignin. In this case the pre-treatment was very successful as the pre-treatment with 21% peracetic acid allowed for approximately a 90% increase in the enzymatic hydrolysis of the lignocellulose.

1.8.3.4 Acidic pre-treatment

Lignocellulosic pre-treatment with acid at ambient temperature is often performed in order to enhance the anaerobic digestion of the biomass: this occurs due to the solubilisation of the hemicellulosic fraction of the lignocellulose by the acid that is being used. The hemicellulose solubilisation increases the accessibility of the cellulose to enzymatic hydrolysis (Hendriks and Zeeman, 2009).

The use of sulphuric acid as a pre-treatment step is well established. Sulphuric acid is used to increase the digestibility of cellulose by the removal of hemicellulose (Brownell and Saddler, 1984; Converse and Grethlein, 1985; Grous *et al.,* 1985; Knappert *et al.,* 1981). Dilute acid pre-treatment of lignocellulose is well established and is generally favoured primarily due to the fact that 80% to 90% of the hemicellulosic sugars are recoverable

(Grohmann *et al.,* 1984, 1985, 1986). In addition to increasing the digestibility of cellulose, sulphuric acid pre-treatment is also employed in a process to recover furfural (Mosier *et al.*, 2005). Similarly to steam pre-treatment, 80-100% of the hemicellulose is solubilized, lignin is redistributed and the cellulosic surface area is increased by partial depolymerisation of the lignocellulose structure (Grethlein, 1985; Tatsumoto *et al.,* 1988; Torget *et al.,* 2000).

As mentioned previously, the choice of pre-treatment is dependent on the downstream process employed in industry. The subsequent chapters discuss the optimization and use of three alkaline pre-treatment processes to enhance the digestability of SCB. Alkaline chemicals used in order to pre-treat SCB to solubilise and remove the lignin without affecting the hemicellulose component of the SCB was of interest in this study, since the synergistic association(s) between hemicellulases and an ancillary enzyme was being investigated.

Chapter 2: Research motivation and hypothesis

2.1 Research motivation

As mentioned previously, there is increasing interest in the potential for lignocellulose to serve as an alternative fuel source. At present, the rate limiting step in the utilisation of lignocellulose is its recalcitrant nature. This makes finding an effective means to hydrolyse this complex substrate to completion challenging. An enhanced rate of enzymatic degradation of lignocellulose has been achieved through the synergistic association of a wide variety of cellulase isozymes; however, the complete hydrolysis of native lignocellulose is still to be achieved. The development of a pre-treatment process that is capable of solubilizing lignin without the formation of inhibitory by-products, the removal of substituents, e.g. acetyl and uronic acid from hemicellulose and the solubilization of the hemicellulose and cellulose fibres is an essential component for the effective degradation of lignocellulose biomass through the synergistic associations of enzymes. The majority of the synergistic relationships established in literature were between cellulases; however, it has become clear that the effective hydrolysis of lignocellulose requires the presence a battery of different enzymes and proteins, such as xylanases, mannanases, arabinofuranosidases, β-glycosidases, etc. The proposed study, investigates the potential synergistic associations between the cellulosomal hemicellulases (mannanase and xylanase) and a non-cellulosomal ancillary enzyme, α-arabinofuranosidase. The proposed research will assist to identify a cost-effective pre-treatment strategy for SCB that will effectively delignify the lignicellulose component and enhance the rate of SCB degradation though the synergistic associations of the different enzymes.

2.2 Hypothesis

There is a synergistic association between cellulosome and non-cellulosome-associated hemicellulases, and that the synergistic degradation of SCB will be further enhanced through the use of appropriate pre-treatment strategies.

2.3 Study aims/objectives

1. To express and purify the recombinant proteins: xylanase A (XynA), mannanase A (ManA) and arabinofuranosidase A (ArfA).
2. To optimise the chemical ($Ca(OH)_2$, NaOH and NH_4OH) pre-treatment of sugarcane bagasse.
3. To determine the synergistic effects of various enzyme combinations on the degradation of sugarcane bagasse.
4. To determine the ideal enzyme combination with the untreated and pre-treated sugarcane bagasse.
5. To determine the chemical composition of the untreated and pre-treated SCB samples.
6. To determine the effect of the alkaline pre-treatments on the chemical composition of SCB.
7. To identify the type of sugar(s) and lignin associated phenolates by-products released during the synergistic hydrolysis and pre-treatment processes.

The objectives of the study were investigated to determine the effect of alkaline pre-treatment on the synergistic hydrolysis of SCB. Using factorial design and response surface methodology (RSM), the pre-treatment factors (time, temperature and chemical loading) were optimized to obtain the maximal production of sugar from the hydrolysis of the hemicellulose moiety of SCB (Chapter 3). Subsequently, the effect of alkaline pre-treatments and the ideal enzyme cocktail required for the synergistic hydrolysis was determined (Chapter 4). The pre-treatments were required to remove the lignin without affecting the holocellulose moiety of the SCB. To determine the efficiency of the pre-treatment, the pre-treatment liquors were analysed for the presence of *ρ*-coumaric and ferulic acid, which covalently bind the lignin to the carbohydrates via ester bonds (Chapter 5). After the SCB samples were hydrolysed, the released sugar(s) were analysed to determine what sugar(s) were released, as the type of sugar would affect the down-stream process employed to produce an economically valuable product (Chapter 5). The effect of the pre-treatments, synergistic degradation, hydrolysis products of the SCB samples and the significance of the study, are discussed in the final chapter (Chapter 6).

Chapter 3: Alkaline pre-treatment optimisation and partial SCB characterisation

3.1 Introduction

SCB is potentially a good source of energy, due to its surplus production. Generally, sugar mills burn SCB as a source of fuel; however, it is also a potential source of cellulose for the production of ethanol via direct fermentation. However, SCB is lignocellulosic in nature making it recalcitrant to complete enzymatic hydrolysis unless it is pre-treated. SCB pre-treated previously with various chemicals for the removal of both the lignin and the hemicellulose moieties, has been used in the paper and pulp industry and for the preparation of cellulose (Pandey *et al.*, 2000).

The basic purpose of pre-treatment is to produce a high sugar yield at a low cost; however, the specific goal of pre-treatment is to increase the enzyme digestibility by disrupting the crystallinity of cellulose by increasing the porosity of the material‟s surface area, thus making it accessible to the cellulase action. Increasing the digestibility of lignocellulose by pre-treatment is performed by removing the lignin and hemicellulose components of lignocellulosic materials (Sun and Cheng, 2002, Mosier *et al.,* 2005). There is a wide variety of pre-treatments that have been used in industry; however, the effects of the different pre-treatments on lignocellulose vary.

As a result of the varying effects of pre-treatment on the substrate, the choice of pre-treatment is dependent on two factors: namely the type of feedstock that is used, and on what fraction of the feedstock is of interest, e.g. cellulose, hemicellulose or lignin (Yang and Wyman, 2008).

The effect of various alkaline solutions on the enzymatically recalcitrant SCB is an important factor to consider, depending on what lignocellulose moiety is of interest. This chapter investigates the potential use of three different alkaline chemicals commonly used in a variety of industrial processes for the removal of lignin with minimal loss in the hemicellulose and cellulose moieties of the lignocellulose. The chemicals that were used to pre-treat the SCB samples that were employed in a subsequent chapter to elucidate potential

synergistic relationships between hemicellulases (XynA and ManA) and the auxiliary enzyme α-arabinofuranosidase (ArfA) included $Ca(OH)_2$, NaOH and NH_4OH. Prior to the synergistic analysis, the pre-treatment of the SCB first had to be optimised in order to determine what the optimal conditions were for removing or redistributing the lignin component of the substrate to increase the accessibility of the enzymes to the hemicellulose component of the lignocellulose.

3.2 Methods and materials

The SCB was kindly donated by Ushukela Milling (Pty) Ltd., Durban, South Africa as a dry by-product of the sugar liquor extraction process. The SCB was finely milled using a bench top blender (Figure 15) prior to analysis. The suppliers for the different chemicals used to pre-treat the SCB and the analysis of the hydrolysis products is listed in Appendix A.

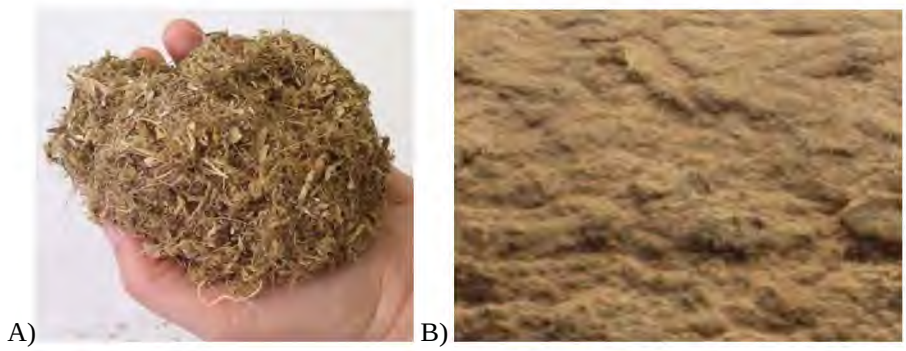


Figure 15: SCB A) before and B) after milling

3.2.1 Chemical pre-treatment optimisations

The chemicals used for the pre-treatment of the SCB were: $Ca(OH)_2$, NaOH and NH_4OH. In order to optimise the pre-treatment of the SCB, a factorial design was set up with central point replicates within each factor (chemical loading / time / temperature) as depicted in Table 3 using response surface methodology (RSM). All the pre-treatments were carried out on 4 g of dried SCB using 100 ml solutions in 500 ml flasks, agitated at 100 rpm.

Table 3: Conditions for the chemical pre-treatment optimisations using calcium hydroxide, sodium hydroxide and ammonium hydroxide

Reactions	Pre-treatment conditions		
	Time (hours)	$Ca(OH)_2$ / NaOH /NH_4OH loading (g/g bagasse)	Temperature (°C)
1	12	0.1	40
2	24		
3	36		
4	12	0.25	
5	24		
6	36		
7	12	0.4	
8	24		
9	36		
10	12	0.1	55
11	24		
12	36		
13	12	0.25	
14	24		
15	36		
16	12	0.4	
17	24		
18	36		
19	12	0.1	70
20	24		
21	36		
22	12	0.25	
23	24		
24	36		
25	12	0.4	
26	24		
27	36		

After the various pre-treatments, the SCB samples were washed with water until a neutral pH was obtained and the samples were odourless.

3.2.2 Determination of the chemical composition of SCB samples

SCB was chosen as a substrate due to the importance of the sugarcane industry to the South African agricultural sector as well as globally. The SCB was finely ground, autoclaved and washed to remove residual sugar and any potential bacterial and/or fungal spores present in the bagasse. The chemical characterisation of the SCB was kindly performed by Mr S. Walford from SMRI and by Dr L. Tyhoda, S. N. Njamela and V. Phumla from the Department of Wood Science, Stellenbosch University, South Africa according to established

National Renewable Energy Laboratory (NREL) protocols (Hames *et al.,* 2005). The SCB samples were dewaxed through three extraction steps using hexane, acetone and ethanol with a Soxhlet apparatus. The extracted material was dried and the samples were hydrolysed with 72% (v/v) sulphuric acid and autoclaved to remove the sugar from the samples. After the hydrolysis the fractions were filtered to remove the insoluble lignin from the sugar solutions. The acid soluble lignin present in the sugar solutions were detected by UV-Vis spectroscopy (Hyman *et al.,* 2007).

3.2.3 Microscopy

3.2.3.1 Scanning electron microscopy

For scanning electron microscopy (SEM), the milled SCB samples (normal, $Ca(OH)_2$, NaOH and NH_4OH pre-treated) were mounted on a metal stub, dried using critical point-drying process and coated with a thin layer of gold prior to SEM analysis (Cross, 2001).

3.2.3.2 Light microscopy

To determine the effect of the various pre-treatment procedures on the SCB lignin, the SCB samples were stained with 10% (w/v) phlorogucinol in 95% (v/v) ethanol. The SCB samples were stained prior to light microscope analysis. The SCB samples were covered with the phlorogucinol solution and incubated at room temperature for 3 min. After the samples were drained, a few drops of concentrated HCl were added to the samples and incubated for 3 min. The excess HCl was removed from the samples. The colour produced from the staining procedure was preserved with a few drops of paraffin (Gahan, 1984). The samples were visualized using an Olympus BX40 light microscope and photographed using an Olympus DP72 digital camera.

3.3 Results

3.3.1 Pre-treatment optimisation

The efficiency of the pre-treatment reactions for the optimisation of the pre-treatment conditions were based on quantification of the total reducing sugars (TRS), which was determined using a modified dinitrosalicylic acid (DNS) method The reactions were performed in triplicate using equal enzyme (xylanase) concentrations (Beukes *et al.*, 2010).

The Design-Expert® 6.0.4 software was utilized to analyse the $Ca(OH)_2$, NaOH and NH_4OH pre-treatment data.

3.3.1.1 Lime pre-treatment

One of the problems associated with the hydrolysis of lignocellulose is the presence of high quantities of lignin, which surrounds the holocellulose moiety of the plant cell wall, acting like a protective sheath. The successful removal of the lignin would facilitate a greater access for the enzymes to their respective substrate components on the lignocellulose, thus increasing the necessity for an effective pre-treatment step to remove the lignin without affecting or removing the holocellulose moiety of the lignocellulose, in this case SCB.

The first chemical pre-treatment made use of $Ca(OH)_2$. The pre-treatment conditions that were considered included: time, $Ca(OH)_2$ loading and temperature (Table 3). Once again the success of the pre-treatment was determined indirectly through the increase in the production of reduced sugars, which was determined using the modified DNS assay (Table 4).

Table 4: The effect of the $Ca(OH)_2$ pre-treatment conditions on SCB weight and TRS yield from the hydrolysis of the pre-treated SCB samples.

Reaction	Pre-treatment conditions	Weight loss (%)	TRS (U)*	Std. Dev.
1	12 h, 40°C, 0.10 g/g	46.07	18.8	1.657
2	24 h, 40°C, 0.10 g/g	40.63	22.3	1.2
3	36 h, 40°C, 0.10 g/g	39.58	27.7	3.3
4	12 h, 40°C, 0.25 g/g	40.74	20.0	1.9
5	24 h, 40°C, 0.25 g/g	42.48	22.5	1.2
6	36 h, 40°C, 0.25g/g	51.63	29.6	0.7
7	12 h, 40°C, 0.40 g/g	46.82	26.2	3.9
8	24 h, 40°C, 0.40 g/g	46.93	28.4	4.1
9	36 h, 40°C, 0.40 g/g	45.4	29.5	2.9
10	12 h, 55°C, 0.10 g/g	49.81	23.4	1.8
11	24 h, 55°C, 0.10 g/g	50.27	26.8	1.1
12	36 h, 55°C, 0.10 g/g	53.51	34.6	1.2
13	12 h, 55°C, 0.25 g/g	40.01	26.7	1.1
14	24 h, 55°C, 0.25 g/g	44.02	27.3	2.1
15	36 h, 55°C, 0.25 g/g	49.51	31.9	2.2
16	12 h, 55°C, 0.40 g/g	39.62	25.0	0.8
17	24 h, 55°C, 0.40 g/g	40.51	28.6	0.8
18	36 h, 55°C, 0.40 g/g	42.52	35.0	1.9
19	12 h, 70°C, 0.10 g/g	45.83	26.5	1.5
20	24 h, 70°C, 0.10 g/g	46.73	32.1	2.26
21	36 h, 70°C, 0.10 g/g	42.68	33.3	0.3
22	12 h, 70°C, 0.25 g/g	45.21	27.8	1.2
23	24 h, 70°C, 0.25 g/g	47.52	30.9	2.0
24	36 h, 70°C, 0.25 g/g	49.01	31.2	1.4
25	**12 h, 70°C, 0.40 g/g**	**41.63**	**39.0**	**4.3**
26	24 h, 70°C, 0.40 g/g	52.85	31.3	2.6
27	36 h, 70°C, 0.40 g/g	46.53	29.4	2.4

*Enzyme activity (U) = average mmol sugar produced per 24 hours

From the data that was obtained hydrolysis of the different pre-treated SCB samples, Table 4 indicated that the greatest amount of sugar was produced by the hydrolysis of the SCB that was pre-treated for 12 hours, at 70°C with 0.40 g $Ca(OH)_2$ /g SCB.

Using Design Expert 6.0, the RSM data obtained from the hydrolysis of the $Ca(OH)_2$ pre-treated SCB was analysed. Different statistical models were tested by testing the sequential model sum of squares (Table 5).

Table 5: Sequential model Sum of squares from the analysis of the data obtained from the hydrolysis of the $Ca(OH)_2$ pre-treated SCB

Source	Sum of Squares	DF	Mean Square	F-Value	Prob > F	Comments
Mean	65089.05	1	65089.05			
Linear	1051.91	3	350.64	30.65	< 0.0001	
2FI	**262.87**	**3**	**87.62**	**10.49**	**< 0.0001**	**Suggested**
Quadratic	47.49	3	15.83	1.97	0.1262	
Cubic	152.1	7	21.73	3.32	0.0044	Aliased
Quartic	87.75	6	14.62	2.57	0.0284	Aliased
Fifth	76.06	3	25.35	5.48	0.0023	Aliased
Sixth	1.78E-04	1	1.78E-04	3.79E-05	0.9951	Aliased
Residual	254.51	54	4.71			
Total	67021.74	81	827.43			

The data from the analysis of the sum of squares (Table 5) indicated that the 2 factorial RSM model best suited the data obtained from the hydrolysis of the $Ca(OH)_2$ pre-treated SCB since it was the model with the highest order polynominal where the model is not aliased and the additional terms were significant.

The one-way analysis of variance (ANOVA) was used to examine the effect of the different pre-treatments. The effects of the different factors and the verification of the model have been depicted in Table 6.

Table 6: ANOVA analysis for the response surface 2FI model for the hydrolysis of the $Ca(OH)_2$ pre-treated SCB

Source	Sum of Squares	DF	Mean Square	F-Value	Prob > F	Comments
Model	1314.78	6	219.13	26.24	< 0.0001	Significant
A – Time	396.64	1	396.64	47.5	< 0.0001	Significant
B - Temperature	533.8	1	533.8	63.93	< 0.0001	Significant
C – $Ca(OH)_2$ loading	121.47	1	121.47	14.55	0.0003	Significant
AB	113.07	1	113.07	13.54	0.0004	Significant
AC	135.96	1	135.96	16.28	0.0001	Significant
BC	13.85	1	13.85	1.66	0.2018	
Residual	617.91	74	8.35			
Lack of Fit	363.39	20	18.17	3.86	< 0.0001	Significant
Pure Error	254.51	54	4.71			
Cor Total	1932.69	80				

For a confidence level of 95% to be achieved, the probability factor would have to be less than 0.05. As depicted (Table 6) the model F-value of 26.24 was significant and that there was only a 0.01% chance that this "Model F-value" could have occurred due to noise. With respect to the different factors, the only factor that was considered insignificant was BC.

The effect of the pre-treatment factors ($Ca(OH)_2$, time and temperature), were illustrated using three dimensional perturbation plots (Figures 16-18) allowing for a visual comparison of the individual effects of the pre-treatment conditions. The effect of $Ca(OH)_2$ on the yield of reducing sugars is seen when comparing the effects of the different concentrations of $Ca(OH)_2$ on the yield of sugar (Figure 16). Figure 16 displays the effect of the different concentrations of $Ca(OH)_2$ on the pre-treatment of SCB, while time (y-axis) and temperature (x-axis) remain constant.

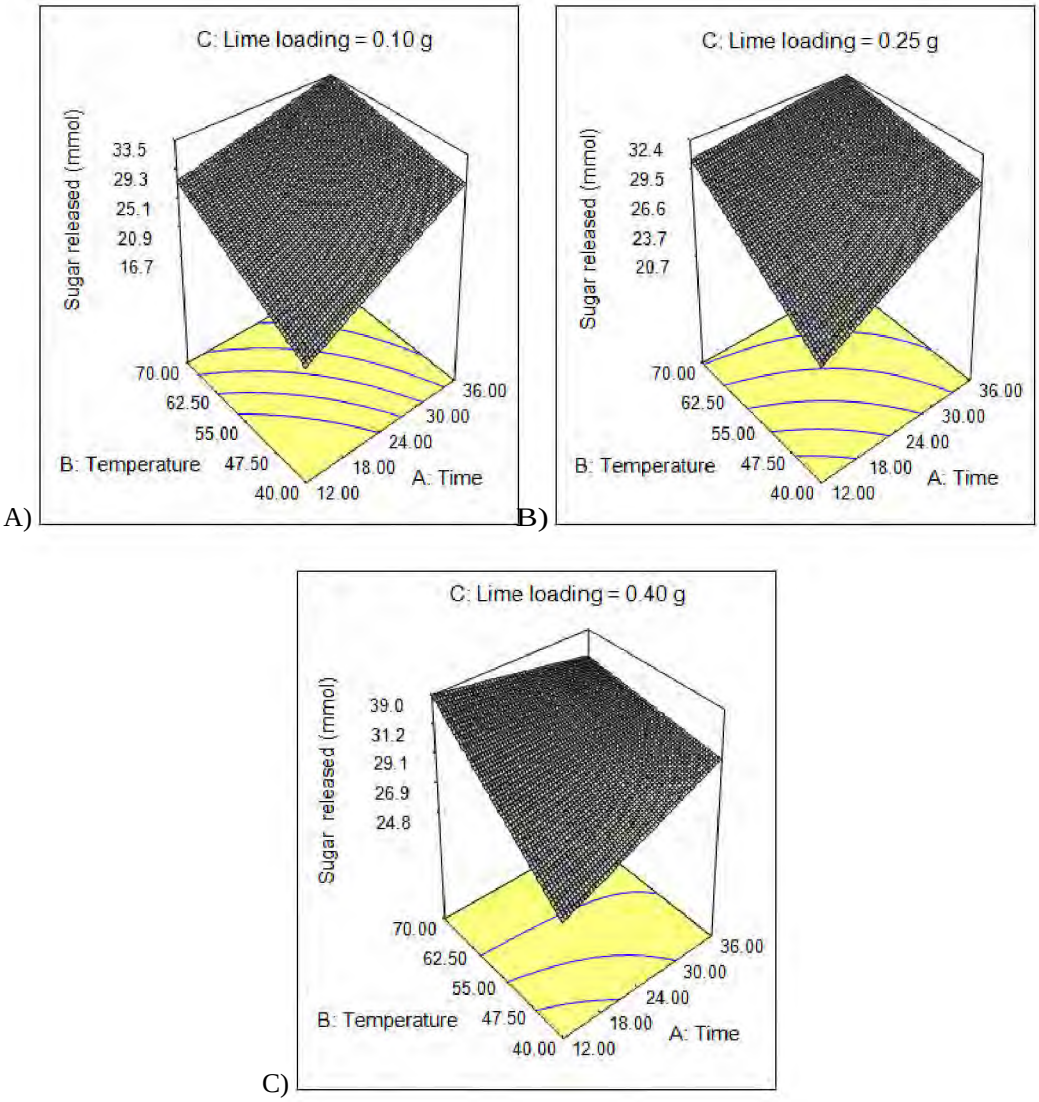


Figure 16: Effect of $Ca(OH)_2$ loading is illustrated with the 3D perturbation plots. A) The first plot illustrates the effect of pre-treating the SCB with 0.10 g $Ca(OH)_2$ /g bagasse with regard to the production of sugar, B) the second 3D plot illustrates the effect of pre-treating the SCB with 0.25 g $Ca(OH)_2$ /g bagasse with regard to the production of sugar and C) the third 3D plot illustrates the effect of pre-treating the SCB with 0.40 g $Ca(OH)_2$ /g bagasse with regard to the production of sugar.

The data obtained from Figure 16 indicated that the largest amount of sugar was released under conditions of 70°C and 12 hours. Maximal release of sugar was obtained when the SCB was pre-treated with 0.40 g $Ca(OH)_2$ per g bagasse; however, similar quantities of sugar were produced from the hydrolysis of the SCB samples that were pre-treated with the different concentration of $Ca(OH)_2$ at 70°C over a longer pre-treatment period.

The second factor that was investigated was time (Figure 17). In this case the $Ca(OH)_2$ (y-axis) and the temperature (x-axis) were kept constant.

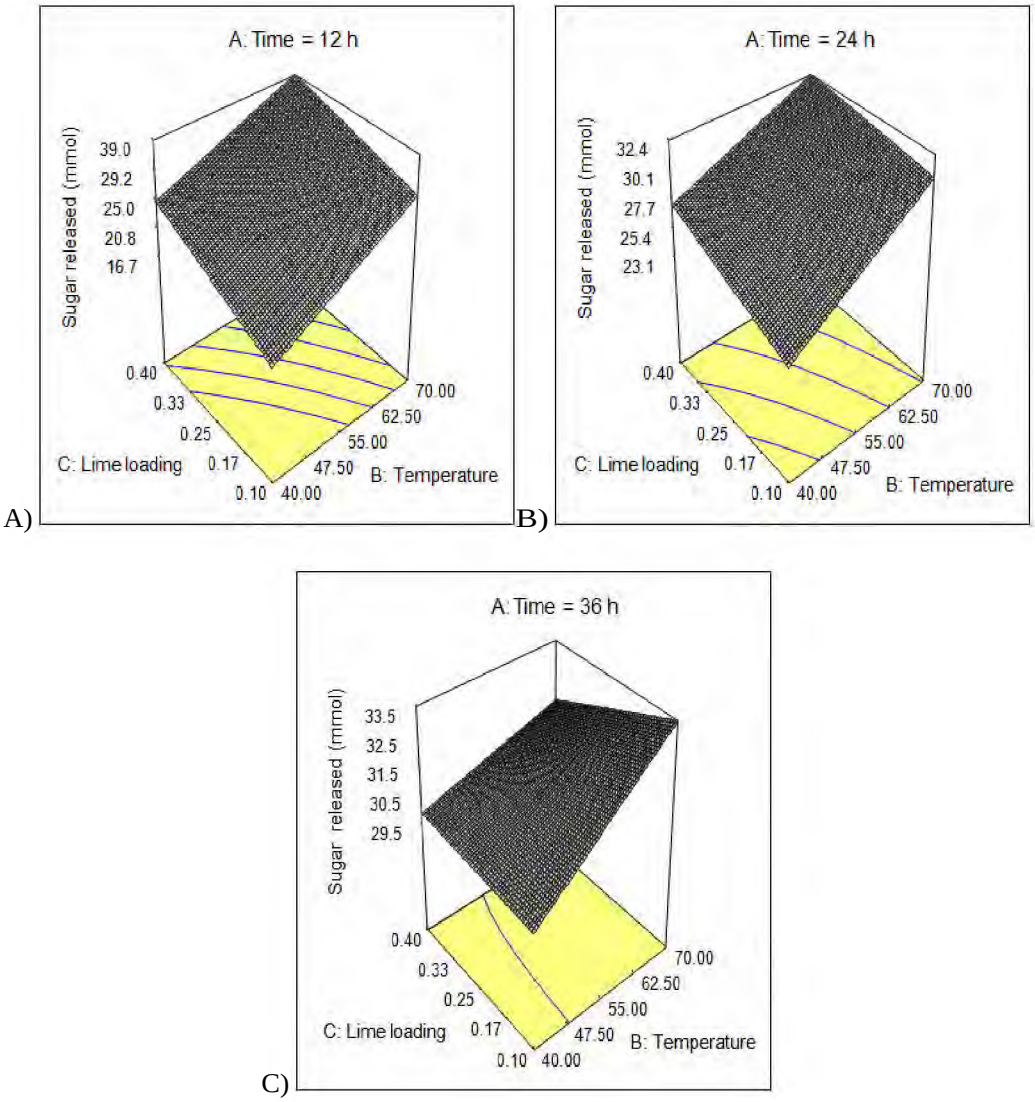


Figure 17: Effect of time on the $Ca(OH)_2$ pre-treatment of SCB is illustrated with the 3D perturbation plots. A) The first plot illustrates the effect of pre-treating the SCB for 12 hours with regard to the production of sugar, B) the second 3D plot illustrates the effect of pre-treating the SCB for 24 hours with regard to the production of sugar and C) the third 3D plot illustrates the effect of pre-treating the SCB for 36 hours with regard to the production of sugar.

The data obtained from the effect of time on the pre-treatment of SCB, indicated that the greatest production of sugar was produced with the conditions of 0.10 g $Ca(OH)_2$/ g SCB, 70°C and 36 hours (Figure 17); however, similar quantites of sugar were produced when the SCB was pre-treated with 0.40 g $Ca(OH)_2$/ g bagasse at 70°C between 12 and 24 hours.

Temperature was the third factor analysed to determine the effect of temperature on the $Ca(OH)_2$ pre-treatment of SCB and to determine the optimal pre-treatment conditions for the effective pre-treatment of SCB (Figure 18).

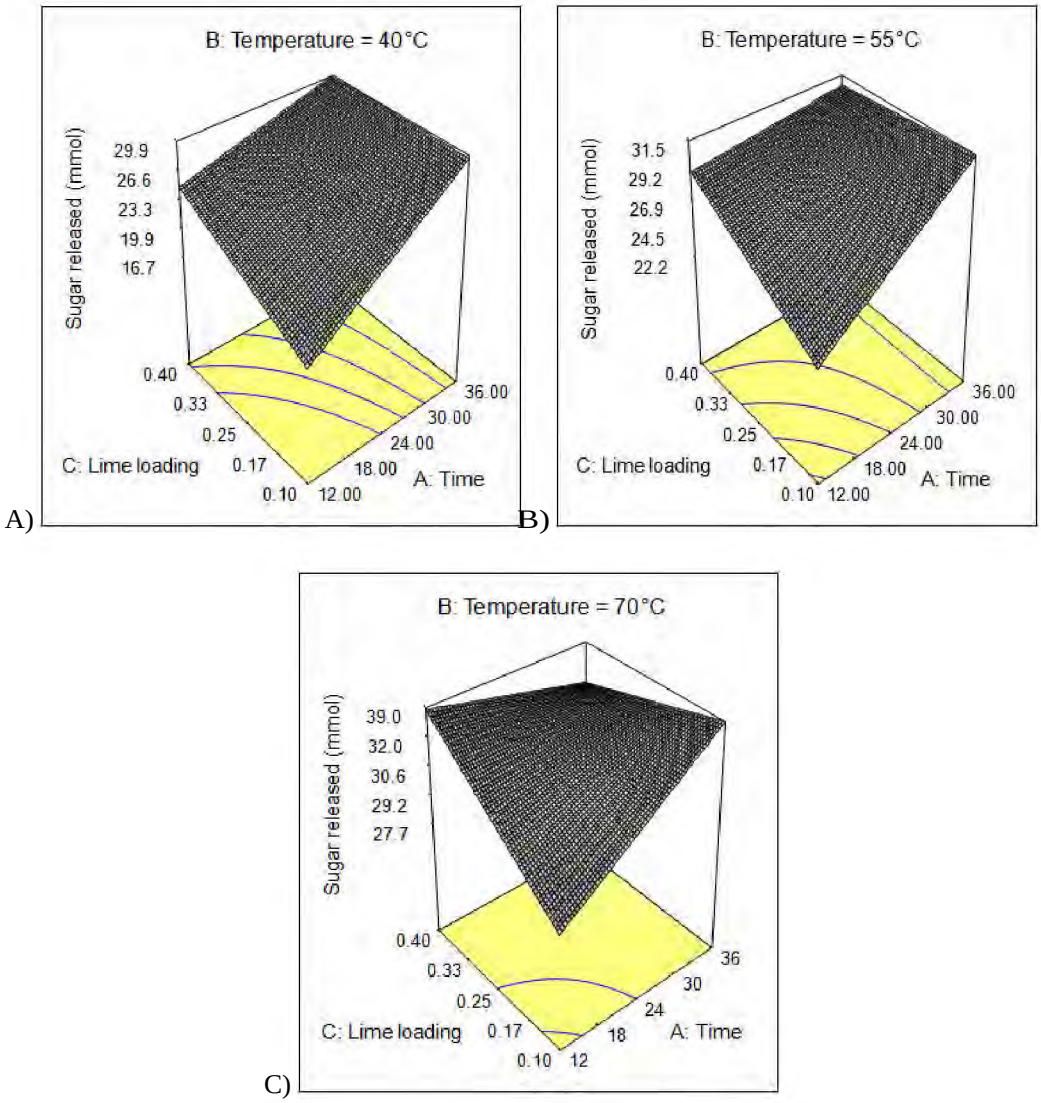


Figure 18: Effect of temperature on the $Ca(OH)_2$ pre-treatment of SCB is illustrated with the 3D perturbation plots. A) The first plot illustrates the effect of pre-treating the SCB at 40°C with regard to the production of sugar, B) the second 3D plot illustrates the effect of pre-treating the SCB at 55°C with regard to the production of sugar and C) the third 3D plot illustrates the effect of pre-treating the SCB at 70°C with regard to the production of sugar.

The effects of temperature on the pre-treatment of SCB using $Ca(OH)_2$ indicated that the greatest amount of sugar was produced by the hydrolysis of SCB sample that was pre-treated at 70°C (Figure 18).

3.3.1.2 Sodium hydroxide pre-treatment

The second alkaline chemical pre-treatment made use of NaOH and the pre-treatment factors that were considered were time, NaOH loading, and temperature as previously described (Table 3). The pre-treatment efficiency was determined by the production of sugar from the hydrolysis of the SCB samples that were pre-treated with different conditions (Table 7).

Table 7: The effect of the NaOH pre-treatment conditions on the SCB weight and TRS yield from the hydrolysis of the pre-treated SCB samples.

Reaction	Pre-treatment conditions	Weight loss (%)	TRS (L))*	Std. dev.
1	12 h, 40°C, 0.10 g/g	39.06	4.1	0.3
2	24 h, 40°C, 0.10 g/g	37.04	4.0	1.2
3	36 h, 40°C, 0.10 g/g	36.14	3.9	0.3
4	12 h, 40°C, 0.25 g/g	38.74	5.1	0.4
5	24 h, 40°C, 0.25 g/g	41.04	7.6	0.3
6	36 h, 40°C, 0.25 g/g	49.05	8.2	1.3
7	12 h, 40°C, 0.40 g/g	78.95	8.0	1.3
8	24 h, 40°C, 0.40 g/g	59.35	9.9	1.7
9	36 h, 40°C, 0.40 g/g	54.4	13.1	1.4
10	12 h, 55°C, 0.10 g/g	39.69	4.7	0.5
11	24 h, 55°C, 0.10 g/g	43.06	21.2	1.0
12	36 h, 55°C, 0.10 g/g	39.94	11.2	0.7
13	12 h, 55°C, 0.25 g/g	45.69	17.3	1.3
14	**24 h, 55°C, 0.25 g/g**	**53.89**	**42.3**	**3.1**
15	36 h, 55°C, 0.25 g/g	57.41	18.2	1.1
16	12 h, 55°C, 0.40 g/g	47.28	19.4	1.0
17	24 h, 55°C, 0.40 g/g	49.72	24.6	2.5
18	36 h, 55°C, 0.40 g/g	51.42	17.3	1.1
19	12 h, 70°C, 0.10 g/g	41.1	4.9	0.8
20	24 h, 70°C, 0.10 g/g	41.31	9.7	1.4
21	36 h, 70°C, 0.10 g/g	41.33	12.5	2.1
22	12 h, 70°C, 0.25 g/g	32.62	5.6	0.5
23	24 h, 70°C, 0.25 g/g	33.85	7.0	0.4
24	36 h, 70°C, 0.25 g/g	39.52	10.4	1.4
25	12 h, 70°C, 0.40 g/g	30.71	7.6	0.7
26	24 h, 70°C, 0.40 g/g	54.96	8.9	1.4
27	36 h, 70°C, 0.40 g/g	60.14	5.9	0.7

*Enzyme activity (L)) = average mmol sugar produced per 24 hours

From the data that was obtained hydrolysis of the different pre-treated SCB samples (Table 7) indicated that the greatest amount of sugar was produced by the hydrolysis of the SCB that was pre-treated for 24 hours, at 55°C with 0.25 g NaOH /g SCB.

The data obtained from the hydrolysis of the NaOH pre-treated SCB was analysed using Design Expert 6. Different statistical models were tested by testing the sequential model sum of squares (Table 8).

Table 8: Sequential model sum of squares from the analysis of the data obtained from the hydrolysis of the NaOH pre-treated SCB

Source	Sum of Squares	DF	Mean Square	F-Value	Prob > F	Comments
Mean	10841.3	1	10841.3			
Linear	353.88	3	117.96	1.72	0.1699	
2FI	183.57	3	61.19	0.89	0.4514	
Quadratic	**3228.85**	**3**	**1076.28**	**40.88**	**< 0.0001**	**Suggested**
Cubic	196.89	7	28.13	1.08	0.3889	Aliased
Quartic	1320.56	6	220.09	36.28	< 0.0001	Aliased
Fifth	50.31	3	16.77	3.06	0.0357	Aliased
Sixth	212.42	1	212.42	128.66	< 0.0001	Aliased
Residual	89.16	54	1.65			
Total	16476.93	81	203.42			

The data from the analysis of the sum of squares (Table 8) indicated that the quadratic RSM model best suited the data obtained from the hydrolysis of the NaOH pre-treated SCB since it is the model with the highest order polynominal where the model is not aliased and the additional terms were significant.

The ANOVA was used to examine the effect of the different pre-treatments. The effects of the different factors and the verification of the model have been depicted in Table 9.

Table 9: ANOVA analysis for the response surface quadratic model for NaOH pre-treated SCB

Source	Sum of Squares	DF	Mean Square	F-Value	Prob > F	Comments
Model	3766.3	9	418.48	15.89	< 0.0001	Significant
A – Time	95.08	1	95.08	3.61	0.0614	
B - Temperature	11.63	1	11.63	0.44	0.5085	
C – NaOH loading	247.17	1	247.17	9.39	0.0031	Significant
A2	479.73	1	479.73	18.22	< 0.0001	Significant
B2	2596.93	1	2596.93	98.63	< 0.0001	Significant
C2	152.19	1	152.19	5.78	0.0188	Significant
AB	1.7	1	1.7	0.065	0.7999	
AC	39.82	1	39.82	1.51	0.2229	
BC	142.05	1	142.05	5.4	0.0231	Significant
Residual	1869.34	71	26.33			
Lack of Fit	1780.18	17	104.72	63.42	< 0.0001	Significant
Pure Error	89.16	54	1.65			
Cor Total	5635.63	80				

For a confidence level of 95% to be achieved, the probability factor would have to be less than 0.05. As depicted (Table 9) the model F-value of 15.89 is significant and that there is only a 0.01% chance that this “Model F-value” could have occurred due to noise. With respect to the different factors, the factors that were considered significant were C, A^2, B^2 C^2 and BC.

The effect of the pre-treatment factors (NaOH, time and temperature), were illustrated using three dimensional perturbation plots (Figures 19-21) allowing for a visual comparison of the individual effects of the pre-treatment conditions. The effect of NaOH on the yield of reducing sugars is seen when comparing the effects of the lowest and highest concentrations of NaOH on the yield of sugar (Figure 19). Two of the three factors in the RSM design were kept constant in order to determine the effect of a specific factor. Figure 19 displays at the effect of the different concentrations of NaOH in the pre-treatment of SCB, while time (y-axis) and temperature (x-axis) remain constant.

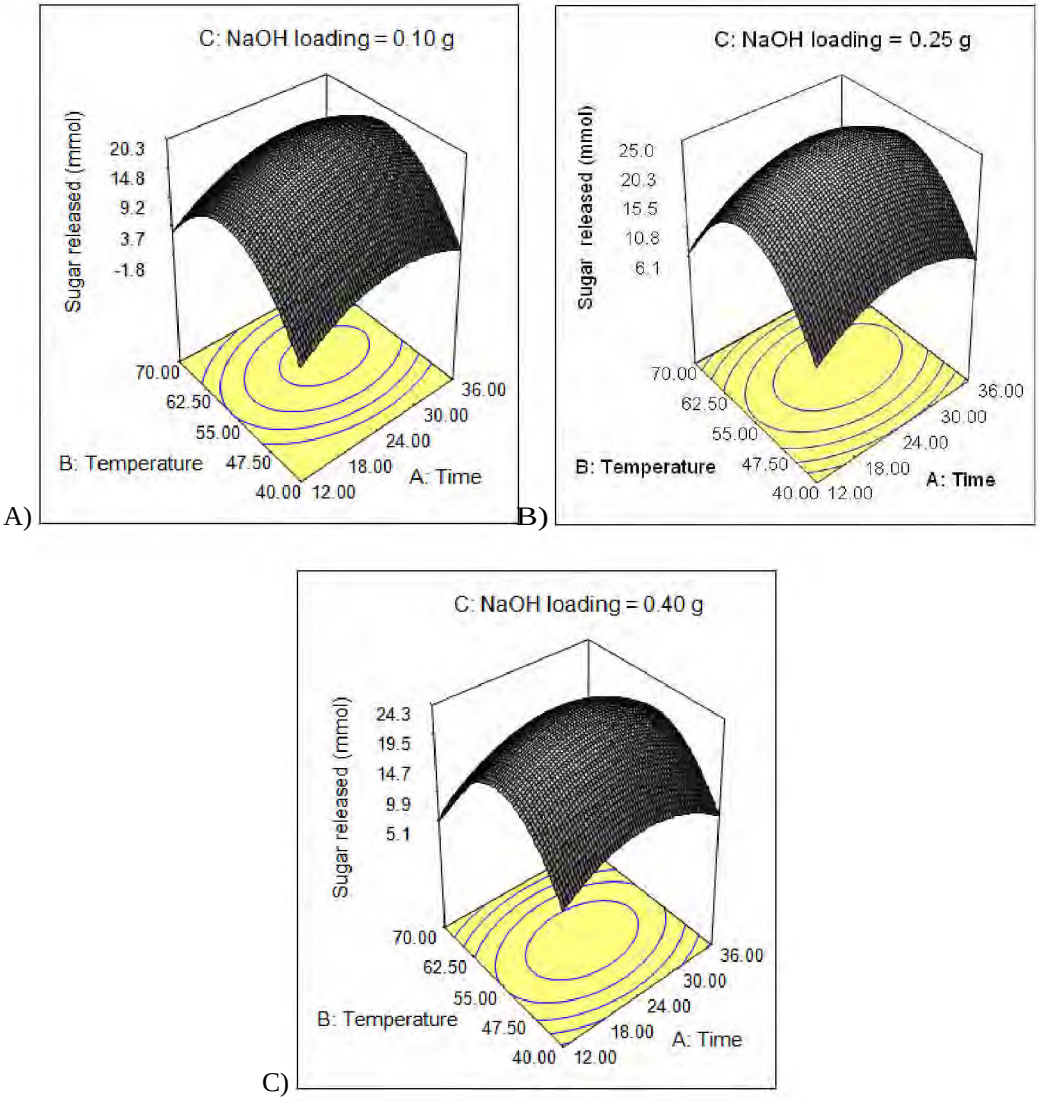


Figure 19: The effect of NaOH loading illustrated with the 3D perturbation plots. A) The first plot illustrates the effect of pre-treating the SCB with 0.10 g NaOH /g bagasse with regard to the production of sugar, B) the second 3D plot illustrates the effect of pre-treating the SCB with 0.25 g NaOH /g bagasse with regard to the production of sugar and C) the third 3D plot illustrates the effect of pre-treating the SCB with 0.40 g NaOH /g bagasse with regard to the production of sugar.

The data obtained from Figure 19 indicated that larger quantities of sugar were produced under conditions of 55°C and 24 hours; however, the greatest amount of sugar was obtained when the SCB was pre-treated with 0.25 g NaOH per g bagasse.

The second factor that was investigated at was time (Figure 20). In this case the NaOH loading (y-axis) and the temperature (x-axis) were kept constant.

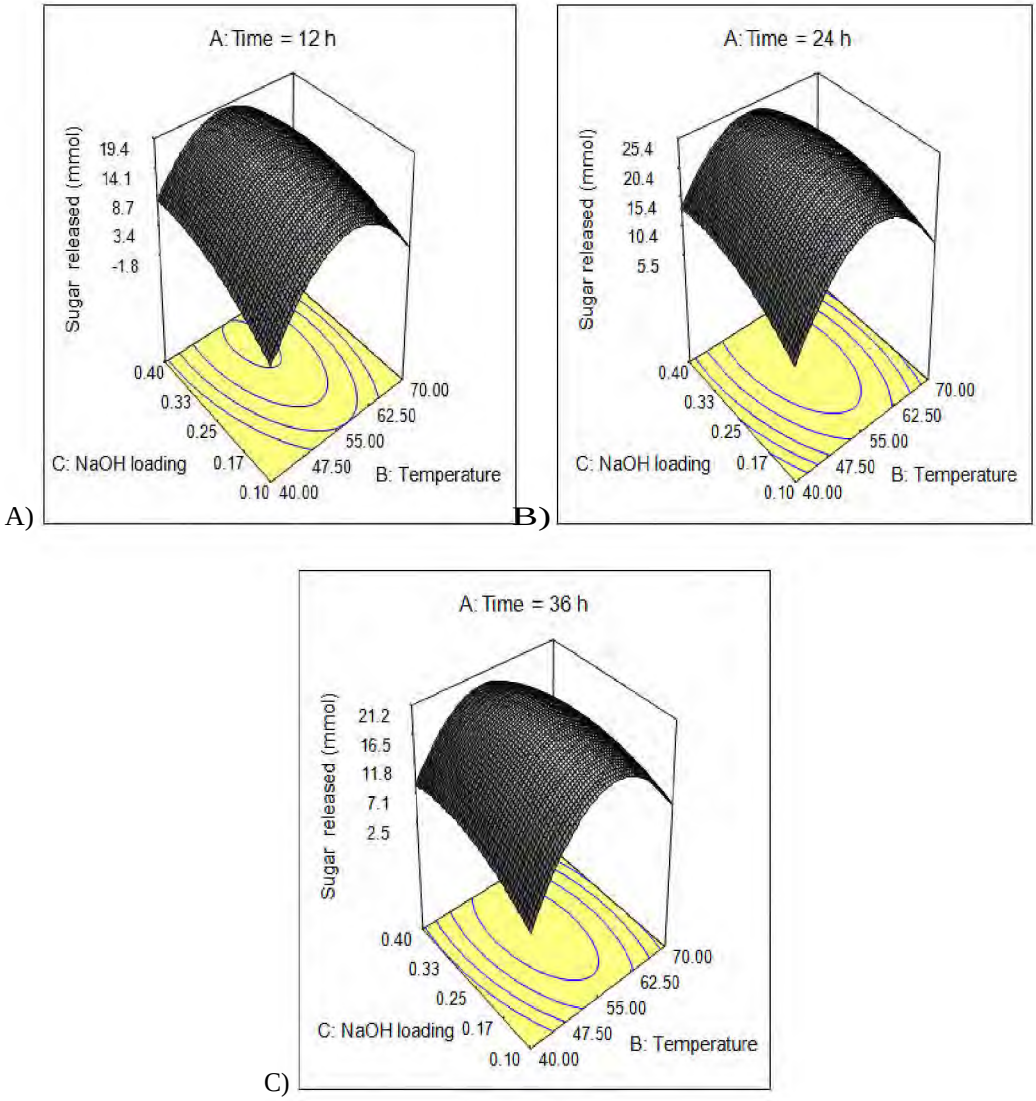


Figure 20: Effect of time on the NaOH pre-treatment of SCB is illustrated with the 3D perturbation plots. A) The first plot illustrates the effect of pre-treating the SCB for 12 hours with regard to the production of sugar, B) the second 3D plot illustrates the effect of pre-treating the SCB for 24 hours with regard to the production of sugar and C) the third 3D plot illustrates the effect of pre-treating the SCB for 36 hours with regard to the production of sugar.

The data obtained from the effect of time on the pre-treatment of SCB indicated that the greatest production of sugar was produced with the harshest conditions of 0.25 g NaOH/ g SCB, 55°C and 24 hours (Figure 20).

Temperature was the third factor analysed to determine the effect of temperature on the NaOH pre-treatment of SCB, and to determine the optimal pre-treatment conditions tested for effective pre-treatment of SCB (Figure 21).

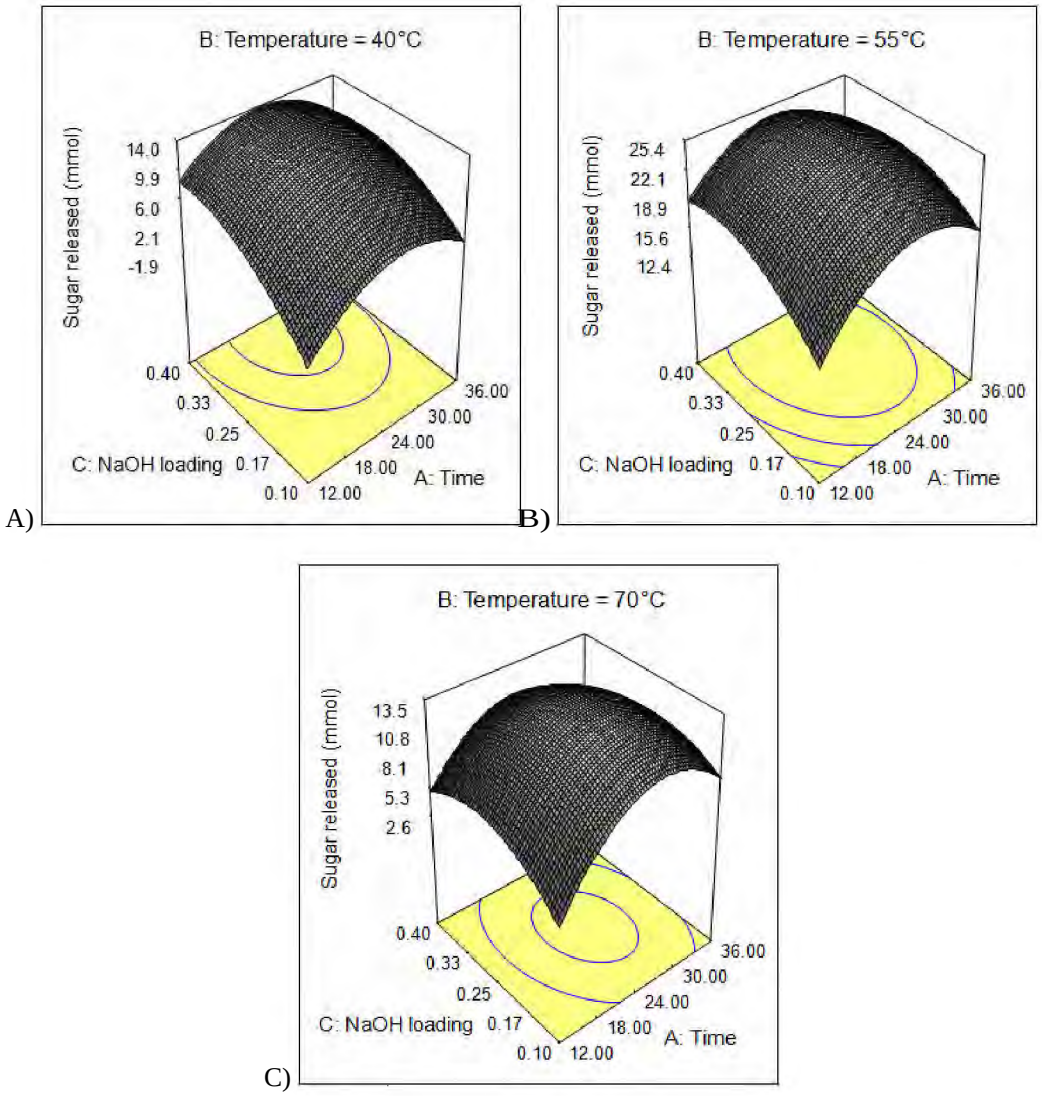


Figure 21: Effect of temperature on the NaOH pre-treatment of SCB is illustrated with the 3D perturbation plots. A) The first plot illustrates the effect of pre-treating the SCB at 40°C with regard to the production of sugar, B) the second 3D plot illustrates the effect of pre-treating the SCB at 55°C with regard to the production of sugar and C) the third 3D plot illustrates the effect of pre-treating the SCB at 70°C with regard to the production of sugar.

Similarily to the effects of NaOH loading and pre-treatment time, the greatest amount of sugar was produced by the hydrolysis of the NaOH fraction that was pre-treated at 55°C, for 24 hours with 0.25g NaOH per gram of SCB (Figure 21).

3.3.1.3 Ammonium hydroxide pre-treatment

For the third pre-treatment, the conditions that were considered included time, NH_4OH loading and temperature (Table 3). These conditions were used to determine the ideal pre-treatment conditions that would successfully remove or solubilise and redistribute the lignin sheath. If the pre-treatment of the SCB had any effect two observations would be expected; firstly there would be an increase in the degree of enzymatic hydrolysis and secondly, there would be a change in SCB mass (Table 10).

Table 10: The effects of the NH_4OH pre-treatment conditions on SCB weight and TRS yield from the hydrolysis of the pre-treated SCB samples.

Reactions	Pre-treatment conditions	Weight loss (%)	TRS (U)*	Std. dev.
1	12 h, 40°C, 0.1 g/g	31.85	17.7	3.7
2	24 h, 40°C, 0.1 g/g	23.15	19.9	2.5
3	36 h, 40°C, 0.1 g/g	20.45	24.2	3.0
4	12 h, 40°C, 0.25 g/g	26.31	12.7	2.4
5	24 h, 40°C, 0.25 g/g	27.12	48.3	5.3
6	36 h, 40°C, 0.25 g/g	29.76	55.6	4.1
7	12 h, 40°C, 0.4 g/g	36.09	50.6	5.9
8	24 h, 40°C, 0.4 g/g	35.98	67.8	2.8
9	36 h, 40°C, 0.4 g/g	38.25	93.6	5.7
10	12 h, 55°C, 0.1 g/g	39.72	60.9	4.0
11	24 h, 55°C, 0.1 g/g	42.27	72.3	7.6
12	36 h, 55°C, 0.1 g/g	43.61	82.5	12.1
13	12 h, 55°C, 0.25 g/g	41.26	80.2	3.1
14	24 h, 55°C, 0.25 g/g	42.18	84.9	3.3
15	36 h, 55°C, 0.25 g/g	43.17	107.1	6.4
16	12 h, 55°C, 0.4 g/g	37.41	72.6	4.3
17	24 h, 55°C, 0.4 g/g	38.05	103.3	10.5
18	36 h, 55°C, 0.4 g/g	43.07	128.5	7.2
19	12 h, 70°C, 0.1 g/g	39.36	70.3	10.5
20	24 h, 70°C, 0.1 g/g	37.32	91.1	11.1
21	36 h, 70°C, 0.1 g/g	34.18	144.0	5.7
22	12 h, 70°C, 0.25 g/g	39.41	103.5	3.6
23	24 h, 70°C, 0.25 g/g	40.13	151.6	5.8
24	36 h, 70°C, 0.25 g/g	41.51	195.2	7.3
25	12 h, 70°C, 0.4 g/g	42.17	160.3	14.5
26	24 h, 70°C, 0.4 g/g	43.97	189.8	12.7
27	**36 h, 70°C, 0.4 g/g**	**46.41**	**254.7**	**16.9**

*Enzyme activity (U) = average mmol sugar produced per 24 hours

Table 10 indicated that the harshest pre-treatment conditions indicated in bold (36 hours, 70°C and 0.114 M NH_4OH/g SCB) resulted in a weight loss of 46.41% and the greatest degree of hydrolysis (TRS release).

Using Design Expert 6.0, the RSM analysis of the data obtained from the hydrolysis of the NH_4OH pre-treated SCB was performed to determine which statistical model best fits

the data. Different statistical models were tested by testing the sequential model sum of squares (Table 11).

Table 11: Sequential Model Sum of Squares for the data obtained from the hydrolysis of the NH_4OH pre-treated SCB

Source	Sum of Squares	DF	Mean Square	F-value	Prob > F	Comments
Mean	7.19E+05	1	7.19E+05			
Linear	2.40E+05	3	79967.67	204.11	< 0.0001	
2FI	14673.84	3	4891.28	23.36	< 0.0001	
Quadratic	**1744.63**	**3**	**581.54**	**3**	**0.0361**	**Suggested**
Cubic	8671.05	7	1238.72	15.61	< 0.0001	Aliased
Quartic	645.55	6	107.59	1.41	0.2272	Aliased
Fifth	425.64	3	141.88	1.95	0.1326	Aliased
Sixth	717.43	1	717.43	11.78	0.0012	Aliased
Residual	3289.26	54	60.91			
Total	9.89E+05	81	12207.05			

The data from the analysis of the sum of squares (Table 11) indicated that the quadratic RSM model best suited the data obtained from the hydrolysis of the NH_4OH pre-treated SCB since it was the model with the highest order polynominal where the model was not aliased and the additional terms were significant.

The ANOVA was used to examine the effect of the different pre-treatments. The effects of the different factors and the verification of the model have been depicted in Table 12.

Table 12: ANOVA analysis for the quadratic RSM from the hydrolysis of the NH_4OH pre-treated SCB

Source	Sum of Squares	DF	Mean Square	F-Value	Prob > F	Comments
Model	2.56E+05	9	28480.17	147.07	< 0.0001	significant
A – Time	34760.96	1	34760.96	179.51	< 0.0001	significant
B - Temperature	1.57E+05	1	1.57E+05	810.01	< 0.0001	significant
C – NH_4OH loading	48285.51	1	48285.51	249.35	< 0.0001	significant
A^2	175.57	1	175.57	0.91	0.3442	
B^2	1531.57	1	1531.57	7.91	0.0064	significant
C^2	37.49	1	37.49	0.19	0.6613	
AB	7016.3	1	7016.3	36.23	< 0.0001	significant
AC	2089.4	1	2089.4	10.79	0.0016	significant
BC	5568.14	1	5568.14	28.75	< 0.0001	significant
Residual	13748.93	71	193.65			
Lack of Fit	10459.67	17	615.27	10.1	< 0.0001	significant
Pure Error	3289.26	54	60.91			
Cor Total	2.70E+05	80				

For a confidence level of 95% to be achieved, the probability factor would have to be less than 0.05. As depicted (in Table 12) the model F-value of 147.07 is significant and there is only a 0.01% chance that this high “Model F-value” could have occurred due to noise. Thus with respect to the different factors, the factors that were not significant were A^2 and C^2.

The effect of the pre-treatment factors (NH_4OH, time and temperature), were illustrated using three dimensional perturbation plots (Figures 22-24) to allow for a visual comparison of the individual effects of the pre-treatment conditions. The effect of NH_4OH on the yield of reducible sugars was seen when comparing the effects of the lowest and highest concentrations of NH_4OH on the yield of sugar (Figure 22). Two of the three factors in the RSM design was kept constant in order to determine the effect of a specific factor. Figure 22 displays the effect of the different concentrations of NH_4OH in the pre-treatment of SCB, while time (y-axis) and temperature (x-axis) remained constant.

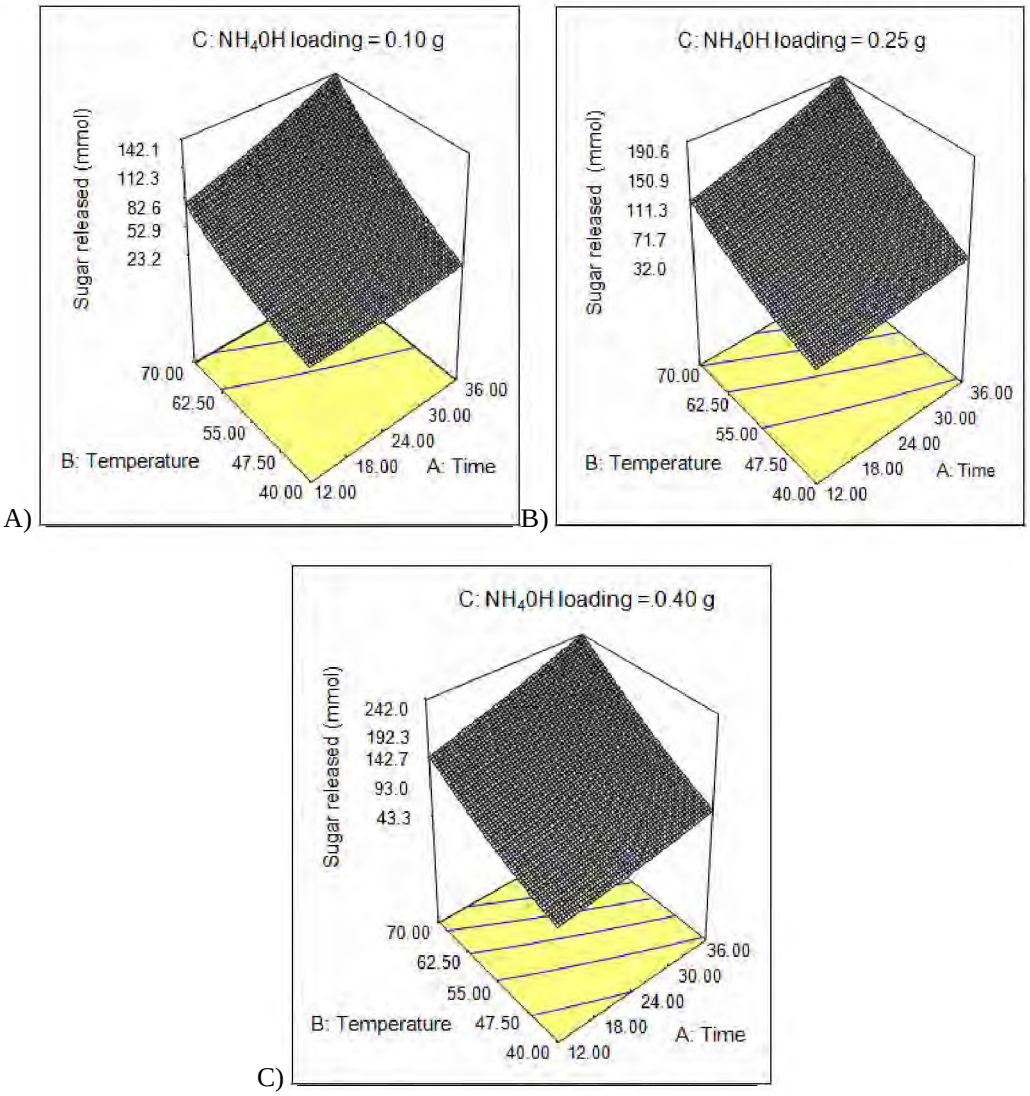
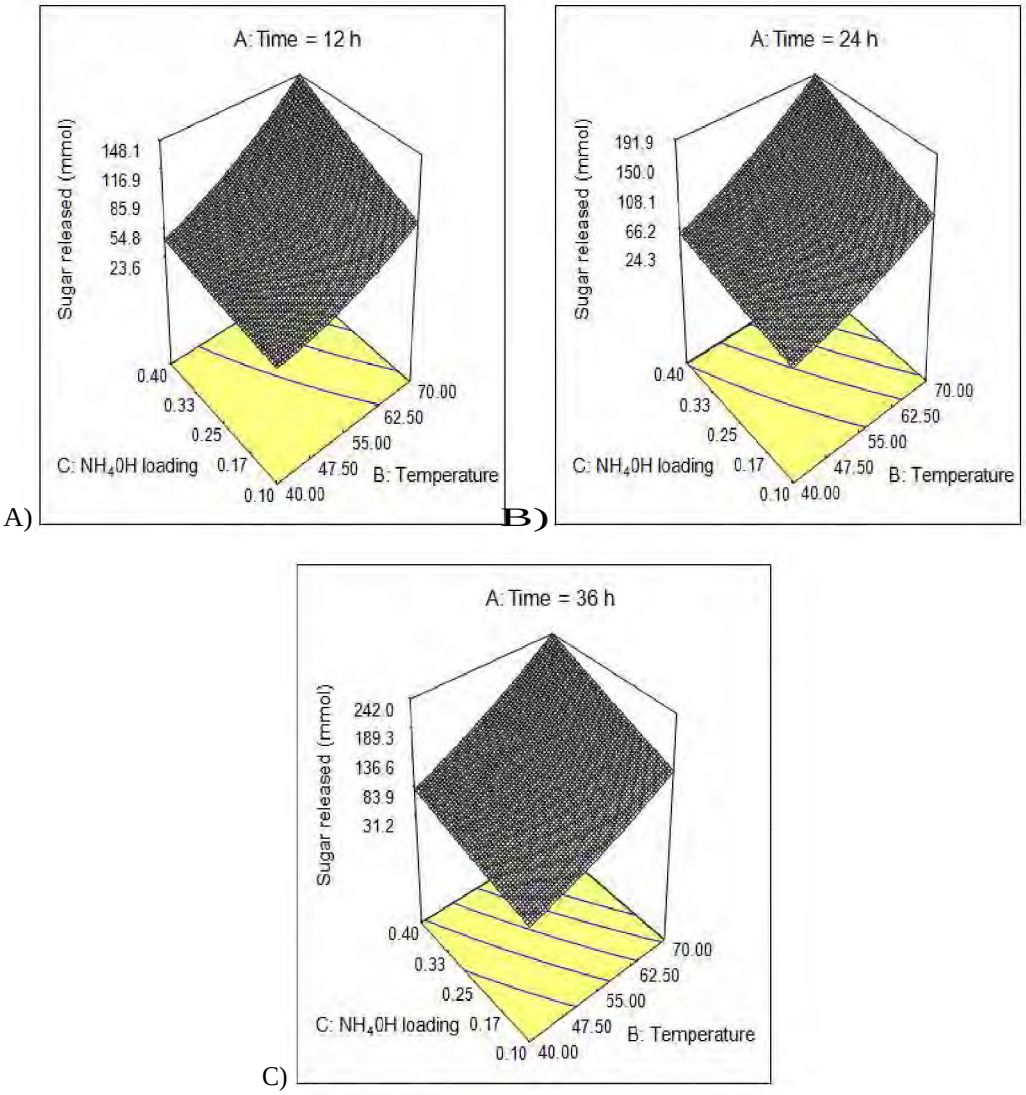


Figure 22: Effect of NH_4OH loading illustrated with the 3D perturbation plots. A) The first plot illustrates the effect of pre-treating the SCB with 0.10 g/g NH_4OH with regard to the production of sugar, B) the second 3D plot illustrates the effect of pre-treating the SCB with 0.25 g/g NH_4OH with regard to the production of sugar and C) the third 3D plot illustrates the effect of pre-treating the SCB with 0.4 g/g NH_4OH with regard to the production of sugar.

The data obtained from Figure 22 indicated that the largest of sugar was produced under conditions of 70°C and 36 hours; however, the maximal level of sugar was obtained using highest concentration of NH_4OH.

The second factor investigated was time (Figure 23). In this case the NH_4OH loading (y-axis) and the temperature (x-axis) were kept constant.



A) B) C)

Figure 23: Effect of time on the NH_4OH pre-treatment of SCB is illustrated with 3D perturbation plots. A) The first plot illustrates the effect of pre-treating the SCB for 12 hours with regard to the production of sugar, B) the second 3D plot illustrates the effect of pre-treating the SCB for 24 hours with regard to the production of sugar and C) the third 3D plot illustrates the effect of pre-treating the SCB for 36 hours with regard to the production of sugar.

The data obtained from the effect of time on the pre-treatment of SCB indicated that the greatest quantity of sugar was produced with the harshest conditions of 0.4 g/g SCB, 70°C and 36 hours (Figure 23). This was consistent with the optimal conditions that were observed when looking at the effect of NH_4OH loading on the pre-treatment of the lignocellulose.

Temperature was the third factor investigated to determine the effect of temperature on the NH_4OH pre-treatment of SCB and to determine the optimal pre-treatment conditions for the effective pre-treatment of SCB (Figure 24).

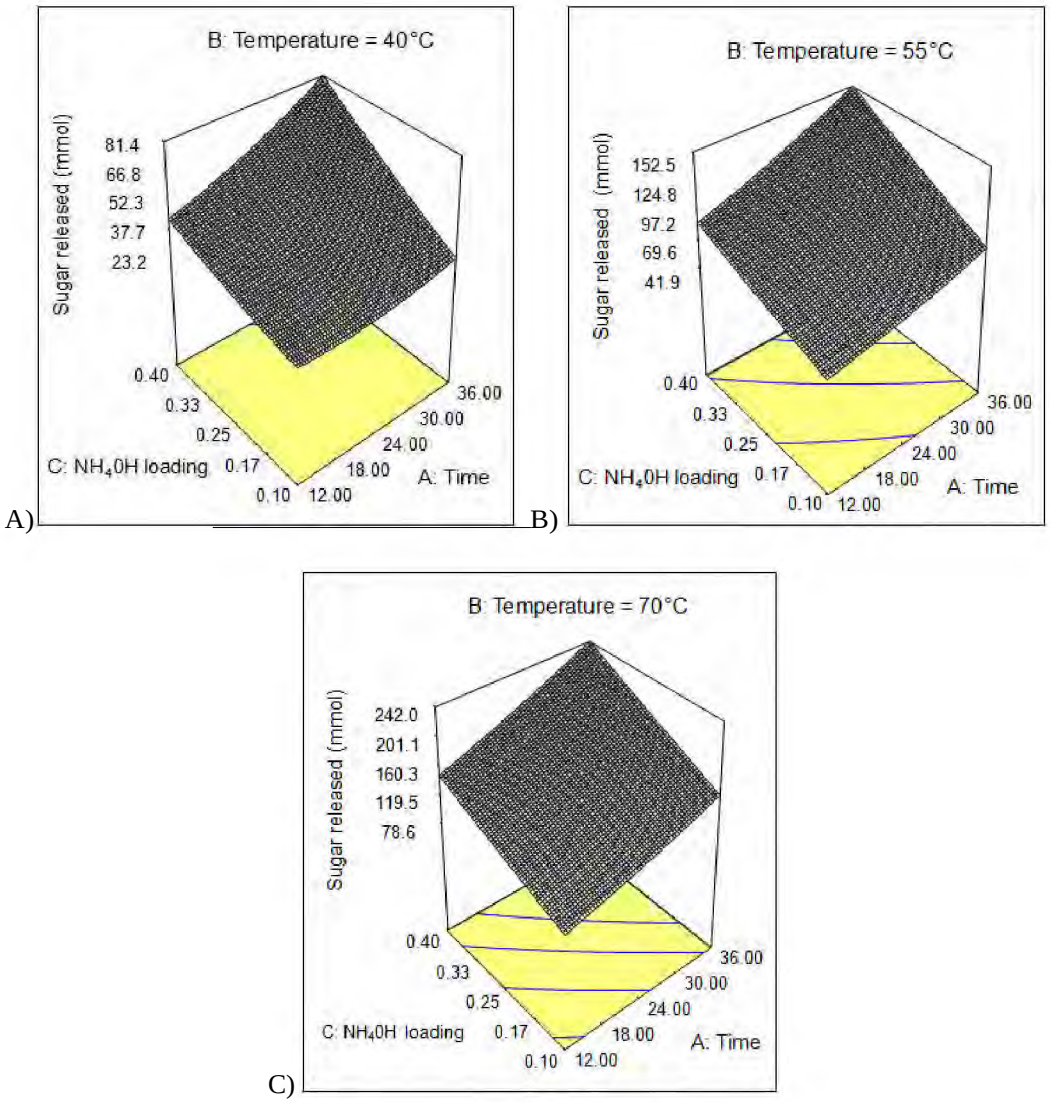


Figure 24: Effect of temperature on the NH_4OH pre-treatment of SCB illustrated with the 3D perturbation plots. A) The first plot illustrates the effect of pre-treating the SCB at 40°C with regard to the production of sugar, B) the second 3D plot illustrates the effect of pre-treating the SCB at 55°C with regard to the production of sugar and C) the third 3D plot illustrates the effect of pre-treating the SCB at 70°C with regard to the production of sugar.

Similarily to the effects of NH_4OH loading and pre-treatment time, the greatest amount of sugar was produced by the hydrolysis of the NH_4OH that was pre-treated for 36 hours, at 70°C using 0.40 g NH_4OH per gram of SCB to pre-treat the substrate (Figure 24).

3.3.2 Chemical characterisation of SCB

In order to determine the efficiency and the effect of the different alkaline pre-treatments on SCB, the determination of the chemical composition of the different samples was essential (Table 13).

Table 13: Comparison of the chemical composition of the different SCB samples

Pre-treated sample	Acid soluble lignin	Acid insoluble lignin	Total lignin	Arabinan	Glucan	Xylan	Total sugar
Untreated SCB	8.5	33.7	42.4	1.1	28.8	14.2	44.0
$Ca(OH)_2$	8.7	24.1	32.8	1	34.0	15.0	50.0
NaOH	Nd	15.6	15.7	0	42.9	17.5	60.4
NH_4OH	Nd	8.8	8.8	1.2	54.6	15.5	71.3

*Results are shown as a % on dry mass and had a mass balance greater than 75%. The acetate fraction represents the acetyl groups present on the arabinose and glucose in the hemicelluloses chains during the acid hydrolysis step.

From the data obtained on the chemical composition of the different SCB samples, it was observed that the chemical pre-treatments had an effect on the composition of the SCB, especially with regard to the removal of lignin (Table 13).

3.3.3 Microscopy

3.3.3.1 Scanning Electron Microscopy analysis

The dry SCB samples were mounted onto metal stubs and coated with a thin layer of gold under vacuum. The samples were analysed under various magnifications using SEM (Figure 25).

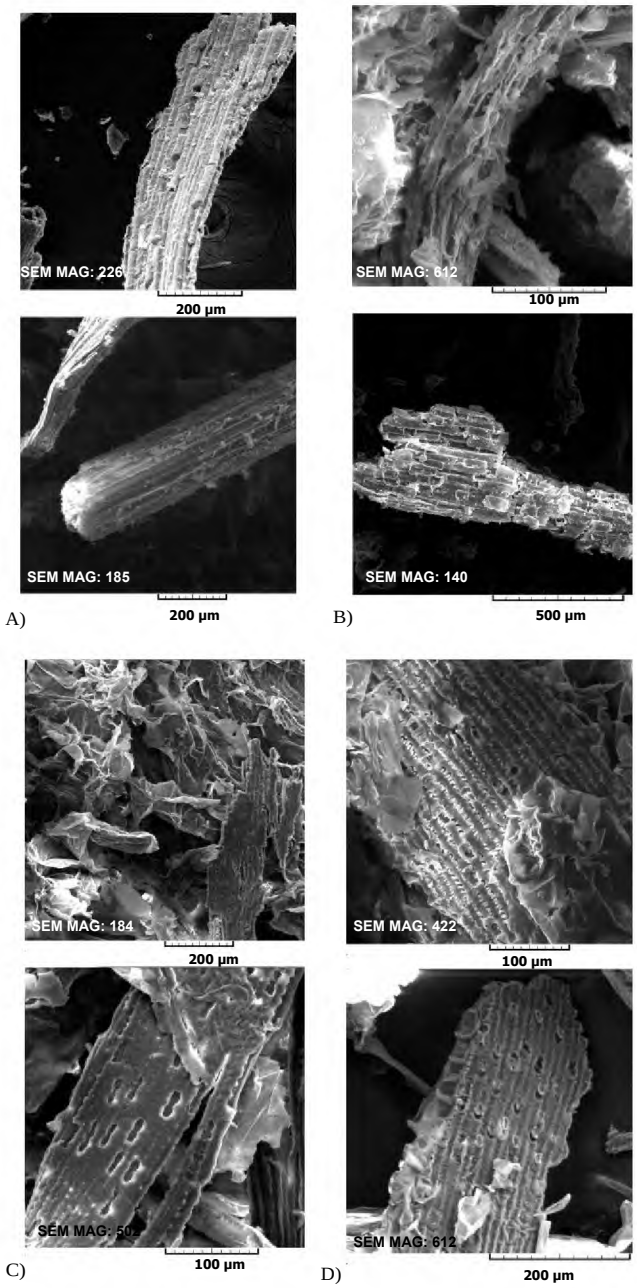


Figure 25: SEM analysis of A) untreated SCB, B) $Ca(OH)_2$ pre-treated SCB, C) NaOH pre-treated SCB and D) NH_4OH pre-treated SCB. The SCB samples were visualised and photographed at various magnifications.

The SEM analysis of the SCB samples after the various chemical pre-treatments (in comparison to the untreated SCB sample that was not exposed to any of the chemicals), indicated that the chemicals had an effect of the structure of the SCB samples (Figure 25).

3.3.3.2 Light microscopy

After the SCB samples were pre-treated with the different alkaline chemicals, the samples were stained for residual lignin (that was not removed with the pre-treatment process) using phlorogucinol (Figure 26).

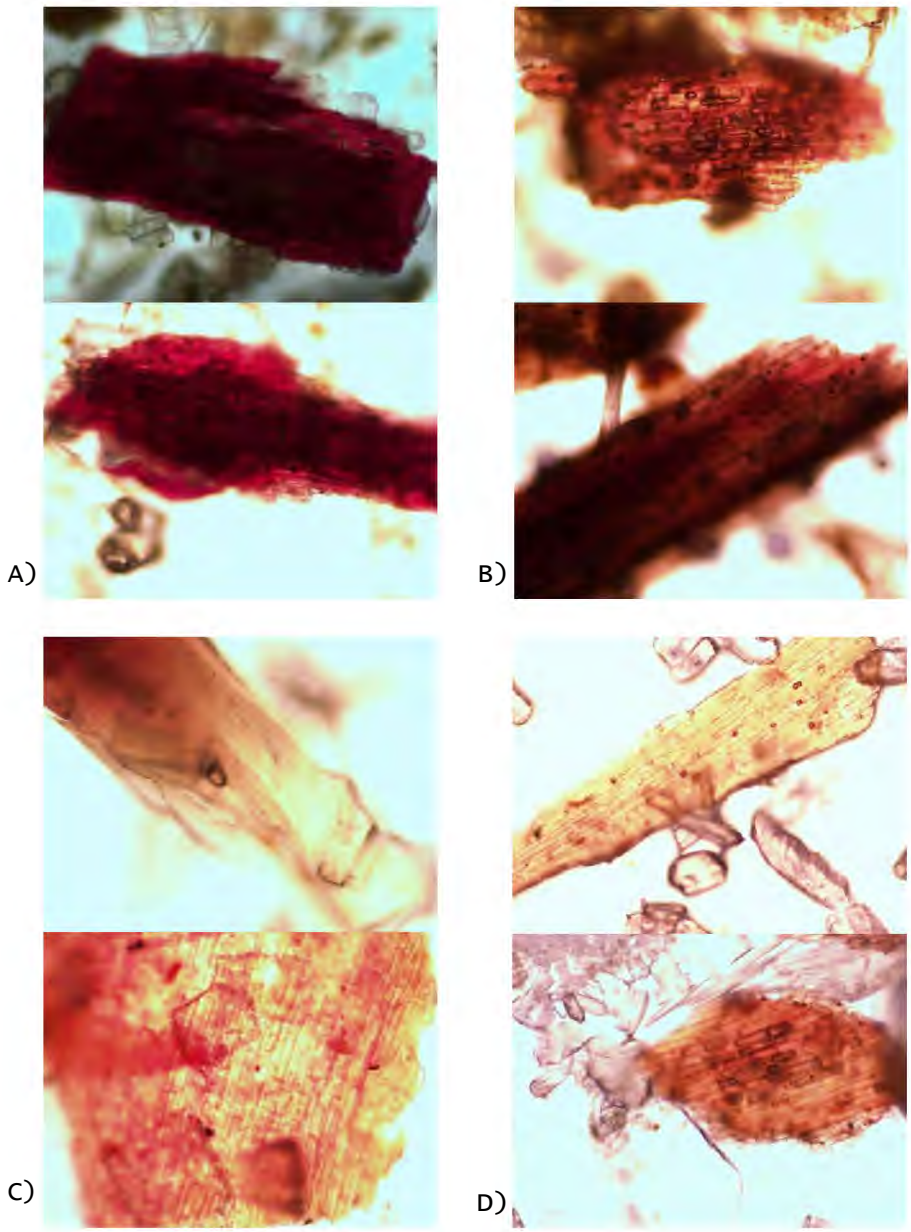


Figure 26: The stained SCB sample visualised at a 40 times magnification with an Olympus BX40 light microscope and photographed using an Olympus DP72 digital. A) Represent the untreated SCB, B) the $Ca(OH)_2$ pre-treated SCB, C) the NaOH pre-treated SCB, and D) the NH_4OH pre-treated SCB.

The light microscope photographs (Figure 26) taken after the SCB samples were pre-treated indicated that the pre-treatment had a distinctive effect on the SCB lignin, which is reflected in the decrease in magenta stain observed on the SCB samples (Figure 26).

3.4 Discussion

Over the last few decades, a great deal of research has been performed to find ways to increase the efficiency of the enzymatic hydrolysis of lignocellulose. However, despite obtaining the enhanced enzymatic hydrolysis of lignocellulose through synergistic relationships between different enzymes, the complete hydrolysis of lignocellulose has not been achieved due to the recalcitrant nature of plant biomass. The reduction of the recalcitrant nature of plant biomass resulting from various pre-treatments is widely documented; however, the choice of pre-treatment is largely dependent on the down stream process of interest.

The choice of pre-treatment, especially for industrial processes, is dependent on whether the pre-treatment method chosen is cost effective or not. The main cost effective lignocellulose pre-treatments for the purification and preparation of cellulose, employed in industry, include steam explosion, liquid hot water and dilute acids (Mosier *et al.,* 2005), which are documented to remove hemicellulose and alter the lignin structure, thus these pre-treatments were not suited for this particular study. Other chemical pre-treatments used in industry include $Ca(OH)_2$, AFEX and ARP, which have been documented to decrystallise the cellulose structure, alter and remove lignin and have a minimal affect on the hemicellulose structure of the lignocellulose substrate (Mosier *et al.,* 2005). However, despite alkaline pre-treatments having some advantages over other pre-treatments, literature has indicated, that the use of alkaline pre-treatments are less attractive for the production of ethanol, due to the possible loss of fermentable sugars and the production of inhibitory products (Hendriks and Zeeman, 2009). As mentioned previously, the chemical pre-treatments discussed in this chapter include $Ca(OH)_2$, NaOH and NH_4OH. In order to ensure that the optimal amount of sugar was produced by the synergistic hydrolysis of the pre-treated SCB, optimisation of the pre-treatment procedure was essential. The pre-treatment of SCB was set up as a factorial design using response surface methodology (RSM). This design used central point replicates within each factor (chemical loading / time / temperature) as shown in Table 3 and the data

was analysed statistically using the sum of squares (Tables 5, 8 and 11) and ANOVA (Tables 6, 9 and 12) to generate 3D perturbation plots (Figures 16 to 24). The data displayed how the different factors contribute to the model with increasing degrees of complexity, and as such, the analysed data can be fitted into one of the models in the model hierarchy. The 3D perturbation plots illustrated the effects of the different factors (concentrations of the chemical, time and temperature). The possible discrepancy illustrated in the ANOVA analysis of the data may potentially be related to the effect of using the harshest conditions to pre-treat the SCB samples.

The first pre-treatment optimised the use of $Ca(OH)_2$. The SCB was pre-treated using 27 different combinations of the three factors under investigation. The efficiency of the pre-treatment was determined by the production of sugar as a result of the hydrolysis of the different $Ca(OH)_2$ pre-treated SCB samples. The data obtained indicated that the greatest amount of sugar (39.03U) was produced by the hydrolysis of the $Ca(OH)_2$ pre-treated SCB was pre-treated with 0.40 g $Ca(OH)_2$/g SCB for 12 hours at 70°C. The data obtained from the sum of squares from the hydrolysis of the $Ca(OH)_2$, indicated that the model that best suited the data was a two factor interaction (2FI) model. Thus, for the subsequent design analysis of the data obtained from the hydrolysis of the $Ca(OH)_2$ pre-treated SCB, a 2FI model was used. The ANOVA analysis of the pre-treatment data verified the model used to examine the effects of the different factors. For a confidence level of 95% to be obtained for the factors that represent the 2FI model, the probability factor would be required to be less than 0.5. The ANOVA analysis of the hydrolysis of the $Ca(OH)_2$ pre-treated SCB (Table 6) indicated that all the factors with the exception of the factor BC (representing temperature vs. $Ca(OH)_2$ loading), had a significant effect on the model. With regard to the 2FI model, temperature (B) appeared to have the greatest effect on the model with a F-value of 63.93, thus indicating that the discrepancy observed with the probability factor of the factor BC may have occurred as an effect of the concentration of $Ca(OH)_2$ used in the pre-treatment process. The perturbation plots for the response surface designs illustrated any changes in the models response (sugar production) in relation to the effect of one of the factors, when the other factors were kept constant.

Similarily to the ANOVA analysis of the $Ca(OH)_2$ pre-treatment, the perturbation plots illustrated the significance of the different factors used in the pre-treatment of SCB.

The perturbation plots depicted the effect of $Ca(OH)_2$ concentration on the pre-treatment of SCB, produced similar quantities (32.4 – 33.5 mmol) of sugar was produced when the SCB was pre-treated for 36 hours at 70°C using 0.25 g $Ca(OH)_2$ /g bagasse or less. However, when SCB was pre-treated with 0.40 g $Ca(OH)_2$ /g bagasse at 70°C for the shortest period of time (12 h), the greatest amount of sugar was produced by the hydrolysis of SCB sample. Potentially indicated that these $Ca(OH)_2$ concentrations may not have a significant effect on the pre-treatment design. The same trend was expected to have been observed, when using a longer digestion time. However, the 3D perturbation plot for the effect of the 0.40 g $Ca(OH)_2$ /g bagasse indicated that the production of sugar decreased with an increase in the digestion time and temperature; thus, if a low temperature is kept constant, the production of sugar would increase with time. The data obtained from the effect of time on the $Ca(OH)_2$ pre-treatment of SCB also indicated that if the pre-treatment process was performed for longer than 24 hours, a decrease in the concentration of $Ca(OH)_2$ is required. At 40°C and a pre-treatment time of 36 hours, the hydrolysis of the SCB samples produced a similar quantity of sugar regarless of the concentration of $Ca(OH)_2$ used in the pre-treatment process (Figure 18). The data also indicated that if the pre-treatment of SCB occurred over a 36-hour period, the concentration of $Ca(OH)_2$ required, would decrease with an increase in temperature, i.e. pre-treatment at the lower temperature required a longer pre-treatment time.

The second alkaline chemical used to pre-treat SCB was NaOH. NaOH is widely used in the paper and pulp industry to prepare wood chips and other plant materials for the production of paper. NaOH in conjuction with sodium sulphide is utilised in the Kraft process, to remove lignin from plant material. Thus, the general effect of the chemical on the chemical composition with regard to sugar content is not regarded as important, as long as the lignin is removed from the biomass (Chakar and Ragauskas, 2004). The pre-treatment was set up as described for the $Ca(OH)_2$ pre-treatment study (Table 3). As in the case with the $Ca(OH)_2$ pre-treatment, the efficiency of the NaOH to pre-treat SCB is reflected in the production of sugar with the hydrolysis of the pre-treated samples (Table 7) and analysed using Design-Expert 6.0 using RSM and a central composite design. A sequential model sum of squares was obtained (Table 8). The data obtained from the sum of squares indicates that the model that best suited the data was a quadratic model. The ANOVA analysis of the NaOH pre-treatment data was used to verify the model that is used to examine the effects of

the different factors (Table 9). As seen from the ANOVA table (Table 9) the factors that had a significant effect on the model were the concentration of NaOH (factor represented by C), A^2, B^2, C^2 and BC. With regard to this model, the quadratic factor of temperature (B^2) appeared to have the greatest effect on the model a F-value of 98.63. As in the case with $Ca(OH)_2$ pre-treated SCB, the effect of the different factors on the NaOH pre-treatment of SCB was illustrated in 3D perturbation plots (Figures 19 - 21). The first set of 3D plots illustrated the effect of the different factors on the pre-treatment of SCB. These results indicated a "bell-shaped" curve, where the highest amount of sugar was produced around the middle range of the different factors. Thus, the contour lines indicated the subtle differences between the different plots. The contour lines for the effect of 0.25g NaOH / g SCB illustrated the broadest and most central contour line, indicating the production of sugar occurred optimally with 0.25g NaOH / g SCB, around 24 hours and 55°C. Similar results were obtained for the 3D plots representing the effects of time (Figure 20) and temperature (Figure 21). All the purturbation plots indicated that the maximal quantity of sugar was produced when the SCB was pre-treated for 0.25g NaOH /g bagasse at 55°C for 24 hours. Literature indicated that NaOH is used in high concentations to pre-treat lignocellulose for the removal of lignin and the enhancement of the cellulolytic hydrolysis of the pre-treated lignocellulose (Damisa *et al.*, 2008).

The final alkaline pre-treatment used was NH_4OH. Similar to the $Ca(OH)_2$ and NaOH pre-treatments, the efficiency of the NH_4OH pre-treatment was related to the production of sugar from the hydrolysis of the pre-treated SCB sample. The data obtained from the hydrolysis of the pre-treated SCB samples was analysed using Design-Expert 6.0 using RSM and a central composite design, a sequential model sum of squares was obtained (Table 11). As in the case with the data obtained with the hydrolysis of the NaOH pre-treated SCB, the sum of squares for the NH_4OH pre-treated SCB indicated that the model that best suits the data was a quadratic model. The data obtained from the hydrolysis of the pre-treated SCB samples also indicated that the recalcitrance of the SCB decreased with an increase in NH_4OH loading, temperature and pre-treatment time. Thus the hydrolysis of SCB that was pre-treated with 0.40 g NH_4OH /g bagasse, for 36 hours at 70°C produced the greatest amount of sugar. The ANOVA analysis of the NaOH pre-treatment data verified the model that was used to examine the effects of the different factors (Table 12). As seen from the

ANOVA table (Table 12) all the factors, with the exception of A^2 and C^2, had a significant effect on the model. With regard to this model, the quadratic factor of temperature (B) appeared to have the greatest effect on the model with the F-value of 810.01, implying that there is only a 0.01% chance that this F-value could have occurred due to noise, thus indicating the importance of all the factors on the pre-treatment of SCB. The 3D perturbation plots illustrated the effect of using different factors on the pre-treatment of the SCB. These plots also indicated that regardless of which factors were analysed, the maximal amount of sugar produced occured when the highest factor conditions (i.e. harshest conditions) to pre-treat the SCB samples was employed.

The SCB samples were dewaxed through three extraction steps using hexane, acetone and ethanol with a Soxhlet apparatus. The extracted material was dried and the samples were hydrolysed with 72% (v/v) sulphuric acid and autoclaved to remove the sugar from the samples. After the hydrolysis the fractions were filtered to remove the insoluble lignin from the sugar solutions. The acid soluble lignin present in the sugar solutions were detected by UV-Vis spectroscopy (Hyman *et al.,* 2007).

Chang and Holtzapple indicated that $Ca(OH)_2$ and other alkaline pre-treatments increase the accessibility of lignocellulose to enzyme hydrolysis by removing the acetyl and uronic acid substituents that may be present on the hemicellulose fraction of lignocellulose (Chang and Holtzapple, 2000). The lignocellulosic pre-treatment with $Ca(OH)_2$ is an effective means to increase the surface area of the lignocellulose, exposing the cellulose structure to enzymes (Mosier *et al.,* 2005). This increase in the accessibility to the cellulose and hemicellulose is believed to be due to $Ca(OH)_2$ altering the structure of lignin, so that the lignin is solubilised and can be removed from the lignocellulose. This was confirmed when comparing the chemical composition of the normal (untreated) and $Ca(OH)_2$ pre-treated bagasse (Table 13).

Unlike the use of steam and acid pre-treatments, alkaline pre-treatments e.g. $Ca(OH)_2$ have been found to solubilise lignin and a small percentage of the hemicellulose (Chang, 2007; Kaar and Holtzapple, 2000). The $Ca(OH)_2$ pre-treated bagasse has approximately 10% less lignin and a higher quantity of sugar relative to percentage of dry mass than that of the untreated lignin (Table 13). As depicted in Table 13, the percentage of arabinose present in

the pre-treated bagasse was less than that found in the untreated bagasse. Table 13 potentially illustrates that the $Ca(OH)_2$ pre-treatment partially solubilised and removed some of the lignin and the arabinan substituents present on the hemicellulose backbone, thus increasing the exposure of the lignocellulose structure to enzymatic hydrolysis. The increase in the solubilisation of the lignin moiety and the removal of the arabinan substituent from the bagasse was expected, since similar results using $Ca(OH)_2$ pre-treatment were obtained by Chang (2007), and Kaar and Holtzapple (2000).

The second chemical used to pre-treat SCB was NaOH. This chemical is widely used in the paper and pulp industry for its ability to aid in the bleaching of paper and in the Kraft process; however, the effect of NaOH on the chemical composition of SCB has not really been established. From the data obtained from the chemical compositions of the different SCB samples (Table 13), NaOH successfully appeared to remove approximately 63% of the lignin present in the SCB, subsequently increasing the relative percentage of the carbohydrate moiety present in the SCB. Unlike the effect of the $Ca(OH)_2$ on the chemical composition of SCB, the NaOH appeared to have removed all the arabinan from the sample. In 1994, Kivaisi and Eliapenda compared the pre-treatment efficiency of HCl, NH_4OH and NaOH to increase the anaerobic digestibility of SCB and coconut fibres. They made use of 1 M solutions of HCl, NH_4OH and NaOH in their pre-treatment protocols. The pre-treatment consisted of incubating the SCB and coconut fibre at 25°C (room temperature) and 30°C, respectively. The results of the pre-treatment study indicated that the fibre digestibility increased by 14% and 11% with NH_4OH and NaOH, respectively. Ju *et al.* (2010) pre-treated the SCB in two steps. For the first step, 1.5 g of SCB was added to 10 ml of different concentrations of NaOH (1- 4 M). The SCB and NaOH mixtures were sterilized at 121°C and 1.5 bar for 30 min. With the second step, the sterilized mixtures were incubated, shaking at 120 rpm for 2 hours at room temperature. The high concentrations of NaOH resulted in the removal of a substantial amount of the hemicellulose and lignin from the SCB. The results also indicated that by increasing the concentrations of NaOH, larger quantities of the hemicellulose and lignin phenolates were removed. However, the glucose that was detected in the liquid fractions produced from the pre-treatment remained more or less constant regardless of the NaOH concentration that was used (Ju *et al.*, 2010). Prior and Day (2008) pre-treated SCB with 0.5 g NH_4OH of a 28% (v/v) per gram of dry biomass at 160°C for 60 min. Their results

(Table 14) indicated that this pre-treatment did not have a dramatic affect on the chemical composition of their SCB; however, the pre-treatment did increase the relative percentage of the glucan moiety.

Table 14: Chemical composition of SCB analysed by Prior and Day (2008)

Sample	Lignin (%)	Glucan (%)	Xylan (%)	Arabinan (%)
SCB (untreated)	25.00	38.50	24.10	1.90
NH_4OH pre-treated SCB	21.00	56.65	24.00	1.20

In contrast, our study indicated a dramatic change in the chemical composition of the SCB samples after optimal NH_4OH pre-treatment conditions were used to pre-treatment the SCB. The total percentage of lignin decreased from 42.4% to 8.8%, thus the percentage of the sugar moiety increased from 44.0% to 71.3%, relative to the total chemical composition of the SCB samples. The data obtained from the chemical composition of the NH_4OH pre-treated samples indicated that the carbohydrate moiety, was not affected by the pre-treatment conditions as indicated by the increase in the relative percentage of the carbohydarate composition. Thus, corresponding to what is reported in literatureregarding the use of the different alkaline chemicals to pre-treatment the lignocellulose.

The external structure of the SCB samples and the effects of the different pre-treatments on the SCB were visualised using SEM (Figure 25) and light microscopy (Figure 26). It has been found that alkaline solutions can solubilise, redistribute and condense lignin (Gregg and Saddler, 1996). Thus, the potential increase in the percentage acid soluble lignin after NH_4OH and NaOH pre-treatment may be due to this phenomenon. During the pre-treatment step, lignin underwent solubilisation, resulting in the formation of lignin monomers. The monomers may have subsequently redistributed and condensed producing phenolate complexes similar to the acid insoluble lignin monomers. The monomer would produce the newly formed phenolate complexes via a variety of different chemical bonds to generate potential more lignin complexes. The removal of lignin in both cases resulted in greater accessibility of the enzymes to the SCB holocellulose moiety.

The alkaline pre-treatments that were performed removed a substantial amount of the lignin in the SCB samples. Thus, it was thought that the remaining acid insoluble lignin, and some of the acid soluble lignin in the case of the NaOH pre-treated sample, was partially

solubilised and subsequently redistributed within the pre-treatment vessel, since the vessel was constantly being shaken. After the various pre-treatments, the solutions were cooled down. The decrease in the temperature may have resulted in the solubilised lignin phenolates condensing and reforming on a different area on the SCB samples. In order to test this theory, the SCB samples were stained with a phloroglucinol:HCl histochemistry stain (Figure 26). The phloroglucinol:HCl stain reacts with aromatic aldehydes that constitute lignin, which is detected by the development of a magenta/ deep purple (Stange *et al.,* 2001). As expected, the normal sample showed the greatest reaction to the stain, thus indicating the presence of aromatic aldehydes in the middle lamella, cell wall and the lignin sheath surrounding the holocellulose moiety (Figure 26A). As expected, the SCB samples that underwent alkaline pre-treatment did not react to the stain to the same extent as the normal SCB, thus indicating that the alkaline pre-treatments partially removed the middle lamella and the lignin surrounding the SCB cell wall (Figures 26B - D); however, in comparison to the NaOH and NH_4OH pre-treated samples (Figure 26C and D), the staining on the $Ca(OH)_2$ pre-treated SCB sample indicated the presence of a greater percentage of lignin (Figure 26B). These results also confirmed the results obtained in Table 13. Figure 26C shows the redistribution of the lignin, confirming the hypothesis that the NaOH pre-treatment conditions resulted in the solubilisation, redistribution and condensation of the lignin phenolates on the SCB after alkaline pre-treatment.

3.5 Conclusions

This chapter described and discussed the optimisation of three alkaline pre-treatments for the purpose of enhancing the hemicellulosic hydrolysis of the different SCB samples. Literature indicates the use of chemical pre-treatments to enhance the enzymatic (cellulase action) hydrolysis of different types of lignocellulose for the production of bioethanol. Thus, to enhance the celluase action, the purpose of those pre-treatment processes was generally to delignify and remove the hemicellulose component of the biomass. As mentioned previously, the choice of pre-treatment is determined by the downstream process that is intended. In this study, the hydrolysis of the hemicellulose was of interest. In conclusion, the pre-treatment of the SCB samples was successful; however, the NH_4OH pretreatment removed the largest percentage of lignin, followed by NaOH and finally, $Ca(OH)_2$. The optimal pre-treament conditions for the different chemical treatments were as follow: $Ca(OH)_2$: 0.40 g $Ca(OH)_2$ /

gram dry SCB at 70°C for 12 hours, NaOH: 0.25 g NaOH / gram dry SCB at 55°C for 24 hours, and NH_4OH: 0.40 g NH_4OH / gram dry SCB at 70°C for 12 hours.

This chapter discussed the utilization of alkaline chemicals to pre-treat SCB and the various effects the chemical pre-treatments had on the composition and structure of SCB. The next chapter discusses the synergistic enzymatic degradation of untreated and pre-treated SCB samples and identifies the effects of the pre-treatments on the combination of enzymes required to efficiently hydrolyse the SCB samples.

Chapter 4: Synergistic enzymatic degradation of sugarcane bagasse

4.1 Introduction

Globally, there has been an exponential surge in the research and development into the discovery and improvement of alternative, environmentally friendly sources of energy to decrease the general dependency and eventual replacement of fossil fuels (Waclawovsky *et al.,* 2010). At present, bioethanol is produced by fermenting the resulting sugars and starch from the partial hydrolysis of different feedstocks; however, the majority of bioethanol is produced from sugarcane and maize (Waclawovsky *et al.,* 2010). The use of maize for bioethanol production is problematic essentially because it is a food crop. With the exception of sugarcane, research into the potential use of sugar beet (*Beta vulgaris*), switch grass (*Panicum virgatum*) and miscanthus (*Miscanthus gigantus*) as biofuel feedstocks is gaining interest. These crops are being studied because they grow to maturity quickly in a cultivation season. They are to a certain extent, drought tolerant, and do not require high temperatures or nutrient inputs, making these biomass feedstocks robust and relatively easy to cultivate (Waclawovsky *et al.,* 2010). Sugarcane is a lignocellulosic perennial grass that is cultivated and harvested primarily for its sucrose content, which can be fermented into ethanol (Lee *et al.,* 2009). After the sugarcane has been harvested, the stalks are crushed to extract the sucrose. The pith and the other solid plant material left after the extraction of sucrose is known as bagasse. The bagasse is subsequently burnt to produce steam and electrical energy; however, the surplus bagasse is not utilised as fuel and is stock piled (Han and Wu, 2004; Rowell and Keany, 1991).

As mentioned previously, the recalcitrant nature and subsequent chemical structure of lignocellulosic biomass is an important feature to consider, if it is to be used industrially for the production of biofuels. Extensive research into the development of an effective means to hydrolyse lignocellulose has been performed; however, the complete hydrolysis of lignocellulose has not been achieved. The inability to completely hydrolyse biomass has led to the development of a variety of pre-treatment strategies that affect the lignocellulose structure. The pre-treatment conditions may increase the surface area of the different components, and may partially or completely remove components of the plant cell walls, e.g. hemicellulose or lignin (Mosier *et al.,* 2005). The choice of the pre-treatment is driven

through the consideration of four main factors: (i) increasing the production of reducing sugars by enzymatic hydrolysis, (ii) preventing the loss of hydrolysable carbohydrates, (iii) preventing the formation and accumulation of inhibitory by-products from the use of the different pre-treatments, and (iv) the pre-treatment strategy(ies) themselves e.g. acid pre-treatments would partially remove both the lignin and a portion of the hemicellulose component of the biomass (Sun and Cheng, 2002).

The main feature of lignocellulose to be considered when using alkaline pre-treatment strategies is the lignin content (Fan *et al.,* 1987; McMillan, 1994). Alkaline solutions are believed to affect the ester bonds that are responsible for the cross-linking between the hemicellulose backbone and lignin (Tarkow and Feist, 1969). The addition of alkaline solutions cause the ester bonds to undergo saponification, and due to the removal of the cross-linking bonds, the porosity of the lignocellulosic material increases (Tarkow and Feist, 1969).

Synergism has been observed in several free enzyme systems; however, the synergy that exists in cellulase systems is the most established. Synergism facilitates an enhanced hydrolytic activity of the enzymes being studied, where the resulting activity is greater than the collective sum of the individual enzyme activities. The synergism that has been observed in cellulase systems have been classified into four groups. These groups are described as being: (i) between endo- and exoglucanases, (ii) between exoglucanases, (iii) between exoglucanases and β-glucosidases, and (iv) between catalytic domains and carbohydrate binding modules (CBMs) (Din *et al.,* 1994; Teeri, 1997). The majority of synergistic systems that have been studied were obtained and purified from aerobic micro-organisms; however, research into synergy in anaerobic environments has grown exponentially with the discovery of the cellulosome.

This study made use of alkaline delignification to remove the majority of the lignin present on the SCB. The partially delignified SCB samples were subsequently treated with varying protein ratios of the purified hemicellulases and an ancillary enzyme. Varying protein ratios of the enzymes would identify the presence of potential synergistic associations between the three enzymes that would enhance the hydrolysis of the SCB samples. The synergy studies were performed in order to establish the optimal combination of the

hemicellulolytic enzymes ArfA, ManA and XynA that would facilitate the efficient means to degradation of native (untreated) and alkaline pre-treated SCB for subsequent artificial/designer cellulosome construction.

4.2 Methods and materials

4.2.1 Expression and purification of recombinant C. cellulovorans enzymes

Three cultures of *E. coli* BL21 (DE3) cells that had been transformed (Appendix B) with one of the recombinant plasmids *pET29-arfA*, *pET 29b-manA* and *pET 29b-xynA* were used to innoculate 2 × YT broth producing the different 5 ml pre-inocula. *E. coli* BL21 (DE3) was grown in 5 ml 2 × YT broth for 12 h at 37ºC, shaking at 200 rpm on a Labcon bench shaker. The pre-inocula were used to inoculate 500 ml of 2 × YT broth, which was incubated at 37°C, shaking at 200 rpm, until an optical density of 0.8 at 600 nm for ArfA and 0.6 at 600 nm was obtained for ManA and XynA, respectively. The recombinant enzymes were expressed after the cultures were induced with the addition of 1 mM IPTG. The cultures were incubated at 18°C for 16 h (Murashima *et al.,* 2002b). The recombinant proteins were purified from the cultures as follows (Appendix C): *E. coli* BL21 (DE3) cells harbouring the recombinant plasmids were harvested by centrifugation at 8, 000 × g for 20 minutes at 4ºC and resuspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 1 mg lysozyme/ml, pH 8.0). The solution was incubated on ice for 30 minutes allowing for partial cell lysis, and the soluble enzymes were subsequently extracted from the cells by sonication. The cells were sonicated 3 times for six seconds with alternate 6 ten second rest intervals. The sonicated cultures were centrifuged at 10, 000 × g for 20 minutes at 4ºC. The enzyme lysates were applied to Protino® Ni-TED (Macherey-Nagel) columns. The flow-through was collected, with two 10 ml washing steps using the washing buffer (50 mM NaH_2PO_4, 300 mM NaCl, pH 7.0), to remove the unbound proteins. The His-tagged enzymes bound to the Ni-NTA columns were eluted with 10 ml elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 7.0) (Murashima *et al.,* 2002b).

4.2.2 Discontinuous SDS-PAGE

Protein samples collected throughout the purification were resolved using SDS-PAGE to determine purity according to the protocol outlined in Mini-Protean® 3 Cell instruction

manual (Bio-Rad, U.S.A.). This protocol (Appendix D) is a modification of that described by Laemmli (1970). The samples collected throughout the purification were added to the SDS sample buffer (0.0625 M Tris-HCl, pH 6.8, 10 % (v/v) glycerol, 2% (v/v) SDS solution, 5% (v/v) β-mercaptoethanol, 0.05% (v/v) bromophenol blue) in a 2 to 1 ratio. The samples were subsequently incubated at 100°C for 5 minutes on a Labnet dry bath. The protein samples were resolved using a 12 % acrylamide resolving gel (Appendix D) for approximately 40 minutes at a constant voltage of 200 V in 500 ml ice cold, 1 × SDS-PAGE running buffer (25 mM Tris-HCl pH 8.8, 192 mM glycine, 1% (w/v) SDS). The SDS-PAGE gels were stained with Coomassie protein stain (0.1% (w/v) Coomassie Brilliant Blue G 250 in 45% methanol and 10% glacial acetic acid) for a minimum of 2 hours. The excess and unbound Coomassie stain was removed with destain solution (45% methanol, 45% distilled water, 10% glacial acetic acid).

4.2.3 Protein determination

The protein concentrations of the different samples obtained during the purification were determined using a protocol based on the method of Bradford (1976) using a protein standard curve and bovine serium albumin (BSA) as the reference protein (Appendix E). The protein concentrations were determined by mixing 5 µl of the various protein samples to 225 µl of the commercial Bradford reagent (Sigma). The protein solutions were incubated at room temperature for 5 minutes to facilitate the binding between the Coomassie Brilliant Blue G250 present in the Bradford reagent to the basic amino acids, arginine and lysine. The samples were shaken gently for 30 seconds in the Powerwave-X spectrophotometer, and the change in colour was determined by measuring the absorbance at 595 nm with Kc Junior software. The standard curve and the data was analysed in Microsoft Excel®.

4.2.4 Synergy assays

To elucidate the presence of synergistic relationships between the two recombinant enzymes, four enzyme combinations were assayed, namely: ArfA (A) and ManA (M); ArfA and XynA (X); ManA and XynA, and a combination of all three enzymes (Table 15). The possible synergistic associations between the hemicellulolytic enzymes were elucidated by combining various protein ratios of the hemicellulolytic enzymes. The degree of synergy that

was obtained was calculated by dividing the sum of the observed activities of the recombinant enzymes obtained with the bagasse, by the theoretical sum of the individual recombinant enzyme activities observed.

Table 15: Enzyme combinations used for the synergy studies involving the three recombinant enzymes

Enzyme protein ratios in the assay combinations (%)	X (µl)	M (µl)	A (µl)	Final enzyme volume (µl)	Enzyme protein ratios in the assay combinations (%)	X (µl)	M (µl)	A (µl)	Final enzyme volume (µl)
X87.5 M12.5	35	5		40	X75 M12.5 A12.5	30	5	5	40
X75 M25	30	10		40	X62.5 M25 A12.5	25	10	5	40
X62.5 M37.5	25	15		40	X50 M37.5 A12.5	20	15	5	40
X50 M50	20	20		40	X37.5 M50 A12.5	15	20	5	40
X37.5 M62.5	15	25		40	X25 M62.5 A12.5	10	35	5	40
X25 M75	10	30		40	X12.5 M75 A12.5	5	30	5	40
X12.5 M87.5	5	35		40	X12.5 M62.5 A25	5	25	10	40
M87.5 A12.5		35	5	40	X12.5 M50 A37.5	5	20	15	40
M75 A25		30	10	40	X12.5 M37.5 A50	5	15	20	40
M62.5 A37.5		25	15	40	X12.5 M25 A62.5	5	10	25	40
M50 A50		20	20	40	X12.5 M12.5 A75	5	5	30	40
M37.5 A62.5		15	25	40	X25 M12.5 A62.5	10	5	25	40
M25 A75		10	30	40	X37.5 M12.5 A50	15	5	20	40
M12.5 A87.5		5	35	40	X50 M12.5 A37.5	20	5	15	40
A87.5 X12.5	5		35	40	X62.5 M12.5 A37.5	25	5	10	40
A75 X25	10		30	40	X50 M25 A25	20	10	10	40
A62.5 X37.5	15		25	40	X37.5 M37.5 A25	15	15	10	40
A50 X50	20		20	40	X25 M50 A25	10	20	10	40
A37.5 X62.5	25		15	40	X25 M37.5 A37.5	10	25	15	40
A25 X75	30		10	40	X25 M25 A50	10	10	20	40
A12.5 X87.5	35		5	40	X37.5 M25 A37.5	15	10	15	40

Once the optimal protein ratios of the enzymes with the different substrates (locust bean gum (LBG): oatspelt xylan (OSX), untreated SCB and pre-treated SCB) were identified, the synergy assays were repeated in order to determine if the synergistic degradation of the different substrates were sequential or simultaneous in nature. The final set of assays performed investigated the effect of time on the synergy assays with regard to the degree of synergy and the activity (i.e. the release of reducing sugar).

4.2.5 General enzyme assay composition

In order to allow a direct comparison between the enzymes activities obtained, the protein concentration (mg/ml) of the enzymes used in the enzyme assays was standardised to the same protein concentration (mg/ml). The molarities of the enzymes were subsequently calculated to be: 0.35 mM ArfA (A), 0.42 mM ManA (M) and 0.33 mM XynA (X). For each of the different assays, 40 ~l of the purified enzyme, 100 ~l of the soluble model substrate LBG:OSX or SCB (untreated and pre-treated) and 50 mM citrate buffer, pH 5.5 (660 ~l of the buffer for the soluble substrate or 760 ~l of the buffer for the complex substrates) was used. The substrates and the buffer were incubated at 40ºC for 5 minutes prior to the addition of the respective purified enzymes. The reaction was performed at 40ºC for 30 minutes for the soluble substrates and for 120 hours for the complex substrates. The reactions were stopped by centrifugation at 16, 000 × g for 1 minute in a Heraeus Biofuge pica micro centrifuge.

4.2.6 Quantification of reducing sugars

To 300 µl of the various concentrations of reducing sugar, 600 µl DNS reagent was added to stop the enzyme reaction. The DNS and reducing sugar mixture was incubated at 100°C on the Labnet dry bath for 5 minutes to develop the colour of the samples, and incubated on ice for 10 minute to stop the colour development. The standard curves (Appendix E) were generated by measuring the change in absorbance, which related to the development of the colour in the different solutions at 540 nm with the Power Wave-X spectrophotometer using Kc Junior software. The standard curves and the data obtained for the enzyme assays were analysed in Microsoft Excel®. The enzyme assays were performed in

triplicate, and the activities were expressed in units (U), where 1 unit was defined as the quantity of enzyme required to release 1 µmol of reducing sugar per min.

4.3 Results

4.3.1 Expression and purification of the C. cellulovorans enzymes

The IPTG induced expressed recombinant proteins were purified using nickel affinity chromatography. The proteins were purified to produce single bands ranging between 47 kDa and 57 kDa, depending on the protein that was being purified (Figures 27-29). The efficiency of the purification of the different proteins was assessed on 12% reducing SDS–PAGE. The first recombinant enzyme that was expressed and purified was ArfA (Figure 27).

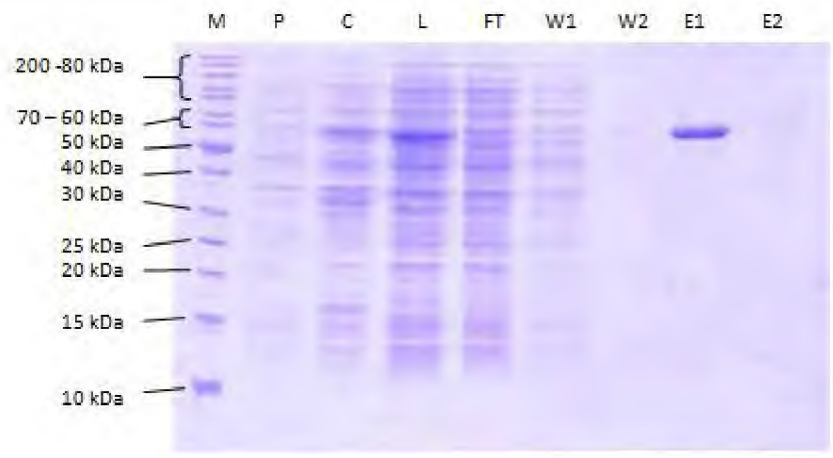


Figure 27: SDS-PAGE for the purification of ArfA. ArfA was induced with the addition of IPTG to a final concentration of 1 mM, incubated at 18°C for 16 h and purified using nickel affinity chromatography. The purification fractions were electrophoresed on a 12% SDS-PAGE gel. The lanes are marked as follows: molecular marker (M), pre-induction sample (P), crude sample after IPTG induction (C), clear enzyme lysate (L), flow through (FT), wash 1 (W1), wash 2 (W2), eluate 1 (E1) and eluate 2 (E2).

The efficiency of the nickel affinity purification of the His-tagged ArfA (Figure 27) indicated that the ancillary enzyme was expressed after the addition of 1 mM IPTG. The His-tagged ArfA bound to the Ni-NTA resin. Contaminating *E. coli* native proteins were removed from the column by washing the column with buffer containing no imidazole. ArfA was eluted from the nickel affinity column with the addition of 250 mM imidazole. As expected, ArfA had a molecular weight of approximately 57 kDa.

The second protein that was expressed and purified was ManA (Figure 28). The efficiency of the nickel affinity purification of the His-tagged ManA (Figure 28) indicated that the ancillary enzyme had been expressed after the addition of 1mM IPTG. The His-tagged ManA was bound to the Ni-NTA resin. Similar to ArfA, the contaminating *E. coli* native proteins were removed from the column by washing the column with buffer containing no imidazole and ManA was eluted from the nickel affinity column with the addition of 250 mM imidazole. As expected, ManA had a molecular weight of approximately 47 kDa.

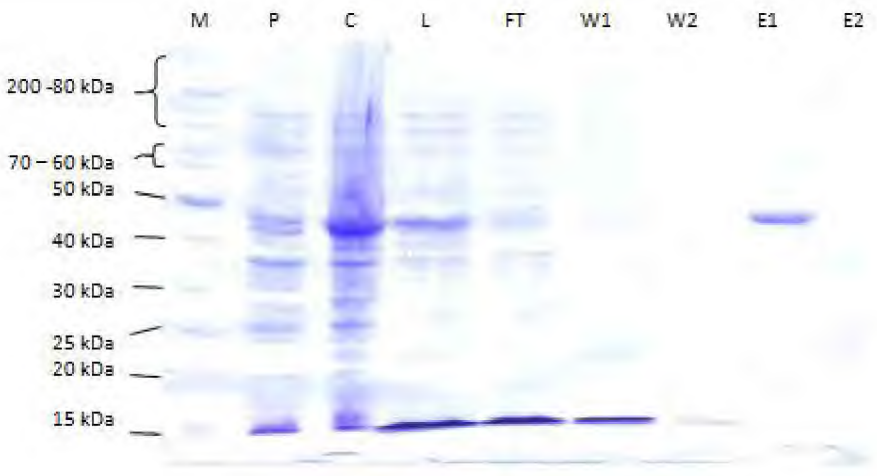


Figure 28: SDS-PAGE for the purification of ManA. ManA was induced with the addition of IPTG to a final concentration of 100 ~g/ml, incubated at 18°C for 16 h and purified using nickel affinity chromatography. The purification fractions were electrophoresed on a 12% SDS-PAGE gel. The lanes are marked as follows: molecular marker (M), pre-induction (P), crude sample (C), clear enzyme lysate (L), flow through (FT), wash 1 (W1), wash 2 (W2), eluate 1 (E1) and eluate 2 (E2).

The third protein that was expressed and purified from the *E. coli* BL21(DE3) cells was XynA (Figure 29). The efficiency of the nickel affinity purification of the His-tagged XynA (Figure 29) indicated that the ancillary enzyme had been expressed after the addition of 1 mM IPTG. Similar to ArfA and ManA, the contaminating *E. coli* native proteins were removed from the column by washing the column with buffer containing no imidazole and bound XynA was eluted from the nickel affinity column with the addition of 250 mM imidazole. As expected, XynA had a molecular weight of approximately 56 kDa.

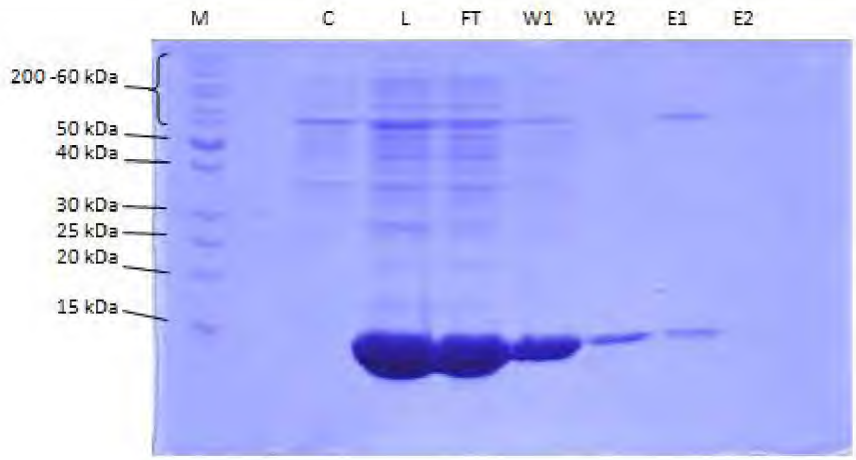


Figure 29: SDS-PAGE for the purification of XynA. XynA was induced with the addition of IPTG to a final concentration of 100 ~g/ml, incubated at 18°C for 16 h and purified using nickel affinity chromatography. The purification fractions were electrophoresed on a 12% SDS-PAGE gel. The lanes are marked as follows: molecular marker (M), crude sample (C), clear enzyme lysate (L), flow through (FT), wash 1 (W1), wash 2 (W2), elution 1 (E1) and elution 2 (E2).

4.3.2 Synergistic analysis

The first batch of assays were performed in order to identify potential synergistic associations between the purified recombinant enzymes. Different combinations of two enzymes, with protein ratios ranging between 0 % and 100 % (Figures 30, 33, 37, 41 and 45) were used in the first set of synergy assays. The second set of synergy assays that were performed used various protein combinations of all three enzymes (Figures 31, 34, 38, 42 and 46). The specific activities and degrees of synergy with 2% (w/v) soluble substrate (LBG:OSX) (Figures 30 and 31), untreated SCB (Figure 33 and 34) and chemically pre-treated SCB (Figure 37 and 38; 41 and 42; 45 and 46) were determined for different enzyme combinations. The synergy studies involving three enzymes were performed in an identical manner to the synergy studies involving two enzymes.

4.3.2.1 Synergy studies with the model substrate (LGB:OSX)

The first synergy assays were performed on a mixture of soluble substrates (2% w/v LBG and 2% w/v OSX) that are easily hydrolysed by the different recombinant enzymes. The first set of synergy assays were performed to elucidate potential synergistic associations between combinations of two of the three enzymes. The enzymes were combined in various

protein ratios ranging from 0 – 100% of each of the different enzymes. The first synergy assays used various combinations of XynA and ManA (Figure 30A), ManA and ArfA (Figure 35B) and ArfA and XynA (Figure 30C).

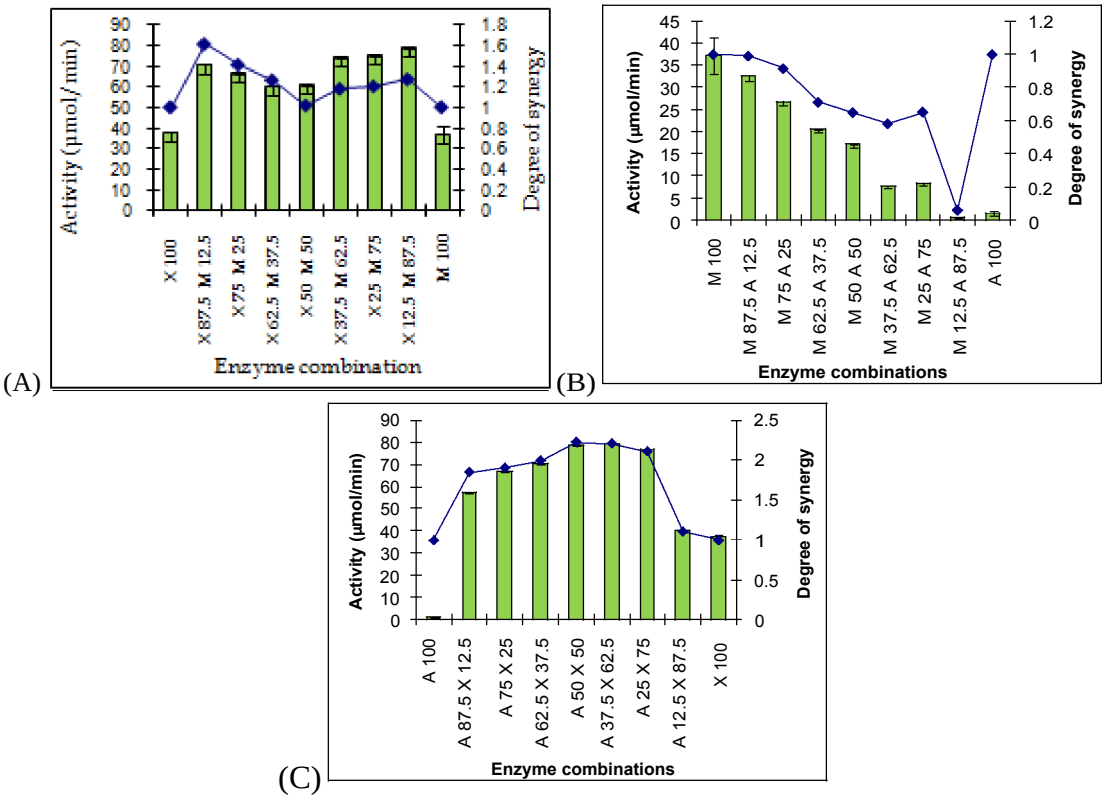


Figure 30: Specific activities (green bars) and the respective synergistic associations (blue lines) with model substrate (2% w/v OSX:LBG) for various combinations of the enzymes. (A) XynA (X) and ManA (M), (B) ManA (M) and ArfA (A), (C) XynA (X) and ArfA (A). Values are presented as mean values ± SD (n=3), but some SD values were too small to be observed.

With the first combination, XynA and ManA (Figure 30A) the largest amount of sugar (78.9 µmol/min), was produced with 12.5% XynA and 87.5% ManA. This combination showed a degree of synergy of 1.27. However, the highest degree of synergy (1.61) was obtained with 87.5% XynA and 12.5% ManA producing 70.9 µmol/min of sugar, which is slightly less than the maximum quantity of sugar produced.

The second set of assays that were performed with the soluble substrate made use of ManA and ArfA (Figure 30B). The highest activity (37.2 µmol/min) was obtained with 100% ManA, therefore indicating an absence of synergy between ManA and ArfA.

The third set of synergy assays performed with the model substrate utilised ArfA and XynA (Figure 30C). The largest amount of sugar and the highest degree of synergy was not obtained with the same combination of enzymes; however, the enzyme activities obtained were similar. The enzyme combination that produced the largest amount of sugar (79.3 μmol/min) and a degree of synergy of 2.20 contained 37.5% ArfA and 62.5% XynA. The enzyme combination containing 50% ArfA and 50% XynA displayed the highest degree of synergy at 2.22 (producing 78.340 μmol reducing sugar/min).

The fourth set of synergy assays that were performed with the 2% (w/v) OSX:LBG model substrate, made use of combinations of all three recombinant enzymes (Figure 31).

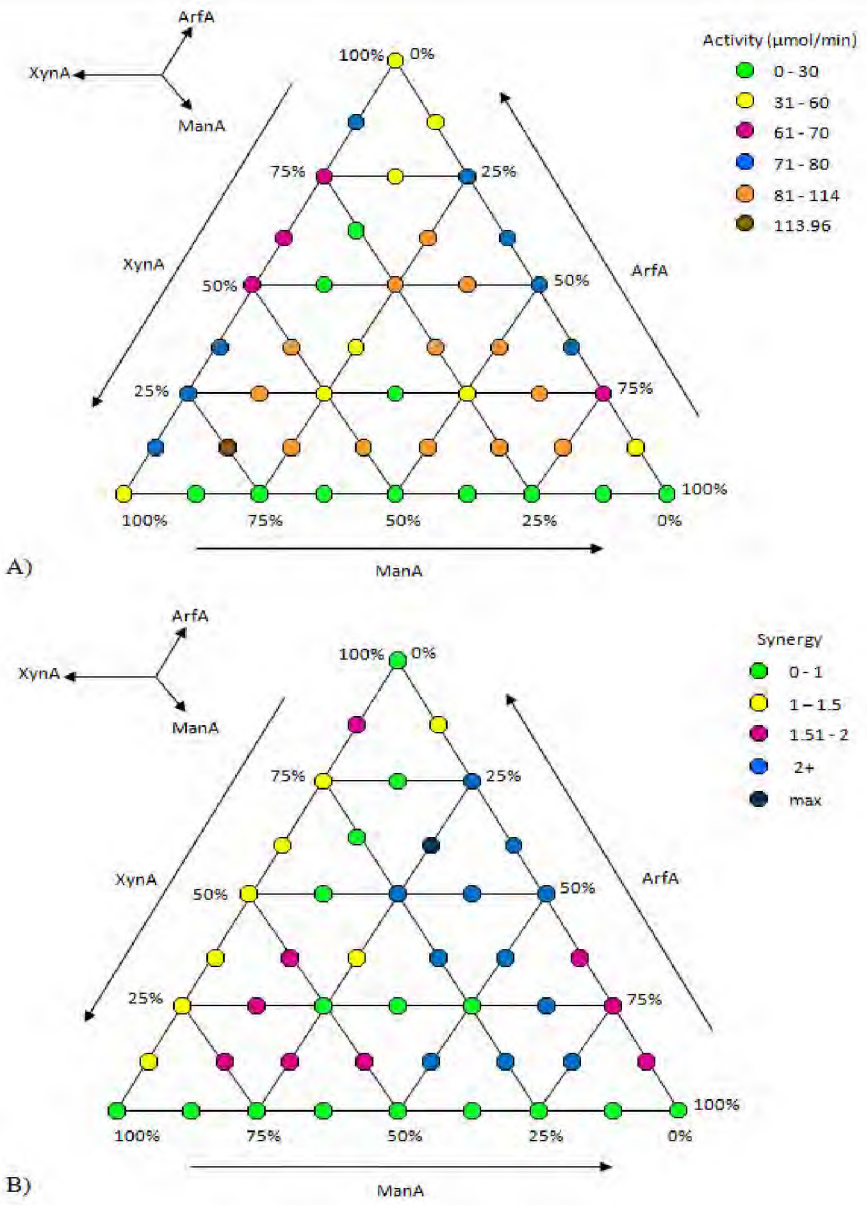
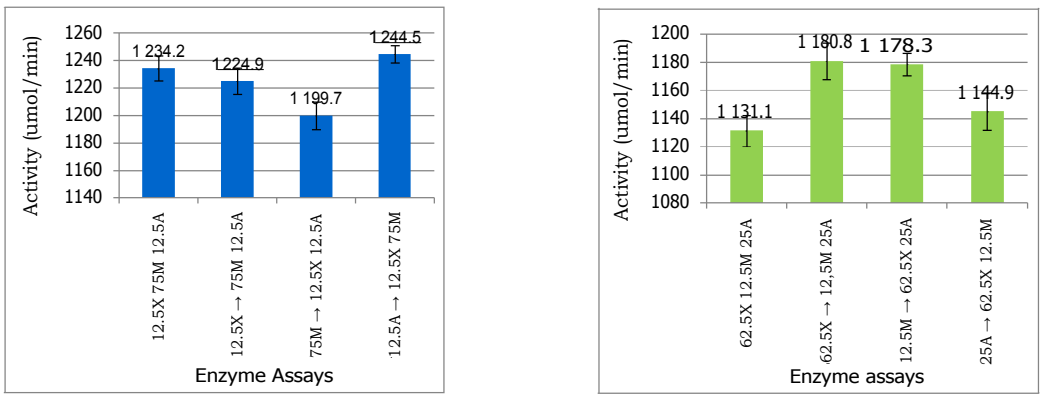


Figure 31: Schematic representation of the enzyme activity and the respective degrees of synergy obtained for the cellulosomal hemicellulases, ManA and XynA, and the noncellulosomal enzyme ArfA against a 2% (w/v) mixture of LBG and OSX. The enzyme activities are represented as the µmol/min sugar produced A) and degree of synergy B) are represented as the different shaded circles, as shown in the figure. The enzymes were mixed in varying compositions shown according to the axis as a protein percentage. The arrows indicate the directions of axis for each enzyme. The degrees of synergy are shown as the actual activities divided by the theoretical activities.

Combining all three recombinant enzymes in the synergy assays increased the total amount of sugar produced, compared to the amount of sugar produced by combining two out of the three enzymes (Figure 30). The greatest quantity of sugar (114.0 µmol/min) and a degree of synergy of 1.94 was produced by combining 75% ManA, 12.5% ArfA and 12.5%

XynA. The highest degree of synergy (2.34) was; however, obtained with the enzyme combination of 12.5% ManA, 25% ArfA and 62.5% XynA. This enzyme combination produced 102.8 µmol/min sugar which was marginally less than the maximum amount of sugar produced with the model substrate.

Using the enzyme combination that produced the maximum amount of sugar and the optimal enzyme combination that produced the greatest degree of synergy, sequential synergy assays were performed (Figure 32). Sequential synergy assays were performed to determine if the synergistic associations between the different enzymes were simultaneous or sequential.



(A) (B)

Figure 32: Sequential synergy with LBG:OSX. The different protein combinations of the enzymes that produced A) the optimal protein ratio that produced the optimal activity and B) the protein ratio combinations for optimal synergy that were obtained during the synergy assays the model substrate (Figure 31).

The first sequential synergy assay performed used the enzyme combination (12.5 % XynA, 75% ManA and 12.5% ArfA) that produced the greatest amount of sugar with the hydrolysis of the model substrate (Figure 32A). As depicted (Figure 32A), the type of synergy that was obtained with the enzyme combination containing 12.5 % XynA, 75% ManA and 12.5% ArfA may vary between simultaneous and sequential. The type of synergy changes from being simultaneous to sequential when the ancillary (ArfA) enzyme was added to the enzyme assay first; however, the sequential synergy increased the production of sugar marginally from 1234.2 µmol/min to 1244.5 µmol/min.

The sequential synergy assays that were performed used the enzyme combination that depicted the greatest degree of synergy (62.5% XynA, 12.5% ManA and 25% ArfA). The data indicated that the synergistic association between the recombinant enzymes at these protein ratios were sequential. An increased production of reducing sugar was observed when the enzymes were added sequentially. The sugar production increased from 1131.1 µmol/min produced by simultaneous synergy assays, to various quantities of sugar ranging from 1144.9 µmol/min to 1180.8 µmol/min that were obtained with the sequential synergy assays.

4.3.2.2 Synergy studies with untreated SCB

SCB is a lignocellulosic biomass composed of intertwined cellulose and hemicellulose components (Lavarack *et al.*, 2002). The hemicellulosic component of the untreated SCB is predominantly composed of a xylan backbone that may contain a variety of substituents. The most common substituents associated with the xylan backbone are glucuronic acid and arabinose (Saavedra *et al*, 1989); however, other sugars may act as additional substituents.

The first lignocellulosic substrate that was hydrolysed to elucidate the presence of potential synergistic associations was untreated SCB. Possible synergistic associations between two out of three of the recombinant enzymes were investigated using varying percentages of A) ManA and ArfA, B) ArfA and XynA, and C) XynA and ManA (Table 15 & Figure 33).

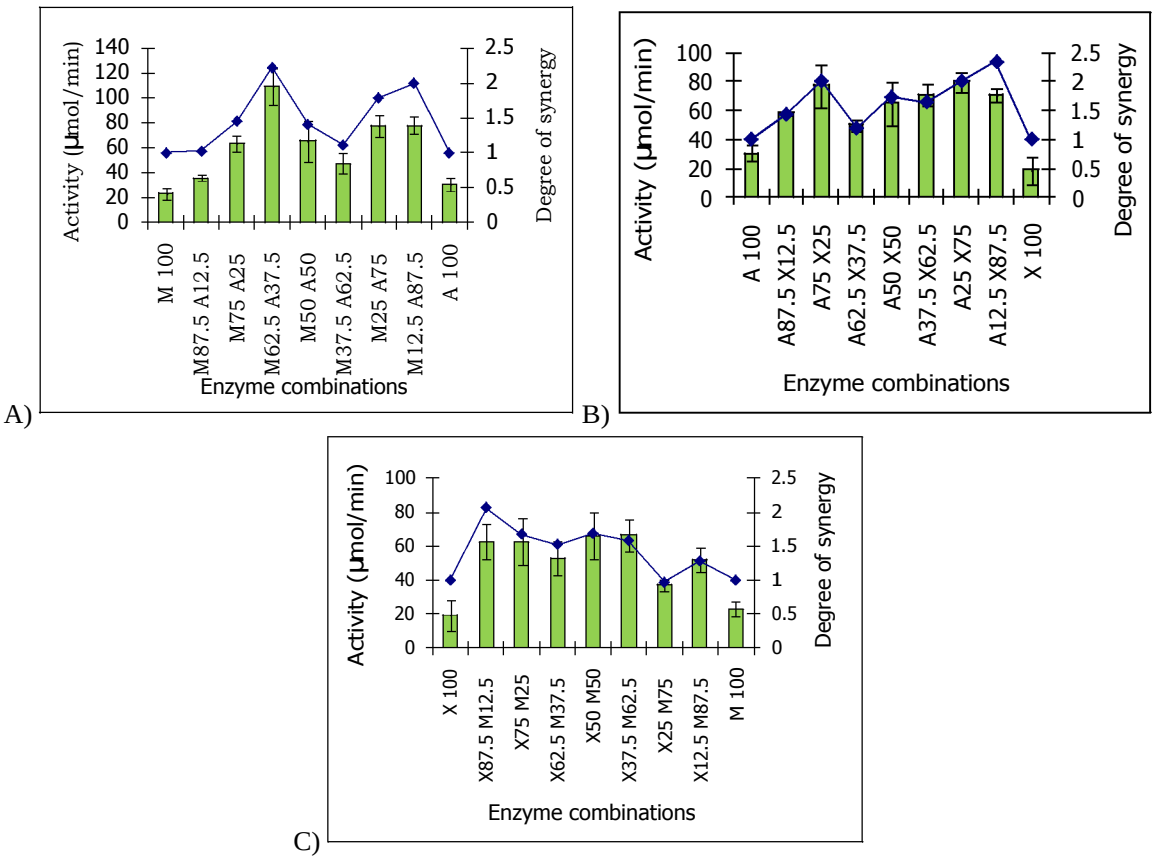


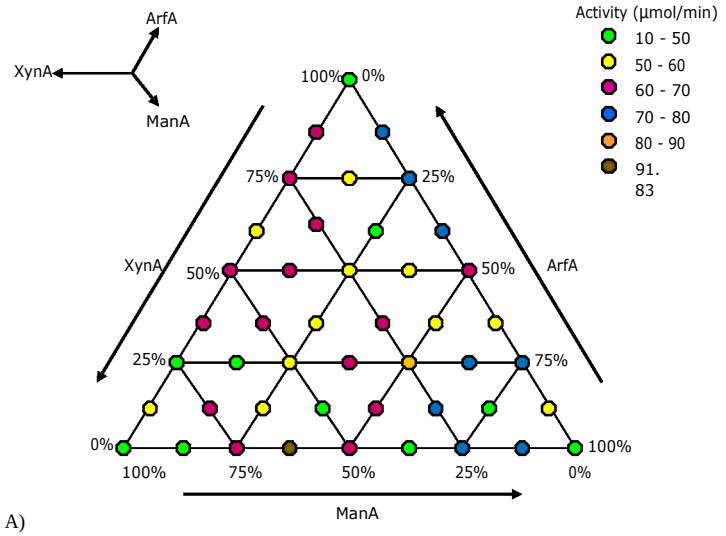
Figure 33: Specific activities (green bars) and the respective synergistic associations (blue lines) between two of the enzymes with the untreated SCB for various combinations of the enzymes. (A) ManA (M) and ArfA (A), (B) ArfA (A) and XynA (X), (C) XynA (X) and ManA (A). Values are presented as mean values ± SD (n=3), but are too small to be visualised.

The first enzyme synergy assay that was performed with the untreated SCB used varying ratios of ManA and AfrA (Figure 33A). The largest amount of sugar and the highest degree of synergy (91.8 µmol/min, 1.9) was obtained with the enzyme combination of 62.5% ManA and 37.5% ArfA.

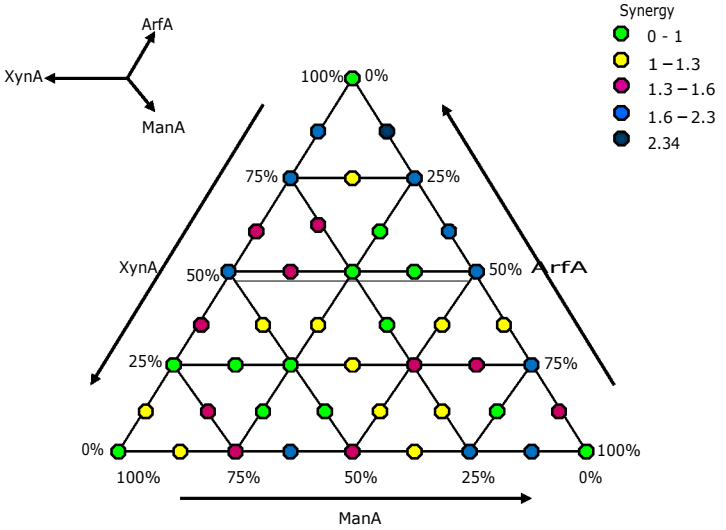
The second enzyme combination using two recombinant enzymes to hydrolyse SCB involved ArfA and XynA (Figure 33B). The largest amount of sugar (79.9 µmol/min) was produced with the combination of 25% ArfA and 75% XynA. This combination displayed a degree of synergy of 2.01. The highest degree of synergy (2.34) was obtained with the enzyme combination of 12.5% ArfA and 87.5% XynA, with a maximal release of 71.2 µmol/min reducing sugar.

The third group of synergy assays performed with untreated SCB used varying combinations of XynA and ManA (Figure 33C). The largest amount of sugar (66.5 μmol/min) with a degree of synergy of 1.58 was produced with the combination of 37.5% XynA and 62.5% ManA. With these two enzymes, the greatest degree of synergy (2.02) was obtained with the enzyme combination of 87.5% XynA and 12.5% ManA, producing 62.8 μmol/min sugar.

The final simultaneous synergy assay that was performed to hydrolyse untreated SCB made use of combinations of all three enzymes (Figure 34). The enzyme combination that produced the optimal degree of synergy was 12.5% ArfA and 87.5% XynA. However, the highest enzyme activity was produced with 62.5% ManA and 37.5% ArfA.



A)

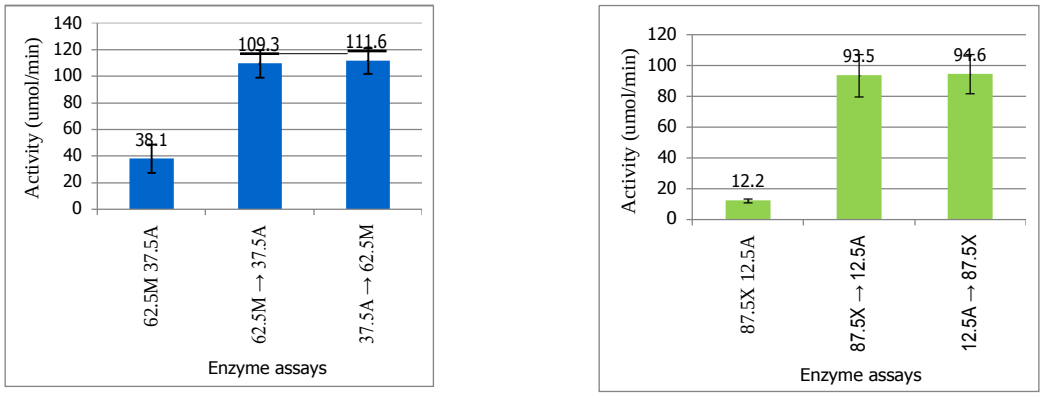


B)

Figure 34: Schematic representation of the enzyme activity and the respective degrees of synergy obtained for the cellulosomal hemicellulases, ManA and XynA, and the noncellulosomal enzyme ArfA with untreated SCB. The enzyme activities are represented as the µmol/min sugar produced A) and degree of synergy B) are represented as the different shaded circles, as shown in the figure. The enzymes were mixed in varying compositions shown according to the axis as a protein percentage. The arrows indicate the directions of axis for each enzyme. The degrees of synergy are shown as the actual activities divided by the theoretical activities.

The data obtained from the simultaneous synergy assays (Figure 34) indicated that to obtain the optimal production of sugar and degree of synergy, different protein ratio enzyme combinations are required. The maximum production of sugar was obtained with the enzyme mixture containing 62.5% ManA and 37.5% ArfA. The greatest degree of synergy with the untreated SCB was achieved with the enzyme combination of 87.5% XynA and 12.5% ArfA.

The combination of 62.5% ManA and 37.5% ArfA producing the maximum amount of sugar and the combination of 87.5% XynA and 12.5% ArfA producing the optimal degree of synergy were used to determine if the synergistic relationship observed between the enzymes were simultaneous or sequential in nature (Figure 35).



(A) (B)

Figure 35: Sequential synergy with the untreated SCB. The different protein combinations of the enzymes that produced (A) the optimal protein ratio that produced the optimal activity and (B) the protein ratio combinations for optimal synergy that were obtained during the synergy assays with the untreated SCB (Figure 33). Values are presented as mean values ± SD (n=3).

The sequential synergy assays (Figure 35) performed with the enzyme combinations 62.5% ManA and 37.5% ArfA (which produced the maximum sugar) and 87.5% XynA and 12.5% ArfA (which produced the optimal degree of synergy), indicated that the quantity of sugar, obtained by hydrolysis of the untreated SCB, increased dramatically when the enzymes were sequentially added into the enzyme reaction.

The effect of time on the sugar production with the SCB substrate (Table 16 and Figure 36) was tested using the enzyme combination that produced the maximum amount of sugar in the synergy assays (62.5% ManA and 37.5% ArfA) (Table 16 and Figure 36).

Table 16: The effect of time on the production of reducing sugars (µmol) with the hydrolysis of the untreated SCB.

Substrate	Enzyme assay	Time (hours)				
		24	**48**	**72**	**96**	**120**
SCB	37.5% ArfA	78.2 ± 9.4	225.8 ± 17.9	290.3 ± 117.6	715.8 ± 54.1	758.0 ± 151.6
	62.5% ManA	332.5 ± 23.7	531.0 ± 191.6	543.4 ± 89.2	62.0 ± 58.1	1295.1 ± 134.3
	37.5% ArfA: 62.5% ManA	494.1 ± 24.5	1091.7 ± 84.5	1271.65 ± 51.8	1692.1 ± 76.6	3535.5± 444.0
	Theoretical value	410.6	756.7	833.6	1344.7	2053.1
	Degree of synergy	1.02	1.44	1.53	1.26	1.72

* Values are presented as mean values ± SD (n=3)

The data obtained for the effect of time (Table 16) on the production of sugar with the hydrolysis of untreated SCB is also represented graphically in Figure 36 for purposes of clarification.

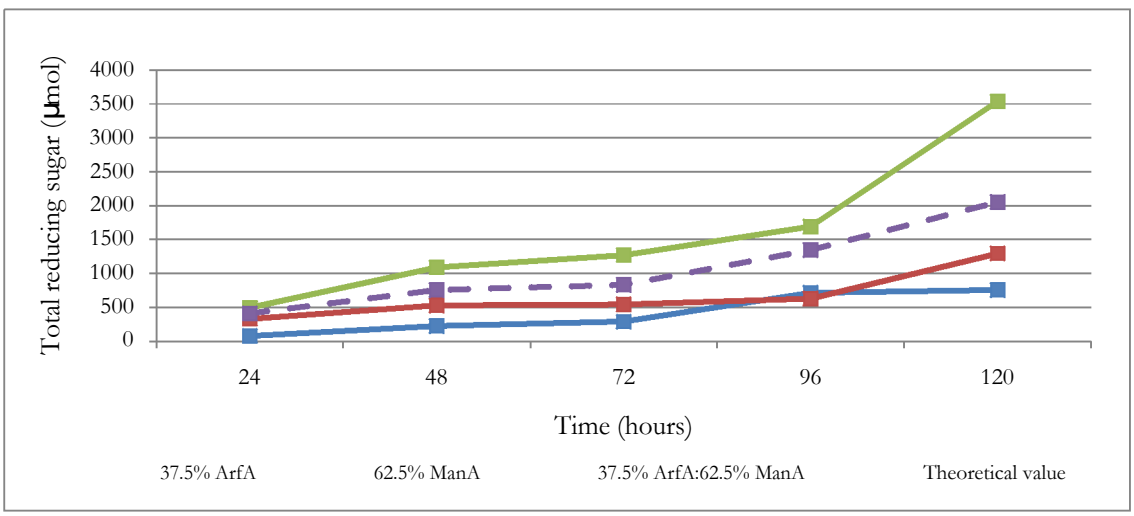


Figure 36: The effect of time on the production of reducing sugars with the untreated SCB. Values are presented as mean values ± SD (n=3), where the error bars at too small to be visualised on the graph .

The data obtained (in Table 16 and Figure 36) with regards to the effect of time on the production of reducing sugars (using the enzyme combination 62.5% ManA and 37.5%

ArfA), confirmed the presence of an increasing synergistic association over time. This data also indicated that the production of sugar (i.e. activity) increased over time.

4.3.2.3 Synergy studies with $Ca(OH)_2$ pre-treated SCB

SCB was pre-treated with three alkaline chemicals: $Ca(OH)_2$, NaOH and NH_4OH. The $Ca(OH)_2$ pre-treated SCB was analysed (using the three recombinant enzymes) for potential synergistic associations as described previously (Table 15). These assays were performed to determine the effect of the $Ca(OH)_2$ pre-treatment on the synergistic associations (degree of synergy) and production of reducing sugars (Figure 37).

Possible synergistic associations between two out of three of the recombinant enzymes were determined using varying ratios of A) ManA and ArfA, B) ArfA and XynA, and C) XynA and ManA (Figure 37).

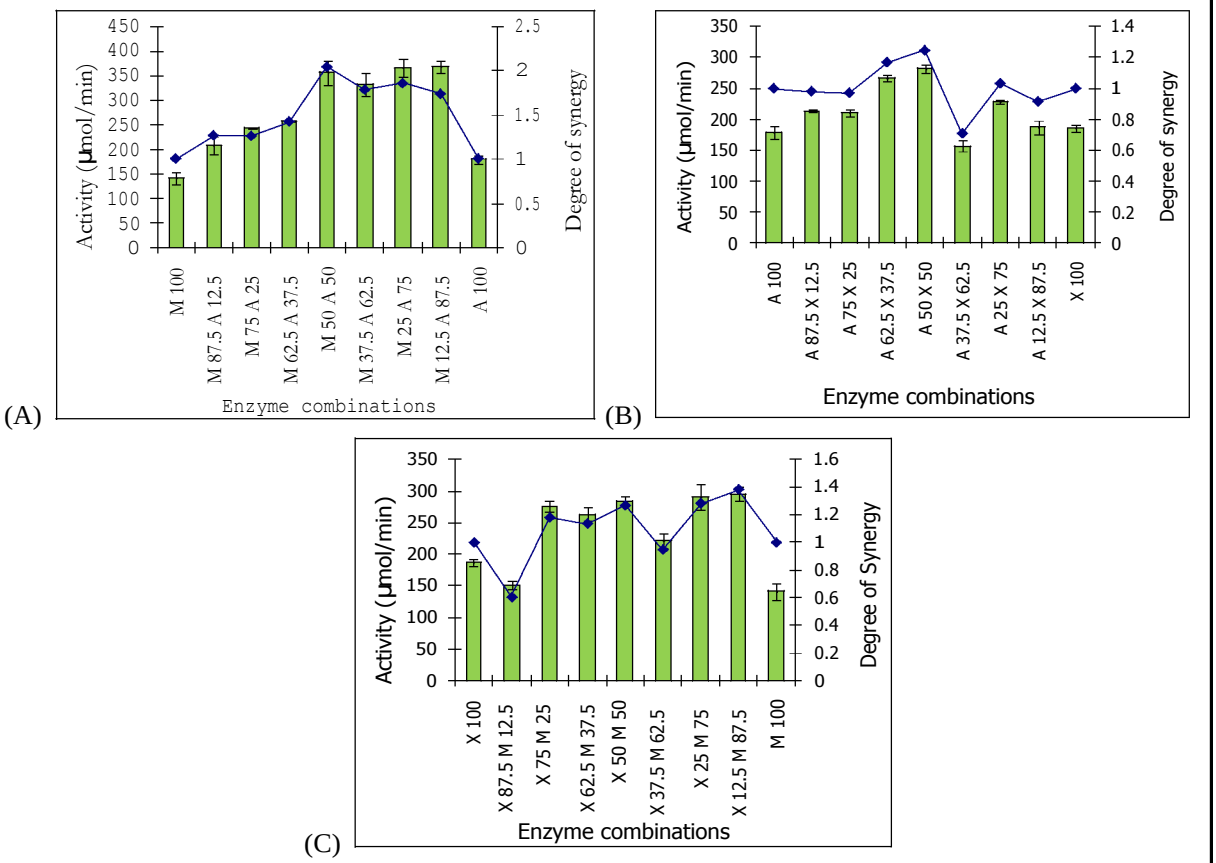


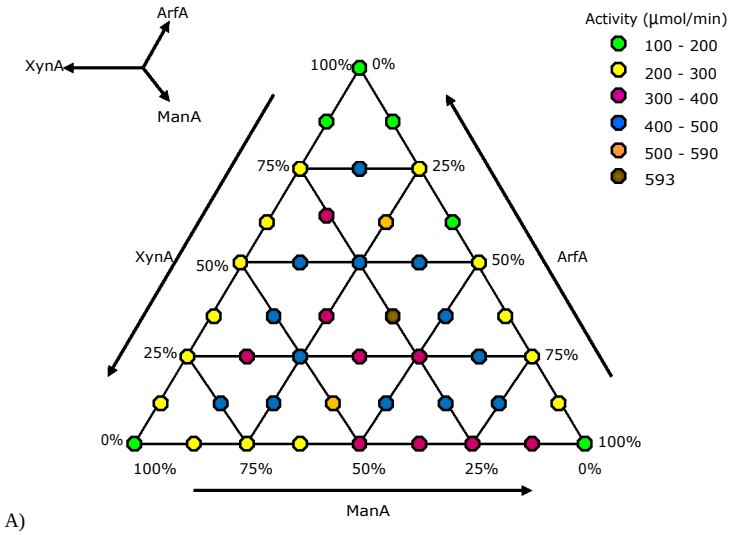
Figure 37: Specific activities (green bars) and the respective synergistic associations (blue lines) against the $Ca(OH)_2$ pre-treated SCB for various combinations of the enzymes. A) ManA (M) and ArfA (A), B) ArfA (A) and XynA (X), C) XynA (X) and ManA (M). Values are presented as mean values ± SD (n=3).

The first set of synergy assays set up to hydrolyse the $Ca(OH)_2$ pre-treated SCB was aimed at elucidating potential synergistic associations between combinations of two out of the three enzymes. The first set of synergy assays involved two enzymes of the three enzymes to degrade the $Ca(OH)_2$ pre-treated SCB combined ManA and ArfA (Figure 37A). The enzyme combination of 12.5% ManA and 87.5% ArfA produced a maximum of 368.5 µmol/min with a degree of synergy of 1.74. However, the greatest degree of synergy (2.05) was obtained with 50% ManA and 50% ArfA and produced 356.225 µmol/min sugar.

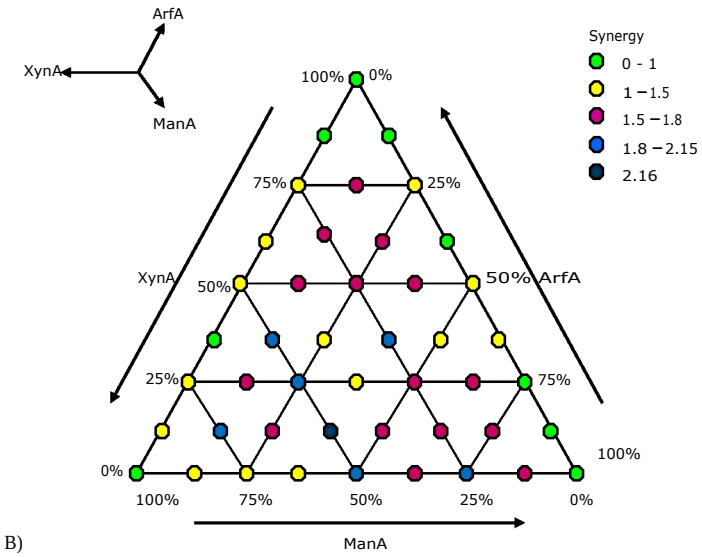
The second set of synergy assays with two of the three enzymes involved ArfA and XynA (Figure 37B). The enzyme combination that produced both the maximum amount of sugar (282.7 µmol/min) and degree of synergy of 1.25 was 50% ArfA and 50% XynA.

The third enzyme combination used to hydrolyse $Ca(OH)_2$ pre-treated SCB combined XynA and ManA (Figure 37C). The enzyme combination of 12.5% XynA and 87.5% ManA produced a maximum of 295.8 μmol/min reducing sugar and the largest degree of synergy of 1.38.

The final set of simultaneous synergy assays performed on the $Ca(OH)_2$ pre-treated SCB was used to elucidate if a synergistic relationship would be obtained by combining all three of the recombinant enzymes (Figure 38).



A)

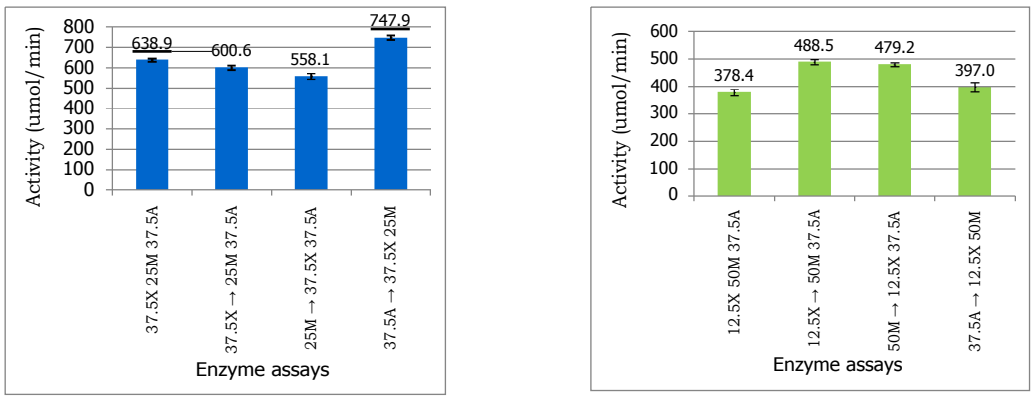


B)

Figure 38: Schematic representation of the enzyme activity and the respective degrees of synergy obtained for the cellulosomal hemicellulases, ManA and XynA, and the ancillary enzyme ArfA with the Ca(OH)$_2$ pre-treated SCB. The enzyme activities are represented as the µmol/min sugar produced A) and degree of synergy B) are represented as the different shaded circles, as shown in the figure. The enzymes were mixed in varying compositions shown according to the axis as a protein percentage. The arrows indicate the directions of axis for each enzyme. The degrees of synergy are shown as the actual activities divided by the theoretical activities.

The data obtained from the simultaneous synergy assays (Figure 38) indicated that to obtain the optimal production of sugar and degree of synergy, different protein ratios of the enzymes are required. The enzyme mixture containing 37.5% ArfA, 25% ManA and 37.5% XynA produced the maximum amount of sugar (593.7 µmol/min) and a degree of synergy of 2.14. However, the enzyme combination that displayed the greatest degree of synergy (2.17)

consisted of 37.5% ArfA, 50% ManA and 12.5% XynA. This combination produced 517.5 µmol/min sugar. These enzyme combinations were used to determine if the synergistic associations between the enzymes were simultaneous or sequential (Figure 39).



A) B)

Figure 39: Sequential synergy against the Ca(OH)$_2$ pre-treated SCB. The different protein combinations of the enzymes that produced A) the optimal protein ratio that produced the optimal activity and B) the protein ratio combinations for optimal synergy (Figure 38). Values are presented as mean values ± SD (n=3).

Using the optimal enzyme combinations from the synergy assays (Figure 38), the effect of pre-treatment on the type of synergy (Figure 39) was determined. From the data obtained regarding the effect of chemical pre-treatment on the type of synergy, different trends were observed (Figure 39).

The results obtained using the enzyme combination 37.5% ArfA, 25% ManA and 37.5% XynA (Figure 39A) only favoured sequential synergy when the arabinofuranosidase was added to the reaction first. When the mannanase and xylanase were the added to the reaction mixtures prior to the other enzymes, less sugar was produced than when the enzymes were added simultaneously.

The data obtained when the second combination (12.5% XynA, 50% ManA and 37.5% ArfA) used indicated that the production of sugar increased when the enzymes were added to the reaction mixture sequentially (Figure 39B).

The final assay performed on $Ca(OH)_2$ pre-treated SCB was the effect of time on activity and degree of synergy. The effect of time on the synergistic relationship between these enzymes was monitored via the production of reducing sugars (Table 17 and Figure 40).

Table 17: The effect of time on the production of reducing sugars (µmol) with the hydrolysis of the $Ca(OH)_2$ pre-treated SCB.

Substrate	Enzyme assay	Time (hours)				
		24	**48**	**72**	**96**	**120**
$Ca(OH)_2$ pre-treated SCB	37.5% ArfA	966.4 ± 99.2	1250.8 ± 241.0	2123.8 ± 27.6	2041.9 ± 128.7	1912.9 ± 156.8
	25% ManA	1473.8 ± 182.8	1084.2 ± 33.7	1860.2 ± 118.3	2314.4 ± 343.2	2870.6 ± 210.4
	37.5% XynA	1786.4 ± 143.5	1730.6 ± 168.1	2203.2 ± 140.7	2358.32 ± 135.6	2652.5 ± 148.2
	37.5% ArfA: 25% ManA: 37.5% XynA	4439.2 ± 44.5	5852.8 ± 5.3	6496.8 ± 1.4	9534.8 ± 162.1	14248.4 ± 371.8
	Theoretical value	4226.5	4065.6	6187.2	6714.6	7436.1
	Degree of synergy	1.05	1.44	1.05	1.42	1.92

*Values are presented as mean values ± SD (n=3)

The data obtained for the effect of time (Table 17) on the production of sugar with the hydrolysis of $Ca(OH)_2$ pre-treated SCB is also represented graphically in Figure 40.

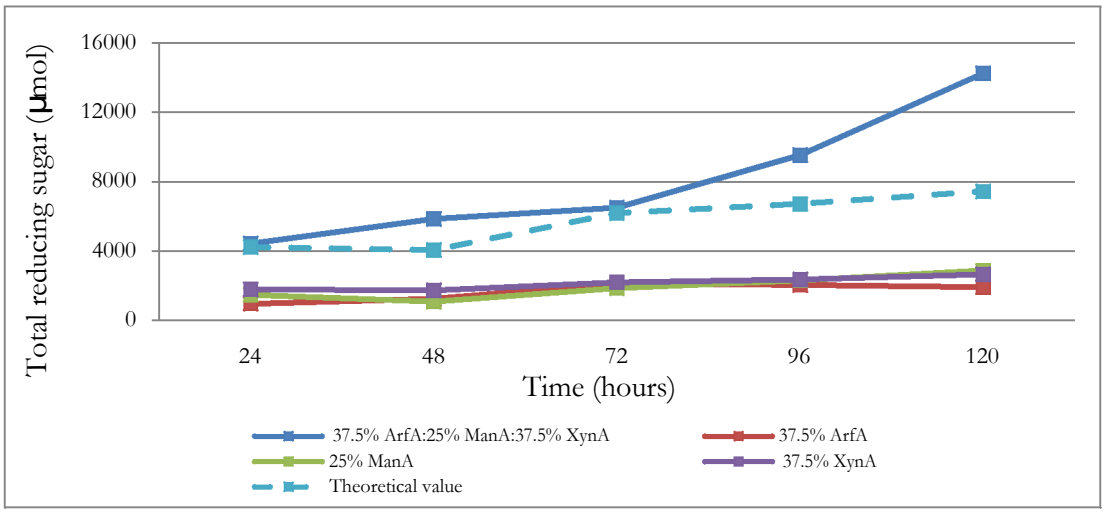


Figure 40: The effect of time on the production of reducing sugars against $Ca(OH)_2$ pre-treated SCB. Values are presented as mean values ± SD (n=3), where the error bars at too small to be visualised on the graph.

As depicted in Table 17 and Figure 40, the production of sugar increased exponentially after 72 hours with the enzyme assays containing all the recombinant enzymes, indicating that the degree of synergy between the recombinant enzymes was maximal after 72 hours. The individual enzymes simulated a gradual increase in the production of sugar for the first 72 hours, after which the amount of additional sugar produced was minimal.

4.3.2.4 Synergy studies with NaOH pre-treated SCB

The second alkaline chemical used to pre-treat SCB was NaOH. Possible synergistic associations between the recombinant hemicellulases and the ancillary enzyme were investigated. These assays were performed to establish the effect of NaOH on the synergistic relationship between the enzymes and the production of sugar. Possible synergistic associations between combinations of two out of the three recombinant enzymes were determined using varying percentages of (A) ManA and ArfA, (B) ArfA and XynA, and (C) XynA and ManA (Figure 41).

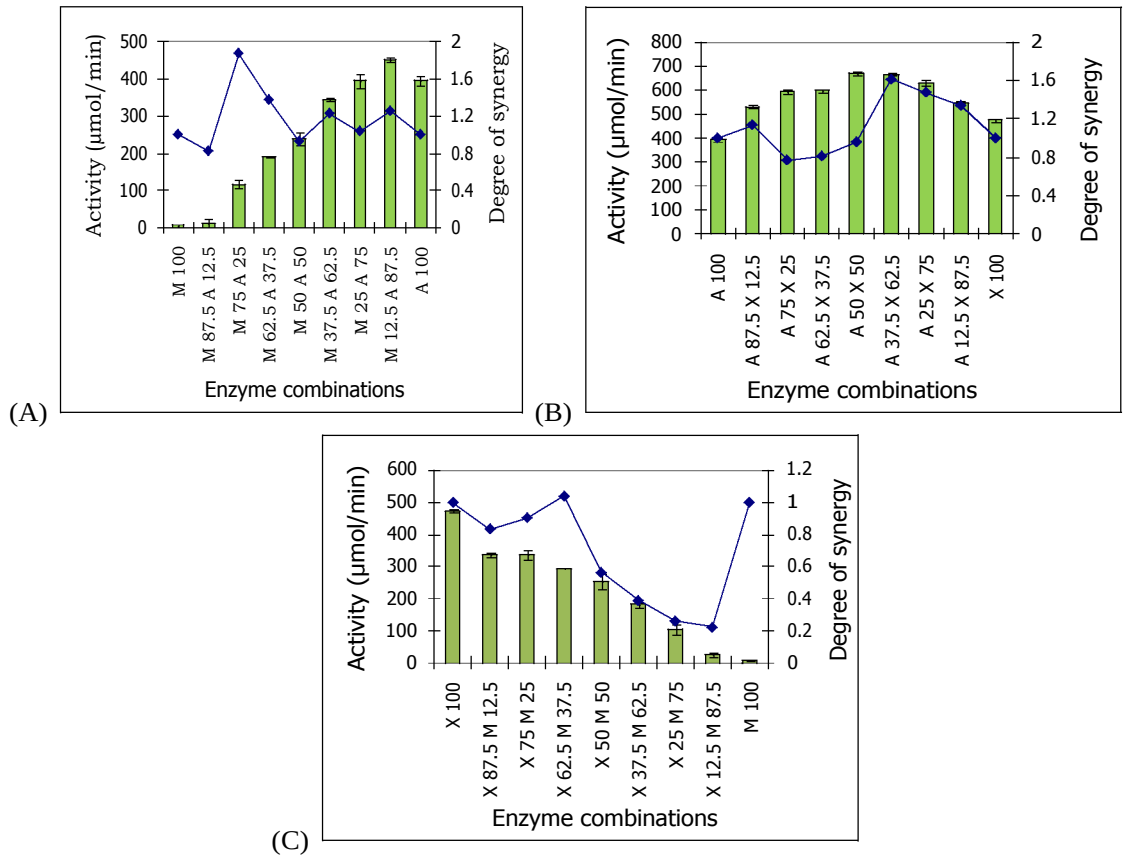


Figure 41: Specific activities (green bars) and the respective synergistic associations (blue lines) with the NaOH pre-treated SCB for various combinations of the enzymes, A) ManA (M) and ArfA (A), B) ArfA (A) and XynA (X), C) XynA (X) and ManA (M). Values are presented as mean values ± SD (n=3).

The first enzyme combination assayed with the NaOH pre-treated SCB utilised ManA and ArfA. The enzyme combination of 12.5% ManA and 87.5% ArfA produced the maximum amount of sugar (451.5 µmol/min) and a degree of synergy of 1.26. The largest degree of synergy (1.88) was obtained with an enzyme combination of 75% ManA and 25% ArfA and produced 116.8 µmol/min sugar (Figure 41A).

The second enzyme combination included ArfA and XynA. The greatest quantity of sugar (669.1 µmol/min) was obtained by combining 50% ArfA and 50% XynA; however, this combination did not display a synergistic association. The enzyme combination of 37.5% ArfA and 62.5% XynA displayed a degree of synergy of 1.61 and produced 667.0 µmol/min sugar (Figure 41B).

The third set of synergy assays used combinations of XynA and ManA to hydrolyse the NaOH pre-treated SCB. The greatest quantity of sugar (474.3 μmol/min) was produced with 100% XynA. The (only and marginal) degree of synergy (1.04) was obtained with 62.5% XynA and 37.5% ManA (Figure 41C).

After the synergy assays using two of the recombinant enzymes with the NaOH pre-treated SCB were performed, the assays were repeated with combinations of all three of the recombinant enzymes (Figure 42).

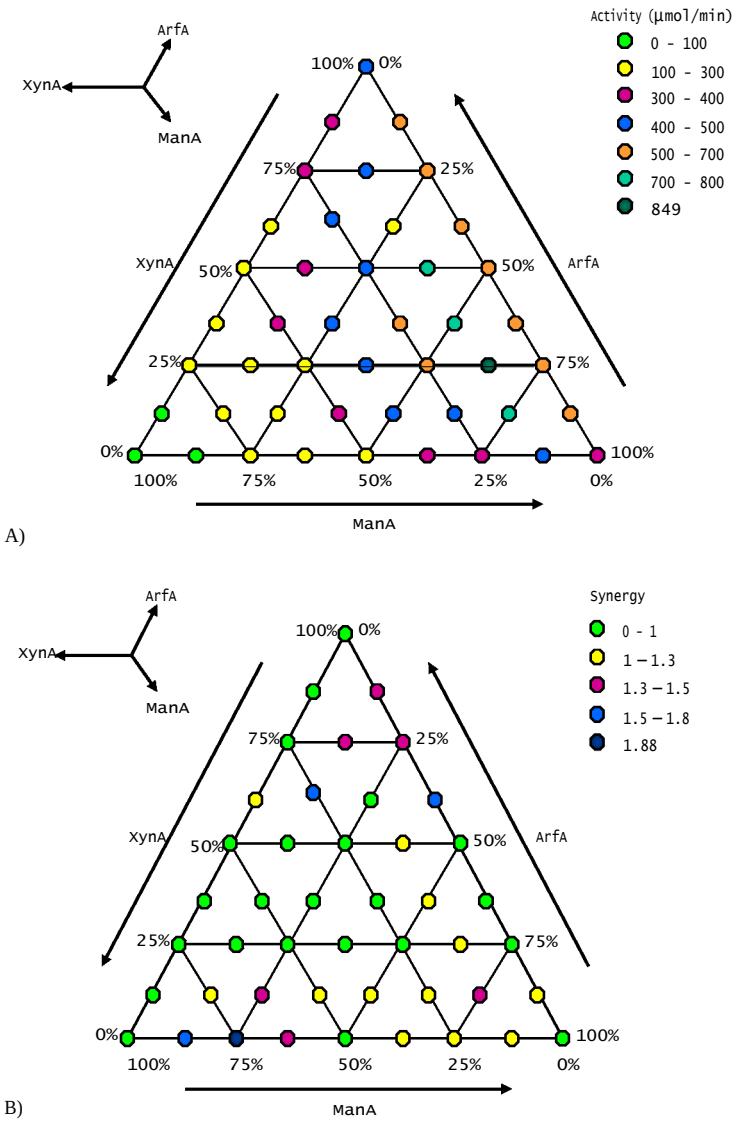


Figure 42: Schematic representation of the enzyme activity and the respective degrees of synergy obtained for the cellulosomal hemicellulases ManA and XynA, and the ancillary enzyme ArfA with the NaOH pre-treated SCB. The enzyme activities are represented as the µmol/min sugar produced (A) and degree of synergy (B) are represented as the different shaded circles, as shown in the figure. The enzymes were mixed in varying compositions shown according to the axis as a protein percentage. The arrows indicate the directions of axis for each enzyme. The degrees of synergy are shown as the actual activities divided by the theoretical activities.

The data obtained from the synergy assays using all the recombinant enzymes (Figure 42) indicated that the largest amount of sugar (849.4 µmol/min) was produced with the enzyme combination containing 25% XynA, 12.5% ManA and 62.5% ArfA, with a degree of synergy of 1.27.

After the simultaneous synergy assay (Figure 42), sequential synergy assays were performed to investigate if the recombinant enzymes were hydrolysing the NaOH pre-treated SCB simultaneously or sequentially. The sequential synergy assays were performed using the enzyme combination of 25% XynA, 12.5% ManA and 62.5% ArfA and the enzyme combination of 75% ManA and 25% ArfA that produced the highest degree of synergy (Figure 43).

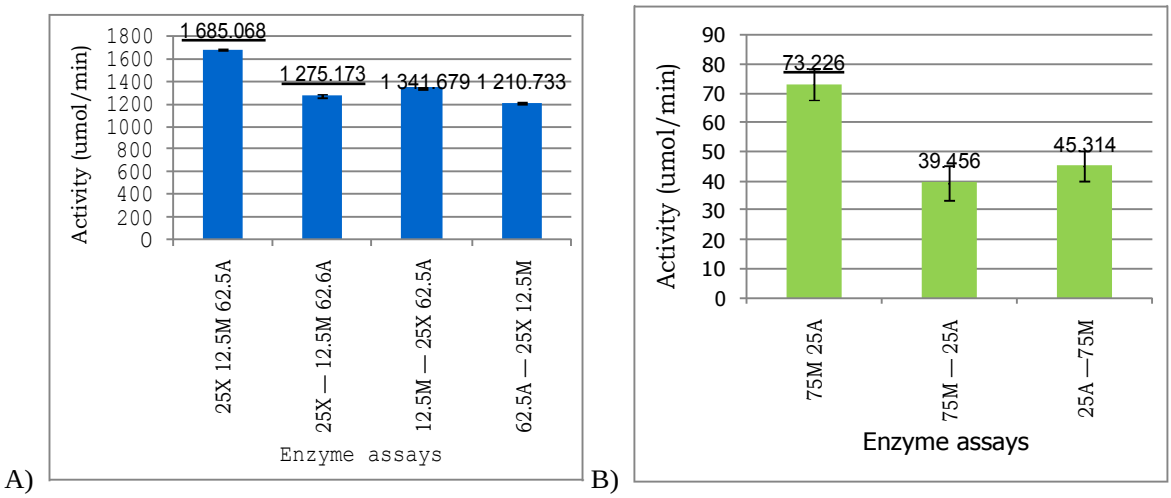


Figure 43: Sequential synergy against the NaOH pre-treated bagasse. The different protein combinations of the enzymes that produced A) the optimal protein ratio that produced the optimal activity and B) the protein ratio combinations for optimal synergy that were obtained during the synergy assays with the NaOH (Figure 42). Values are presented as mean values ± SD (n=3).

The results for both enzyme combinations indicated that the quantity of sugar obtained by hydrolysis of the NaOH pre-treated SCB, increased dramatically when the enzymes were added simultaneously into the enzyme reaction (Figure 43).

The effect of time (Figure 44) on the synergistic associations was determined using the enzyme combination that produced the greatest quantity of sugar on the NaOH pre-treated SCB (Table 18 and Figure 44).

Table 18: The effect of time on the production of reducing sugars (µmol) with the hydrolysis of the NaOH pre-treated SCB.

Substrate	Enzyme assay	Time (hours)				
		24	**48**	**72**	**96**	**120**
NaOH pre-treated SCB	62.5% ArfA	749.3 ± 7.6	1520.9 ± 10.2	2158.5 ± 12.0	2331.0 ± 120.6	3306.3 ± 35.2
	12.5% ManA	255.6 ± 23.8	271.7 ± 13.4	416.8 ± 61.2	518.6 ± 95.4	575.6 ± 123.7
	25% XynA	640.2 ± 68.9	1060.7 ± 127.3	1238.1 ± 444.8	2942.6 ± 187.6	6158.0 ± 281.5
	62.5% ArfA: 12.5% ManA: 25% XynA	3180.7 ± 48.4	4283.6 ± 150.9	4840.6 ± 418.4	6553.8 ± 848.2	12417.8 ± 575.4
	Theoretical value	1645.0	2853.2	3813.4	5792.1	10040.0
	Degree of synergy	1.93	1.50	1.27	1.13	1.24

*Values are presented as mean values ± SD (n=3)

The data obtained for the effect of time (Table 18) on the production of sugar with the hydrolysis of NaOH pre-treated SCB is also represented graphically in Figure 44 for clarification.

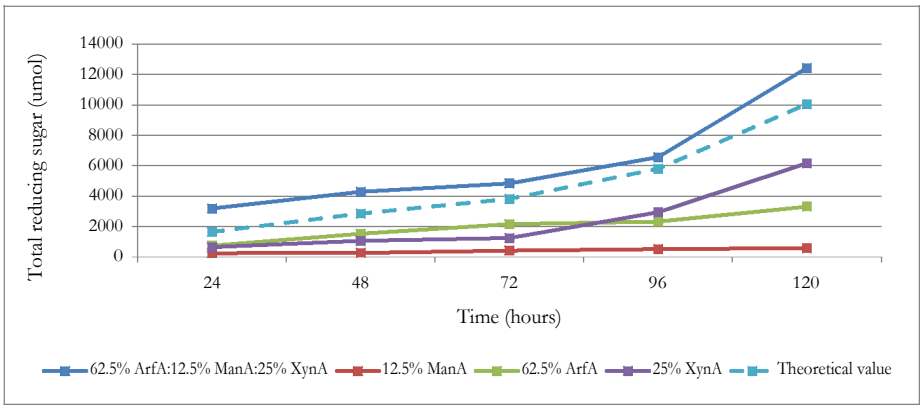


Figure 44: The effect of time on the production of reducing sugars with NaOH pre-treated SCB Values are presented as mean values ± SD (n=3), where the error bars at too small to be visualised on the graph.

As depicted in Table 18 and Figure 44, the production of sugar increased exponentially after 72 hours when the SCB was hydrolysed with the enzyme cocktail, indicating that the production of sugar between the recombinant enzymes was highest after 72 hours. However, the degree of synergy appeared to decrease over time as the degree of synergy decreased from 1.93 to 1.24.

4.3.2.5 Synergy studies with NH_4OH pre-treated SCB

The third alkaline chemical used to pre-treat SCB was NH_4OH. Synergy assays were performed on the NH_4OH pre-treated SCB to determine if the alkaline chemical had an effect on the amount of sugar produced from the hydrolysis of the biomass and to determine if the chemical pre-treatment had an effect on the combination of enzymes required to effectively hydrolyse the pre-treated SCB (Figure 45). The possible synergistic associations between two out of three of the recombinant enzymes were determined using varying ratios of A) ManA and ArfA, B) ArfA and XynA, and C) XynA and ManA (Figure 45).

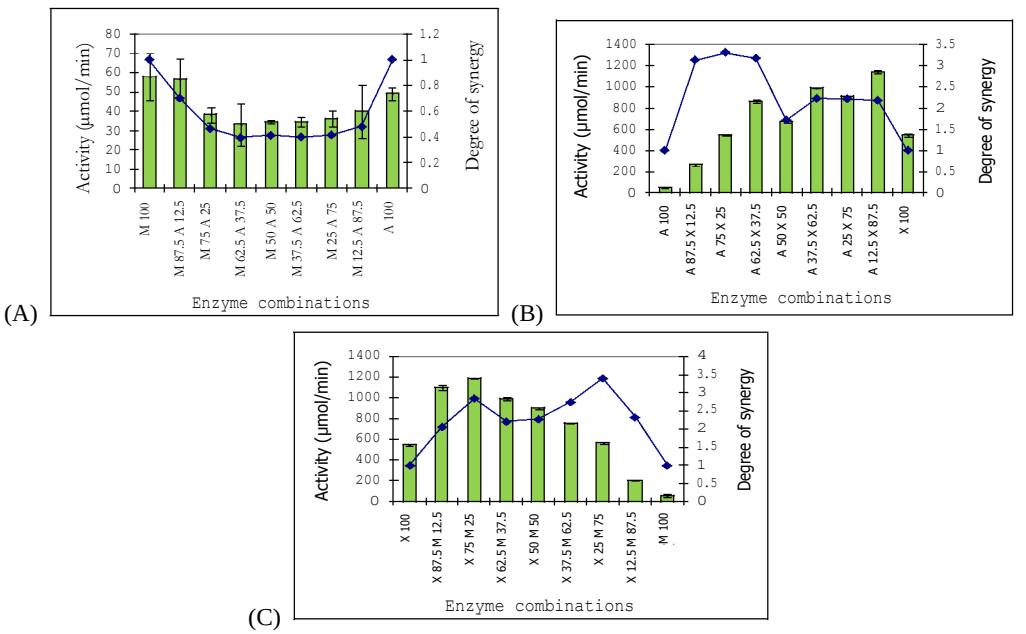


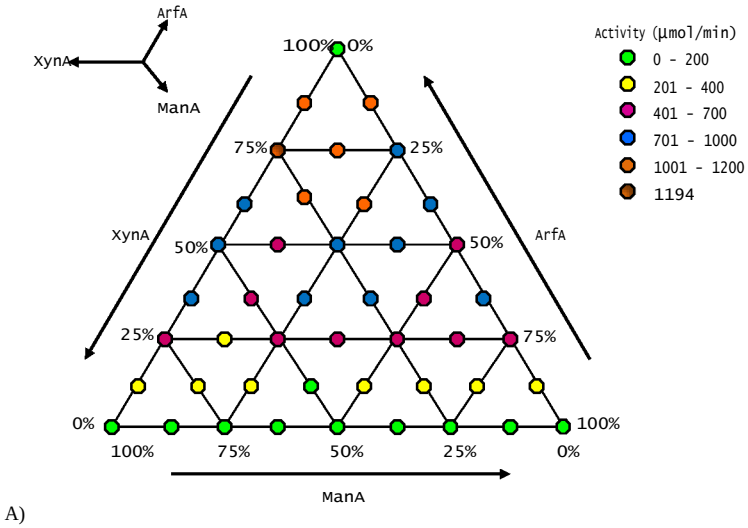
Figure 45: Specific activities (green bars) and the respective synergistic associations (blue lines) with the NH_4OH pre-treated SCB for various combinations of the enzymes. (A) ManA (M) and ArfA (A), (B) ArfA (A) and XynA (X), (C) XynA (X) and ManA (M). Values are presented as mean values ± SD (n=3), but are too small to be visualised.

The first enzyme combination was used to identify possible synergistic associations between the hemicellulases, ManA and ArfA. All the ManA and ArfA combinations tested to determine possible synergistic associations did not produce a synergistic association. The combination of 87.5% ManA and 12.5% ArfA produced a maximum of 56.7 μmol/min sugar, which is marginally less than the sugar produced (58.1 μmol/min) by hydrolysing the NH_4OH pre-treated SCB with 100% ManA (Figure 45A).

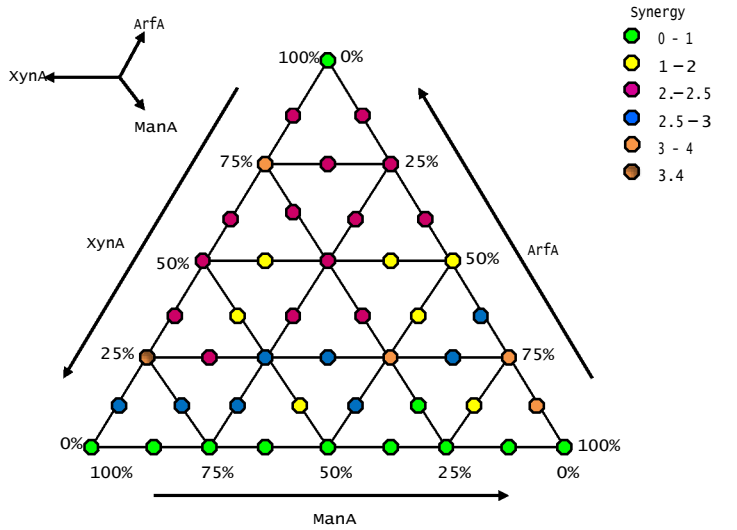
The second set of synergy assays using combinations of two enzymes were performed using various combinations of ArfA and XynA. The largest amount of sugar (1147.3 μmol/min) was produced using the combination of 12.5% ArfA and 87.5% XynA. With this combination, the observed degree of synergy was 2.18. The highest degree of synergy (3.31) was observed using 75% ArfA and 25% XynA producing 544.804 μmol/min sugar (Figure 45B).

The third enzyme synergy study using combinations of two of the three enzymes with the NH_4OH pre-treated SCB employed XynA and ManA. The largest amount of sugar produced (1194.0 μmol sugar/min with a degree of synergy of 2.85) was produced by combining 75% XynA and 25% ManA. The largest degree of synergy (3.40) was observed with an enzyme combination of 25% XynA and 75% ManA which produced 564.7 μmol/min sugar (Figure 45C).

The last set of simultaneous synergy assays that were performed used various combinations of all three enzymes (Figure 46).



A)

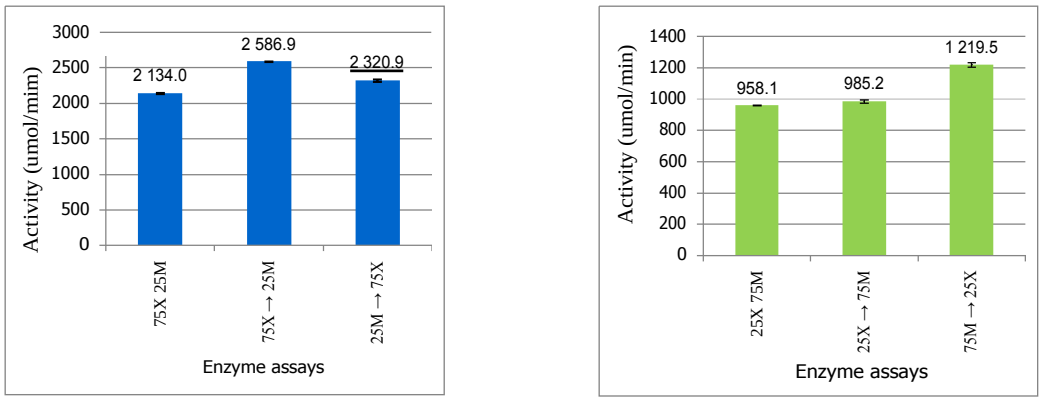


B)

Figure 46: Schematic representation of the enzyme activity and the respective degrees of synergy obtained for the cellulosomal hemicellulases ManA and XynA, and the ancillary enzyme ArfA with the NH_4OH pre-treated SCB. The enzyme activities are represented as the µmol/min sugar produced (A) and degree of synergy (B) are represented as the different shaded circles, as shown in the figure. The enzymes were mixed in varying compositions shown according to the axis as a protein percentage. The arrows indicate the directions of axis for each enzyme. The degrees of synergy are shown as the actual activities divided by the theoretical activities.

The data obtained from the simultaneous synergy assays involving all three recombinant enzymes (Figure 46) indicated that the largest amount of sugar (1194.0 µmol/min) was produced by the enzyme combination of 75% XynA and 25% ManA.

To determine if the enzymes interact in a simultaneous or sequential manner, the enzymes that produced the largest amount of sugar and optimal degree of synergy in the simultaneous synergy assays (Figure 46) were employed. The enzyme assays were performed by sequentially adding the enzymes (Figure 47) to the reaction assay.



(A) (B)

Figure 47: Sequential synergy with the NH_4OH pre-treated bagasse. The different protein combinations of the enzymes that produced (A) the optimal protein ratio that produced the optimal activity and (B) the protein ratio combinations for optimal synergy that were obtained during the synergy assays the NH_4OH pre-treated SCB (Figure 45). Values are presented as mean values ± SD (n=3).

The data from the enzyme synergy assays indicated the the synergistic association between the xylanase (75% XynA) and mannanase (25% ManA) was enhanced when the enzymes were added sequentially (Figure 52A). A similar result was observed with 25% XynA and 75% ManA (Figure 47B).

The effect of time on the synergistic associations was determined using the enzyme combination that produced the largest amount of sugar with the NH_4OH pre-treated SCB (Table 19 and Figure 48).

Table 19: The effect of time on the production of reducing sugars (µmol) with the hydrolysis of NH_4OH SCB.

Substrate	Enzyme assay	Time (hours)				
		24	**48**	**72**	**96**	**120**
NH_4OH pre-treated SCB	25% ManA	58.3 ± 9.0	43.4 ± 21.5	156.2 ± 52.0	171.6 ± 15.4	224.6 ± 18.8
	75% XynA	2199.5 ± 148.1	2484.8 ± 64.1	2442.6 ± 97.1	3235.5 ± 146.7	4210.4 ± 157.1
	25% ManA: 75% XynA	5438.5 ± 138.5	6422.3 ± 166.1	7213.7 ± 322.1	8347.7 ± 730.3	12396.7 ± 961.1
	Theoretical value	2257.8	2528.2	2598.8	3407.0	4434.9
	Degree of synergy	2.41	2.54	2.78	2.45	2.80

* Values are presented as mean values ± SD (n=3)

The data obtained for the effect of time (Table 19) on the production of sugar with the hydrolysis of NH_4OH pre-treated SCB is also represented graphically in Figure 48.

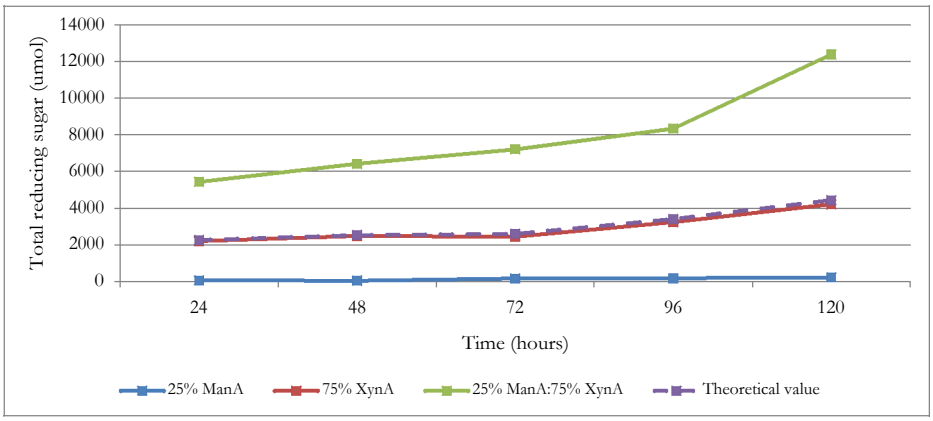


Figure 48: The effect of time on production of reducing sugars with NH_4OH pre-treated SCB.

As depicted in Table 19 and Figure 48, the production of sugar increased exponentially after 96 hours, indicating that the degree of synergy between the recombinant enzymes required for the optimal production of sugar was the highest after 96 hours.

4.4 Discussion

The synergistic associations between the recombinant enzymes (ArfA, ManA and XynA) were determined through the quantification of the reducing sugars released during the degradation of untreated SCB and alkaline pre-treated SCB. The various enzyme concentrations were standardized to the same protein concentration (mg/ml) and the assay volume remained constant throughout. The synergistic relationships between ArfA, ManA and XynA were also determined by varying the ratios of the enzymes used in the hydrolysis of the different substrates. The most effective combination of enzymes was determined by quantifying the amount of reduced sugar present in solution, and the greatest degrees of synergy were determined by dividing the observed activities of the recombinant enzymes obtained with the enzyme assays with the different substrates, by the theoretical sum of the individual recombinant enzyme activities. The synergistic associations between the different combinations of the recombinant enzymes required the degree of synergy to be greater than 1.

To test the validity of the synergy assays the recombinant enzymes were purified and assayed with a mixed model substrate (2% (w/v) LBG:OSX) to elucidate the presence of any synergistic associations that may have developed between the three enzymes. The model substrate that was used in the synergy studies was a mixture of a galactomannan (LBG) and an arabinoxylan (OSX). Galactomannans have a backbone composed of 1,4-β-L-mannose residues with α-D-galactose substituents. The general structure of galactomannans are similar, the only difference between the different galactomannans is the degree of substitution. LBG has a substitution ratio of 1 galactose residue to 4 mannose residues (Figure 49A). LBG is considered to have a low degree of substitution in comparison to other galactomannans. The galactose substituents are bound to the mannose backbone via the formation a glycosidic bond at the C-1 position of the galactose residue and the C-6 position of the mannose residue (Samil-Kok *et al.,* 2007). An arabinoxylan backbone is composed of 1,4-β-D-xylose chain, with arabinose substituents (Figure 49B). The arabinose side chains are typically bound to the O-2 and the O-3 position on the xylose residues, depending on the degree of substitution. However, the substitution primarily occurs at the O-2 position (Pell *et al.,* 2004; Taylor *et al.,* 2006).

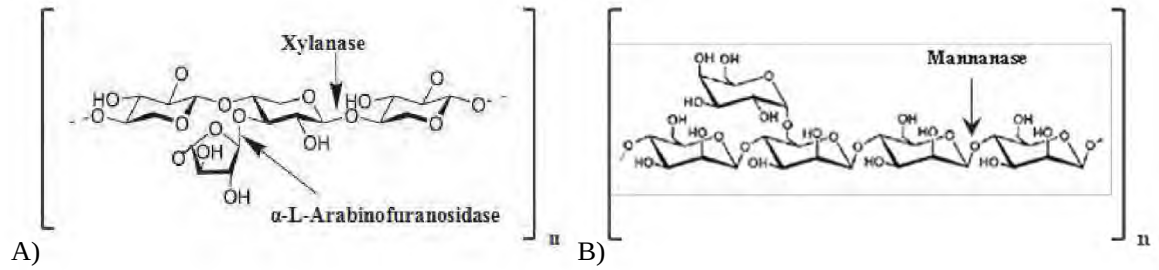


Figure 49: Schematic representation of the structure of A) LBG and B) arabinoxylan (adapted from Taylor *et al.*, 2006 & Zhang *et al.*, 2008).

LBG is composed of a galactose substituated mannan, in a ratio of 1 galactose residue per 4 mannose residues, as previously mentioned, thus the galactomannan is comprised of 80% mannose, thus the model substrate would contain 40% mannan. There is limited information available on the structure of the commercial OSX with the regard to the amount of arabinose substituents that are present on the xylose backbone. The data obtained from the hydrolysis of the model substrate using XynA and ManA, indicated that the individual enzymes had a similar activity. From this it could be speculated that the OSX may have an identical substituent ratio as the LBG, i.e. 1 arabinose residue per 4 xylose residues. The data obtained from the synergy assays confirmed that the hemicellulases were essential for the effective hydrolysis of the model substrate. Therefore, the hydrolysis of the 2% (w/v) LBG:OSX mixed model substrate yielded the expected results. The hydrolytic activity of the hemicellulases contributed to the majority of the released sugar, which was expected since mannan and xylan constituted approximately 80% of the composition of the model substrates composition, whereas the arabinose side chains on the OSX would constitute approximately 10% of the substrate. Therefore, the low ArfA activity was also expected. The largest amount of sugar was produced with the simultaneous synergistic association between the three enzymes; however, the production of sugar increased and displayed a sequential synergistic association when the ancillary enzyme was first added to the reaction. This would occur if the initial reaction involved the removal of the arabinose side chains with the addition of ArfA, with the subsequent addition of the remaining enzymes. This result is confirmed by literature, where increased activity is generally obtained by the removal of side chains that may hinder

the accessibility of the glycosidic bonds to hydrolysis.The data obtained from the hydrolysis of the model substrate with various combination of ArfA and XynA confirmed that a synergistic association occurs between the ancillary enzyme and the hemicellulase which has been observed in previous studies by Kosugi *et al.* (2002a), Koukiekolo *et al.* (2005) and Beukes *et al.* (2010).

To test the efficiency of the different alkaline pre-treatments on the production of sugar and potential synergistic association(s) between the different enzymes, various synergy assays were performed with native or untreated SCB. The synergistic analysis of the untreated SCB provided the basal enzyme activities and synergistic association for comparison with the results observed with the synergistic analysis of the pre-treated SCB samples. SCB is a lignocellulosic biomass, thus it has a high percentage of lignin present in its chemical composition increasing the recalcitrance of SCB to enzymatic hydrolysis and further downstream processing (including fermentation). The lignin forms a protective sheath around the carbohydrate moiety of the SCB; however, as a result of sugarcane harvesting and processing to remove the sucrose content, the sugarcane is milled, crushed and air-dried. This essentially affects the structure of the SCB, creating "holes" where the sugarcane had been bent or broken during the extraction of sucrose. The "holes" allow the recombinant enzymes to gain access to and hydrolyse the glycosidic bonds of their respective substrates on the untreated SCB. Ideally, the enzyme combination that would produce the largest amount of sugar should also have the optimal degree of synergy; however, this was not the case. The largest amount of sugar (91.8 μmol/min) was produced with 37.5% ArfA and 62.5% ManA, with a degree of synergy of 1.83 indicating, that the hemicellulose, xylan backbone was embedded further within the untreated SCB cell walls protected from enzymatic hydrolysis. The hydrolysis of the xylan backbone appeared to be hindered by the presence of arabinose, mannosyl and other substituents. The optimal degree of synergy (2.35) was obtained with 12.5% ArfA and 87.5% XynA, producing 71.2 μmol/min sugar, which was expected, since these two enzymes are well known to associate and act synergistically in the degradation of xylan (Kosugi *et al.,* 2002a; Koukiekolo *et al.,* 2005). The final synergy assays that were performed attempted to identify the type of synergistc relationship was present between the combinations of 1) 37.5% ArfA and 62.5% ManA and 2) 12.5% ArfA and 87.5% XynA. In both cases, the production of sugar increased when the enzymes were added sequentially.

With regard to the combination of 62.5% ManA and 37.5% ArfA, the sequential synergistic relationship may have occured primarily because mannose and arabinose units are generally not directly associated with each other in the hemicellulose fraction. Thus, the removal of the side chains with the sequential assays may have removed the steric hindrance between the sugars, indicating that these sugars are in close proximity on the SCB structure. The final assay that was performed with the SCB determined the effect of time on the production of sugar. As expected, the production of sugar increased gradually over time, whereas time did not dramatically affect the degree of synergy.

The SCB was pre-treated with alkaline chemicals for a number of reasons. Alkaline pre-treatments generally proceed at ambient temperatures and at low pressures, eliminating the cost of maintaining these pre-treatment comditions. However, alkaline pre-treatments may require more time in comparison to the pre-treatment technologies ($Ca(OH)_2$, NaOH and NH_4OH) discussed in this and the previous chapter. During alkaline pre-treatment lignocelluloses undergoes two reactions, namely solvation and saponification, causing the lignocelluloses structure to swell, decreasing the degree of polymerization, and making the lignocellulosic components more accessible to enzymatic hydrolysis (Hendriks and Zeeman, 2009). The first alkaline chemical used to pre-treat the SCB was $Ca(OH)_2$. The hydrolysis of the $Ca(OH)_2$ pre-treated SCB increased 6.5 fold. Unlike the hydrolysis of the untreated SCB, the pre-treated SCB required the use of all three enzymes. The maximum amount of sugar (593.7 μmol/min) was produced with 37.5% ArfA, 25% ManA and 37.5% XynA, with an observed degree of synergy of 2.14. The combination of 37.5% ArfA, 50% ManA and 12.5% XynA achieved the optimal degree of synergy (2.17) with the hydrolysis of the $Ca(OH)_2$ pre-treated SCB, producing approximately 517.5 μmol sugar per min. The increase in enzyme activity was potentially due to the disruption/partial removal of lignin sheath, thus exposing the xylan and mannan residues to ManA and XynA hydrolysis, respectively. However, despite the increase in enzymatic activity with $Ca(OH)_2$ pre-treated bagasse, the maximum degree of synergy remained unaffected. Once the optimal enzyme combinations were obtained for the simultaneous synergy assays, the type of synergy was investigated. As in the case with the hydrolysis of the untreated SCB, the optimal amount of sugar was produced when the ArfA was added to reaction to cleave off any arabinose side chains prior to the addition of the other enzymes. However; this chemical pre-treatment did not really affect the

optimal degree of synergy. After the synergy assays were performed, the effect of time on the production of sugar with the hydrolysis of the $Ca(OH)_2$ pre-treated SCB was investigated. Time had a definite effect on the degrees of synergy obtained with the hydrolysis of the $Ca(OH)_2$ pre-treated SCB, doubling the degree of synergy. As in the case with the hydrolysis of the untreated SCB, a gradual increase in the enzyme activity with the majority of the enzymes was observed thus indicating that time may have an important role to play in the formation of synergistic relationships between different enzymes for the optimal production of reducing sugar. Thus it may be hypothesised that the enzymes may require a certain amount of time to bind and hydrolyse the glycosidic bonds of the respective carbohydrate moieties within the cell walls.

The hydrolysis of the NaOH and NH_4OH pre-treated SCB produced both expected and unexpected results. NaOH was the second chemical used to pre-treat the SCB, and is commonly used to delignify biomass in various industrial processes, e.g. the Kraft process. In comparison to the untreated SCB (91.8 μmol/min), the NaOH pre-treatment enhanced the digestibility of the SCB as indicated by the 9.24-fold increase in the enzymatic hydrolysis of the SCB 9.24-fold, producing a maximum of 849.4 μmol/min of sugar. The enzyme combination of 25% XynA, 12.5% ManA and 62.5% ArfA produced the maximum amount of sugar (849.4 μmol/min) with the NaOH pre-treated SCB, and displayed a degree of synergy of 1.27. The third alkaline chemical that was used to pre-treat the SCB was NH_4OH. The highest activity (1194.9 μmol/min) was produced with the enzyme combination of 75% XynA and 25% ManA and displayed a degree of synergy of 2.85. The results obtained from the synergy assays with the NaOH pre-treated SCB were unexpected. With the removal of the arabinan from the SCB (discussed in the previous chapter), ArfA appeared to be able to hydrolyse the exposed 1,4-β-glycosidic bonds of the smaller xylan oligosaccharides produced the xylanase activity. This activity was unexpected since ArfA traditionally hydrolyses the (1--+3)- and the (1--+5)-α-arabinosyl linkages between the arabinose substituents present on some of the xylose monomers that constitute the xylan backbone (Greve *et al.,* 1984). A similar finding was observed by Kosugi *et al.* (2002c), who showed that ArfA had a very low degree of activity with birchwood xylan. With regard to the NH_4OH pre-treated SCB, the higher degree of hydrolysis is thought to be predominantly due to the presence of the xylanase (XynA). It was expected that the majority of the hydrolysis of the NH_4OH pre-

treated SCB was due to the action of XynA since the main type of hemicellulose present in the SCB samples is xylan. Thus, the higher activities that were observed would have been as a direct result of the hydrolysis of the xylan in the substrate. However, the low ManA activity observed potentially indicated that the SCB sample contained a low percentage of mannose side chains, thus it was not expected to significantly affect the total amount of sugar produced during the hydrolysis of the NH_4OH pre-treated SCB.

It has been documented that in some cases, the rotation around the 1,3-α-glycosyl linkages between the arabinose side chains and the xylan backbone, may be forced into a different conformation, resembling the 1,4-β-xylosidic bonds between xylose residues (Utt *et al.,* 1991). We propose that NaOH not only delignified the SCB, but also compromised the structural integrity of the plant cell walls. The disruption of the structural integrity of the SCB may have lead to increased tension on the 1,3 or 1.5-α-glycosyl linkages resulting in conformational changes and in some of the 1,3 or 1,5-α-glycosidic bonds resembling the 1,4-β-glycosidic bonds of the hemicelluloses backbone. This hypothesis supports the theory that the hemicellulolytic enzymes used in this study are bifunctional and are capable to a certain degree to hydrolyse the different glycosidic bonds present in the SCB samples to a certain degree which is also supported by findings by Utt et al. (1991) and Kosugi et al. (2002a). The potential bifunctionality of ArfA and the disruption of the structural integrity (discussed in a previous chapter) of the NaOH SCB may have been responsible for the high ArfA activity. It is also hypothesised, that the ManA may be capable of randomly hydrolysing of the 1,4-β-glycosidic bonds of the hemicellulose xylan backbone after NH_4OH pre-treatment, increasing the amount of sugar that was released with the hydrolysis of the SCB sample. In some cases, a degree of synergy below 1.0 was obtained; indicating that the theoretical sum of the individual enzyme activities in the enzyme cocktail was greater than the actual activity that was observed. The lack of a synergistic association between ArfA and the ManA indicated that the respective hydrolysis targets (α-arabinosyl linkages and β-mannosyl linkages) may not have been present, potentially due to the possible removal of the α-arabinosyl and β-mannosyl substituents during the pre-treatment steps. Thus, it is thought that this may have occurred as a result of the potential bifunctionality of the enzymes, resulting in the enzymes competing for the same substrate cleavage site.

Once the optimal enzyme combinations were established, the type of synergy was indentified. The data obtained from the hydrolysis of the NaOH pre-treated SCB indicated that the enzyme association(s) between the enzyme combinations were simultaneous, unlike the synergistic associations obtained with untreated and $Ca(OH)_2$ pretreated SCB. The hydrolysis of NH_4OH pre-treated SCB increased slightly with the sequential assays; however, the increase in the production of sugar was minimal in comparison to the hydrolysis observed with simultaneous addition of the enzymes. This indicates that the hydrolysis of the NH_4OH pre-treated SCB would occur efficiently regardless of how the enzymes are introducted into the reaction system.

4.5 Conclusions

The main objective of this study was to identify the presence of any synergistic associations between the different enzymes that would facilitate the efficient hydrolysis of SCB, and to determine the effect(s) of the various alkaline pre-treatments of SCB on the synergistic relationships and hydrolysis of the different SCB samples. The synergistic analyses using the hemicellulases (ManA and XynA) and the ancillary enzyme (ArfA) yielded both expected and unexpected results.

From the results obtained for the hydrolysis of the untreated SCB, the following was concluded:

1) The maximum activity (91.8 µmol/min) was obtained with an enzyme combination of 37.5% ArfA and 62.5% ManA.
2) The optimal degree of synergy (2.35) was obtained with an enzyme combination 12.5% ArfA and 87.5% XynA.
3) Time did not appear to affect the degree of synergy; however, the production of sugar increased over time.
4) The largest amount of sugar was produced under conditions of sequential synergy.

From the results obtained for the hydrolysis of $Ca(OH)_2$ pre-treated SCB, the following was concluded:

1) $Ca(OH)_2$ pre-treatment increased enzyme activity ± 6.5 fold. Maximum sugar (593.7 µmol/min) was produced at a combination of 37.5% ArfA, 25% ManA and 37.5% XynA.
2) The optimal degree of synergy (2.17) was obtained with an enzyme combination of 37.5% ArfA, 50% ManA and 12.5% XynA.
3) The increase in enzyme activity was potentially due to the disruption/partial removal of lignin sheath, thus increasing the hemicellulose surface area to hydrolysis.
4) Time affected the degree of synergy, and thus may play a role in the formation of synergistic relationships between different enzymes for the optimal production of reducing sugar.

From the results obtained for the hydrolysis of the the NaOH pre-treated SCB, the following was concluded:

1) The hydrolysis of NaOH pre-treated SCB indicated a 9.24-fold increase in the overall production of sugar.
2) The maximum amount of sugar (849.4 µmol/min) was produced under conditions of simultaneous synergistic hydrolysis of the pre-treated SCB with 62.5% ArfA, 12.5% ManA and 25% XynA.
3) The optimal degree of synergy (1.88) was obtained with 25% ArfA and 75% ManA producing 116.8 µmol sugar/min.
4) With regard to the effect of time, there was a gradual increase in the production of sugar; however, the degree of synergy decreased over time.

From the results obtained for the hydrolysis of the the NH_4OH pre-treated SCB, the following was concluded:

1) The maximal production of sugar (1194.0 µmol/min) was produced by the combination of 75% XynA and 25% ManA displaying a 13.01-fold increase in enzyme activity.
2) The optimal synergy (3.40) was obtained with 75% ManA and 25% XynA producing 564.7 µmol/min.

3) The production of sugar increases over time; however, time appeared to affect the degree of synergy.
4) Maximum sugar was produced under conditions of sequential synergy; however, the difference between the production of sugar with the sequential and simultaneous synergy was minimal.

Thus, in summary, it was concluded that, the alkaline pre-treatments proved to be effective with the partial removal of lignin under moderate conditions, therefore rendering the process more cost effective. Synergistic associations between the different enzyme combinations were established. A substantial increase in the hydrolysis of the SCB and the production of sugars for downstream processing was achieved, predominantly under conditions of sequential synergy. The synergy assays performed on some of the pre-treated SCB samples indicated the possibility that the hemicellulases and the ancillary enzyme are bifunctional under certain conditions, enabling these enzymes to cleave the glycosidic bonds that they do not normally cleave. The most efficient means to chemically pre-treat SCB appeared to be NH_4OH, facilitating a 13-fold increase in the production of sugar with 25% ManA and 75% XynA.

This chapter identified the different combinations of enzymes required for the synergistically hydrolysis of untreated and pre-treated SCB. The subsequent chapter describes the basic analysis of the sugar produced by the synergistic enzymatic hydrolysis of the different SCB samples and the by-products of obtained by the alkaline pre-treatment processes.

Chapter 5: Basic analyses of chemical pre-treatment products and enzymatic hydrolysates

5.1 Introduction

The most common renewable biofuel is bioethanol, which is traditionally derived from starch (composed of glucose) obtained from maize and sucrose from SCB (Gray *et al.*, 2006). Unlike cellulose, hemicellulose is heterogeneous mixture of predominantly pentose sugars (xylose and arabinose) and hexose sugars (mannose, glucose, and galactose) and sugar acids. Not all of the lignocellulose sugars can be fermented for biofuel production, e.g. xylose is generally used for the production of xylitol. Due to the wide variey of potential sugars that may be produced from the enzymatic hydrolysis of the lignocellulose, it is potentially important to know what sugars are produced, as the resultant sugars may affect the type of downstream processes that are employed. The analysis of sugars is generally limited to the use of traditional techniques, such as nuclear magnetic resonance (NMR), chromatography and capillary electrophoresis, due to their general lack of chromophoric or fluorophoric functional groups and low extinction coefficients (O'Shea *et al.*, 1998; Starr *et al.*, 1996). This study makes use of thin layer chromatography (TLC) and fluorophore-assisted carbohydrate electrophoresis (FACE), which has been reported to separate different sugars efficiently for putative identification.

Lignin is characterised as a heterogeneous plant polymer, produced through the dehydrogenative polymerisation of *p*-coumaryl, coniferyl and sinapyl alcohols; however, when the mentioned above cinnamyl alcohols are incorporated into lignin they are referred to as *ρ*-hydroxyphenyl, gauiacyl and syringyl lignin units (Boerjan *et al.*, 2003; Sederoff *et al.*, 1999). These phenolates have a variety of potential bonding motifs, creating the heterogeneity and aromaticity of the lignin structure. The extensive carbon-crosslinking of the phenolates increases the recalcitrance of lignin (Chang, 2007; Ralph *et al.*, 2004). Lignin can be covalently linked to the hemicellulose moiety of the plant biomass via ferulic acid ester linkages (Gray *et al.*, 2006). This chapter makes use high performance liquid chromatography (HPLC) and mass spectroscopy to separate the different phenolates extracted from the pre-treatment liquors for putative identification.

5.2 Methods and materials

5.2.1 High performance liquid chromatography and mass spectroscopy analysis of the pre-treatment liquors

5.2.1.1 HPLC sample preparation

After the SCB was pre-treated, the SCB was filtered through cheesecloth to remove the pre-treatment liquor (filtrate). The filtrate was filtered through a 0.45 µm nylon filter and the pH was slowly adjusted to pH 2.0 using phosphoric acid. Any subsequent insoluble precipitates were removed by filtration as described before. The compounds were extracted from the filtrate using Oasis HLB solid phase extraction (SPE) cartridges from Waters. The extraction procedure was as follows:

1) The SPE cartridges were equilibrated by washing the cartridges with 3 ml of a methanol:diethyl ether (10:90 v/v) solution. The cartridges were rinsed with 2 ml methanol and 2 ml dH_2O.
2) After the SPE cartridges had been equilibrated, 20 ml of the filtrate was applied to the cartridges. The cartridges were subsequently washed with 1 ml dH_2O.
3) The phenolic samples were eluted from the SPE cartridges using 2 ml methanol. The sample was aliquoted into 20 µl fractions and the methanol was evaporated using N_2 gas to further concentrate the eluted sample. The aliquots were stored at -20°C until analysed.

5.2.1.2 HPLC analysis of pre-treatment liquor

The pre-treatment liquor samples were analysed on a Waters C18 column. The samples were resolved on Beckman Gold HLPC system, using a diode array detector. The mobile phases used to separate the different compounds were: Solvent A was 90% (v/v) acetonitrile, acidified using 1% (v/v) phosphoric acid, and Solvent B was 23% (v/v) methanol, acidified using 1% (v/v) phosphoric acid. A gradient elution was used with a flow rate of 1 ml per minute and a wavelength of 260 nm was used resolve the different compounds (Table 20).

Table 20: Running conditions for HPLC elution

Time (minutes)	Module	Function	%B	Duration (minutes)
0	Pump	%B	100	0.5
0.5	Det 168	Autozero		
0.5	Pump	%B	100	5
5.5	Pump	%B	0	45.5
51	Pump	%B	0	20
71	Det 168	Stop Data		

5.2.1.3 HPLC-MS analysis of pre-treatment liquor

The analysis of the pre-treatment liquor samples was performed on a Waters UPLC SYNAPT G1 HDMS QTOF system using a Waters HSS T3 analytical column (150 x 2.1 mm; 1.7 um). The peaks were detected with a PDA detector and a SYNAPT G1 HDMS QTOF mass spectrometer. The HPLC-MS system was equilibrated as follows: the starting eluting buffer was 85% (v/v) water, acidified with 0.1% (v/v) formic acid and 15% (v/v) acetonitrile. A linear gradient was used to ramp the mobile phase conditions to 10% (v/v) water: 90% (v/v) acetonitrile after 10 minutes. These conditions were kept constant for two minutes and a fast gradient back to the initial starting conditions prepared the system for the next injection.

The column temperature was kept at 50°C and a flow rate of 0.45 ml/min was used. The samples were run for 15 min. The MS detection was performed in ESI negative mode. The full scan data was collected between 100 and 1000 Da at a scan rate of 100 ms. Leucine Enkephalin was used as lockmass signal and mass accuracy was on average 2 -5 mDa.

Accurate mass data were used to calculate the best empirical formulae and submitted to the ChemSpider database; and the EI spectra were submitted to the NIST 2005 mass spectral library for identification.

5.2.2 Analysis of hydrolysis products

5.2.2.1 FACE analysis

The hydrolysis products were analysed using modified protocols from Jackson (1990) and Volpi and Maccari (2005). The hydrolysis products were derivatised with the fluorophore, 8-aminonaphthalene-1,3,6-trisulphonate (ANTS). The sugar samples were lyophylised overnight. The lyophylised sugar samples were dissolved in 5µl of 0.2 M ANTS prepared in acetic acid:water (3:17, v/v) and 1 M sodium cyanoborohydride ($NaCNBH_3$) prepared in dimethyl sulphoxide (DMSO). The sugar solutions were derivatised for 15 hours at 37°C and vacuum dried. The derivatised sugar samples were redissolved in 0.5 M Tris-HCl (pH 6.60) and stored at -20°C when not used. The derivatised sugar samples were electrophoresed on a native PAGE gel, using T25%/C3.75% resolving gels and T5%/C1.5% stacking gels (Appendix D). The derivatised sugars were electrophoresed on ice using the following parameters: prior to loading the sugar samples, the native PAGE gels were electophoresed for 10 min at 100 V, after which the samples were loaded, and the gels were electrophoresed for 10 min at 100 V, and for 40 min at 300V (Appendix D). The gels were visualised under UV light.

5.2.2.2 TLC analysis

TLC was used to putatively identify the sugars released from the synergistic enzymatic hydrolysis of the different SCB samples over a period of 5 days. The hydrolysis products were hydrolysed using 2 M triflouroacetic acid (TFA) and analysed on TLC, as follows: To 50 µl of the hydrolyasates, 300 µl of 2 M TFA was added and incubated at 110°C for 1 hour. The acid hydrolysed samples were vacuum-dried at 40°C for 15 hours and separated on a TLC plate. The TLC mobile phase used to resolve the sugars was 90% (v/v) acetonitrile. After the TLC plate was run and air-dried, the TLC plate was briefly covered with a solution containing 90% (v/v) absolute ethanol and 10% (v/v) of a 20% (v/v) sulphuric acid solution and air dried. The samples were visualised by incubating the TLC plate at 110°C.

5.3 Results

5.3.1 Analysis of the pre-treatment liquor

5.3.1.1 HPLC analysis

The compounds extracted from the different pre-treatment liquors were reconstituted in 1 ml of methanol and filtered through a 0.2 µm syringe filter. The filtered samples were injected into the HPLC system and resolved on a $_{C18}$ reverse phase column with gradient elution using a combination of mobile phases containing methanol and acetonitrile (Figure 50-52).

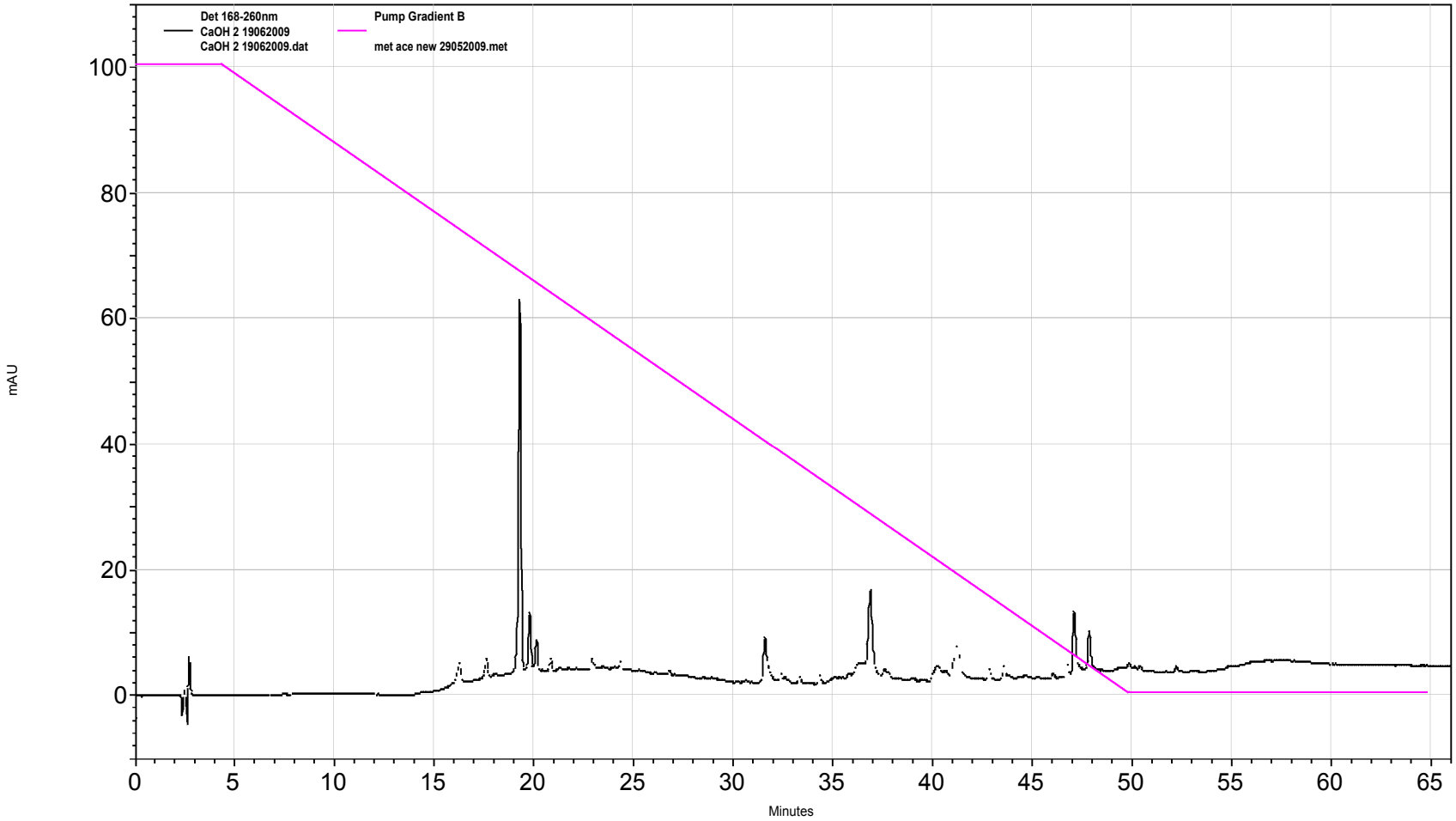


Figure 50: HPLC elution profile for the sample obtained from the $Ca(OH)_2$ pre-treatment liquor. The pink line represents the gradient elution profile.

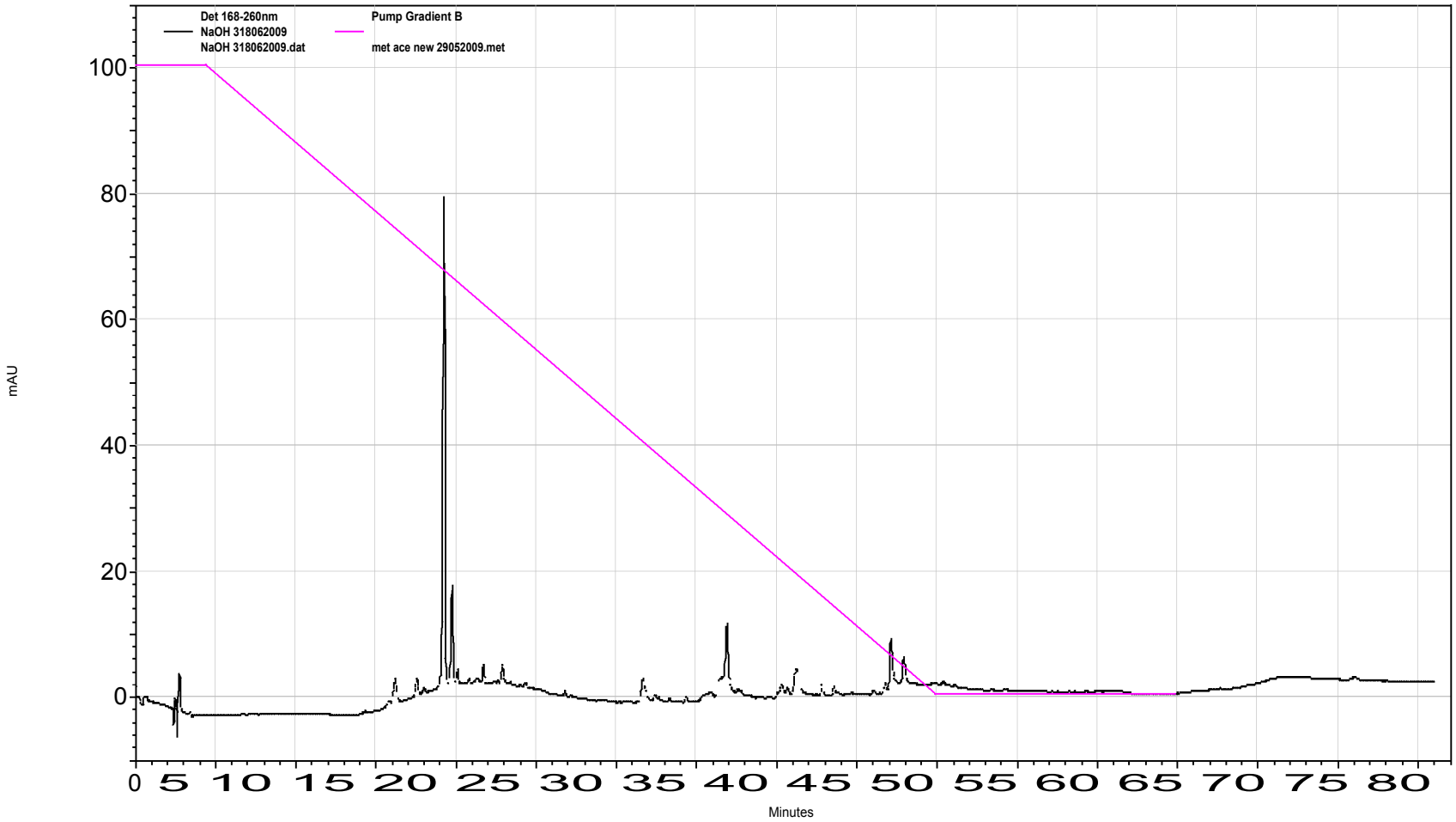


Figure 51: HPLC elution profile for the sample obtained from the NaOH pre-treatment liquor. The pink line represents the gradient elution profile.

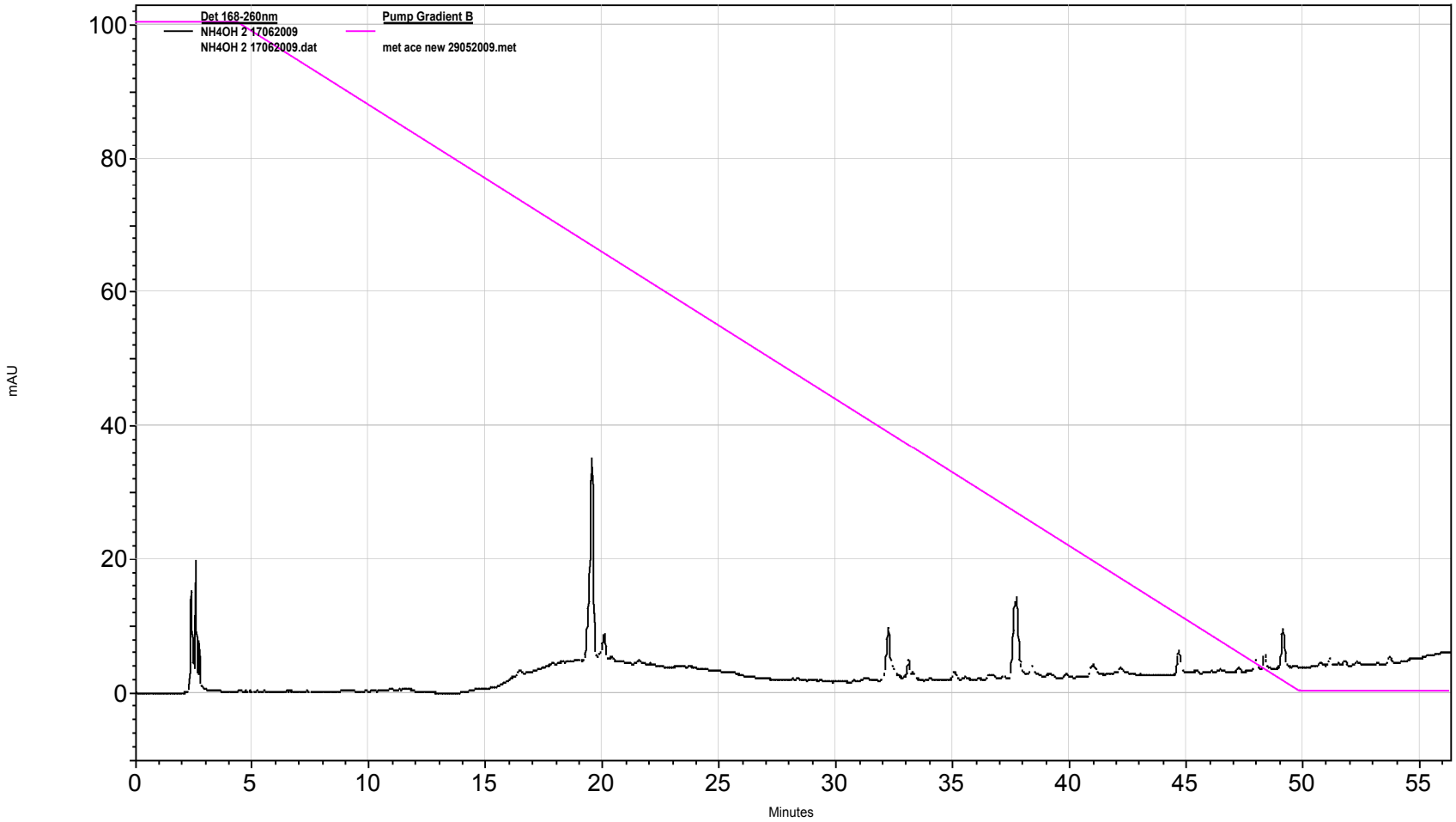


Figure 52: The HPLC elution profile for the compounds extracted from the NH_4OH pre-treatment liquor. The pink line represents the radient elution profile.

From the chromatograms for the different pre-treatment liquors (Figure 51-52), indicated a similar elution profiles were obtained from the different pre-treatment liquors, especially with regard to the elution profiles obtained for the NaOH and $Ca(OH)_2$ pre-treatment liquors.

5.3.1.2 LC-MS analysis

After the samples were resolved on the C18 reverse phase column, the samples were applied on to a UPLC SYNAPT G1 HDMS QTOF system using a Waters HSS T3 analytical column. The compound peaks were detected using a PDA detector and a SYNAPT G1 HDMS QTOF mass spectrometer (Figures 53 – 54, Tables 21 - 23).

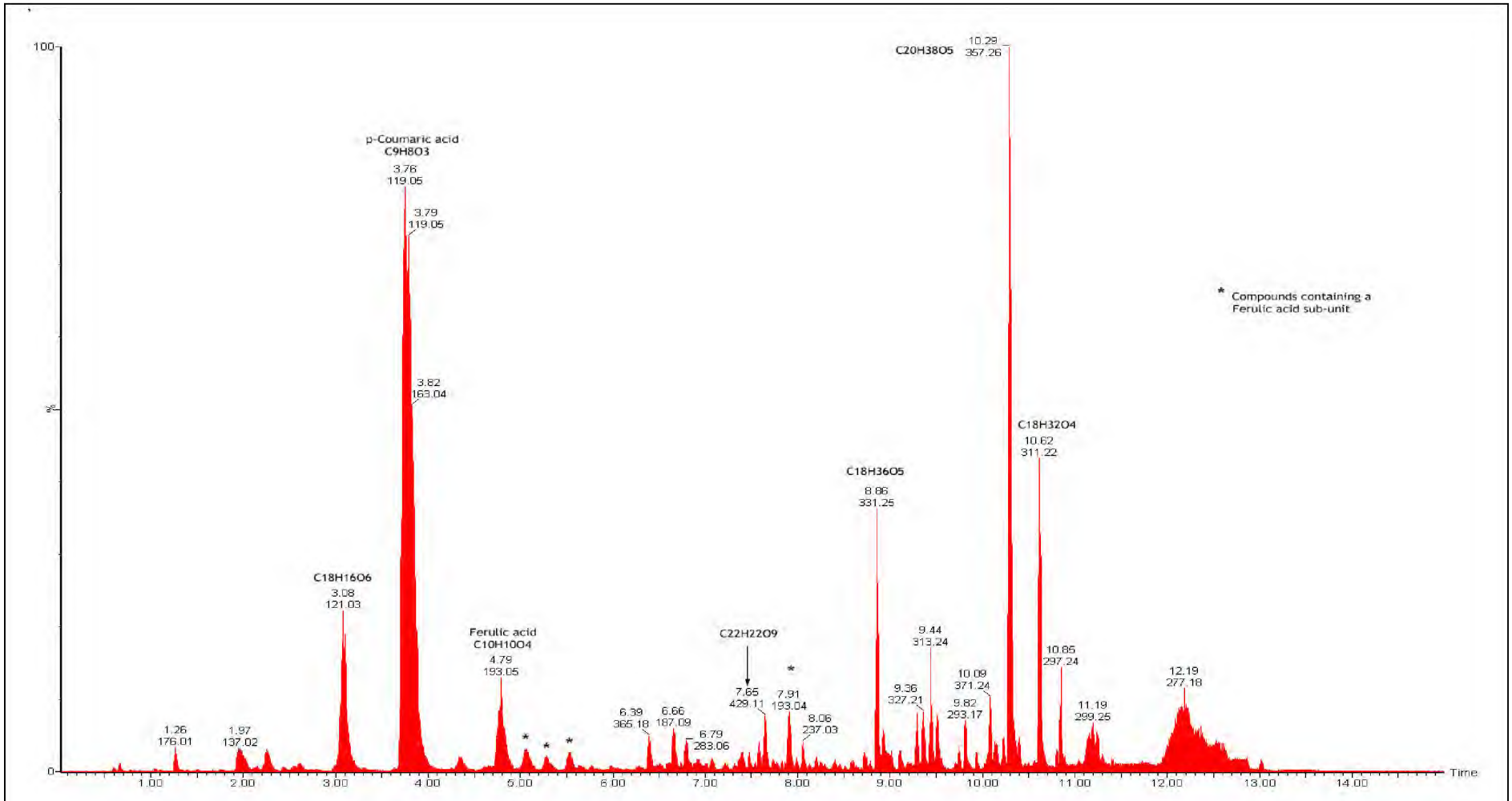


Figure 53: LC-MS for the liquor obtained from the $Ca(OH)_2$ pre-treated SCB. VTOF ESINeg 85%A2: 15%B2 @0.4, T= 50, C8, CV = 20. 1:TOF MS ESI-BPI 2.21e4.

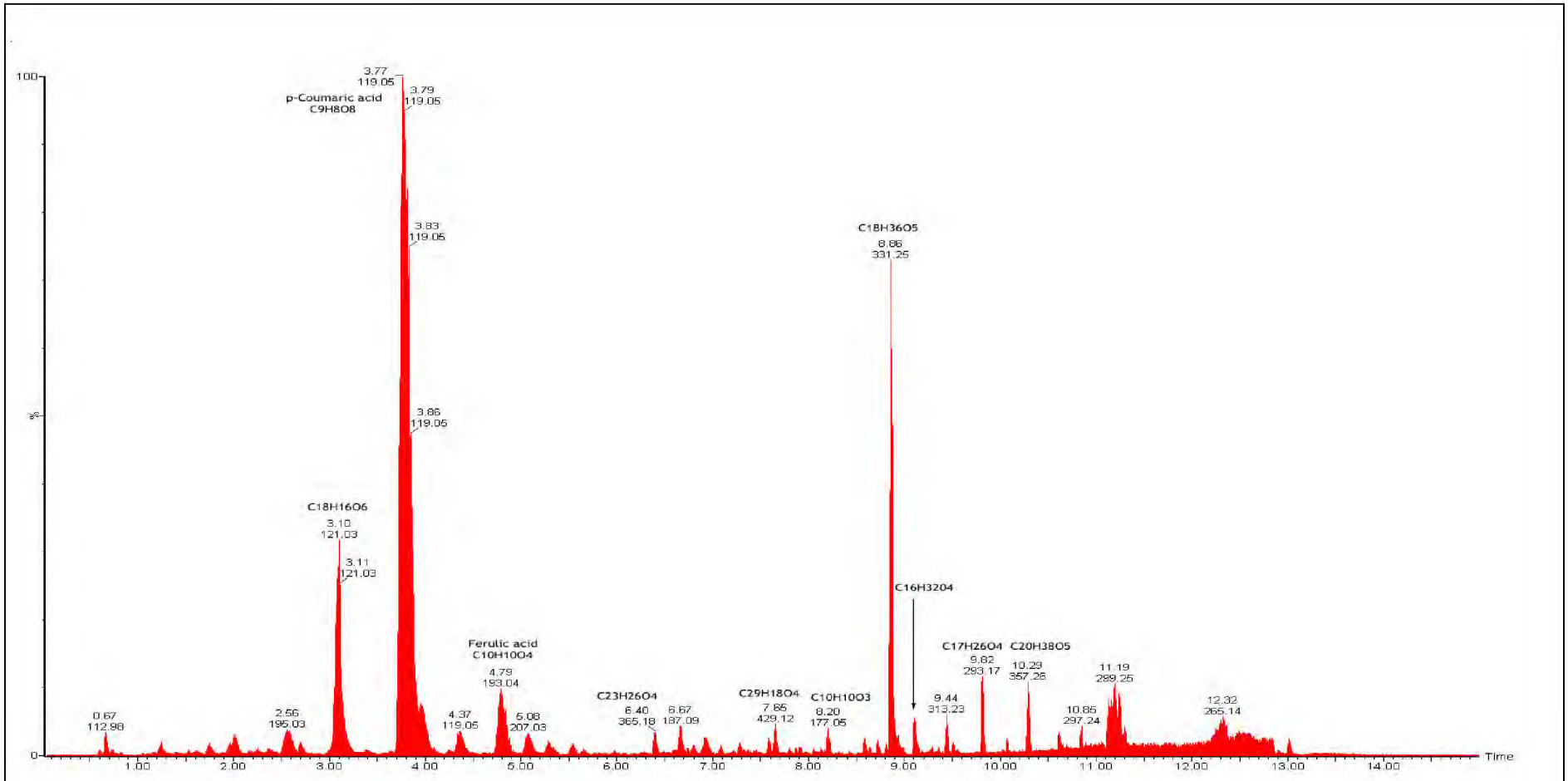


Figure 54: LC-MS for the liquor obtained from the NaOH pre-treated SCB. VTOF ESINeg 85%A2: 15%B2 @0.4, T= 50, C8, CV = 20. 1:TOF MS ESI-BPI 1.49e4.

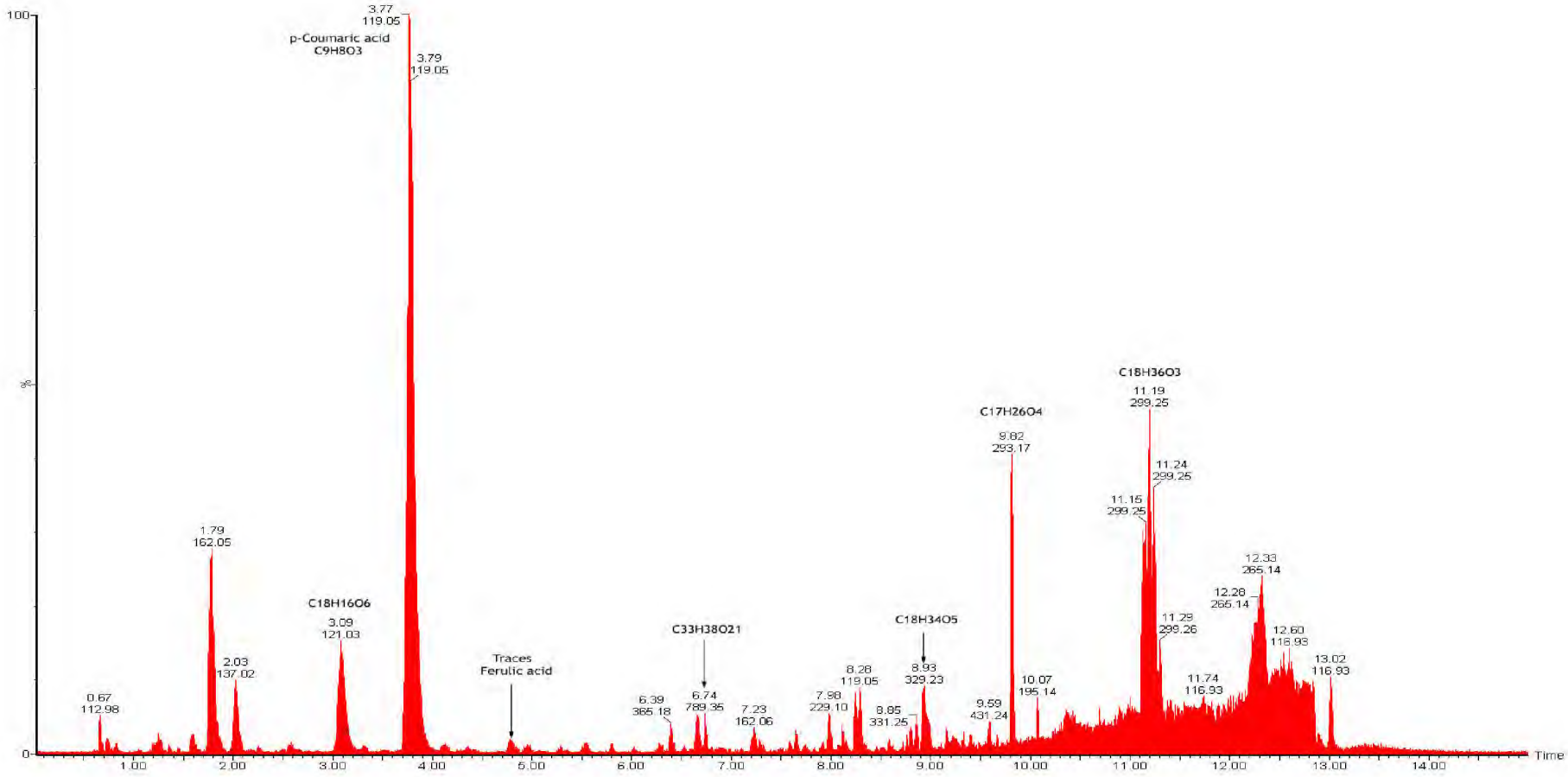


Figure 55: LC-MS for the liquor obtained from the NH_4OH pre-treated SCB. VTOF ESINeg 85%A2: 15%B2 @0.4, T= 50, C8, CV = 20. 1:TOF MS ESI-BPI 3.90e3.

Table 21: List of the possible compounds extracted from the $Ca(OH)_2$ pre-treated SCB liquor separated and detected on the HPLC-MS

$Ca(OH)_2$ pre-treatment liquor		
Compound	**RT (min)**	**m/z**
Vinyl phenol	3.76 / 3.79	119.05
Ethyl phenol ($C_{18}H_{16}O_6$)	3.08	121.03
Methyl guaiacol	1.97	137.02
p-Coumaric acid ($C_9H_8O_3$)	3.82	163.04
	1.26	176.01
	6.66	187.09
Ferulic acid ($C_{10}H_{10}O_4$)	4.79	193.05
Propenyl syringol (contains ferulic acid subunit)	7.91	193.05
Vinyl phenol dimer	8.06	237.03
Methoxycatechol dimer	12.19	277.18
	6.79	283.06
p-Coumaryldehyde dimer	9.82	293.17
p-Coumaryl or Vinyl guacol dimer	10.85	297.24
	11.19	299.25
Coniferyl alcohol and Syringol complex ($C_{18}H_{32}O_4$)	10.62	311.22
	9.44	313.24
p-Coumaryl alcohol and Conferyl alcohol complex	9.36	327.21
Coniferyl alcohol and Syringol complex ($C_{18}H_{36}O_5$)	8.86	331.25
Coniferyl alcohol dimer ($C_{20}H_{38}O_5$)	10.29	357.26
	6.39	365.18
	10.09	371.24
$C_{22}H_{22}O_9$	7.65	429.11

Table 22: List of the possible compounds extracted from the NaOH pre-treated SCB liquor separated and detected on the HPLC-MS

NaOH pre-treatment liquor		
Compound	**RT (min)**	**m/z**
	0.67	112.98
Vinyl phenol ($C_9H_8O_3$)	3.77 / 3.79	119.05
Vinyl phenol	3.86	119.05
Vinyl phenol	4.37	119.05
Ethyl phenol ($C_{18}H_{16}O_6$)	3.1 / 3.11	121.03
Coniferaldehyde ($C_{10}H_{10}O_3$)	8.2	177.05
	6.67	187.09
Ferulic acid ($C_{10}H_{10}O_4$)	4.79	193.04
Acetosyringone	2.56	195.03
Sinapaldehyde	5.08	207.03
Propenyl phenol dimer	12.32	265.14
$C_{16}H_{32}O_4$	9.1	288.42
p-Coumaryl alcohol dimer ($C_{17}H_{26}O_4$)	9.82	293.17
p-Coumaryl alcohol or Vinyl guaiacol dimer	10.85	297.24
	11.19	299.25
	9.44	313.23
Coniferyl alcohol and Syringol complex ($C_{18}H_{36}O_5$)	8.86	331.25
Coniferyl alcohol dimer ($C_{20}H_{38}O_5$)	10.29	357.26
$C_{23}H_{26}O_4$	6.4	365.18
$C_{29}H_{18}O_4$	7.65	429.12

Table 23: List of the possible compounds extracted from the NH_4OH pre-treated SCB liquor separated and detected on the HPLC-MS

NH_4OH pre-treatment liquor		
Compound	**RT (min)**	**m/z**
	0.67	112.98
Indole benzeneacetonitrile	11.74	116.93
Indole benzeneacetonitrile	12.6	116.93
Indole benzeneacetonitrile	13.02	116.93
Vinyl phenol	3.77 / 3.79	119.05
Vinyl phenol	8.28	119.05
Ethyl phenol ($C_{18}H_{16}O_6$)	3.09	121.03
Methyl guaiacol	2.03	137.02
	1.79	162.05
	7.23	162.06
Traces Ferulic acid ($C_{10}H_{10}O_4$)	4.79	193.05
Acetosyringone	10.07	195.14
	7.89	229.1
Propenyl phenol dimer	12.28 / 12.33	265.14
p-Coumaraldehye dimer ($C_{17}H_{26}O_4$)	9.82	293.17
$C_{18}H_{36}O_3$	11.15 /11.19 /11.24	299.25
Coniferl alcohol and vanillin complex ($C_{18}H_{34}O_5$)	8.93	329.23
Coniferyl alcohol and Syringol complex	8.85	331.25
	6.39	365.18
	9.59	431.24
Tetramer of Ferulic acid ($C_{33}H_{38}O_{21}$)	6.74	789.35

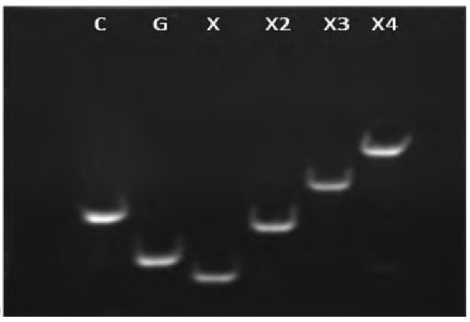
Similar to the chromatograms obtained from the HPLC analysis of the different pre-treatment liquors, the EI spectra obtained from the HPLC-MS analysis of the different samples indicated the presence of similar peaks in the different spectra (Figures 53 - 55). The variety of possible binding combinations that may occur between the different cinnamyl alcohols (p-coumaryl, coniferyl and sinapyl) in lignin increases the difficulty in identifying the products released during the pre-treatments. The data obtained from the HPLC-MS analysis of the pre-treatment liquors (Figures 53 - 55, Tables 21 - 23) identified p-coumaryl and ferulic acid. The other compounds that were

detected may have been a combination of polymerised compounds producing a variety of possible different phenolates (Tables 21 - 23).

5.3.1.3 FACE analysis

The use of fluorophores in carbohydrate analysis allows the different sugars to be separated according to size on PAGE. The combined use of PAGE and fluorophores is termed FACE. This technique is a relatively fast way to analyse sugars inrelation to their degrees of polymerisation (DP) and potentially identify the different carbohydrates.

To determine if the FACE gels would be able to separate different monomeric sugars with varying DP: glucose, cellobiose, xylose, xylobiose, xylotriose and xylotetrarose were electrophoresed (Figure 56).

A) 

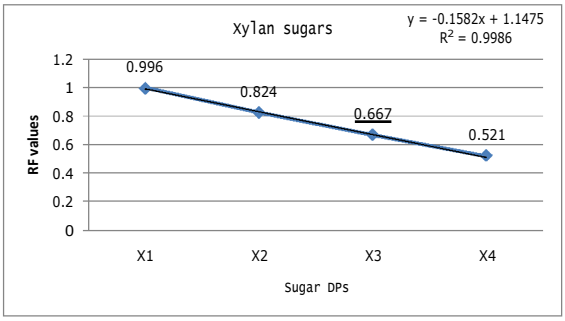
B) 

Figure 56: A) Standard sugars were electrophoresed on a FACE gel and B) the mobility of the different xylose DPs were analysed using Microsoft Excel®. The standard sugars electrophoresed on FACE were glucose (G), cellobiose (C), xylose (X), xylobiose (X2), xylotriose (X3) and xylotetrarose (X4).

Figure 56A, illustrated that the FACE gels separated pentose and hexose and their respective polysaccharides with sufficient resolution that would allow a putative analysis. The mobility of the xylose sugars with the different DPs were analysed using Microsoft Excel® (Figure 56B). The data obtained from the graph exhibited an R-squared value of 0.9986, illustrating that the different DP sugars underwent a linear separation (Figure 56B).

The sugar products produced from the hydrolysis of the untreated SCB sample with the enzyme combination 37.5% ArfA: 62.5% ManA and the respective individual enzymes were analysed using FACE (Figure 57).

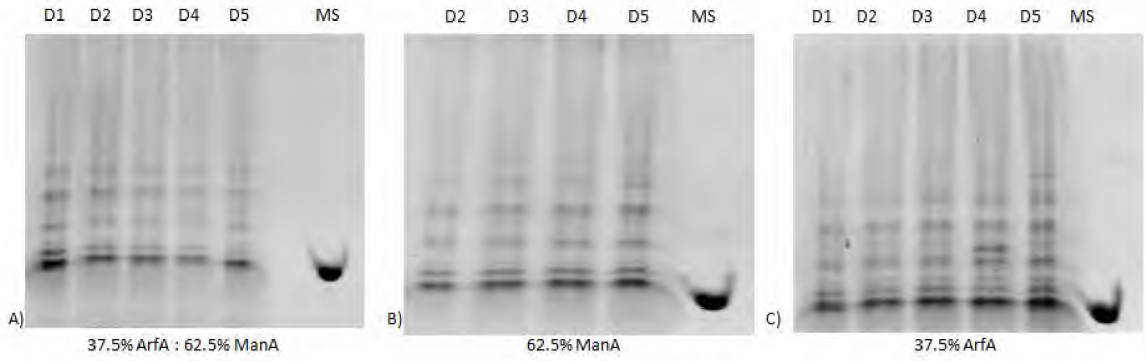


Figure 57: FACE analysis of A) the enzyme combination that produced the maximum amount of sugar with the hydrolysis of the untreated SCB sample and B & C) the individual enzymes. From the different enzyme assays, samples were taken on a daily basis (D1-D5). The daily hydrolysates were compared to a xylose standard (MS).

The data obtained from the separation patterns for the different enzyme assays illustrated a distinct similarity between the sugars samples taken on a daily basis. When comparing the separation patterns of the samples taken daily to that of the monomeric standard (xylose), the FACE gels indicated that the smallest sugar produced from the hydrolysis of the SCB sample had a DP of 2 (Figure 57).

The second set of SCB hydrolysates that were analysed on FACE gels was obtained from the hydrolysis of the $Ca(OH)_2$ pre-treated SCB (Figure 58). The $Ca(OH)_2$ pre-treated SCB was hydrolysed with the enzyme combination A) 37.5% ArfA:25% ManA:37.5% XynA, B) 37.5% ArfA and C) 25% ManA and D) XynA (Figure 58).

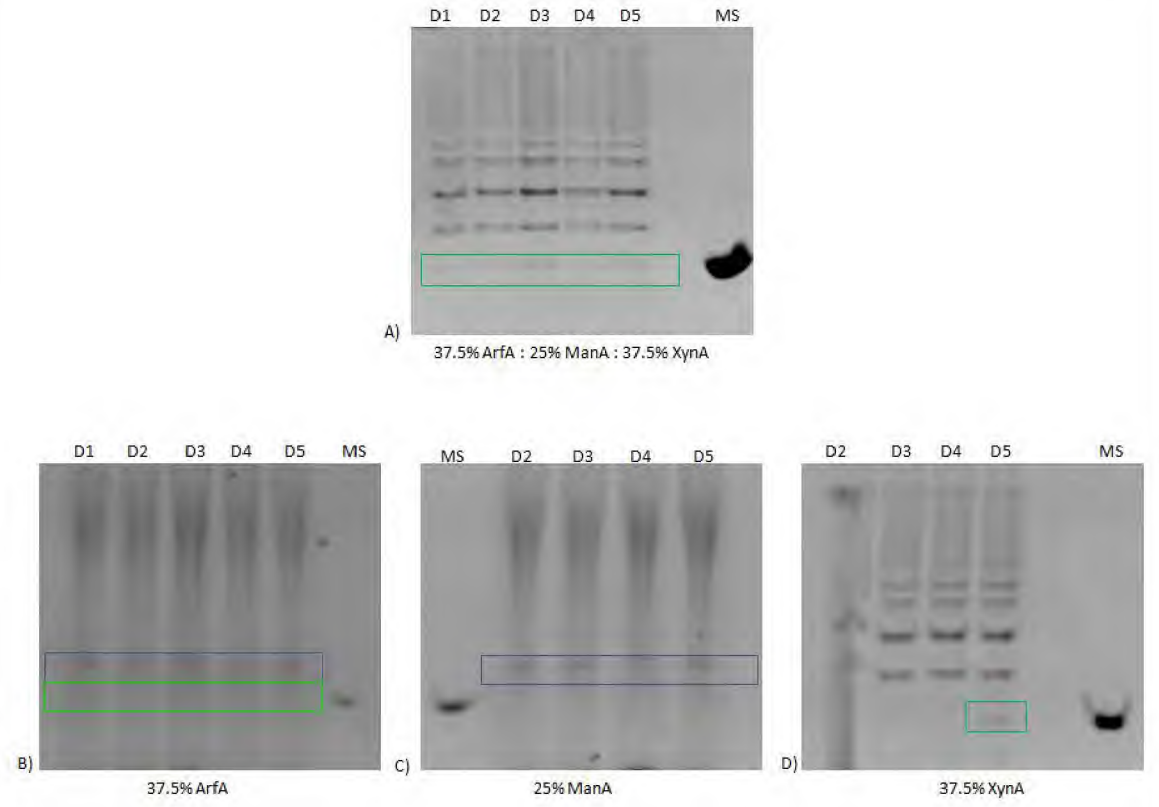


Figure 58: FACE analysis of A) the enzyme combination that produced the maximum amount of sugar with the hydrolysis of the $Ca(OH)_2$ pre-treated SCB sample and B - D) the individual enzymes. From the different enzyme assays, samples were taken on a daily basis (D1-D5). The daily hydrolysates were compared to a xylose standard (MS)**.** The blue boxes indicate the presence of feint DP2 sugars, and the green boxes indicate the presence of feint monomeric sugars.

Unlike the separation pattern obtained from the hydrolysis of the untreated SCB (Figure 57), the separation patterns obtained from the hydrolysis of the $Ca(OH)_2$ pre-treated SCB indicated that the majority of the hydrolysis was due to the action of the xylanase (Figure 58). The hydrolysis of the $Ca(OH)_2$ pre-treated SCB using the enzyme combination (Figure 58A) was similar to the separation pattern obtained from the hydrolysis of the $Ca(OH)_2$ pre-treated SCB using XynA (Figure 58D), whereas the sugar samples obtained from the hydrolysis of the $Ca(OH)_2$ pre-treated SCB using 37.5% ArfA (Figure 58B) and 25% ManA (Figure 58C) were similar. The hydrolysis of the SCB sample using ArfA and ManA produced low concentrations of dimeric and monomeric sugars (Figures 58B & C).

The third set of SCB hydrolysates that were analysed on FACE gels were obtained from the hydrolysis of the NaOH pre-treated SCB (Figure 59). The NaOH pre-treated SCB was hydrolysed with the enzyme combination A) 62.5% ArfA:12.5% ManA:25% XynA, B) 62.5% ArfA, C) 12.5% ManA and D) 25% XynA (Figure 59).

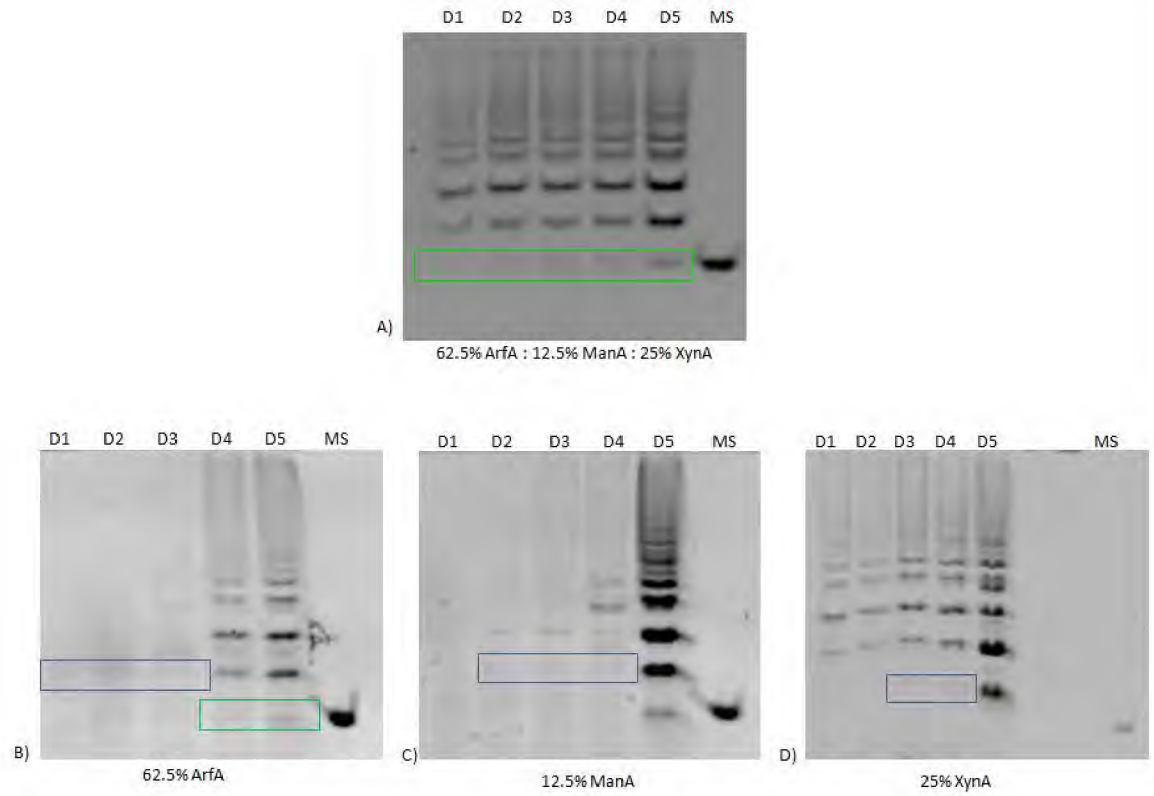


Figure 59: FACE analysis of A) the enzyme combination that produced the maximum amount of sugar with the hydrolysis of the NaOH pre-treated SCB sample and B - D) the individual enzymes. From the different enzyme assays, samples were taken on a daily basis (D1-D5). The daily hydrolysates were compared to a xylose standard (MS)**.** The blue boxes indicate the presence of feint DP2 sugars, and the green boxes indicate the presence of feint monomeric sugars.

The separation pattern obtained from the hydrolysis of the NaOH pre-treated SCB using the enzyme combination (Figure 59A) was similar to that obtained from the hydrolysis of the $Ca(OH)_2$ pre-treated SCB (Figure 59A). The analysis of the sugar samples obtained from the hydrolysis of the NaOH pre-treated SCB indicated that the majority of the enzymatic hydrolysis was due to the action of the xylanase. The XynA

hydrolysed the SCB efficiently over the 5-day period, as indicated by the gradual increase in the sugar (Figure 59D). The hydrolysis of the NaOH pre-treated SCB using ArfA and ManA did not appear to produce sugar with the first 24 hours. However, the FACE gels indicated a gradual increase in the production of sugar with time after the initial 24 hours (Figures 59 B & C).

The final chemical pre-treatment that was analysed using FACE gels displayed the hydrolysis of the NH_4OH pre-treated SCB (Figure 60). The NH_4OH pre-treated SCB was hydrolysed with A) 25% ManA: 75% XynA, B) 25% ManA and C) 75% XynA.

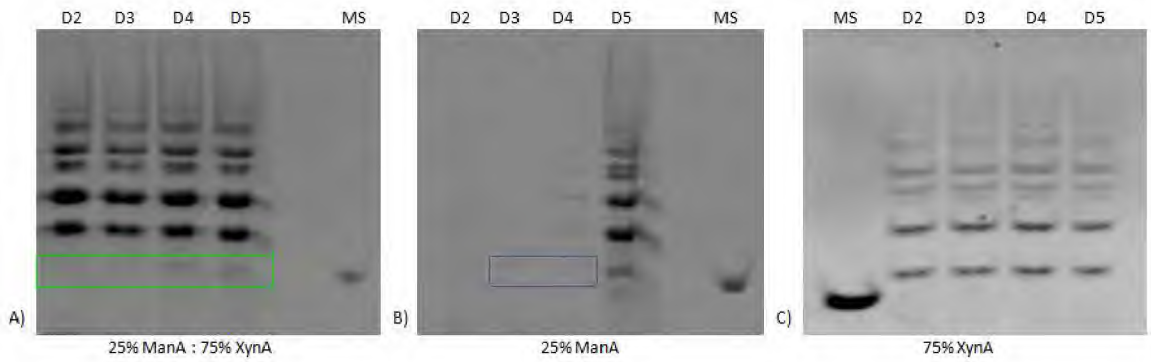


Figure 60: FACE analysis of A) the enzyme combination that produced the maximum amount of sugar with the hydrolysis of the NH_4OH pre-treated SCB sample and B & C) the individual enzymes. From the different enzyme assays, samples were taken on a daily basis (D1-D5). The daily hydrolysates were compared to a xylose standard (MS). The blue boxes indicate the presence of feint DP2 sugars, and the green boxes indicate the presence of feint monomeric sugars.

The hydrolysis of the NH_4OH pre-treated SCB using the combination of the enzymes (Figure 60A) and XynA (Figure 60C) were similar to that of the NaOH pre-treated SCB sample (Figure 59). FACE gels also indicated that the XynA hydrolysed the SCB over the 5-day period, producing sugars with a range of DPs; however, the smallest sugar produced may have been xylobiose (Figure 60C). Unlike the hydrolysis of the SCB with the xylanase, the ManA didn‟t appear to hydrolyse the SCB within the first 2 days; however, from day 3, the production of sugar increased over time. On the final day, the

mannanse hydrolysed the SCB to produce sugars with a variety of DPs, including monomers (Figure 60B).

5.3.1.4 TLC analysis

After FACE analysis of the hydrolysis products from the synergy studies was performed, the samples were hydrolysed using TFA, producing the respective monomers and analysed further on TLC (Figure 61).

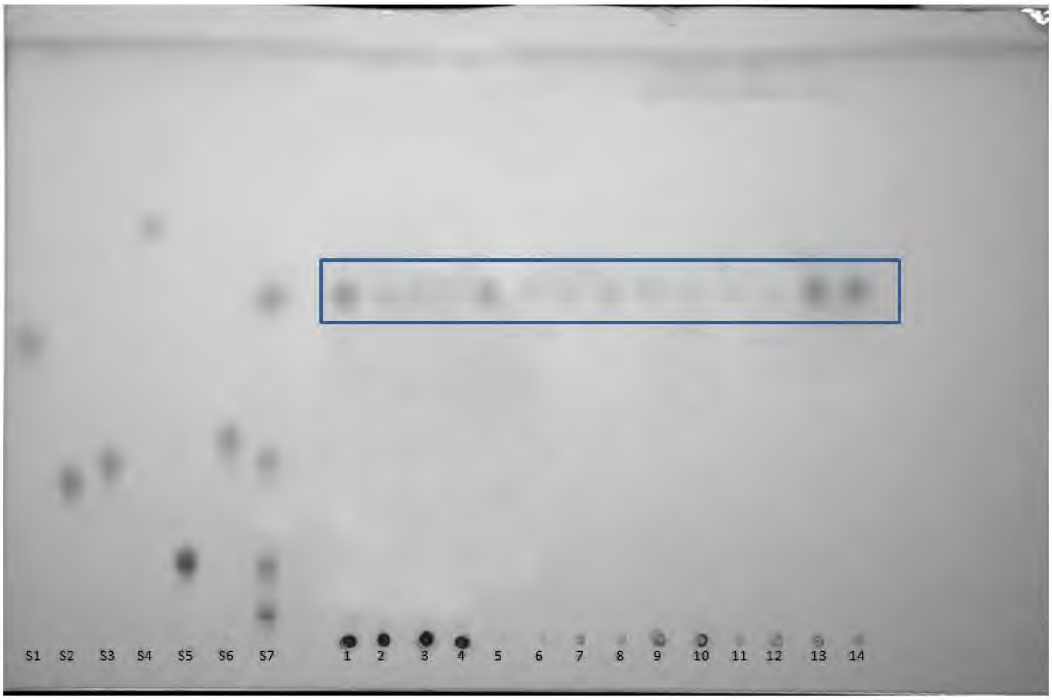


Figure 61: TLC analysis of the different sugar samples obtained from the synergy studies on the SCB and the pre-treated SCB samples. The standard sugars used in the TLC analysis: S1) arabinose, S2) galactose, S3) glucose, S4) mannose, S5) rhamnose, S6) sucrose and S7) in decending order – xylose, xylobiose, xylotriose and xylotetrarose. From the hydrolysis of the $Ca(OH)_2$ pre-treated SCB, the sugar samples that was analysed were: 1) 37.5% ArfA: 25% ManA: 37.5% XynA, 2) 37.5% ArfA, 3) 25% ManA, 4) 37.5% XynA. From the hydrolysis of the NaOH pre-treated SCB, the sugar samples that was analysed were: 5) 62.5% ArfA: 12.5% ManA: 25% XynA, 6) 62.5% ArfA, 7) 12.5% ManA and 8) 25% XynA. From the hydrolysis of the untreated SCB sample, the sugar samples that was analysed were: 9) 37.5% ArfA, 10) 62.5% ManA and 11) 37.5% ArfA and 62.5% ManA. From the hydrolysis of the NH_4OH pre-treated SCB, the sugar samples that was analysed were: 12) 25% ManA, 13) 75% XynA, 14) 25% ManA: 75% XynA.

The TLC plate illustrated the mobilities of the different sugar standards; it was observed that the monomeric sugars had different mobilities. The difference in the mobilities would subsequently allow for a putative identification of the TFA hydrolysed samples (Figure 61). The samples obtained for the hydrolysis of the untreated SCB and the different pre-treated SCB samples were hydrolysed using TFA to produce monomers. As indicated by the TLC data, the TFA hydrolysed samples exhibited had similar mobilities to that of xylose, thus, indicating that the only sugars produced by the hydrolysis of the different SCB samples were xyloses with various DPs (Figure 61).

5.4 Discussion

The complete utilisation and hydrolysis of SCB and other types of lignocellulose are hindered by the presence of lignin. As a result, extensive research has gone into establishing ways to remove the lignin from the plant cell walls without affecting the carbohydrate moiety. It has been well documented that lignin is generally attached to the plant cell wall carbohydrates via ester bonds between the ρ-coumaric and ferulic acids (Hartley *et al.,* 1990; Max *et al.,* 2009). Thus it may be suggested that, if the cleavage of the ester bonds can be achieved without the use of harsh pre-treatment conditions, it may facilitate delignification and enhance the hydrolysis of the carbohydrates. The pre-treatment processes performed in this study pre-treated the SCB with a combination of oxygenation and alkaline chemicals at what may be considered mild pre-treatment conditions. The analysed pre-treatment conditions illustrated that they were effective in facilitating the partial delignification of the SCB samples due to the presence of ρ-coumaric and ferulic acids. However, the other phenolates that were removed from the SCB samples could not be positively identified from m/z data and the NMR molecular weight database established for lignin and cell wall components, suggesting that the basic cinnamyl alcohols that constitute lignin may have polymerised into a variety of different and potentially novel phenolate complexes.

As mentioned previously, the wide variey of sugars that may be produced from the enzymatic hydrolysis of SCB is an important factor to consider since the resultant sugars

may affect the type of downstream processes that are employed, e.g. if the majority of the sugar produced is glucose, the sugar can be fermented to bioethanol. However, several traditionally non-fermentable sugars may be produced with the hydrolysis of SCB, e.g. arabinose, fructose and xylose (Pauly and Keegstra, 2008). The use of FACE and TLC illustrated that individual sugars and polysaccharides had distinctive mobilities, allowing for the different polysaccharides to be putatively identified. The data obtained from the analysis of the SCB hydrolysis products provide some insight into how the hemicellulases and the ancillary enzyme (ArfA) hydrolysed the different SCB samples. The hydrolytic behaviour of XynA with regard to the hydrolysis of the different SCB samples was expected. The random cleavage of the xylan backbone producing a variety of polysaccharide with a range of DPs and a minimium DP of 2 was observed. However, the hydrolytic behaviour of the mananase and α-arabinofuranosidase varied according to what has been previously documented. It would have been expected that different sugars may have been produced by the hydrolysis of the SCB samples due to use of three different enzymes; however, TLC analysis of the different sugar samples indicated that xylose was the only sugar produced by the hydrolysis of the SCB samples regardless of the enzyme(s) used. ManA, and especially ArfA, appeared to cleave the 1,4-β-glycosidic bonds between the xylose residues of the oligosaccharides with lower DP values.

5.5 Conclusion

In conclusion from the limited data obtained from the analysis of the pre-treatment liquors, it may be speculated that the presence of *ρ*-coumaric and and ferulic acid indicated that the pre-treatments successfully cleaved the ester bonds between the lignin and the carbohydrate moieties.

From the analysis of the sugar products obtained with the hydrolysis of the different SCB samples, it was also concluded that the majority of the sugar arose from the hydrolytic activity of XynA. TLC analysis indicated that the only sugar produced by the

hydrolysis of the SCB samples was xylose. ArfA and ManA in most cases appeared to have aided in the hydrolysis of the xylan backbone by cleaving the xylan in a similar fashion to that of endo- and exoglucanases. This suggests that the pre-treatment of the SCB samples may have altered the structure and the conformation of the carbohydrate moiety sufficiently to enable the hydrolysis of the SCB samples by enzymes that are not usually known to cleave the the 1,4-β-glycosidic bonds between the xylose residues.

Chapter 6: General discussion and conclusions

Collectively, the global community relies predominantly on the various types of fossil fuels for the generation of the different types of electrical energy and petrochemicals that drive both industrial processes and domestic use. According to the EIA, the average global fossil fuel based energy demand is set to increase at approximately 1.1% per annum until the year 2030; however, this value is country dependent (Shafiee and Topal, 2008). The last decade has seen an increase in the demand of and an increase in volatility in fossil fuel prices (Shafiee and Topal, 2010). Along with the high, fluctuating fossil fuel prices and the adverse environmental effects from the use of fossil fuels, the WEC has urged, especially the first world countries to reduce their dependence on fossil fuels and increase their usage of renewable energy sources. At present, renewable energy sources contribute a small percentage to global energy sources; however, this is expected to increase to 14% by the year 2030 (Energy Information Administration, 2006).

The prospect of using renewable energy sources (lignocellulosic biomass) for biofuel production has attracted a lot of attention. However, the complete hydrolysis of plant biomass has yet to be achieved, primarily due to the heterogeneic complexity and composition that contributes to the recalcitrant nature of lignocellulose (Tamaru *et al.*, 2000). As a result, much research has revolved around several questions; Firstly, what can be used to decrease the recalcitrance of lignocellulose, secondly, what is needed to facilitate the complete and efficient hydrolysis of lignocellulose, thirdly, can all the lignocellulosic components be utilized and can the complete utilization of lignocellulose be economically viable as a replacement for fossil fuels?

At face value, the concept of using lignocellulose or agricultural wastes that contain a high percentage of fermentable sugars for biofuel production appears to be straight forward, as it is essentially a three step process. These steps would include: the delignification of the biomass, hydrolysis of the carbohydrate moiety and finally the fermentation of the hydrolysed sugars, producing the biofuel. However, in reality this

process is not that simple. Sugarcane is a valuable South African agricultural crop that is generally grown in surplus. After the sucrose has been extracted from the sugarcane, the remaining pith and other plant cell wall fibers is subsequently referred to as the SCB and is discarded as waste. SCB is produced in surplus annually by over 100 countries (Waclwovsky *et al.,* 2010), and is generally burnt in sugar mill furnaces. In the last few decades, SCB has become one of the most popular renewable energy sources, second only to maize, for the production of bioethanol. SCB is a lignocellulosic biomass, which is one of the main problems associated with using SCB as a renewable energy source. SCB is a valuable agricultural waste product used in the production of bioethanol for a number of reasons. Possibly the most important reason is that SCB unlike maize is not considered a food source. The high percentage of lignin present in its chemical composition increases the recalcitrance of SCB to downstream processes that involve hydrolysis and fermentation. Another important factor that has to be considered for facilitating the effective hydrolysis of lignocellulose after delignification is the chemical composition. In general, lignocellulose consists of cellulose, pectin and a variety of hemicellulose components, thus a variety of enzymes would be required to effectively hydrolyse the biomass. Unlike cellulose, the composition of the hemicellulosic component of the plant biomass varies depending on the type of biomass that is being studied. Xylan may have several types of sugar substituents attached to the polysaccharide backbone, increasing the complexity of the xylan (Kosugi *et al.,* 2002b). Therefore, several enzymes are required to degrade xylan depending on its sugar composition. Xylanases, mannanases and pectinases will hydrolyse the polysaccharides that constitute the hemicellulose backbone; however, side chain-cleaving enzymes such as L-arabinofuranosidase (ArfA) and feruloyl esterases would also be required (Aspinall, 1980; Koukiekolo *et al.,* 2005; Thomson, 1993).

Most of the research performed on SCB, has focused on the utilization of cellulose for the production of bioethanol. As a result, the hemicellulosic components are generally disregarded. As mentioned previously, this study investigated the following:

1) The use of alkaline pre-treatments to delignify SCB without affecting the holocellulosic fraction of the lignocelluloses.
2) Optimizing various pre-treatment processes to maximize the hydrolysis of the SCB holocellulose.
3) The synergistic associations between two cellulosomal hemicellulases and a non-cellulosomal ancillary enzyme that would facilitate the maximal production of sugar from the hydrolysis of the different pre-treated SCB samples.

As mentioned previously, knowing the chemical composition of the lignocellulose (in this case SCB) is important, thus the chemical composition of one of the South African cultivars was determined. The chemical composition of the native (untreated) SCB was subsequently used as a basis for comparison with the alkaline pre-treated SCB samples. The SCB was pre-treated with three chemicals that are widely used in different industries. The first SCB chemical pre-treatment utilised $Ca(OH)_2$. The data obtained from the optimization of the $Ca(OH)_2$ indicated that $Ca(OH)_2$ concentration, temperature and time are significant factors for consideration prior to pre-treating the SCB. It was found that when the pre-treatment occured longer than 12 hours, the $Ca(OH)_2$ concentration should be decreased and the pre-treatment temperature increased accordingly. The $Ca(OH)_2$ pre-treatment partially solubilised and removed some of the lignin and the arabinan substituents present on the hemicellulose backbone, thus increasing the exposure of the hemicellulose structure as indicated by the 6.5-fold increase in the enzymatic hydrolysis, and the SEM and phloroglucinol:HCl histochemical data of the SCB structure obtained.

The second chemical pre-treatment employed NaOH, which is widely used in the paper and pulp industry. Unlike the $Ca(OH)_2$ pre-treatment factors that allowed a certain

degree of flexibility in the pre-treatment conditions, the NaOH illustrated a correlation between the different factors. The optimization data obtained indicated that the mildest conditions (0.10g NaOH/ g SCB; 12 hours and 40°C) was unsuccessful in lignin removal. On the other hand, the harshest conditions (0.40g NaOH/ g SCB; 36 hours and 70°C) may have successfully solubilised and removed/redistributed the lignin; however, the low enzyme activity indicated that the hemicellulose may also have been hydrolysed. It has been found that alkaline solutions can solubilise, redistribute and condense lignin (Gregg and Saddler, 1996). After pre-treatment, the SCB/NaOH slurry was cooled to a safe handing temperature. The decrease in the temperature resulted in the solubilized lignin phenolates condensing and reforming on a different area on the SCB samples (as observed when the SCB samples were stained with a phloroglucinol:HCl histochemistry stain).

The third chemical pre-treatment employed NH_4OH, which is commonly used as part of AFEX and APR pre-treatment processes. The results obtained from the pre-treatment optimization highlighted the significance of the different factors, indicating that increasing the different factor conditions individually or in combination would increase the removal of lignin. Unlike the $Ca(OH)_2$ and NaOH pre-treatments, the NH_4OH did not display adverse effects with the conditions tested, i.e. regardless of the conditions used, the digestability of the SCB was enhanced. The increase in the digestability was reflected by a 13-fold increase in the enzymatic hydrolysis of the pre-treated SCB in comparison to the untreated SCB.

In general, the chemical pre-treatments successfully increased the digestibility of the SCB; however, the sugar analysis results obtained for the synergy studies were unexpected, yielding interesting results. The majority of the hydrolysis of the SCB samples was expected to be due to the action of the xylanase (XynA), since the main type of hemicellulose present is xylan. However, the enzyme cocktails used to produce the maximum quantity of sugar from the different SCB samples used a small percentage of XynA. Normally these results would be expected, since it is well established that xylan may have a wide variety of sugar substituents depending on the type and source of the

lignocellulose. From the data obtained from the sugar analysis of the synergy hydrolysis products, two observations about these enzymes were made. Firstly, these enzymes predominantly behave as endohydrolases, randomly cleaving the glycosidic bonds, producing oligosaccharides with a range of DPs. Secondly, under certain conditions they behave as exohydrolases producing monomeric sugars. The interesting results obtained from sugar analysis was not typical of the hydrolytic activity of these enzymes, as the only sugar that appeared to have been produced was xylose. This indicated that these enzymes were not hydrolyzing the glycosidic bonds they are documented to hydrolyze (α-arabinosyl linkages and β-mannosyl linkages). ArfA appeared to be able to hydrolyse the exposed 1,4-β-glycosidic bonds of the smaller xylan oligosaccharides, producing the xylanase activity, which was unexpected, since ArfA hydrolyses the (1--+3)- and the (1--+5)-α-arabinosyl linkages between the arabinose substituent present on some of the xylose monomers that constitute the xylan backbone (Greve *et al.,* 1984). Generally, ManA had a low degree of activity in the hydrolysis of the SCB when it was the only enzyme used. It was therefore not expected to significantly affect the total amount of sugar produced during the hydrolysis of the SCB samples.

From this study the following was concluded:

1) Alkaline pre-treatment successfully solublized, redistributed and removed lignin from the SCB, increasing SCB digestability.
2) The most effective pre-treatment employed 0.40 g NH_4OH / g SCB at 70° for 36 hours, removing approximately 79% of the lignin without affecting the hemicellulose moiety.
3) The presence of *ρ*-coumaric and and ferulic acid in the pre-treatment liquors indicated that the pre-treatments successfully cleaved the ester bonds between the lignin and the carbohydrate moieties.
4) During pre-treatment, lignin solubilizes, producing lignin monomers that subsequently redistribute and condense producing phenolate complexes both in solution and on the SCB. The monomers would produce the newly formed

phenolate complexes via a variety of different chemical bonds to generate more potential novel lignin phenolates.

5) The production of sugar increased approximately 13-fold using an enzyme cocktail containing 25% ManA and 75% XynA.
6) The only sugar produced from the hydrolysis of the SCB was xylose, indicating that the enzymes used may be bifunctional under certain conditions.

Thus, in relation to the following three questions mentioned in the beginning of this chapter:

1) What can be used to decrease the recalcitrance of lignocellulose?

Alkaline pre-treatments (at what would industrially be classified as mild pre-treatment conditions) would effectively redistribute and/or remove lignin, reducing the recalcitrance and subsequently increasing the digestibility of lignocellulose.

2) What is needed to facilitate the complete and efficient hydrolysis of lignocellulose?

A suitable pre-treatment, and the action of a variety of cellulolytic and hemicellulolytic enzymes would be required.

3) Can all the lignocellulosic components be utilized and can the complete utilization of lignocellulose be economically viable as a replacement for fossil fuels?

The majority of the sugars, including xylose which is not normally used for bioethanol production, can be fermented. It is not really economically viable to use xylose (being the only hydrolysis product found in this study) for the production of biofuel. However, xylose is very important industrially as it is used in the production of a variety of other chemicals and products, e.g. xylitol.

Future recommendations

Based on this study the following future recomendations are suggested:

1) The solubilisation, redistribution and condensation of the phenolates under moderate pre-treatment conditions may prove to be beneficial in the development of consolidated bioprocesses. Thus, it may be useful to investigate the solubilisation, redistribution and condensation of the phenolates, specifically looking at methods to detect and quantify the production of potentially inhibitory compounds associated with lignin.
2) A more indepth study into how substrate conformational changes may induce a bifunctional response in ArfA, ManA and XynA with regard to ther active sites.
3) Perform additional synergy studies, making use of cellulases and other ancillary enzymes such as β-glucosidases, and ferulic esterases etc.
4) A pilot study on how to design a bioreactor based system that would factilitate the simultaneous production of sugar alcohols e.g. xylitol and bioethanol, depending on market requirements.

Appendices

Appendix A: Chemicals and suppliers

(D)-Arabinose	Sigma (Cat No. A3131)
(D)-Manose	Sigma (Cat. No. M2069)
(D)-Glucose	Saarchem (Cat. No. 2676020)
(D)-Xylose	Sigma (Cat No. X3877)
(D)-Galactose	Sigma (Cat. No. 112593)
(L)-Rhamnose	Sigma (Cat. No. R3875)
Sucrose	Merck (Cat. No. 107687025)
Xylobiose	Megazyme (Cat. No. O-XBI)
Xylotriose	Megazyme (Cat. No. O-XTR)
Xylotetraose	Megazyme (Cat. No. O-XTE)
2-mercaptoethanol	Fluka (Cat. No. 63700)
3, 4-dinitrosalicylic acid	Sigma (Cat No. D0550)
8-aminonaphthalene-1,3,6-trisulphonate	Sigma (Cat. No. 08658)
Acetonitrile	Merck (Cat. No. 114291250)
Acrylamide	Sigma (Cat No. A8887)
Ammonia	Merck (Cat. No. 1054322500)
Ammonium persulphate	Sigma Aldrich (Cat No. A3678)
Bacteriological agar	Biolab (Cat. No. BX1)
Birchwood xylan	Fluka (Cat. No. 95588)
Bradfords reagent	Sigma (Cat. No. B6916)
Bromophenol blue	Sigma (Cat. No. B8026)
Calcium chloride	Saarchem (Cat. No.1524900)
Calcium carbonate	Merck (Cat. No. 1020660250)
Carboxymethyl cellulose	Calbiochem (Cat. No. 217277)
Citric acid	Merck (Cat. No. 1.00244)
Coomassie Brilliant Blue R250	Merck (Cat. No. 1.12553)
Disodium hydrogen orthophosphate	Saarchem (Cat. No. 5822860)

Dimethyl sulphoxide	Merck (Cat. No. 1029522511)
Diethyl ether	Merck (Cat. No. 1009212500)
Ethanol	Merck (Cat. No. 8.18700)
Gaunidinium chloride	Merck (Cat. No. 1.04219)
Glacial acetic acid	Merck (Cat No. 1.00063)
Glycerol	Saarchem (Cat. No.2676520)
Glycine	Merck (Cat. No. 1.04169)
Hyrochloric acid	Saarchem (Cat. No. 3063040)
Imidazole	Merck (Cat. No. 1.04716)
Isopropylthiogalactoside	Calbiochem (Cat. No. 420322)
Kanamycin	Roche (Cat. No. 106801)
Locust bean gum	Fluka (Cat. No. 62631)
Lysozyme	Fluka (Cat. No. 62971)
Magnesium chloride	ACE
Methanol	Merck (Cat. No. 8.22283)
Methanol (HPLC grade)	Merck (Cat. No. 10601842500)
N,N,N',N'-tetramethylethylene diamine	Sigma Aldrich (Cat. No. T9281)
N,N-methylenebisacrylamine	Sigma (Cat. No.M7279)
Nickel (II) sulphate	Merck (Cat. No. 1.06727)
Ni-NTA agarose	Qiagen (Cat. No. 30210)
peqGold protein marker (IV)	peqLab (Cat. No. 27-2110)
Phenol	Sigma (Cat.No. P3653)
Phlorogucinol	Sigma (Cat. No. P3502)
ortho-Phosphoric acid	Merck (Cat. no. 100573250)
Phosphoric acid (HPLC grade)	Sigma (Cat. no. 1005520250)
Polyethylene glycol 20 000	Merck (Cat. No. 8.18897)
SDS	BDH biochemicals (Cat. No. 301754)
Sodium azide	Merck (Cat. No. 8.22335)
Sodium chloride	Saarchem (Cat. No. 5822320)

Sodium cyanoborohydride	Sigma (Cat. No. 156159)
Sodium dihydrogen phosphate	Saarchem (Cat. No. 3822650)
Sodium hydroxide	Saarchem (Cat. No. 5823200)
Sodium metasulfite	Sigma Aldrich (Cat. No. 255556)
Sodium potassium tartrate	Merck (Cat. No. 1.08087)
Sodium sulphate	Saarchem (Cat. No. 5825200)
Sodium sulphite	Saarchem (Cat. No. 5825400)
Sulphuric acid	Merck (Cat. No.1120802500)
Tris (hydroxymethyl) aminomethane	Merck (Cat. No. 1.08382)
Tri-sodium citrate dehydrate	Merck (Cat. No. 1.06448)
Trifluoroacetic acid	Sigma (Cat. No. T6508)
Tryptone	Fluka (Cat. No. 70169)
Yeast extract	Biolab (Cat. No. BX6)

Appendix B: Preparation and transformation of E. coli BL21 (DE3) cells

Preparation of competent E. coli BL21(DE3) cells

Prior to the transformation of the *E. coli* strain BL21(DE3), the cells were made chemically competent. Using 20 µl of *E. coli* BL21 (DE3) cells, 5 ml of yeast-tryptone (2 ×YT) broth was inoculated. Incubating at 37°C, the inoculum was shaken at 200 rpm on a Labcon bench top shaker for approximately 12 hours. The overnight culture was diluted with fresh 2 ×YT broth (50 ml) in the respective ratio of 1 to 200. At 37°C, and shaking at 200 rpm, the new inoculum was incubated for approximately 3 hours, where an optical density at 260 $_{(OD260)}$ of between 0.3 and 0.6 was reached. The cells were harvested in sterile centrifugation tubes, at 5, 000 × g for 5 minutes at 4°C in a Beckman Avanti centrifuge. The *E. coli* BL21 (DE3) cells were kept on ice for the remainder of the protocol. The cell pellet was resuspended in 50 ml ice cold, sterile 0.1 M $MgCl_2$, and kept on ice for 20 minutes. The cells were harvested in sterile centrifugation tubes, at 5, 000 × g for 5 minutes at 4°C, and the pellet was resuspended in 25 ml ice cold, sterile 0.1 M $CaCl_2$. The cells remained on ice for a period of 2 hours. The cells were harvested as before, and the pellet resuspended in 5 ml ice cold, sterile 0.1 M $CaCl_2$, and the cells were mixed with 5 ml 30 % sterile glycerol, and stored at -70°C in suitable aliquots.

Transformation of competent E. coli BL21(DE3) cells

The plasmid constructs for the recombinant enzymes were kindly donated by Prof. R. H. Doi. (University of Davis California,USA). Growth of *E. coli* BL 21(DE3) harbouring the gene construct, for the protein of interest was performed at 30°C in 2 × Yeast-Tryptone (YT) broth.

2 ×YT broth:

10 g Yeast extract
16 g Tryptone (Pancreatic digest of casein)
5 g Sodium chloride

The components were dissolved in 1 L distilled MilliQ water, and autoclaved at 121°C for 20 minutes. The medium was allowed to cool and using sterile techniques, the appropriate antibiotic was added prior to inoculation with the *E. coli* BL 21(DE3) harbouring the gene construct for the protein of interest. The genes of interest were kindly donated by Prof. R.H. Doi in the form of plasmids. The hemicellulases (ManA & XynA) and the ancillary enzyme (ArfA) genes had been cloned into pET29a(+) vectors (Figure 62)

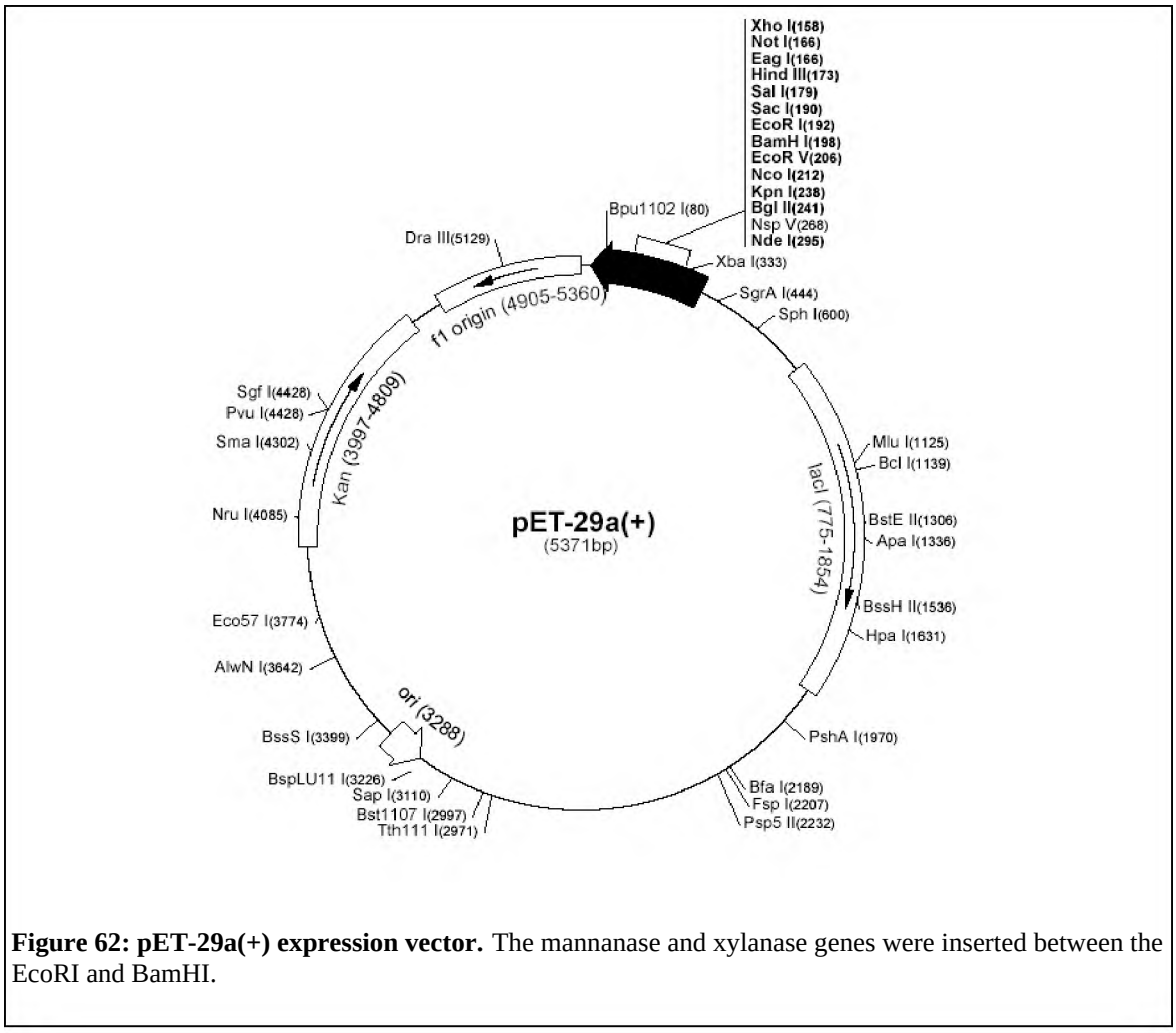


Figure 62: pET-29a(+) expression vector. The mannanase and xylanase genes were inserted between the EcoRI and BamHI.

Appendix C: Purification of recombinant proteins

1. *Lysis Buffer (pH 7.0)*

50 mM Sodium dihydrogen phosphate
300 mM Sodium chloride
10 mM Imidazole
8 mg/ml Lysozyme

Once the pH of the solution had been corrected, the buffer was stored at 4°C.

2. *Wash Buffer (pH 7.0)*

50 mM Sodium dihydrogen phosphate
300 mM Sodium chloride
10 mM Imidazole

Once the pH of the solution had been corrected, the buffer was stored at 4°C.

3. *Elution Buffer (pH 7.0)*

50 mM Sodium dihydrogen phosphate
300 mM Sodium chloride
250 mM Imidazole

Once the pH of the solution had been corrected, the buffer was stored at 4°C.

The recombinant proteins were expressed in an *E. coli* BL21 (DE3) strain. The *E. coli* cells containing the appropriate gene construct were used to inoculate 250 ml of 2 × YT broth containing either ampicillin in the case of pET22b vector or kanamycin in the case on the pET29a vector. The inoculum was incubated at 30°C, shaking at 200 rpm, until the optical density at 600nm $_{(OD600nm)}$ was between 0.5 and 0.6. During the growth of the *E. coli* harbouring the ampicillin based vector, the medium was spiked with

ampicillin every 2 hours. Once the correct $_{OD600nm}$ was obtained, the expression of the recombinant enzymes were initiated by the addition of IPTG to produce a final concentration of 400 μg/ml for the pET 22b vector and 100 μg/ml for the pET29a vector. Subsequent to the addition of the inducer, the cultures were incubated at 19°C for 14 hours.

The cells were harvested by centrifugation at 8 000 × g using a Beckman Avanti JA 14 rotor for 20 minutes. The cell pellet was resuspended in 10 ml Lysis buffer (pH7) and incubated at 4°C for 20 minutes. The sample was sonicated at 50 Hz six times for 10 seconds with 10 second rest intervals. The post sonicated sample was centrifuged at 10 000 × g using a Beckman Avanti JA 14 rotor for 20 minutes to produce a clear enzyme lysate. The lysate was applied to a Protino® Ni-TED (Macherey-Nagel) columns The column was washed twice with 10 ml of the wash buffer (pH 7.0) and the protein was eluted with the addition of three 10 ml washes with elution buffer (pH 7.0). The purified recombinant protein was desalted through over night (O/N) dialysis in a 50 mM sodium phosphate buffer (pH 7.0). Protein fractions were subsequently analysed for purity using SDS-PAGE, enzyme activity assays and protein assays.

Appendix D: Electrophoresis

Discontinuous Gel Electrophoresis

The purity of the recombinant proteins, which were purified using nickel-affinity chromatography were analysed using sodium docecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) according to the Mini-Protean® 3 Cell instruction manual (modified according to Laemmli, 1970).

The SDS-PAGE required the following components:

1. *10 x SDS Page Running buffer:*

30.3 g Tris(Hydroxymethyl)Aminomethane
144.0 g Glycine
10.0 g Sodium dodecyl sulphate (SDS)

The three chemicals were dissolved in distilled MilliQ water to 1 L, the pH was not adjusted. Once dissolved the 10 × running buffer was stores at 4°C. Prior to use, the 10 × running buffer was diluted to a 1 × running buffer by diluting 50 ml 10 × running buffer with 450 ml distilled MilliQ water.

2. *10% SDS Stock Solution*

1 g SDS was dissolved in 10 ml distilled water

3. *10% Ammonium Persulphate (APS) Stock solution*

0.1 g APS was dissolved in 1ml distilled water (Prepared freshly)

4. 30% Acrylamide Stock Solution (100ml)

29.8 g Acrylamide
0.2 g Bis-Acrylamide

Made up to 100ml with distilled MilliQ water in a dark bottle or a bottle covered with aluminium foil.

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

Dissolved 6.00 g Tris(Hydroxymethyl)Aminomethane (Tris) in 100 ml of distilled MilliQ water and adjusted the pH of the buffer to 6.80 with hydrochloric acid (HCl).

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

Dissolved 18.15 g Tris in 100 ml distilled MilliQ water, and adjusted the pH of the buffer to 8.8 with hydrochloric acid (HCl).

7. SDS reducing buffer

7.10 ml distilled water
2.50 ml 0.5M Tris-HCl buffer (pH 6.8)
5.00 ml glycerol
4.00 ml 10% SDS solution (w/v)
0.4 ml 0.5% bromophenol blue (w/v)

The reducing buffer was stored at room temperature. Prior to use, 50 µl β-mercaptoethanol was added to 950 µl SDS reducing buffer. The protein samples were diluted in a 1:2 ratio with the sample buffer, and boiled for 5 minutes prior to loading the protein samples onto the SDS-PAGE gel. For long term storage, the boiled samples were stored at -20°C.

8. Coomassie Brilliant Blue Protein Staining

The Comassie Brilliant Blue protein stain was prepared by mixing 0.075 % (w/v) Coomassie Brilliant Blue R250 to a solution of 40% methanol and 0.7% glacial acetic acid. The polyacrylamide gels were stained for a minimum of one hour with shaking.

9. Coomassie destain solution

The destain solution was prepared by mixing 45% methanol, 45 % distilled MilliQ water and 10 % glacial acetic acid and mixed by inversion. The polyacrylamide gels were destained until the stained protein bands were visible against a clear background.

10. Gel drying solution

7% Glycerol
10% Ethanol

The solution was made up to 1 L by the addition of distilled MilliQ water and stored at room temperature. After the polyacrylamide gels have been destained, the gels were placed gels in enough gel drying solution to cover gels for a minimum of 30 minutes before the gels were dried.

11. Preparation of SDS-PAGE gels

The 12% SDS-PAGE resolving gel was prepared by the addition of the following solutions in the following sequence:

3.4 ml distilled MilliQ water
4.0 ml 30% Acrylamide stock solution
2.5 ml 1.5 M Tris-HCl buffer (pH 8.8)
0.1 ml 10% SDS solution
0.1 ml 10% APS solution
0.05 ml TEMED

Once the gel solution had been poured into the gel apparatus, the gel was covered with 0.3 ml isopropanol and allowed to set. Prior to pouring the stacking gel, the isopropanol was removed with filter paper.

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

6.1 ml distilled MilliQ water
1.3 ml 30% Acrylamide stock solution
2.5 ml 1.5 M Tris-HCl buffer (pH 8.8)
0.1 ml 10% SDS solution
0.1 ml 10% APS solution
0.05 ml TEMED

Once the stacking gel solution had been poured, plastic combs were inserted into the gel to form the wells and the gel was allowed to set.

Fluorophore-assisted carbohyrdate electrophoresis

1. *Sample and sugar standard preparation:*

The sugar standards and hydrolysis products were derivatised with ANTS as follows:

The sugar samples and hydrolysis products were lyophylised overnight. The lyophylised sugar samples were dissolved in 5µl of 0.2 M ANTS prepared in acetic acid:water (3:17, v/v) and 1 M sodium cyanoborohydride ($NaCNBH_3$) prepared in dimethyl sulphoxide (DMSO) and incubated 15 hours at 37°C and vacuum dried. The derivatised sugar samples were redissolved in 0.5 M Tris-HCl (pH 6.60) and stored at -20°C when not used

2. *Preparation of acrylamide stock solutions (100ml):*

The T50%/C7.5% resolving gel acrylamide stock solution was prepared by mixing 45.25 g acrylamide and 3.75 g bis-acrylamide to 50 ml water. The solution was slowly heated using warm water until the acrylamide and bis-acrylamide dissolved.

The T50%/C15% stacking gel acrylamide stock solution was prepared by mixing 45.25 g acrylamide and 7.50 g bis-acrylamide to 50 ml water.

3. *Preparation of native PAGE running buffer:*

30.3 g Tris(Hydroxymethyl)Aminomethane
144.0 g Glycine

The three chemicals were dissolved in distilled MilliQ water to 1 L, the pH was not adjusted. Once dissolved the 10 × running buffer was stores at 4°C. Prior to use, the 10 × running buffer was diluted to a 1 × running buffer by diluting 50 ml 10 × running buffer with 450 ml distilled MilliQ water.

4. Preparation of FACE gels:

The derivatised sugar samples were electrophoresed on a native PAGE gel, using T25%/C3.75% resolving gels and T5%/C1.5% stacking gels.

The T25%/C3.75% resolving gel was prepared by the addition of the following solutions in the following sequence:

2.5 ml distilled MilliQ water
5.0 ml 30% Acrylamide stock solution
2.5 ml 1.5 M Tris-HCl buffer (pH 8.8)
0.1 ml 10% APS solution
0.05 ml TEMED

Once the gel solution had been poured into the gel apparatus, the gel was covered with 0.3 ml isopropanol and allowed to set. Prior to pouring the stacking gel, the isopropanol was removed with filter paper.

The T5%/C1.5% stacking gel was prepared by the addition of the following solutions in the following sequence:

6.4 ml distilled MilliQ water
1.0 ml 30% Acrylamide stock solution
2.5 ml 1.5 M Tris-HCl buffer (pH 8.8)
0.1 ml 10% APS solution
0.05 ml TEMED

Once the stacking gel solution had been poured, plastic combs were inserted into the gel to form the wells and the gel was allowed to set.

5. FACE analysis:

. The derivatised sugars were electrophoresed on ice using the following parameters: prior to loading the sugar samples, the native PAGE gels were electophoresed for 10 min at 100 V, after which the samples were loaded, and the gels were electrophoresed for 10 min at 100 V, and for 40 min at 300V. The gels were visualised under UV light.

Appendix E: Standard curves for the determination of protein concentration and enzyme activity

Protein standard curve

The protein standard curve was generated using a modified Bradford protein assay (Bradford, 1976) and bovine serum albumin (BSA) (Sigma) as the reference protein (Figure 63). Various concentrations of BSA were made ranging from 0 and 0.4 mg/ml. The standard curve was generated by mixing 5 µl of the protein standard to 225 µl of the Bradford‟s reagent and allowed to stand at room temperature for 5 minutes. The samples were gently shaken for 30 seconds, and the change in colour was determined at 595 nm in the Power Wave X microplate reader from Bio-Tek Instruments using Kcjunior software. The standard curve was generated in a Microsoft Excel® worksheet (Figure 63).

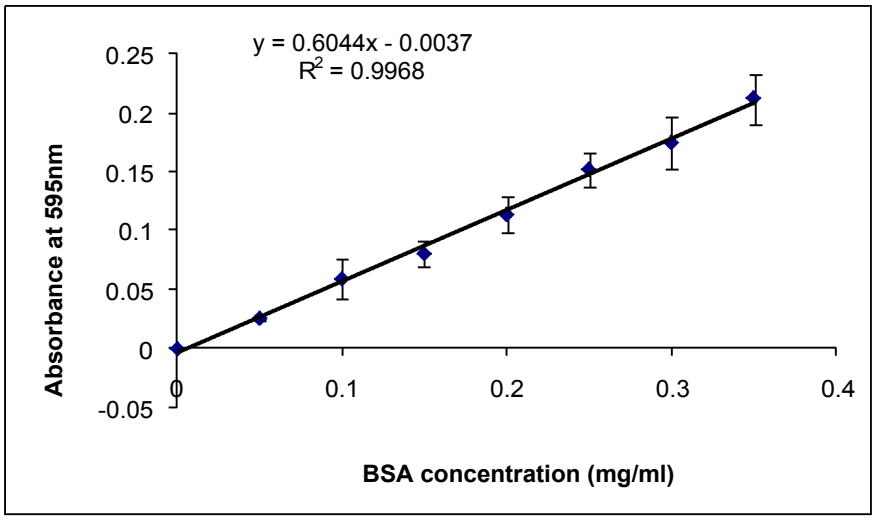


Figure 63: Protein standard curve generated using the Bradford protein assay using various concentrations of BSA as the reference protein (SD±3).

Sugar standard curves

DNS reagent:

100 ml 2% (w/v) NaOH

2 g Dinitrosalicylic acid (DNS)

40 g Sodium potassium tartrate (Roschelle salts)

0.1g Phenol

0.1 g Sodium Metasulfite

100 ml Distilled MilliQ water

Once all the components were dissolved, nitrogen gas (Afrox) was flushed through the solution for 30 minutes to remove any oxygen in solution.

The enzyme activities of the recombinant enzymes were determined by quantification of the reducing sugars liberated through the degradation of the various substrates. The quantity of reducing sugars was determined through the use of the DNS carbohydrate assay. Standard curves were generated using a modified (scaled down) assay based on the carbohydrate assay described by Miller (1959). Sugar standards (glucose, mannose and xylose) were prepared, ranging in concentration between 0 and 10 mM, of which, 300 µl of each was added to 600 µl DNS reagent. The sugar-DNS reagent mixture was incubated at 100°C for 5 minutes, followed by 5 minute incubation on ice. The change in colour was determined at 540 nm in a Power Wave X microplate reader from Bio-Tek Instruments using Kcjunior software. Three sugar standard curves were generated using glucose, mannose and xylanose in a Microsoft Excel® worksheet (See Figures 64– 66).

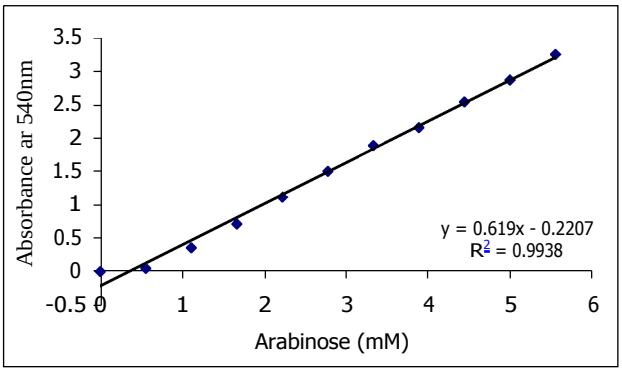


Figure 64: Arabinose standard curve generated using the DNS carbohydrate assay. All the readings were performed in triplicate. (n = 3, SD were too small to be visualized).

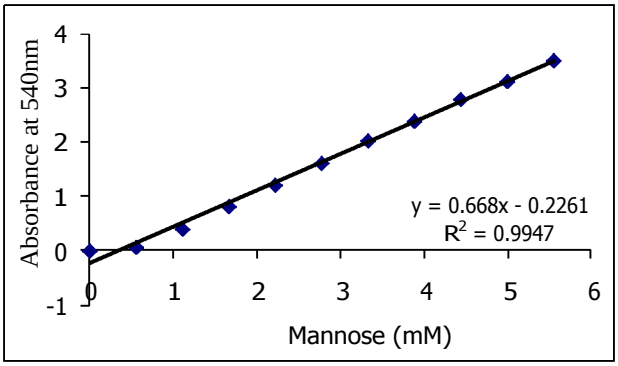


Figure 65: Mannose standard curve generated using the DNS carbohydrate assay. All the readings were performed in triplicate. (n = 3, SD were too small to be visualized).

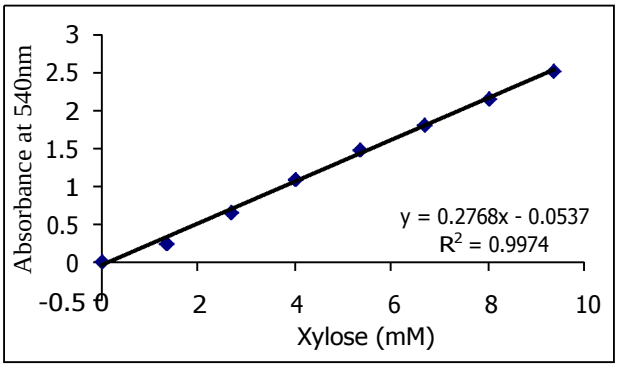


Figure 66: Xylose standard curve generated using the DNS carbohydrate assay. All the readings were performed in triplicate. (n = 3, SD are too small to be visualized).

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