Enhancing the saccharolytic phase of sugar beet pulp via hemicellulase synergy

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By

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Abstract

The sugar beet (*Beta vulgaris)* plant has in recent years been added to the Biofuel Industrial Strategy (Department of Minerals and Energy, 2007) by the South African government as a crop grown for the production of bio-ethanol. Sugar beet is commonly grown in Europe for the production of sucrose and has recently been cultivated in Cradock and the surrounding areas (Engineering News, 2008). The biofuel industry usually ferments the sucrose with *Saccharomyces cerevisiae* to yield bio-ethanol. However, researchers are presented with a critical role to increase current yields as there are concerns over the process costs from industrial biotechnologists.

The beet factories produce a pulp by-product removed of all sucrose. The hemicellulose-rich pulp can be degraded by microbial enzymes to simple sugars that can be subsequently fermented to bio-ethanol. Thus, the pulp represents a potential source for second generation biofuel.

The process of utilising microbial hemicellulases requires an initial chemical pre-treatment step to delignify the sugar beet pulp (SBP). An alkaline pre-treatment with 'slake lime' (calcium hydroxide) was investigated using a 2^3 factorial design and the factors examined were: lime load; temperature and time. The analysed results showed the highest release of reducing sugars at the pre-treatment conditions of: 0.4 g lime / g SBP; 40°C and 36 hours.

A partial characterisation of the *Clostridium cellulovorans* hemicellulases was carried out to verify the optimal activity conditions stated in literature. The highest release of reducing sugars was measured at pH 6.5 – 7.0 and at 45°C for arabinofuranosidase A (ArfA); at pH 5.5 and 40°C for mannanase A (ManA) and pH 5.0 – 6.0 and 45°C for xylanase A (XynA). Temperature studies showed that a complete loss of enzymatic activity occurred after 11 hours for ManA; and 84-96 hours for ArfA. XynA was still active after 120 hours.

The optimised lime pre-treated SBP was subsequently degraded using various combinations and percentages of *C. cellulovorans* ArfA, ManA and XynA to determine the maximal release of reducing sugars. Synergistically, the highest synergy was observed at 75% ArfA and 25% ManA, with a specific activity of 2.9 μmol/min/g protein. However, the highest release of sugars was observed at 4.2 μmol/min/g protein at 100% ArfA.

This study has initiated the research within South Africa on SBP and its degradation by *C. cellulovorans.* Preliminary studies show that SBP has the potential to be utilised as a second generation biofuel source.

For the glory of the risen Lord,
For whilst I was still a sinner He died
So, that I may live.

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List of Abbreviations

APD	Ammonia pressurization depressurisation
APS	Ammonium persulphate
ArfA	Arabinofuranosidase A
BSA	Bovine serum albumin
BX	Birchwood xylan
CAX	Cellulose / arabinoxylan substrate
CbpA	Cellulose binding protein A
CBD	Cellulose binding domain
CBM	Carbohydrate binding module
CD	Cohesion domain
DNS	Dinitrosalicylic acid
GH	Glycosyl hydrolase
IPTG	Isopropylthiogalactoside
LB	Luria broth
LBG	Locust bean gum
ManA	Mannanase A
µg	Microgram
mg	Milligram
µl	Microliter
ml	Milliliter
mM	Millimolar
nm	Nanometer
OD	Optical density
*p*NPA*f*	*p*-nitrophenyl-α-L-arabinofuranoside
SBP	Sugar beet pulp
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	Scanning Electron Microscope

SLH	Surface layer homology
TEMED	*N*,*N*,*N*’,*N*’-tetramethylethylenediamine
Tris	Tris(Hydroxymethyl) Aminomethane
w/v	Weight / Volume
v/v	Volume / Volume
XynA	Xylanase A
2 x YT	Yeast-Tryptone

List of Research Outputs

A. POSTER PRESENTATION

Dredge, R.A. and Pletschke, B.I. (2010). Saccharification of sugar beet pulp with hemicellullase synergy with an optimised pretreatment step. 22nd South African Society for Biochemistry and Molecular Biology (SASBMB) Congress. Ilanga Estate. Bloemfontein. 18-20 January 2010.

B. ANTICIPATED PUBLICATION IN A PEER-REVIEWED SCIENTIFIC JOURNAL

Dredge, R.A. and Pletschke, B.I. (2010). Optimised lime pre-treatment and *Clostridium cellulovorans* hemicellulase synergy with sugar beet pulp. Article in preparation for submission to "Biotechnology Letters".

1.1 Biofuel

Climate changes have been a concern both politically as well as within the environmental community (Halleux *et al.*, 2008). The increase in greenhouse gas emissions (CO_2, CO, CH_4 and N_2O) from industry and transportation has been linked to changes in the climate (İçöz *et al.*, 2009). This concern, coupled with limited fossil fuel reserves, has initiated a global refocus on energy resources and use.

Biofuels have attracted a great deal of attention as part of the solution to the energy crisis. A liquid, gaseous or solid fuel produced primarily from biomass is termed biofuel. In comparison to the other renewable energy sources, the use of biomass has been shown to have the most potential (Demirbas, 2008). This is due to the accessibility to biomass and the fact that it can be supplied to a location distant to its source. Another benefit is that it has less of an environmental impact in comparison to fossil fuels due to its low sulphur and nitrogen content (Demirbas, 2008).

Biofuel production has potential advantage in improving rural and agricultural economies, benefiting agricultural and industrial sectors by using by-products (such as wood wastes, oil waste products and municipal solid wastes), as well as a decrease in dependence on oil imports and therefore providing some energy security (Demirbas *et al.*, 2008; Demirbas, 2009; Taylor, 2008). However, despite the advantages of biofuels, there are areas of concern that also need to be addressed: will the biofuel price be competitive with the price of fossil fuel; and with agricultural land switching to biofuel production would food prices increase (Murphy and Power, 2009)? The complexity of this bio-energy argument has been exemplified by the questions asked by Schwinle in the preface to Halleux *et al.* (2008). Schwinle questioned how the carbon emissions should be calculated on land converted to producing biofuels. Firstly, he questioned how should this be related to land that once was utilized for biofuel production and is now being used for food production and vice versa. Secondly, with limiting agricultural land, biofuel production could push farmers to convert natural habitats into agricultural land thus should this be considered as a biofuel carbon emissions? The last critical question posed by Schwinle is how does all this affect biodiversity? General consensus across

environmental and political communities is that a greater efficiency is required in the conversion of plant matter to biofuel with less economic demand. Thus, research into increasing the efficiency of this energy conversion is critical (Mosier *et al.*, 2005).

1.2 Lignocellulosic Biomass

The most abundant component of plant material is ligno-cellulose (Bayer *et al.*, 1998), with an approximate mass content of 40-50% cellulose, 25-35% hemicellulose, 15-20% lignin and a small fraction of acids, salts and minerals (Hamelinck *et al.*, 2005; Wyman *et al.*, 2005). The basic chemical structures of these three components of biomass are presented in Figure 1.1.

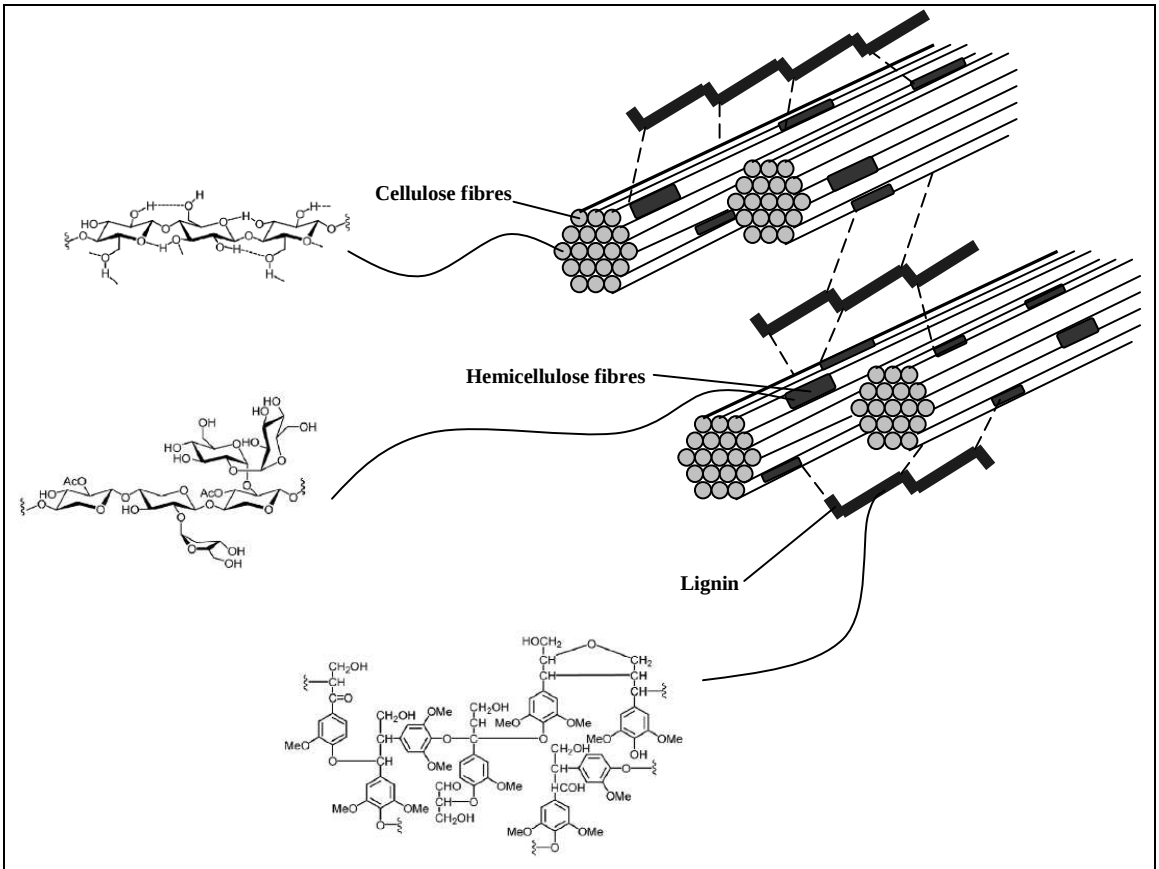

Figure 1.1 Structures of cellulose, hemicellulose and lignin The cellulose chains have β-1,4-linkages that allow for hydrogen-bonding (dashed lines) between each cellulose strand. Hemicellulose is composed of either a pentose or hexose backbone with linear and branched side-chains. Lignin forms a complex structure of aromatic rings and polysaccharide polymers with various bonds (Modified from Chang 2007; Khandeparker and Numan, 2008).

1.2.1 Cellulose

Cellulose has a simple chemical composition of glucose residues linked with *β*-1,4-glycosidic bonds that form cellulose fibrils of 500-14,000 linked glucose residues (Chang *et al.,* 2007). It is the high degree of hydrogen-bonding between these cellulose 'bundles' that creates a crystalline structure. The crystallinity of cellulose is determined with X-ray scattering, the non-crystalline fraction is termed amorphous. The degree of polymerization increases from primary to secondary cell walls (O'Sullivan 1997).

Six cellulose polymorphs have been discovered: Iα; Iβ; II; IIIα; IIIβ; IVα and IVβ (O'Sullivan 1997). Cellulose in nature is found as cellulose I, the two polymorphs Iα and Iβ were determined as two distinct forms by the NMR work of Atalla and Van der Hart (1984). Cellulose Iα is the dominant form in algae and bacteria whilst cellulose Iβ is dominant in secondary walls of higher plants (Atalla and Van der Hart, 1984). Cellulose II – IV are all formed through various pre-treatment methods (such as NaOH, ammonia and heating) (O'Sullivan 1997).

1.2.2 Hemicellulose

Interlinking the cellulose fibrils are hemicellulose structures containing a diverse matrix of linear and branched pentose (D-xylose, D-arabinose), hexose (D-mannose, D-glucose, D-galactose) and uronic acid (4-O-methylglucuronic, D-glucuronic and D-galactouronic acid) (Kumar *et al.,* 2009). The hemicellulose polymers feature β-1,3-bonded as well as β-1,4-bonded backbones that form shorter lateral chains compared to cellulose (Hendriks and Zeeman, 2009).

Xylan is the most common of the hemicelluloses (Saha, 2003). The xylose residues form a xylan backbone with β-1,4-linkages and may contain various side-chains (Figure 1.2). Hardwood xylans consist of methylated glucuronic acid and acetic acid sidechains, whilst in softwood xylans consist of uronic acid and L-arabinofuranose sidechains and in grasses the L-arabinofuranose is substituted with *p*-cumaric acid and ferulic acid (Shallom and Shoham, 2003).

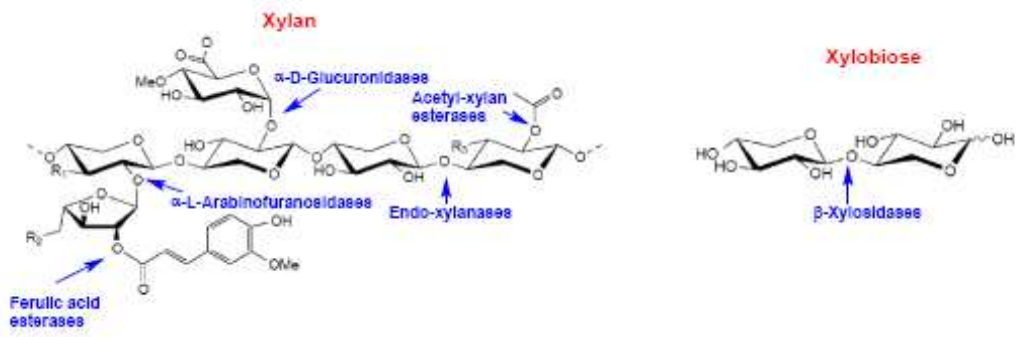

Figure 1.2. Xylan and xylobiose chemical structures. The xylan backbone and various side-chains are cleaved by a number of hemicellulases: α-D-glucuronidase; acetyl-xylan esterase; ferulic acid esterase; α-L-arabinofuranosidase; endo-xylanase and β-xylosidase. Me: methyl; R_1OH: arabinofuranoside residue; R_2OH: ferulic acid; R_3OH: acetic acid (Shallom and Shoham, 2003)

The mannan structure is composed of a backbone of β-1,4-linked mannose units or with mannose and glucose residues. Galactoglucomannan has a mannan backbone and side-chains of galactose and acetate residues (Shallom and Shoham, 2003) (Figure 1.3).

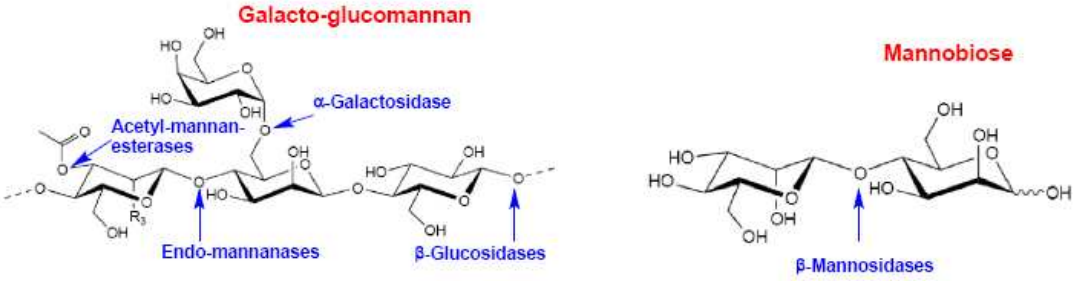

Figure 1.3. Galacto-glucomannan and mannobiose chemical structures. The hemicellulases necessary to degrade galacto-glucomannan are: acetyl-mannan-esterases; α-galactosidease; endo-mannanases; β-glucosidases and β-mannosidases. R_3OH: acetic acid (Shallom and Shoham, 2003)

The arabinose sugar is present in arabinogalactan and L-arabinan. Arabinogalactan has a galactose backbone with a variety of side-chains such as β-1,6-bonded galactose and α-1,3-bonded L-arabinofuranosyl. L-arabinan has an L-arabinofuranosyl backbone with further L-arabinofuranosides linked as side-chains (Shallom and Shoham, 2003) (Figure 1.4).

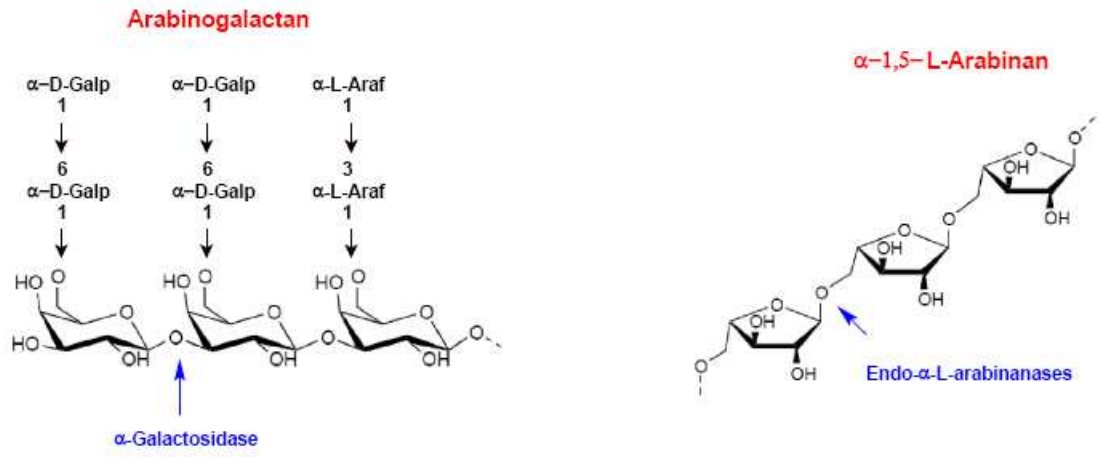

Figure 1.4. Arabinogalactan and arabinan Arabinogalactan is degraded by the hemicellulases α-galactosidase and endo-α-L-arabinanase. α-D-Galp: α-Galactopyranose; α-L-Araf: α-Arabinofuranosyl (Shallom and Shoham, 2003).

Hemicellulose, in comparison to cellulose, is easily hydrolysable (Kumar *et al.*, 2009). Research has shown that the solubilities of these hemicellulose polymers vary in ascending order: galactose; arabinose; glucose; xylose and mannose (Gray *et al.*, 2003).

1.2.3 Lignin

Lignin is the component of the plant cell wall that exists to provide structural support. It is an amorphous structure composed of hydroxyphenylpropanoid units with various linkages to other polymers. This heteropolymer is significant for impermeabiltiy as well as resistance to microbial attack (Hendricks and Zeeman, 2009).

1.3. Sugar beet plant

Sugar beet (*Beta vulgaris)* is grown in temperate climates to produce sucrose, particularly in Europe and the United States (Foster *et al.*, 2001, Vaccari *et al.*, 2008). The other major sucrose producing crop is sugarcane, grown in the tropics and sub-tropical climates. Sugarcane supplies 65% of sucrose worldwide compared to the 35% supplied by sugar beet (Foster *et al.*, 2001).

In the South African context, sugar beet has been included as one of the crops for the production of bio-ethanol. The Biofuels Industrial Strategy (Department of Minerals and

Energy, 2007) proposed an introduction of 2% liquid fuel (400 million litres biofuel per annum) into the national fuel supply. The Strategy encouraged biofuel production with fuel tax exemptions of 50% for biodiesel and 100% for bio-ethanol production. To date the only sugarbeet farming currently taking place in South Africa is that in the Eastern Cape (Engineering News, 2008).

The most common means of producing bio-ethanol from sugar beet comes from fermenting the beet sugar syrup (İçöz *et al.,* 2009; Leiper *et al.,* 2006). However, with the increased interest in biofuel production, even by-products are being considered. Currently the sugar beet pulp (residue remaining after the sucrose has been extracted) is being used as a pectin source for the food industry (Turquois *et al.,* 1999), animal feed (Deaville *et al.,* 1994; Serena and Knudsen, 2007) and in the production of paper (Vaccari *et al,* 2005). Due to the abundant and low value of the carbohydrate-rich sugar beet pulp, this by-product could also be utilized as a source of renewable biomass for biofuel (Foster *et al.,* 2001).

The chemical composition of the sugar beet pulp expressed as a percentage of dry weight of total solids shows a slightly larger hemicellulose fraction (45-61%) than cellulose (20-24%), as well as 7-8% protein and 1-2% lignin (Foster *et al.,* 2001). A gas liquid chromatography (GLC) analysis detected a greater breakdown of the constituents of the sugar beet pulp (Figure 1.5).

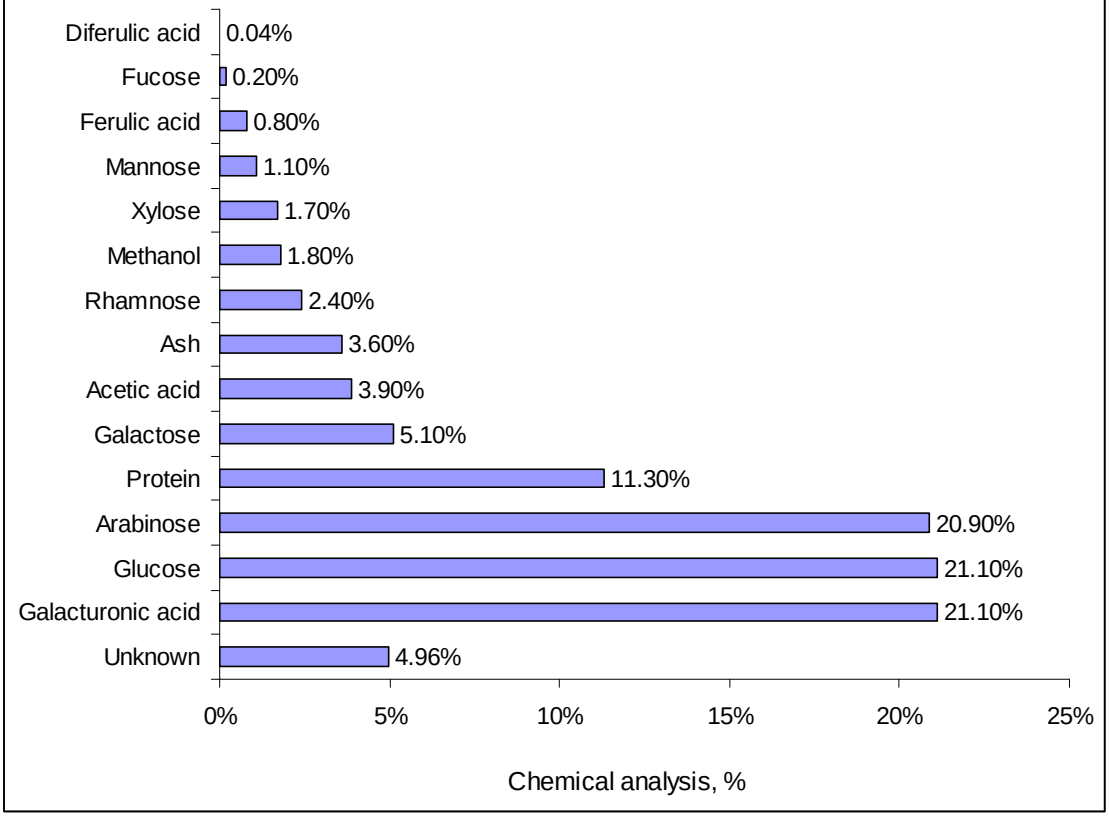

Figure 1.5. Sugar beet pulp chemical composition (% dry weight)
(Modified from Micard *et al.*, 1996)

De Boer and Den Uil (1997) discussed how the chemical composition can only be an approximation, since it can be influenced by growth area, fertilizer type, the time of harvesting and conditions of storage. Thus, not only do these factors need to be considered but also the hemicellulose extraction procedures will differ and therefore there will be a variation in the results obtained. An example of this was observed in Fares *et al.* (2004) where the pectin extraction was followed by a 1 M NaOH and 4 M NaOH hemicellulose extraction. The 1 M NaOH yield of monosaccharides included 31-33% arabinose, 6% mannose and 24-27% xylose, whereas the 4 M NaOH yield was 35-37% arabinose, 8% mannose and 16-17% xylose. Levigne *et al.* (2002) mentioned that the pectin composition was higher in fresh sugar beet than in sugar beet pulp, their researches effectively showed the effect of pH, time and temperature on the pectin yield. However, the type of acid used had no influence.

The effective utilization of the sugar beet pulp will require a selective hydrolysis that will produce pentoses and hexoses that can be subsequently fermented to produce ethanol (Chamy *et al.*, 1994).

1.4. Pre-treatments

Cellulose fibres formed by linked glucose residues are a perfect substrate for conversion to fuel; however, lignocelullose present in plant biomass is recalcitrant to hydrolysis, due to its rigidity and heterogeneity (Bayer *et al.*, 2007). Consequently, the pre-treatment steps are recognised to be the key features in increasing the efficiency of conversion of cellulosic biomass into biofuel. Selecting the optimal pre-treatment conditions for each biomass is critical, though, as it can be the most costly step of the process design, where it can account for up to 20% of the total cost (Yang and Wyman, 2007).

A simple depiction of how the complex network of the lignocellulosic biomass needs to be disrupted for effective enzymatic hydrolysis to occur is presented in Figure 1.6.

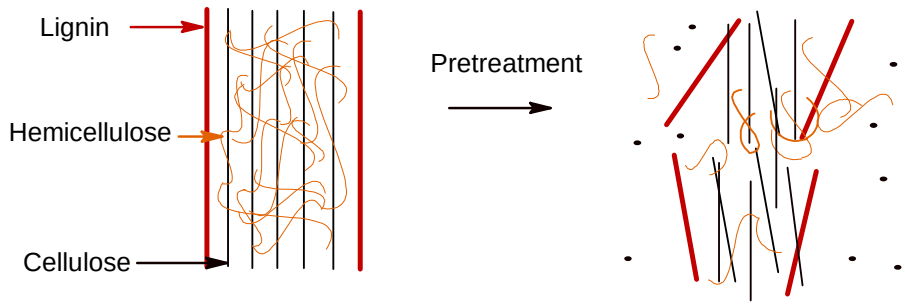

Figure 1.6. Pre-treatment technologies disrupting or altering the structure of biomass (Modified from Mosier *et al.*, 2005)

In choosing a suitable pre-treatment method, the resulting biomass should yield a 90% or higher cellulose digestibility in 3-5 days and a small particle size reduction (as a mechanical pre-treatment can be costly with energy requirements). The pre-treatment technology has to take into consideration the following: release of high yields of fermentable polysaccharides; bioreactors with a low cost design able to withstand

corrosive pre-treatment conditions and low energy demands. As chemical pre-treatments have been shown to be the most promising with regard to high yields and low costing, the factors that need to be taken into account here are: inexpensive chemicals; neutralisation before fermentation and formation of chemical products that are problematic in subsequent processing steps (Yang and Wyman, 2007).

Pre-treatments include a variety of physical, thermal and chemical methods as well as a combination of these technologies (Table 1.1). Physical or mechanical pre-treatment involves dry or wet milling of the plant biomass. The process reduces the particle size and crystalline structure (degree of polymerization), which therefore, enhances the surface area (Hendriks and Zeeman, 2009). A thermal pre-treatment of passing high flow rates of steam over the biomass will disrupt the cellulose chains, remove the hemicellulose fraction and some of the lignin (Yang and Wyman, 2007). Temperatures above 160°C will hydrolyse the hemicellulose to form acids which in turn hydrolyse the hemicellulose further (Hendriks and Zeeman, 2009). Chemical pre-treatments utilize chemical compounds, most frequently acids and bases, to remove hemicellulose or lignin from the lignocellulosic biomass (Mosier *et al.*, 2005).

Table 1.1. The effect various pre-treatment technologies have on cellulosic biomass

	Increase accessible surface area	Decrystallisation cellulose	Solubilisation hemicellulose	Solubilisation lignin	Alteration lignin structure	*Formation furfural / HMF
Physical Pre-treatment						
Mechanical	+ + +	+ + +				
Thermal Pre-treatment						
Steam pretreatment / Steam explosion	+ + +		+ + +	+	+ + +	+ + +
Liquid hot water (batch)	+ + +	ND	+ + +	+	+	+
Liquid hot water (flow through)	+ + +	ND	+ + +	+ +	+	+
Chemical Pre-treatment						
Acid	+ + +		+ + +	+	+ + +	+ + +
Alkaline	+ + +			+ +	+ + +	+
Oxidative	+ + +	ND		+ +	+ + +	+
Combination Pre-treatment				+ +		
Thermal + Acid	+ + +	ND	+ + +	+ +	+ + +	+ + +
Thermal + Alkaline (lime)	+ + +	ND	+	+ +	+ + +	+
Thermal + Oxidative	+ + +	ND	+	+ +	+ + +	+
Thermal + Alkaline + Oxidative	+ + +	ND	+	+ +	+ + +	+
Ammonia (AFEX)	+ + +	+ + +	+	+ + +	+ + +	+
CO2 explosion	+ + +		+ + +			

* Formation of inhibitors such as furfual and hydroxymethylfurfural
+ + + Major effect, + + Possible effect, + Minor effect, ND Not detected
(Modified from Mosier *et al.*, 2005; Hendriks and Zeeman, 2009).

Sugarbeet pre-treatment methods are less stringent due to the low lignin content of the beet. Chamy *et al.* (1994) investigated the use of acid hydrolysis which, depending on the concentration of the acid, will solubilize hemicellulose and precipitate solubilized lignin (Hendriks and Zeeman, 2009). The pre-treatment method on sugarbeet was optimal at: 55 g/l load of 32-50 mesh sugar beet pulp (SBP), 1.1 g H_2SO_4/ g SBP and 80°C for 90 minutes at 400 rpm. Ammonia pressurization-depressurization (APD) (a modification of ammonia fiber explosion, AFEX) was the pre-treatment method researched by Foster *et al.* (2001). APD involves treating the SBP with elevated pressures and liquid ammonia and then a sudden depressurization and evaporation of the ammonia. The effectiveness of the pre-treatment is due to the ammonia's ability to decrystalize the cellulose fibers and tear the fibers apart by the sudden evaporation of the ammonia. Foster *et al.* (2001) showed an enhanced cellulase activity but no effect with hemicellulases and pectinases. They concluded that the APD possibly degraded all hemicellulose and pectin.

An effective pre-treatment for subsequent fermentation of the sugarbeet pulp would require a treatment where there is a disruption of the lignin and hemicellulose structures without the formation of any inhibitory products. As there is a low lignin composition in SBP this would not need to be a stringent method, so this could include any of the following means of pre-treatment: alkaline, thermal and AFEX or a combination of these methods with oxygenation.

1.5. Cellulosomes

The microbial degradation of plant cell walls has been observed to occur in two well-studied ways, firstly, where the free cellulases are secreted by mostly aerobic microbes and secondly, where the cellulases are bound within a multi-enzyme complex known as a cellulosome (Wilson, 2008). A third, less well-studied, means of degrading cellulose proposes a mechanism whereby starch degrading enzymes are present in the periplasmic space of the microbe (Cho and Salyers, 2001; Wilson, 2008).

The cellulosome system present in many anaerobes is believed to have been developed due to the harsh anaerobic conditions (Bayer *et al.*, 2004). The cellulosome of *Clostridium cellulovorans* 743B (ATCC 35296, Tamaru *et al.*, 2010) as shown in Figure 1.7 is organised around a non-enzymatic scaffolding protein or scaffoldin, which forms the base for several enzymatic subunits to bind to as well as other cellulosomal modules (Doi *et al.*, 2003).

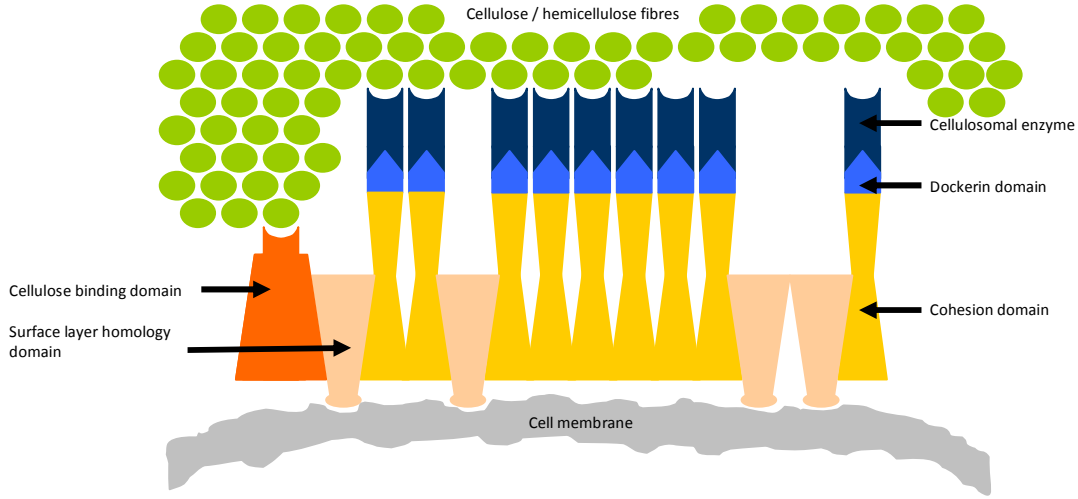

Figure 1.7. Cellulosome structure from *Clostridium cellulovorans*. The cellulose binding protein A (CbpA) consists of a cellulose binding domain that attaches to the cellulose residues; several surface layer homology domains that bind the CbpA to the cell surface and cohesion domains that interact with the dockerin domains or duplicated sequences of the cellulosomal enzymes. (Modified from Doi and Tamaru, 2000).

1.5.1 Cellulose binding domain

The cellulose binding domain (CBD) or carbohydrate binding module (CBM) has been shown to increase catalytic activity, due to closer proximity and prolonged association of the enzyme with the cellulose substrate that it facilitates (Shoseyov *et al.*, 2006). CBMs have been categorized into 52 families based on protein sequence similarity (Coutinho and Henrissat, 1999). The various CBMs recognise and bind to crystalline cellulose, non-crystalline cellulose, xlyan, chitin, mannan, glactan and starch. Therefore, the CBMs are further categorized as surface-binding (Type A), glycan-binding (Type B) and small sugar-binding (Type C) proteins (Boraston *et al.*, 2004).

The cellulose-binding protein A (CbpA) produced by *C. cellulovorans* binds specificially to crystalline cellulose (Goldstein *et al.,* 1993) and through mutation analysis it was shown that the entire CBD is necessary for maximum binding (Goldstein and Doi, 1994). The *C. cellulovorans* CbpA has been classified as a family 3 CBD (Goldstein and Doi, 1994) and as Type A as it binds to crystalline polysaccharides.

1.5.2 Cohesion domains

The cohesion domains on the scaffolding protein form a 'plug-and-socket' interaction for the dockerin domains on the cellulosomal enzymes. This cohesion-dockerin interaction is calcium-dependent (Bayer *et al.,* 2004). A classification system based on sequence homology has categorised cohesions into a Type I, Type II or Type III cohesion (Bayer *et al.,* 2004).

The cellulosome of *C. cellulovorans* has 9 cohesion domains that are conserved hydrophobic regions. The dockerin domains of each cellulosomal enzyme have a duplicated sequence of approximately 22 amino acid residues (Doi and Tamaru, 2000).

1.5.3 Surface layer homology domain

The attachment to the cell surface is mediated with the surface layer homology (SLH) domain or hydrophilic domain (HLD). Some cellulosomal scaffolding proteins have SLHs, whilst other proteins, containing a SLH region, are known as anchoring proteins and these interact with the cellulosomal protein (Bayer *et al.,* 2004).

Within the *C. cellulovorans* CbpA sequence there are four conserved SLHs, homologous to surface layer proteins that are present on the cell wall (Shoseyov *et al.,* 1992). The endoglucanase E has a unique structure with a dockerin domain as well as 3 N-terminal SLHs, and thus it is able to bind to the cellulosome and the cell surface simultaneously (Figure 1.7) (Kosugi *et al.,* 2002a ; Tamaru and Doi, 1999).

1.6. Hemicellulases

The glycosyl hydrolases (GH) are classified into families according to their primary structures (Henrissat and Bairoch, 1996). The hydrolysis of the glycoside bond occurs via general acid-base catalysis through one of two alternative mechansims. The first possible reaction mechanism (Figure 1.8A) involves an inversion of the anomeric configuration and the second reaction mechanism (Figure 1.8B) involves a net retention of the anomeric configuration (McCarter and Withers, 1994).

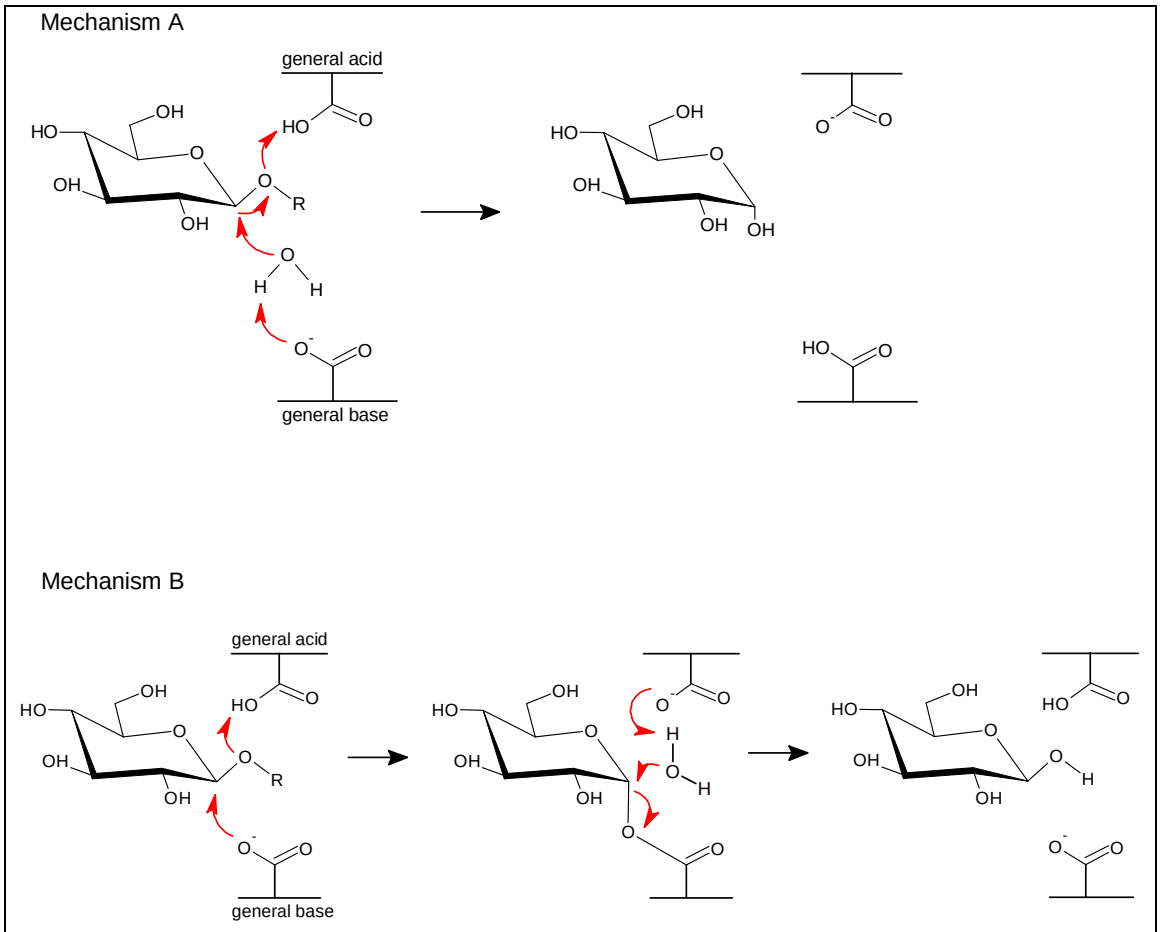

Figure 1.8. Glycosidase reaction mechanisms. Mechanism A: A net inversion with a direct displacement with water, Mechanism B: A net retention with a double-displacement with water (Modified from McCarter and Withers, 1994).

1.6.1 Hemicellulases: Arabinofuranosidases

The hemicellulase enzyme α-L-arabinofuranosidase (Arf), according to its primary structure, falls into the glycosyl hydrolase (GH) families 43, 51, 54 and 62 (Henrissat and Bairoch, 1996). Family 43 GH's are able to degrade the α-1,5-arabinanose bonds via an inversion of the anomeric configuration (Nurizzo *et al.*, 2002). Cleavage has also been shown to be 'processive' in that once a bond is broken by the arabinanase it then proceeds to cleave to release arabinotrioses (Nagy *et al.*, 2002). Family 51 is the largest family of the α-L-arabinofuranosidases and members have been shown to have a stereo-retaining catalytic mechanism (Shallom *et al.*, 2002a; Shallom *et al.*, 2002b) also present in the GH 4/7 superfamily (Debeche *et al.*, 2002). Family 54 GH also have a net retention mechanism (Pitson *et al.*, 1996). There is no published data on the catalytic mechanism of Family 62 (Shallom *et al.*, 2002a).

Although the pH optimum for Arf activity is between pH 3.0-6.9 and the temperature optimum is between 40-75^{0}C (Saha, 2000), there are several microbial Arf's that have activity beyond these mentioned ranges. Substrate specificity varies between α-1,3 and α-1,5 arabinosyl linked terminal residues (Saha, 2000).

Fritz *et al.* (2008) noted that some microbes produce more than one α-L-arabinofuranosidase. The fungi *Aspergillus* produce two Arf isoenzymes in *A. awamori*a and *A. niger* (Kaneko *et al.*, 1998; Rombouts *et al.*, 1988), whilst *Penicillium* produces 2 Arf isoenzymes in *P. capsulatum*, *P. canescens* and *P. chrysogenum* (Filho *et al.*, 1996; Sakamoto and Kawasaki, 2003; Sinitsyna *et al.*, 2003). Three Arf isoenzymes are produced by *A. terreus* (Le Clinche *et al.*, 1997; Luonteri *et al.*, 1995) and *P. purpurogenum* (De Ionnes *et al.*, 2000). Some microorganisms also produce enzymes that are able to exhibit both xylanase and arabinofuranosidase activity, examples include: *Clostridium stercorarium* (Sakka *et al.*, 1993) and *Prevotella ruminicola* (Gasparic *et al.*, 1995). The biotechnology applications of α-L-arabinofuranosidases are numerous and displayed in Table 1.2.

Table 1.2. Potential biotechnology applications of α-L-arabinofuranosidases

Agricultural Feeds - Increased digestibility to increase the conversion of feeds into energy
Food and Drink Applications Bread - Increased quality and shelf life Wine - α-L-arabinofuranosidase and glycosidases improve flavour with the release of terpenols Juice - Preventing the formation of haze
Industrial Bio-ethanol production - enzyme pre-treatment of hemicellulose to yield sugars for subsequent microbial fermentation to ethanol Paper - delignification and bleaching of the pulp
Medicinal Antimetastic / anticarcinogenic properties - α-L-arabinofuranosidase hydrolysis of the root of *Panax ginseng* produces a compound that has anti-cancer effects Antiglycemic properties - α-L-arabinofuranosidase releases the sweet flavoured L-arabinose. The sugar has a competitive absorption to sucrose as well as a low absorption in the body, thus decreasing the energy yield of sucrose.

(Modified from Numan and Bhosle, 2006; Saha, 2000)

Research has shown that the enzyme expression is largely dependent on the growth conditions of the microorganism (Han *et al.*, 2005). *C. cellulovorans* produced a more enzymatically active crude supernatant when grown on a pectin-rich substrate (Tamaru *et al.*, 2002) and no Arf production was seen when the microorganism was grown on cellulose (Kosugi *et al.*, 2002c). *Bacillus stearothermophilus* T-6 expressed an Arf when grown on sugar beet or oatspelt xylan; however, no expression was observed when *B. stearothermophilus* was grown on xylose or glucose (Gilead and Shoham, 1995).

Sugar beet pulp is commonly used as an inducer of Arf formation in fungal and bacterial cultures (Table 1.3), due to the high L-arabinose chemical composition of the pulp.

Table 1.3. Microbial α-L-arabinofuranosidase production with sugar beet pulp

Organism		Reference
Aspergillus nidulans	Fungus	Fernandez-Espinar *et al.*, 1994
Aspergillus niger	Fungus	Flipphi *et al.*,1994
Aspergillus terreus	Fungus	Luonteri *et al.*, 1995
Aureobasidium pullulans	Fungus	Saha and Bothast, 1998
Pencillium purpurogenum	Fungus	De Loannes *et al.*, 2000
Trichoderma aurantiacus	Fungus	Roche *et al.*, 1994; Roche and Durand, 1996
Trichoderma reesei	Fungus	Roche *et al.*, 1995
Bacillus stearothermophilus	Bacterium	Gilead and Shoham, 1995
Bacillus subtilis	Bacterium	Weinstein and Albersheim, 1979
Rhodotorula flava	Bacterium	Uesaka *et al.*,1978

1.6.1.1 *Clostridium cellulovorans*

The *C. cellulovorans* α-L-arabinofuranosidase or ArfA is a non-cellulosomal family 51 glycosyl hydrolase. The *arfA* gene encodes the ArfA protein with 493 amino acids and a molecular weight of 55.731 kDa (Kosugi *et al.*, 2002c). It is interesting to note that while the ArfA amino acid sequence in comparison with other family 51 proteins within the SWISS-PROT database shows a higher homology of 64% identity with BH1874 of *Bacillus halodurans*, the phylogenetic analysis (CLUSTAL W) shows the closest evolutionary relationship with ArfB of *Clostridium stercorarium* (Figure 1.9).

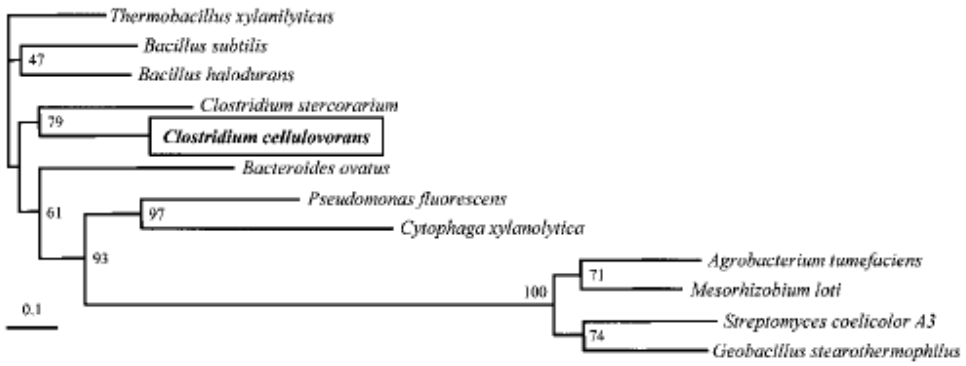

Figure 1.9. Glycosyl family 51 phylogenetic tree indicating the position of *C. cellulovorans* ArfA. The phylogenetic tree was constructed with CLUSTAL W and the closest in homology to the *C. cellulovorans* ArfA was ArfB from *Clostridium stercorarium* (Kosugi *et al.*, 2002c).

Characterisation of ArfA by Kosugi and co-workers (2002c) has shown that the pH optimum was 6.0 and temperature optimum was between 40 - 50^{0}C with all activity being lost after incubation at 80^{0}C for 20 minutes. Stability studies indicated that ArfA had a greater stability at pH 8.0 - 9.0 compared to pH 7.0. The kinetic parameters of the recombinant rArfA with the substrate *p*-nitrophenyl α-L-arabinofuranoside included a K_m of 0.71 mM and V_{max} of 75.8 U/mg of protein. Enzyme activity was demonstrated to remain unchanged upon the addition of Ca^{2+}, Mg^{2+} and Mn^{2+}, but was inhibited by Cu^{2+}, Hg^{2+} and Zn^{2+}.

The arabinofuranosidase mechanism of ArfA involves general acid-base catalysis. The ArfA of *Geobacillus stearothermophilus* was shown to have a Glu^{175} as the acid-base residue and Glu^{294} as the nucleophile (Shallom *et al.,* 2002b), while *Thermobacillus xylanilyticus* was shown to have Glu^{176} as the acid-base residue and Glu^{298} as the nucleophile (Debeche *et al.,* 2002). Thus, with reference to the *C. cellulovorans* amino acid sequence from SWISS-PROT, it can be suggested that Glu^{173} is the acid-base catalytic residue and Glu^{296} the catalytic nucleophile.

Another noncellulosomal enzyme from *C. cellulovorans* was found to be associated with the arabinose degradation of the plant cell wall. The gene for BgaA is located next to *arfA* and the protein has a molecular mass of 76.414 kDa (Kosugi *et al.,* 2002c). In contrast to ArfA, BgaA has activity for *p*-nitrophenyl α-L-arabinopyranoside as well as *p*-nitrophenyl β-D-galactopyranoside activity.

1.6.2 Hemicellulases: Mannanases

Mannan is composed of a β-1,4-linked mannose backbone with various β-1,4-linked glucose and α-1,6-linked galactose side chains. The range of enzymes necessary to degrade mannan are: β-mannanases (EC 3.2.1.78); β-mannosidases (EC 3.2.1.25); β-glucosidase (EC 3.2.1.21); acetyl mannan esterase (EC 3.1.1.6) and α-galactosidase (EC 3.2.1.22) (Moreira and Filho, 2008).

The mannan backbone-hydrolyzing enzymes are β-mannanases and β-mannosidases, each with a stereochemical mechanism B as shown in Figure 1.8 (Moreira and Filho, 2008). The β-mannanases fall into the GH family 5 found in bacterial, fungal and eukaryotic sources and GH family 26 found almost exclusively in bacteria and a few fungi (Dhawan and Kaur, 2007). The endohydrolase (β-mannanase) cleaves β-1,4-linked mannan chains randomly and the exohydrolase (β-mannosidase) cleaves to release the mannose residues (Moreira and Filho, 2008). The β-mannosidases fall into the GH families 1, 2 and 5 (Shallom and Shoham, 2003).

The ubiquity of mannanases in nature and their growing industrial applications have increased subsequent scientific research into these enzymes. Some of these commercial applications are indicated in Table 1.4 below.

Table 1.4. Potential biotechnology applications of β-mannanases

Agricultural Feeds - β-mannans release mannooligosaccharides, thus decreasing viscosity and providing a food source for the beneficial microflora of poultry
Food and Drink Applications Coffee - β-mannanase hydrolysis of coffee extract to reduce viscosity (instant coffee processing) Coconut Oil - utilised in coconut oil extraction
Industrial Paper - softwood pulp bleaching Detergent - stain (gums) removal in laundry detergents Textiles - preparation of textile fibers for the manufacture of clothing Drilling Oil/ Gas - hydrolysis of guar gum, increases the flow of oil in drilling pipes
Medicinal Probiotic microflora - β-mannanases release mannooligosaccharides from foods that can not be hydrolysed by the digestive microflora, these sugars are then utilised by the beneficial bacteria.

(Modified from Dhawan and Kaur, 2007)

1.6.2.1 *Clostridium cellulovorans*

The *manA* gene was first discovered by Tamaru and Doi (2000) in investigation of the cell wall degrading subunits of the cellulosome. Several sections of the *C. cellulovorans* cellulosome were sequenced before the entire gene cluster was published by Tamaru *et al.* (2010).

The gene *manA* produces a family 5 glycosyl hydrolase. The enzyme with a molecular mass of 47.156 kDa (425 amino acids) has a catalytic site between residues 88-425 and includes a dockerin domain allowing it to bind to the scaffolding protein of the cellulosome (Tamaru and Doi, 2000). The optimal activity of ManA occurs at pH 7.0 and 45°C. The highest specific activity was noted with glucomannan and locust bean gum while the lowest activity was noted with guar gum and β-mannan (Tamaru and Doi, 2000).

1.6.3 Hemicellulases: Xylanases

Xylan comprises of a backbone of β-1,4-linked xylopyranosyl resides including various side groups such as acetyl, L-arabinofuranosyl and O-methylglucuronyl (Biely *et al.*, 1997). Xylananases (EC 3.2.1.8) hydrolyzing the xylan backbone into xylooligomers are found mostly in GH families 10 and 11. A higher catalytic diversity has been shown with endo-xylanases in family 10 and it has been proposed that these xylanases have a smaller substrate binding site than those in family 11 (Biely *et al.*, 1997). A smaller number of xylanases have been discovered that fall into GH families 5, 8 and 43 (Shallom and Shoham, 2003). The β-xylosidases (EC 3.2.1.37) hydrolyse the xylooligomers produced by the xylanases into individual xylose residues. The GH families that contain the β-xylosidases fall into families 3, 39, 43, 52 and 54 (Shallom and Shoham, 2003). The most promising industrial applications for xylanases are presented in Table 1.5.

Table 1.5. Potential biotechnology applications of xylanases

Agricultural Feed - reduce viscosity and increase digestibility
Food and Drink Applications Sweetner - xyliooligosaccharides (released from xylan), useful in diabetic products Flour - enhancement of the quality of wheat flour
Industrial Bio-ethanol production - Xylanase hydrolysis of lignocellulosic biomass to sugars, for subsequent fermentation to bio-ethanol / bio-methanol
Medicinal Probiotic microflora - xylanases release xylooligosaccharides a food source for beneficial bacteria Anti-obesity - xylooligosaccharides have a low calorie value Cholesterol - xylooligosaccharides bind to bile acids and are passed out the human body, thus lowering cholesterol levels

(Modified from Khandeparker and Numan, 2008)

1.6.3.1 *C. cellulovorans*

The *xynA* gene from *C. cellulovorans* produces a family 11 endo-1,4-β-xylanase (XynA), a protein of 520 amino acids with a molecular mass of 57.038 kDa (Koukiekolo *et al.*, 2005). XynA contains a catalytic domain that degrades β-1,4-xylans (Shallom and Shoham, 2003), a dockerin domain and a nodulation protein domain (NodB domain) that encodes acetyl xylan esterase with a broad substrate range (Kosugi *et al.*, 2002b). The properties of XynA include an pH optimum of 5.0 and temperature optimum of 60°C (Kosugi *et al.*, 2002b).

In the degradation of cellulose/arabinoxylan (CAX) substrate, XynA was observed to be the rate-limiting enzyme, as there was a higher degree of synergy in the sequential reactions when the XynA-mini-CbpA was added before EngL-mini-CbpA (Koukiekolo *et al.*, 2005). Therefore, hemicellulases are significant within cellulosomes.

1.7. Enzyme synergy

Purification of the active cellulosomal enzymatic and non-enzymatic proteins once bound to the cellulosome, is difficult (Choi and Ljunghahl, 1996). Murashima *et al.* (2002) noted that this inability to work with purified cellulosomes has prevented a comprehensive understanding of the cellulomosal mode of action and thus artificial cellulosomes have been designed to study the mechanism of cellulosomes on cellulose (Cho *et al.,* 2004; Murashima *et al.,* 2002). The complexity of this study is increased by a synergistic relationship that exists between the bound enzymes (Klyosov, 1990). Synergistic enzyme activity occurs when a mixture of enzymes has a larger activity than the sum (total) displayed activity by the individual enzymes (Koukiekolo *et al.,* 2005). This definition can be known as the degree of synergy or the synergism coefficient.

The synergistic associations of unbound or free α-L-arabinofuranosidase and xylanases have been well studied (Table 1.6). Variations in synergistic effects have been noted to be related to the percentage of arabinose in each substrate. Vincent *et al.* (1997) showed a higher synergistic relationship with L-arabinofuranosidase (Abr), xylanase A (XlnA) and xylanase C (XlnC) from *Streptomyces lividans* with xylan from wheat and rye flour (arabinose 41-49%) compared to oatspelt xylan (arabinose 9%).

Table 1.6. Synergistic studies on arabinose and xylanase

Enzyme	Substrate	Pre-treatment	Degree of Synergism*	Reference
α-arabinose and xylanase (*Trichoderma reesei)*	Wheat straw arabinoxylan	10% NaOH	Yes	Poutanen, 1988
α -arabinose and β-1,4-xylanase (*Ruminococcus albus* 8)	Alfalfa cell wall (grass) - ACW	Extracted x3 with 1L chloroform-methanol (3:1), extraction x1 with 500ml acetone.	Yes (5.2)	Greve *et al.*,1984
α-arabinose (*R. albus 8*) and polygalacturonase (*Fusarium oxysporum cubense)*	Alfalfa cell wall (grass) - ACW		Yes (5.2)	
α-arabinose (*R. albus 8*) and polygalacturonase (*Fusarium oxysporum cubense)*	Alfalfa cell wall (grass) - PGSC	ACW treatment and pebble milled, washed with 0.1M $NaCH_3C00$ (pH5.0 at 37°C), filtered and rinsed, digested with polygalacturonase from *Fusarium oxysporum cubense*, *EtOH 80% vol/vol*, centrifuged pellet	Yes (2.6)	
α-arabinose (*R. albus 8*) and β-1,4-xylanase (*R. albus* 8)	Alfalfa cell wall (grass) - BSC	ACW treatment and pebbled milled, washed with 0.1M $NaCH_3C00$ (pH5.0 at 37°C), filtered and rinsed, digested with polygalacturonase from *Fusarium oxysporum cubense*, centrifuged pellet, suspended in 1N NaOH + 1mg/ml NaBH4, centrifuged, EtOH 90% vol/vol, centrifuged, washed EtOH 90% wt/vol, dried.	Yes (2.7)	
α-arabinose B and xylanase A, α-arabinose B and xylanase C (*Streptomyces lividans)*	Wheat flour	Substrate purchased**	Yes	Vincent *et al.*, 1997
	Oat spelt xylan	Substrate purchased**	None	
α-L-arabinofuranosidase and xylanase (*Bacillus stearothermophilus)*	Oat spelt xylan	Substrate purchased**	Yes	Gilead and Shoham, 1995

*The degrees of synergy are cited in parenthesis where published. Degree of synergy is the ratio of α-arabinose and xylanase activity to the sum of the individual α-arabinose and xylanase activities.
**Substrate purchased from Sigma / Megazyme

Research on synergy between *C. cellulovorans* ArfA and XynA was initiated in 2002 by Kosugi *et al.* as they investigated the possible interactions between noncellulosomal and cellulosomal enzymes (Figure 1.10). This study was continued by the same research group to investigate various other aspects of synergy (Koukiekolo *et al.*, 2005). Firstly, an increase in the reaction time (5 hours to 20 hours) showed an increase in the degree of synergy (1.1 ro 2.6) between ArfA and XynA. Secondly, synergy was determined to be related to the arabinoxylan content of the substrate, with similar findings to Kosugi *et al.* (2002c), where the reaction was incubated for 20 and not 15 hours as shown in Figure 1.11. A higher arabinoxylan percentage of 68% cellulose/arabinoxylan (CAX) resulted in a higher synergy than corn stem fiber (20% arabinoxylan).

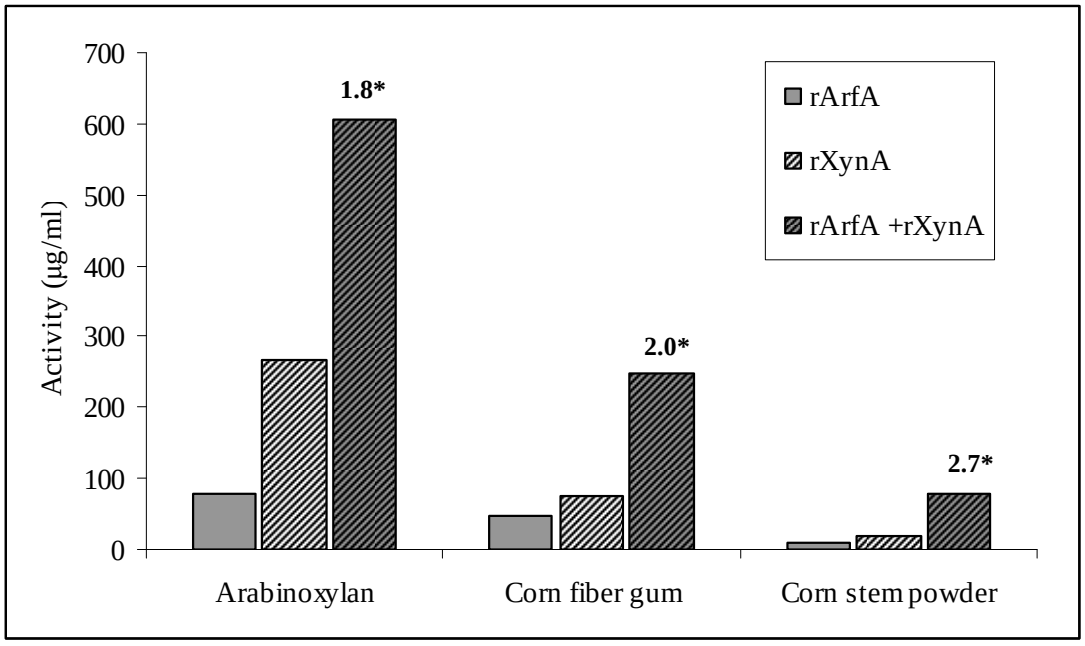

Figure 1.10. Substrate specificity of *C. cellulovorans* ArfA and XynA and degree of synergy *Synergism coefficient is the ratio of rArfA and rXynA activity to the sum of the individual rArfA and rXynA activities. (Modified from Kosugi *et al.*, 2002c).

Sequential synergistic effects were observed where the reactions were set up with one enzyme for a set period (5 or 15 hours) and then the second enzyme was added (5 or 15 hours), so that the total reaction time was 20 hours (Koukiekolo *et al.*, 2005). The results are shown in Figure 1.11. The most significant result is the finding that the highest degree of synergy was observed when XynA was employed as the first enzyme.

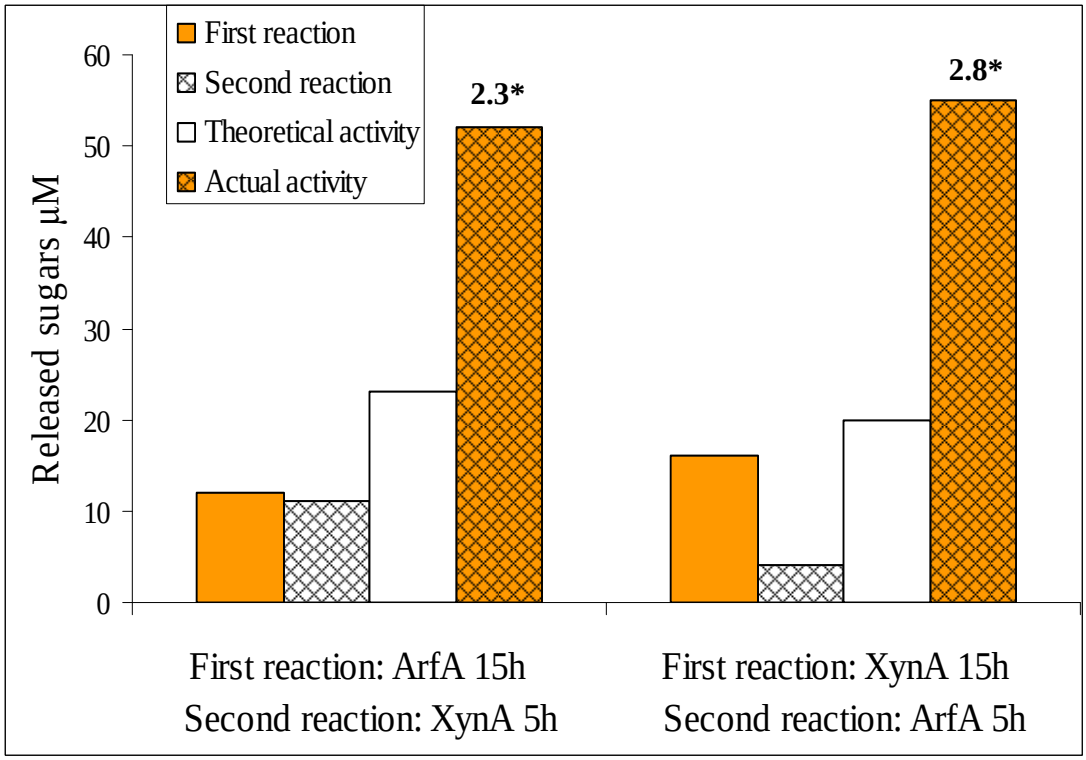

Figure 1.11. Sequential reactions with varying times with *C. cellulovorans* ArfA and XynA [a] The release of sugars was monitored with the sequential additional of enzymes (0.5 µM of each) on the substrate 0.5% CAX at 37°C. *Degree of synergy is measured as the combined activity of ArfA and XynA divided by the total of the individual activities of ArfA and XynA. (Modified from Koukiekolo *et al.*, 2005).

Within this study on the saccharification of the SBP, a partial characterisation of the *C. cellulovorans* hemicellulase ArfA, ManA and XynA will be performed and, in subsequent chapters, the optimisation of the lime pre-treatment step and finally, the enzyme synergy will be discussed. The degradation of the SBP will be measured by the release of reducing sugars.

Chapter II: Research Motivation and Hypothesis

2.1 Motivation

Attention on the biofuel industry has increased as the global energy requirements are anticipated to grow by 50% by the year 2025 (Ragauskas *et al.*, 2006). Cellulose is the most abundant carbohydrate resource in the world, and when degraded to simple glucose molecules, it can be fermented to bio-ethanol. The heterogeneity and rigidity of cellulose make it recalcitrant to enzymatic hydrolysis.

The *Beta vulgaris* plant, better known as sugar beet, is grown commercially to produce 35% of the total global sucrose (Foster *et al.*, 2001). In South Africa, the sugar beet plant is one of the five crops grown for bio-ethanol production (Department of Minerals and Energy, 2007). The beet sugar syrup is extracted from the plant and utilised in a fermentation process to produce first generation bio-ethanol (Leiper *et al.*, 2006). Once the commercially valuable part of the sugar beet is removed the 'pulp' is sold cheaply as a by-product. The sugar beet pulp (SBP) is therefore a potentially cheap carbohydrate-rich source for second generation bio-ethanol. The chemical composition of the SBP shows a large hemicellulose fraction of 45-61% dry weight (Micard *et al.*, 1996). These hemicellulose fibres intertwine the cellulose fibrils giving support to the plant cell wall and increasing the recalcitrant nature of cellulose (Kumar *et al.*, 2009).

In bio-ethanol production, a pre-treatment step is critical in disrupting the lignocellulosic biomass for subsequent hydrolysis. However, the pre-treatment costs can account for up to 20% of the cost of process design (Yang and Wyman, 2007). It is therefore important that there is a careful selection and optimisation of one or a combination of pre-treatment methods. The largest fraction of the SBP is hemicellulose, and thus the focus of this study is on the enzymatic degradation of hemicellulose. The pre-treatment of choice (to remove the lignin sheath and partially degrade the hemicellulose structure) is slaked lime (calcium hydroxide). As the conditions for a lime pre-treatment on SBP have not been reported previously, there is a need to establish the optimal pre-treatment conditions.

Clostridium cellulovorans is an anaerobic bacterium known to produce efficient plant cell wall degrading extracellular enzymes, some of which are associated with a scaffolding protein to form a cellulosome (Murashima *et al.*, 2003). A combination of hemicellulases will often synergistically degrade the complex nature of biomass as the hemicellulose fibres overlap and weave between each other and the cellulose fibrils. Arabinose, as the highest percentage component of the hemicellulose, will be hydrolysed from arabinan, arabinogalactan and arabinoxylan by *C. cellulovorans* arabinofuranosidase A (ArfA). Mannan and xylan may be degraded by *C. cellulovorans* mannanase A (ManA) and xylanase A (XynA).

To date, no research has been performed on the digestion of SBP by *C. cellulovorans* enzymes. The synergistic enzyme activity of the three hemicellulases (ArfA, ManA and XynA) will be examined with the aim of releasing the highest amount of reducing sugar.

2.2 Hypothesis

Selected *C. cellulovorans* hemicellulases can act synergistically to degrade sugar beet pulp efficiently for subsequent fermentation into bio-ethanol.

2.3 Aims and Objectives

1. To over-express cloned constructs of *C. cellulovorans* ArfA and ManA, XynA
2. To purify and partially characterize the above enzymes
3. To optimise lime pre-treatment on sugar beet pulp, and
4. To determine the optimal enzyme ratios for maximal synergy on sugar beet pulp (with and without a lime pre-treatment)

2.4 Overview of Thesis

The hypothesis that *C. cellulovorans* hemicellulases synergistically degrades sugar beet pulp was explored and developed in work described in the subsequent four chapters in this thesis. The methodology used to meet these aims and objectives is discussed in Chapter III.

The over-expression of the hemicellulase genes, in addition to the purification and partial enzyme characterisation, is described in Chapter IV. As part of the partial characterisation, the pH and temperature optima of the enzymes were established as well as their thermal stability.

Chapter V discusses the mechanical and chemical pre-treatment on the sugar beet. A 2^3 factorial design examined the factors of lime load, exposure time and temperature on increasing the response of hemicellulase-released sugars. A one-way analysis of variance (ANOVA) determined the optimal conditions for the lime pre-treatment on sugar beet pulp.

Chapter VI describes the combined hemicellulase saccarification of the untreated and pretreated beet pulp. The synergistic associations of various percentage combinations of these hemicellulases are assessed.

A concluding general discussion of this thesis is provided in Chapter VII with a focus on recommendations for future research related to this work.

Chapter III: Methodology

The *Clostridium cellulovorans* plasmid constructs of the recombinant hemicellulase enzymes ArfA, ManA and XynA used in this synergy study were kindly donated by Professor Roy H. Doi from the University of California, USA.

3.1 Preparation of competent cells

The competent *Escherichia coli* BL21 (DE3) (Novagen) cells were prepared as follows: *E. coli* BL21 cells were inoculated into double strength Yeast-Tryptone (YT) broth in a ratio of 1:250 (20 µl : 5 ml) and incubated at 37°C for 12 hours shaking at 200 rpm (Labcon shaking incubator). An additional 50 ml 2 x YT broth was added to the overnight culture for approximately 3 hours (at 37°C for 12 hours shaking at 200 rpm) until an optical density (OD) of 0.3 – 0.6 was observed at 260 nm. The *E. coli* BL21 cells were harvested by centrifuging at 5,000 x g (Beckman Coulter Avanti J-E centrifuge) for 5 minutes at 4°C. The pelleted cells were transferred to ice for the rest of the protocol and all reagents used kept on ice and using sterile conditions. The cell pellet was resuspended in 50 ml 0.1 M $MgCl_2$ and incubated on ice for 20 minutes. The cells were centrifuged at 5,000 x g for 5 minutes at 4°C and the pelleted cells resuspended in 25 ml 0.1 M $CaCl_2$ and incubated on ice for 2 hours. The cells were centrifuged at 5,000 x g for 5 minutes at 4°C and the cell pellet resuspended in 5 ml 0.1 M $CaCl_2$. The resuspended cells were added to 5 ml 30% (v/v) glycerol and stored in aliquots at -70°C.

3.2 Transformation of recombinant proteins

The competent *E. coli* cells were transformed with plasmid constructs in a ratio of 1:100 (2 µl : 200 µl) and placed on ice for 20 minutes. The cells were heat shocked at 42ºC (Labnet AccuBlock™ digital dry bath) for 50 seconds before placed on ice for 2 minutes. The cells were cultivated in 950 µl 2 x YT broth and placed at 37ºC, shaking at 200 rpm (Labcon shaking incubator) for 2 hours. The cells were collected by centrifugation at 16,000 x g (Heraeus Biofuge pica micro centrifuge) for 2 minutes. The cells were

resuspended in 150 µl 2 x YT broth and cultivated on 2 x YT agar with 50 µg/ml kanamycin.

3.3 Expression of recombinant proteins

The transformed cells were cultivated in 250 ml Luria broth (LB) containing 50 µg/ml kanamycin at 30ºC, shaking at 200 rpm (Labcon shaking incubator) to an optical density at 600 nm (Power Wave$_x$ Spectroquant from Bio-Tek Instruments on Kc Junior software) of approximately 0.8. The expression of the recombinant proteins were induced by the addition of isopropylthiogalactoside (IPTG) to a final concentration of 1.0 mM for rArfA (Kosugi *et al.,* 2002c), rManA and rXynA (Beukes *et al.,* 2008). The culture was incubated at 18ºC, shaking at 150 rpm for 6 hours.

3.4 Purification of recombinant proteins

A 1 ml crude enzyme fraction of the *E. coli* culture was collected before the first centrifugation step for analysis and stored at 4ºC. The cells were collected by centrifugation at 8,000 x g (Beckman Avanti centrifuge) for 10 minutes and resuspended in 10 ml lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, lysozyme was added to a final concentration of 8 mg/ml; pH 7.0). The resuspended cells were placed on ice for a period of 30 minutes, sonicated (Vibra Cell Sonics Materials) three times at 40 Hz for 10 seconds with a 10 second interval in between. A centrifugation step of 12,000 x g for 10 minutes and a 1 ml fraction of the supernatant was collected and stored for analysis.

A Ni-TED (using a Protino® Ni-TED 2000 packed column) purification of the supernatant was performed. The flow-through was collected and the column was washed twice with 4 ml washing buffer (50 mM NaH_2PO_4, 300 mM NaCl; pH 8.0). The proteins were eluted twice with 4 ml elution buffer (50 mM NaH_2PO_4, 250 mM imidazole, 300 mM NaCl; pH 8.0). The various fractions were collected and stored at 4°C for analysis.

3.5 Discontinuous denaturing gel electrophoresis

Protein purity was determined by analyzing the fractions collected (during the purification methods) with the Mini-Protein® 3 cell protocol (Bio-Rad). The collected enzyme fractions were added in a 2:1 ratio to SDS (sodium dodecyl sulfate) reducing buffer (0.0625 M Tris-HCl pH 6.8, 10% (v/v) glycerol, 2% (v/v) SDS, 5% (v/v) β-mercaptoethanol, 0.05% (v/v) bromophenol blue) and placed at 100ºC (Labnet AccuBlock™ digital dry bath) for 5 minutes. The protein samples were run on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The 12% acrylamide resolving gel was made as follows: 0.375 M Tris-HCl (pH 8.8), 0.1% (v/v) SDS, 12% acrylamide solution and just prior to pouring 0.1% (v/v) ammonium persulphate (APS) and 0.025% (v/v) *N,N,N',N'*-tetramethylethylenediamine (TEMED) were added. The gel was placed in the tank with SDS-PAGE running buffer (25 mM Tris base, 192 mM glycine, 1% (w/v) SDS) for approximately 40 minutes at a constant voltage of 180 V (Bio-Rad Power Pac™ Basic). The gel was Coomassie-stained for 3 hours with 0.1% (w/v) Coomassie Brilliant Blue G250, 45% (w/v) methanol and 10% glacial acetic acid. The gel was then destained with 10% glacial acetic acid, 45% methanol and 45% distilled water.

3.6 Protein determination

The protein concentration was determined as described by Bradford (1976) and the modified protocol was as follows: 5 µl of protein sample was added to 225 µl Bradford reagent and left to stand for 5 minutes at room temperature. The samples were lightly shaken and the readings were taken at 595 nm with the Power $Wave_x$ Spectroquant on Kc Junior software. Bovine serum albumin (BSA) was used as a suitable standard.

3.7 Enzymatic activity determination

3.7.1 Reducing sugar analysis

The quantity of reducing sugar released was measured with the DNS (dinitrosalicylic acid) method described by Miller (1959). The assay reaction mixture in a ratio of 1:2:6 with 40 µl sample: 80 µl substrate: 240 µl 50 mM sodium citrate buffer (pH 5.5). The

reaction was conducted for 30 minutes at 50°C (Labnet AccuBlock™ digital dry bath) for 30 minutes. The reaction mixture was then centrifuged at 13,000 x g (Heraeus Biofuge pica micro centrifuge) for 1 minute. The DNS protocol was modified as follows; 150 µl sample added to 300 µl DNS reagent and placed at 100 ºC (Labnet AccuBlock™ digital dry bath) for 5 minutes and then immediately on to ice for 5 minutes. The total reducing sugar released was measured with readings were taken at 540 nm with the Power $Wave_x$ Spectroquant on Kc Junior software. Enzyme activity was measured in units (U), with 1 Unit defined as the quantity of enzyme releasing 1 µmol of reducing sugar per minute. Glucose, mannose and xylose were used as standards. The substrates for rManA were 2% (w/v) locust bean gum (LBG) and for rXynA were 2% (w/v) birchwood xylan (BX).

3.7.2 Arabinofuranosidase assay

Specific activity for the rArfA was measured by the hydrolysis of *p*-nitrophenyl-α-L-arabinofuranoside (*p*NPA*f*) as described by Kosugi *et al.* (2002c). The assay reaction mixture was modified to a ratio of 1:1:8 of 50 µl sample; 50 µl 0.5 mM *p*NPA*f* and 400 µl 50 mM sodium citrate buffer (pH 5.5). The reaction was conducted at a 50°C for 30 minutes and terminated by an equal volume (500 µl) of 2 M sodium carbonate. The released *p*-nitrophenyl product was measured at 410 nm with Power $Wave_x$ Spectroquant on Kc Junior software. *p*-Nitrophenyl was used as a suitable standard.

3.8 pH studies

A range of pH values (pH 3-11) was used to determine the optimal pH (Inolab pH meter) for the individual rArfA, rManA and rXynA hemicellulase enzymes. The buffers were: 50 mM sodium citrate (pH 3.0 - 5.5); 50 mM potassium phosphate (pH 6.0 - 8.0) and 50 mM glycine (pH 9.0 – 11.0). The enzyme assays were performed in triplicate for 30 minutes at 50°C (Labnet AccuBlock™ digital dry bath). rManA and rXynA activity was monitored with the DNS method as mentioned above in section 3.7.1. Glycosyl hydrolase activity for rArfA was determined with the arabinofuranosidase assay as described in section 3.7.2.

3.9 Temperature studies

The temperature optima for the rArfA, rManA and rXynA activities were determined in the range of 20-80ºC (Labnet AccuBlock™ digital dry bath) at pH 5.5 (50 mM sodium citrate). The assays were run in triplicate for 30 minutes. The activities of rManA and rXynA were measured with the DNS method as described in section 3.7.1 and the activity of rArfA with the arabinofuranosidase assay as described in section 3.7.2.

3.10 Thermal stability studies

The stabilities of the individual recombinant hemicellulase enzymes were investigated in triplicate over time at 40ºC (Labnet AccuBlock™ digital dry bath) at pH 5.5 (50 mM sodium citrate). Each hemicellulase was incubated at 40ºC for a selected time period, substrate was added for 30 minutes and the residual enzymatic activity was subsequently determined with the DNS method (see section 3.7.1) for rManA and rXynA and the arabinofuranosidase method (see section 3.7.2) for rArfA.

3.11 Pre-treatment Optimisation

3.11.1 Preparation of the sugar beet pulp

The sugar beet was initially prepared by cutting by hand, blending and air-drying at 40°C. Multiple washes and filtering steps with water were performed until the filtrate showed no reaction with the DNS assay (see section 3.7.1) and the pH was raised to pH 7.0. This sugar beet was defined as ‘sugar beet pulp’ (SBP).

The second method of sugar beet preparation included cutting the sugar beet into pieces of approximately 5 cm x 3 cm x 3 cm, shredding with an industrial vegetable peeler to a size of < 1cm and then blending to a size of < 0.3 cm. The pulp was washed in the same way until the filtrate showed no activity with the DNS method (see section 3.7.1) and raised to pH 7.0. The SBP was air-dried at room temperature for 24 hours and stored at room temperature. This method was utilised for the untreated SBP and in the optimisation of the lime pre-treatment strategy.

3.11.2 Pre-treatment Optimisation

An alkaline (lime) pre-treatment step was performed on the SBP to determine the optimal production of reducing sugars with hemicellulase enzymatic degradation. A lack of published data on optimal lime pre-treatment conditions for SBP lead to a choice of conditions based on that used for sugarcane bagasse (Rabelo *et al.*, 2008). The following pre-treatment factors were investigated: lime load, temperature and time. A 2^3 factorial design was set up with central point replicates as shown in Table 3.1

Table 3.1 Design matrix of lime Pre-treatment on sugar beet pulp

Run No.	Lime (g/g)	Time (h)	Temp (°C)
1	0.10	12	40
2	0.40	12	40
3	0.10	36	40
4	0.40	36	40
5	0.10	12	70
6	0.40	12	70
7	0.10	36	70
8	0.40	36	70
9	0.25	24	55
10	0.25	24	55
11	0.25	24	55

The design matrix was carried out on the prepared sugar beet pulp (4 g) and then several wash-and-filter steps followed to remove all the lime from the pulp. The pH of the filtrate was monitored until it was lowered to pH 7.0 and there were no further reducing sugar detected with the DNS method (see section 3.7.1). The pretreated SBP was dried at room temperature for 24 hours.

The response used in the design matrix was the release of reducing sugars by enzymatic degradation. The assays were run in triplicate with equal concentrations (5 μg/ml) of the hemicellulases on each of the runs for 24 hours at 37°C and pH 5.5 (50 mM sodium citrate). The total reducing sugar released was analysed by the DNS method (see section 3.7.1)

3.12 Scanning Electron Microscopy

The un-treated and lime pre-treated sugar beet pulp (SBP) samples (runs no. 4 and 8) were airdried at room temperature. Sample preparation for the Scanning Electron Microscope (SEM) included a sputter coating of gold (Balzers Union Sputtering Device) on the various SBP samples and examination in the SEM.

3.13 Synergy studies

The reactions were analyzed for enzyme synergy, whereby each enzyme (rArfA; rManA; rXynA) within the reaction was tested at 12.5%, 25%, 37.5%, 50%, 62.5%, 75% and 87.5% of the total enzyme protein concentration (40 μg/ml). The assays were set up on the un-treated and optimised lime pre-treated sugar beet pulp (0.4 g lime / g SBP; 36 hours; 40ºC) and run in triplicate at 40ºC for 5 days. The reaction assays were made up to a final volume of 500 μl with 50 mM sodium citrate (pH 5.5). Enzymatic activity was measured with the DNS method (see section 3.7.1). The degree of synergy was determined by dividing the activity of the recombinant hemicellulases by the sum of the individual theoretical activities of these same enzymes when assayed separately.

Chapter IV: Purification and Partial Enzyme Characterisation

Introduction

Sugar beet pulp (SBP) generally has a large hemicellulose fraction of 45-61% dry weight (Micard *et al.,* 1996), which necessitates degradation and removal for effective cellulase saccharification. Research into *Clostridium cellulovorans* has shown that growth conditions can greatly influence the expression of various enzymes, in particular hemicellulases, as no arabinofuranosidase A (ArfA) production was noted when the organism was cultured on cellulose (Tamaru *et al.,* 2002). Other studies on inducing arabinofuranosidase formation utilise SBP as a common medium (Table 1.4).

C. cellulovorans ArfA was thus chosen for this research study since the sugar beet pulp has a high arabinose chemical composition of 20.9% (Micard *et al.,* 1996). The arabinose backbone is xylan and therefore the second hemicellulase of choice was *C. cellulovorans* xylanase A (XynA). Several synergy studies have previously been completed on ArfA and XynA (Kosugi *et al.,* 2002c; Koukiekolo *et al.,* 2005); however, the *C. cellulovorans* mannase A (ManA) has not been studied in combination with ArfA and XynA.

The plasmid constructs of the three *C. cellulovorans* recombinant hemicellulases were successfully transformed into *Escherichia coli* BL21 (DE3) (see section 3.2). Expression (see section 3.3) of *arfA*, *manA* and *xynA* was observed and active proteins of each of these genes were purified (see section 3.4) for partial enzyme characterisation.

C. cellulovorans hemicellulases were studied to determine their pH optima, temperature optima and stabilities over time. pH profiles were determined at 50°C (see section 3.8) and temperature profiles were determined at pH 5.5 (see section 3.9), both sets of conditions were chosen according to optimal values of all three hemicellulases from published data. Individual enzyme thermal stabilities over time (see section 3.10) were investigated at 40°C and pH 5.5. These selected conditions (40°C, pH 5.5) fell within the optimal ranges of this present study.

4.1 *Clostridium cellulovorans* Arabinofuranosidase (ArfA)

4.1.1 ArfA Expression and Purification

The successful expression of recombinant *arfA* and purification of ArfA is shown in the 12% SDS-PAGE gel in Figure 4.1. The 55.7 kDa ArfA protein was expressed with isopropylthiogalactoside (IPTG) induction (seen in lane 3). The purification protocol used Ni-TED (Nickel tris-carboxymethyl ethylene diamine) purification columns (Protino® Ni-TED 2000) based on Ni^{2+} ion affinity chromatography (see section 3.4). This method was based on previous research in the group and similar to the Ni-NTA (Nickel nitrilotriacetic acid) purification columns (QIAGEN) utilised by Murashima *et al.* (2002). The polyhistidine tag on the ArfA was immobilised onto the Ni^{2+} ions and then displaced by the addition of 250 mM imidazole. The Ni-TED purification succeeded in purifying the lysate to a single protein, ArfA, as shown clearly in lane 8.

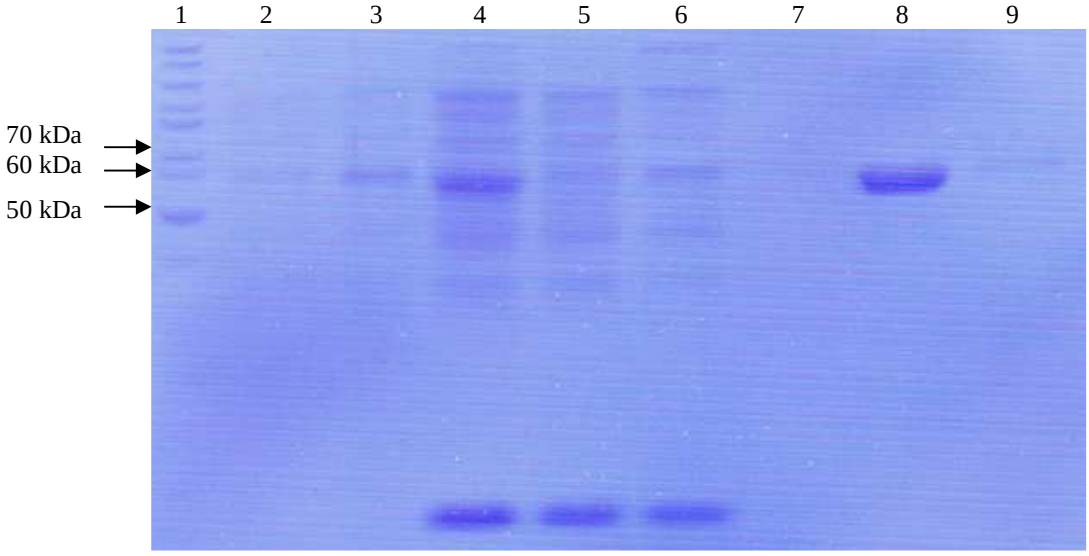

Figure 4.1 Purification of ArfA with a 12% SDS-PAGE gel The 55.7 kDa ArfA protein is shown to have been effectively purified as seen in Lane 8. The gel was loaded as follows: Lane 1: Molecular Marker; Lane 2: Crude; Lane 3: Induced Culture; Lane 4: Lysate; Lane 5: Flow-through; Lane 6: Wash I; Lane 7: Wash II; Lane 8: Elution I and Lane 9: Elution II.

The activity of the ArfA was determined with the arabinofuranosidase assay and the purification table (Table 4.1) demonstrates the successful purification of active ArfA.

Table 4.1 Purification table for ArfA using the arabinofuranosidase assay

Fraction	Volume (ml)	Protein Conc. (mg/ml)	Total protein (mg)	Enzyme Activity (U/ml)	Total Activity (U)	Specific Activity (U/mg)	Yield (%)	Fold Purification
Induced Culture	250	0.27	67.50	2.34	585.50	8.67	100.0	1.0
Lysate	10	1.56	15.63	2.01	20.05	1.28	3.4	0.1
Flow Through	10	1.32	13.17	5.96	59.58	4.52	10.2	0.5
Wash I	4	0.65	2.60	4.82	19.26	7.41	3.3	0.9
Wash II	4	0.07	0.28	2.17	8.69	31.04	1.5	3.6
Elution I	4	0.15	0.60	5.81	23.23	38.98	4.0	4.5
Elution II	4	0.03	0.10	1.82	7.27	72.68	1.2	8.4

U = nmol arabinose released per minute

As observed from the 12% SDS-PAGE gel the Ni-TED column was highly effective in purifying the ArfA. This is supported in Table 4.1 by the high fold purification values of 4.5 (Elution I) and 8.4 (Elution II) that were achieved. Thus it was to be expected that a higher percentage yield was noted from the first elution step compared to the second (4.0% compared to 1.2%).

4.1.2 ArfA Partial Characterisation

The initial characterisation of the ArfA involved determining the pH optimum. The pH study (Figure 4.2) was carried out at 50°C as this fell within the optimal range of the work reported by Kosugi *et al.* (2002c).

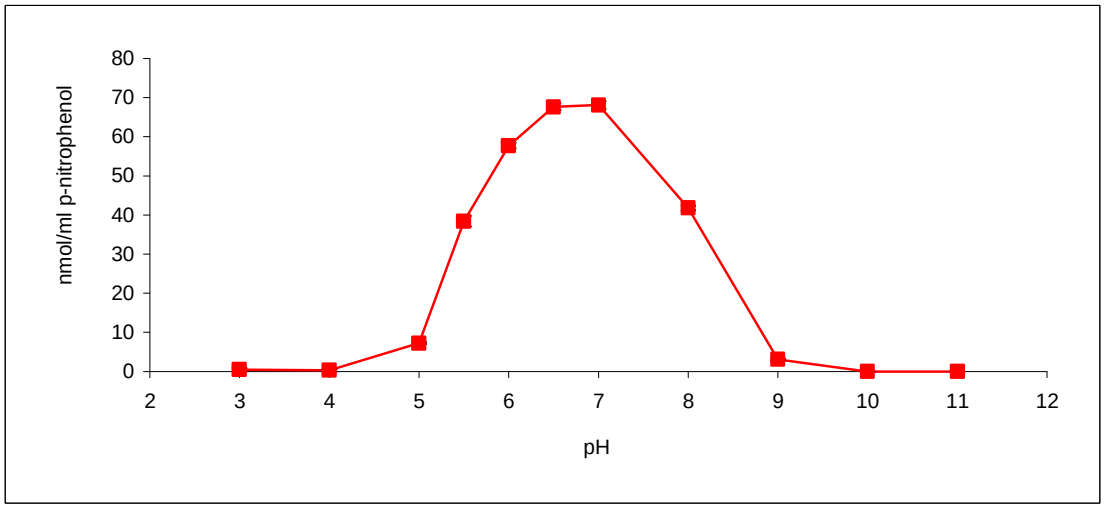

Figure 4.2 pH profile for ArfA using the arabinofuranosidase assay Reaction assays were carried out in triplicate for 30 minutes at 50°C. Buffers were: 50 mM sodium citrate (pH 3.0 – 5.5); 50 mM potassium phosphate (pH 6.0 – 8.0) and 50 mM glycine (pH 9.0 – 11.0). The optimal range extended from pH 5.5 – 8.0, with a pH optimum at pH 6.5 – 7.0. Values represent means ± SD, n=3.

The pH optimum of 6.5 – 7.0 correlated with the published data of pH 6.0 (Kosugi *et al.*, 2002c).

The temperature profile for ArfA was determined. A pH of 5.5 was selected based on the pH optimum ranges reported for all three recombinant hemicellulases in the literature. Figure 4.3 illustrates the broad temperature optimum range for activity, ranging from 20 - 55°C. A maximal release of 78.0 nmol/ml *p*-nitrophenol product was released at 45°C. This is in agreement with the 40 - 50°C temperature optimum reported by Kosugi *et al.* (2002c). At 80°C ArfA still exhibited activity, whilst it was reported by Kosugi *et al.* (2002c) that activity was lost after 20 minutes at 80°C. Since the assay was run for 30 minutes, it is possible that for the first 20 minutes the ArfA produced a product and then subsequently lost all activity.

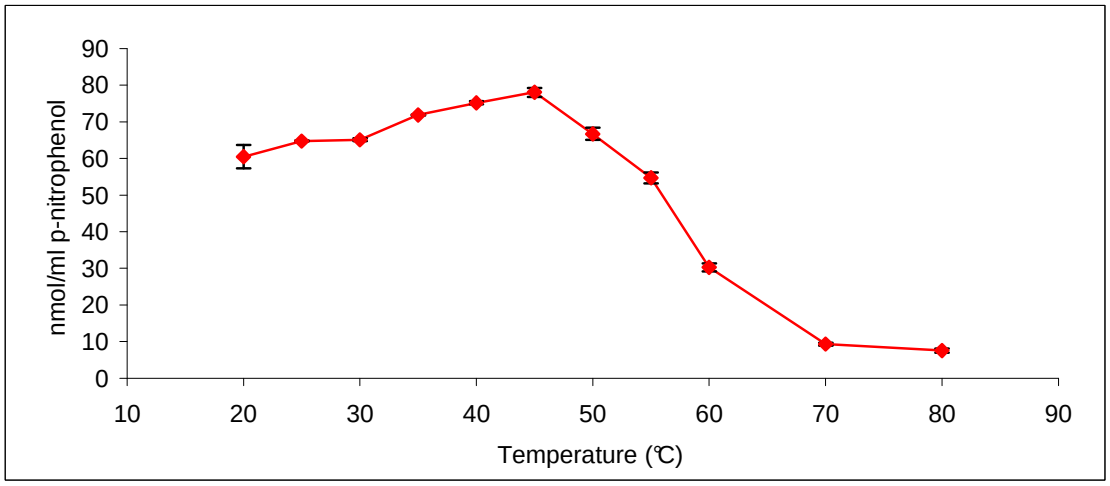

Figure 4.3 Temperature profile for ArfA using the arabinofuranosidase assay Reaction assays were carried out in triplicate for 30 minutes in 50 mM sodium citrate (pH 5.5). ArfA displayed optimal activity over a broad temperature range of 20 - 55°C. Values represent means ± SD, n=3.

ArfA stability was investigated over time at 40°C. Aliquots were removed at various time intervals and the ArfA was incubated with substrate and the assay conditions were carried out at 40°C for 30 minutes. The ArfA stability profile is shown in Figure 4.4.

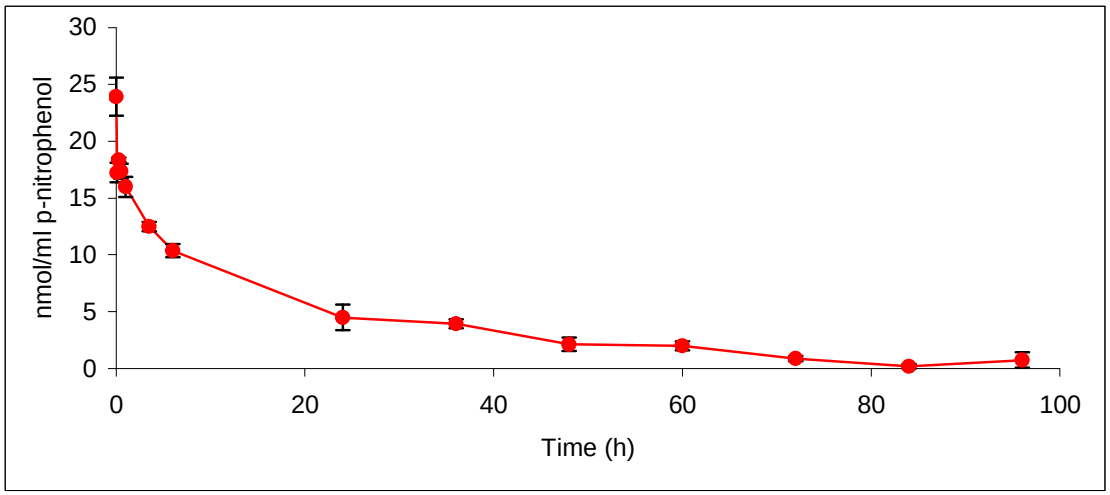

Figure 4.4 Enzyme stability over time (at 40°C) for ArfA Reaction assays were performed in triplicate at 40°C at pH 5.5 (50 mM sodium citrate). A sudden decrease in ArfA activity was noted within the first 6 hours followed by a plateau after 20 – 30 hours. Values represent means ± SD, n=3.

There was a significant decrease in ArfA activity within the initial 6 hours of incubation followed by a plateau from 24 – 84 hours. It would appear that some structural integrity was retained (Fields, 2001) since there was still some residual activity after 24 hours.

4.2 *Clostridium cellulovorans* Mannanase (ManA)

4.2.1 ManA Expression and Purification

The recombinant *manA* gene was effectively expressed with the induction of IPTG even though leaky expression of the gene was noted in the fraction of the crude culture in the 12% SDS-PAGE gel (Figure 4.5). The 47.2 kDa protein is circled in lane 2. The Protino® Ni-TED purification column was successfully used to elute the ManA within the first elution (lane 8), so that there was no ManA remaining on the column for the second elution (lane 9). The amount of ManA was in excess of the number of available Ni^{2+} ions binding sites, and thus ManA eluted through the Protino® Ni-TED column and is observed in the flow-through (lane 5) and wash I (lane 6). The ManA activity was tested using the DNS method and the substrate locust bean gum (2% w/v). Table 4.2 represents the purification table obtained for the purification of ManA.

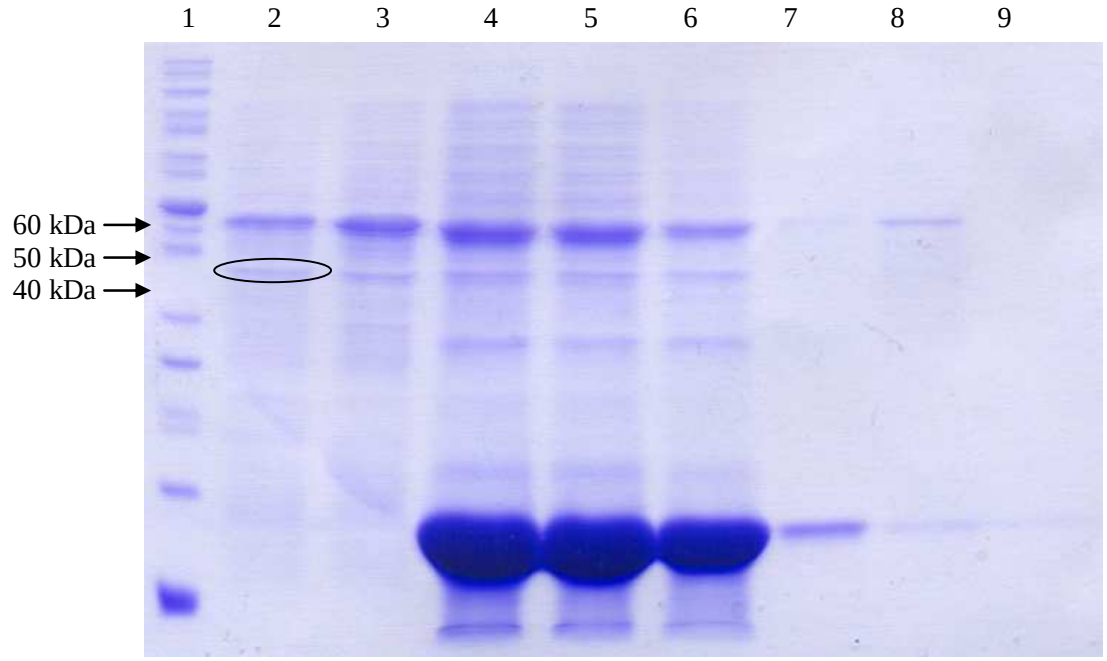

Figure 4.5 Purification of ManA with a 12% SDS-PAGE gel A leaky expression of the ManA is observed with the highlighted band in Lane 2 before induction. The gel was loaded as follows: Lane 1: Molecular Marker; Lane 2: Crude; Lane 3: Induced Culture; Lane 4: Lysate; Lane 5: Flow-through; Lane 6: Wash I; Lane 7: Wash II; Lane 8: Elution I and Lane 9: Elution II.

Table 4.2 Purification table for ManA using the DNS method

Fraction	Volume (ml)	Protein Conc. (mg/ml)	Total protein (mg)	Enzyme Activity (U/ml)	Total Activity (U)	Specific Activity (U/mg)	Yield (%)	Fold Purification
Induced Culture	250	0.15	36.50	0.36	90.50	2.48	100.0	1.0
Lysate	10	1.75	17.49	0.86	8.57	0.49	9.5	0.2
Flow Through	10	1.41	14.06	0.87	8.72	0.62	9.6	0.3
Wash I	4	0.86	3.43	0.85	3.38	0.99	3.7	0.4
Wash II	4	0.04	0.15	0.22	0.90	6.05	1.0	2.4
Elution I	4	0.20	0.81	0.49	1.94	2.40	2.1	1.0
Elution II	4	0.00	0.00	0.00	0.00	0.00	0.0	0.0

U = μmol mannose released per min

There was a surprising higher specific activity value in the wash II fraction at 6.05 U/mg compared to elution I at 2.4 U/mg, even though there was a larger band representing ManA observed in lane 7 (Wash II) compared to lane 6 (Wash I) in Figure 4.5. This

discrepancy resulted in lower fold purification with the elution step, while the yield increased slightly from 1.0 to 2.1%.

4.2.2 ManA Partial Characterisation

The characterisation of ManA was initiated by Tamaru and Doi (2000) as the *manA* gene was discovered as part of the *C. cellulovorans* cellulosome. The pH profile for the recombinant ManA is illustrated in Figure 4.6. The pH optimum was observed at pH 5.5 with 1.77 µmol/ml of mannose released compared to 1.50 µmol/ml at pH 5.0 and 1.59 µmol/ml at pH 6.0. Tamaru and Doi (2000) however, reported a higher pH optimum of 7.0.

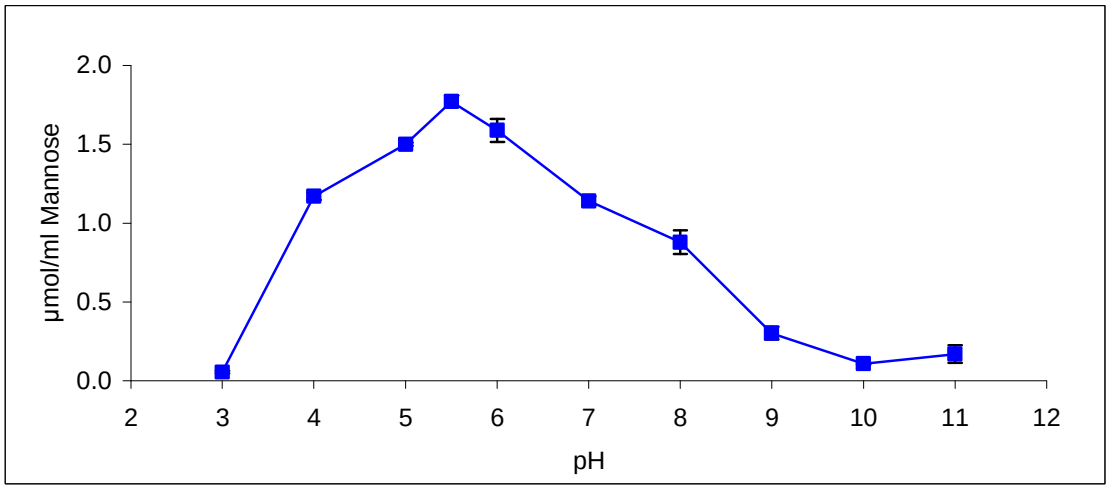

Figure 4.6 pH profile for ManA using the DNS method Reaction assays were carried out in triplicate for 30 minutes at 50°C. Buffers were: 50 mM sodium citrate (pH 3.0 – 5.5); 50 mM potassium phosphate (pH 6.0 – 8.0) and 50 mM glycine (pH 9.0 – 11.0). A steady increase in activity from pH 3.0 to a pH optimum of 5.5 was observed. Values represent means ± SD, n=3.

The ManA temperature profile in Figure 4.7 showed a similar level of activity (2.80 – 3.00 µmol/ml mannose released) over the temperature range of 20 - 35°C. There was a sudden increase to 3.62 µmol/ml of mannose released at 40°C and then a rapid decline of activity. The optimum temperature of 40°C correlated well with the 45°C published by Tamaru and Doi (2000).

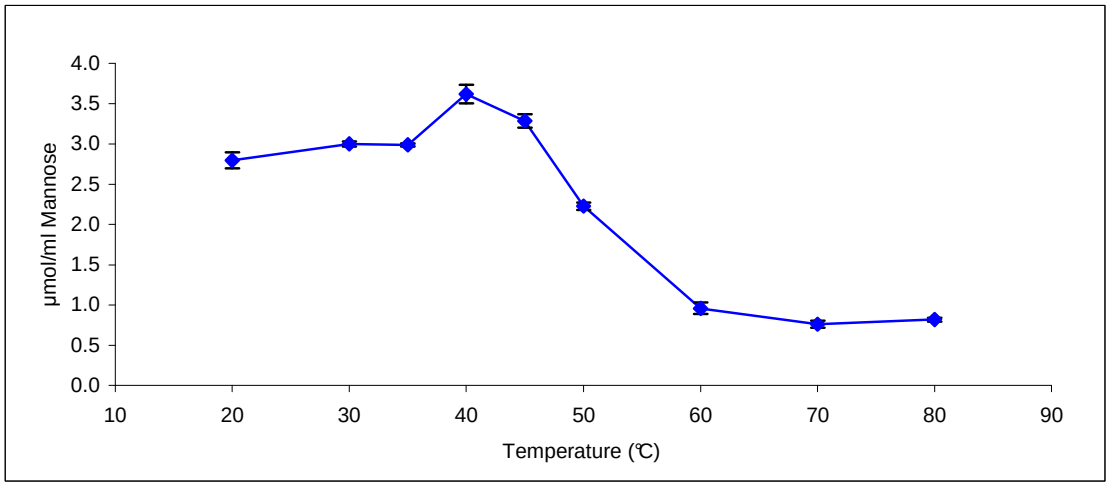

Figure 4.7 Temperature profile for ManA Reaction assays were carried out in triplicate for 30 minutes in 50 mM sodium citrate (pH 5.5). A constant level of activity was observed between 20 - 35°C with a temperature optimum at 40°C. Values represent means ± SD, n=3.

The stability of ManA was significantly less than that observed for ArfA, with a complete loss of activity noted at 11 hours at 40°C. There was a sudden initial loss of activity from 4.44 µmol/ml of mannose released (0 minutes) to 2.82 µmol/ml (after 30 minutes), followed by a gradual loss of activity over the remainder of the time study. Data on the stability of the ManA has not been published previously.

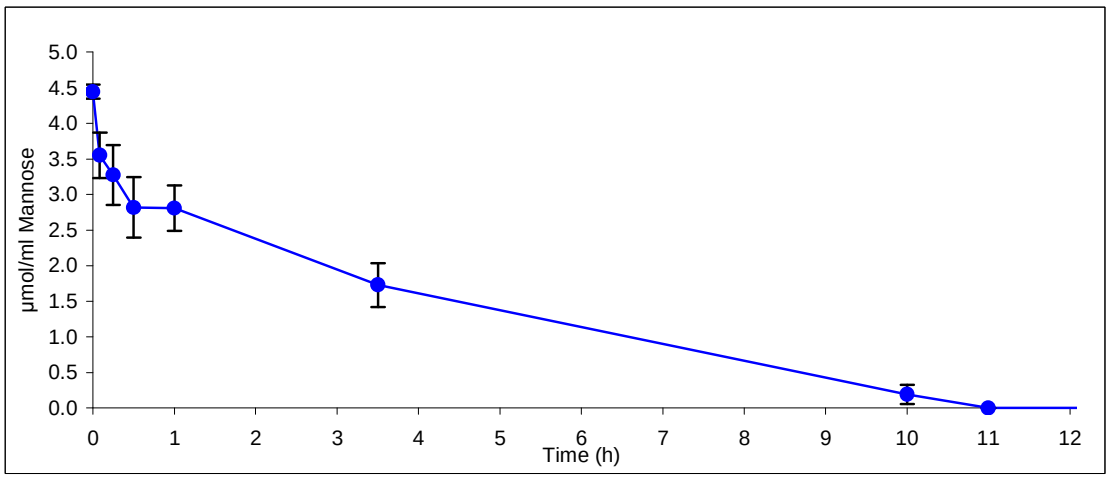

Figure 4.8 Enzyme stability over time (at 40°C) for ManA Reaction assays were performed in triplicate at 40°C at pH 5.5 (50 mM sodium citrate). A significant decrease in ManA activity occurred within the first 30 minutes. Values represent means ± SD, n=3.

4.3 *Clostridium cellulovorans* Xylanase (XynA)

4.3.1 XynA Expression and Purification

The recombinant *xynA* was expressed as a 57.0 kDa protein, the *xynA* gene was observed to have a leaky expression similar to *manA.* The addition of IPTG to the cell culture increased the expression of the *xynA* gene as observed in lane 3 of the 12% SDS-PAGE gel in Figure 4.9. Protino® Ni-TED chromatography successfully purified the XynA and displayed as a thick band in lane 8 and a single protein in lane 9.

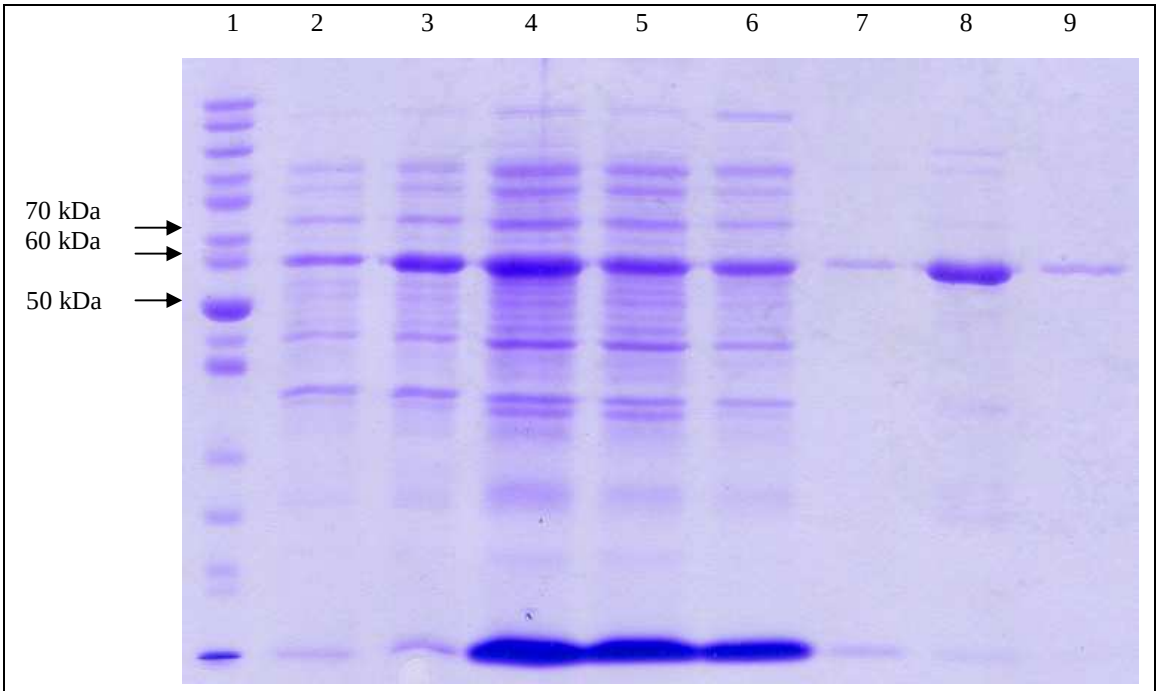

Figure 4.9 Purification of XynA with a 12% SDS-PAGE gel The successful induction of the XynA. The gel was loaded as follows: Lane 1: Molecular Marker; Lane 2: Crude; Lane 3: Induced Culture; Lane 4: Lysate; Lane 5: Flow-through; Lane 6: Wash I; Lane 7: Wash II; Lane 8: Elution I and Lane 9: Elution II.

The purification table (Table 4.3) showed high yields in fractions of elution I and II, of 34.8% and 24.8%, respectively.

Table 4.3 Purification table for XynA

Fraction	Volume (ml)	Protein Conc. (mg/ml)	Total protein (mg)	Enzyme Activity (U/ml)	Total Activity (U)	Specific Activity (U/mg)	Yield (%)	Fold Purification
Induced Culture	250	0.13	33.00	0.45	112.25	3.40	100.0	1.0
Lysate	10	1.94	19.42	18.70	187.04	9.63	166.6	2.8
Flow Through	10	1.55	15.52	9.50	95.02	6.12	84.7	1.8
Wash I	4	0.68	2.72	6.03	24.11	8.88	21.5	2.6
Wash II	4	0.22	0.86	3.87	15.46	17.98	13.8	5.3
Elution I	4	0.88	3.53	9.77	39.08	11.07	34.8	3.3
Elution II	4	0.68	2.73	6.97	27.87	10.22	24.8	3.0

U = µmol xylose released per min

4.3.2 XynA Partial Characterisation

Cellulosomal XynA is a significant hemicellulase in biomass degradation by *C. cellulovorans*. The hydrolytic properties of XynA were investigated by Kosugi *et al.* (2002b) to show xylanase, as well as acetyl xylan esterase, activity. The pH profile for XynA is illustrated in Figure 4.10 with activity displayed over a broad range. A pH optimum of pH 5.0 – 6.0 was previously reported by Kosugi *et al.* (2002b).

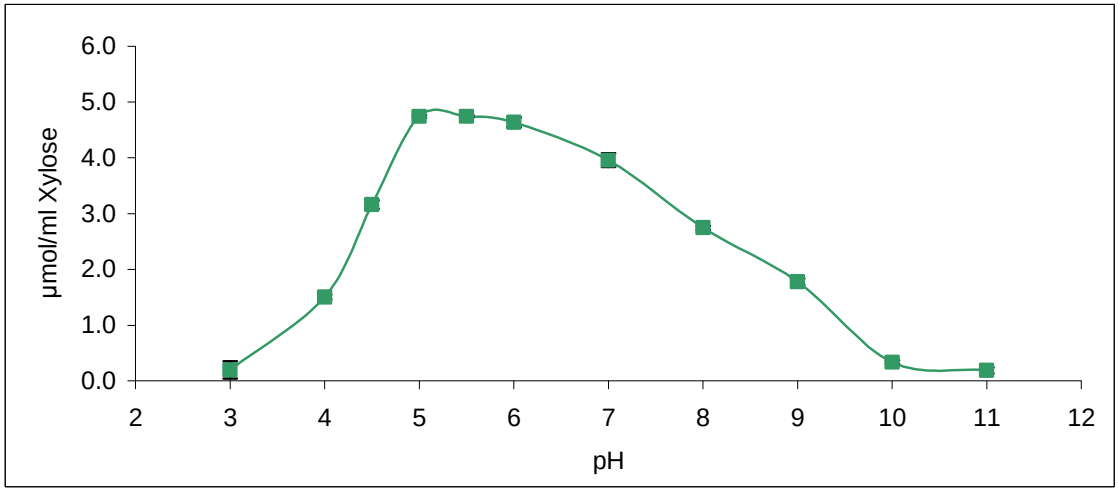

Figure 4.10 pH profile for XynA Reaction assays were carried out in triplicate for 30 minutes at 50°C. Buffers were: 50 mM sodium citrate (pH 3.0 – 5.5); 50 mM potassium phosphate (pH 6.0 – 8.0) and 50 mM glycine (pH 9.0 – 11.0). The XynA exhibited an optimal pH range of pH 5.0 – 6.0. Values represent means ± SD, n=3.

XynA exhibited high activity over a large temperature range of 20 - 50°C (Figure 4.11), with a temperature optimum at 45°C with 4.99 µmol/ml of xylose released. A 60% loss in activity was observed between 50°C and 60°C; however, there was still some activity remaining at 80°C. This data disagrees with the published temperature optimum of 60°C published by Kosugi *et al.* (2002b).

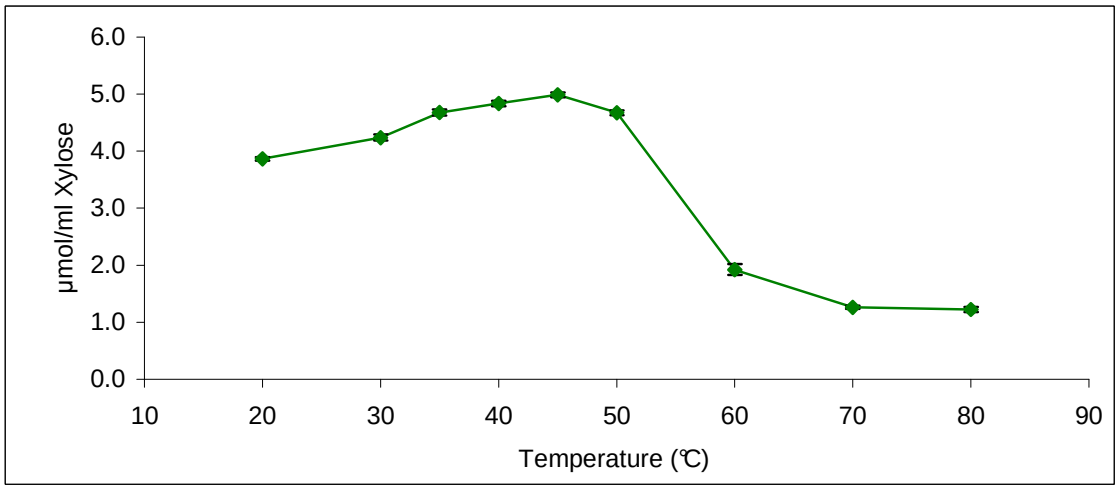

Figure 4.11 Temperature profile for XynA Reaction assays were carried out in triplicate for 30 minutes in 50 mM sodium citrate (pH 5.5). Values represent means ± SD, n=3.

A graph of XynA stability versus time is presented in Figure 4.12. A sudden decrease in hemicellulase activity was noted within the initial 3.5 hours followed by a stable release of between 0.37 – 1.27 µmol/ml xylose between 12 – 120 hours. Kosugi *et al.* (2002b) reported stability at 30°C for 12 hours over a pH range of 2.0 - 7.0.

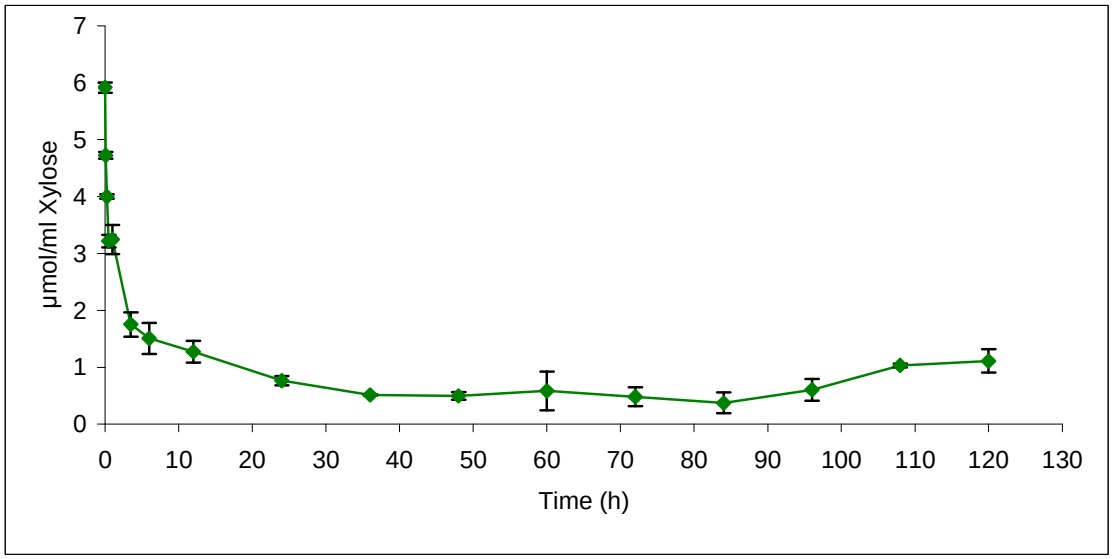

Figure 4.12 Enzyme stability over time (at 40°C) for XynA using the DNS method
Reaction assays were performed in triplicate at 40°C at pH 5.5 (50 mM sodium citrate). A significant decrease in activity within the initial 3.5 hours followed by a steady level of stability of the enzyme. Values represent means ± SD, n=3.

Conclusion

Similar conclusions were drawn from our pH and temperature optima data compared to that previously published, despite using different substrates. ArfA was maximal at pH 6.5 – 7.0 and 45°C (Kosugi *et al.*, 2002c, and this study). The optimal properties of the ManA from our study were pH 5.5 and 40°C; however, these were slightly different to those reported by Tamaru and Doi (2000) of pH 7.0 and 45°C. Data for XynA confirmed the optimal activity at pH 5.0 – 6.0 but were in disagreement with a temperature optimum at 40 - 50°C obtained in our study compared to a reported temperature optimum of 60°C (Kosugi *et al.*, 2002b).

The temperature stability studies indicated a complete loss of activity in ManA after 11 hours and in ArfA after 84-96 hours at 40°C. However, XynA was still active after 120 hours. It is worth noting that these studies investigated enzyme stability in the absence of substrate and thus the stability of these enzymes could be significantly higher in the presence of substrate. This temperature stability data has significance for interpreting the optimal lime pre-treatment studies (Chapter 5) as well as the synergy studies (Chapter 6).

The characterisation of each hemicellulase led to an improved understanding of the optimal conditions for ArfA, ManA and XynA activity. The optimised conditions from this chapter were utilised to degrade the SBP substrate in the next chapter. However, the lignocellulose nature of the SBP is recalcitrant to hemicellulose degradation and will require an optimised pre-treatment step to release the reducing sugars.

Chapter V: Pre-treatment Optimisation

Introduction

The lignocellulose nature of the SBP is recalcitrant to hemicellulase degradation and requires a pre-treatment step to release the cellulose and hemicellulose fibres. The alkaline process of choice for the SBP was slaked lime (calcium hydroxide). The selection was based on the fact that alkaline pre-treatments degrade sugars to a lesser degree than acid pre-treatment (necessary for subsequent fermentation), and that calcium hydroxide is inexpensive and can be recovered through neutralising to insoluble calcium carbonate and regeneration through lime kiln technology (Kaar and Holtzapple, 2000).

As the optimal lime pre-treatment conditions for SBP are not recorded in published data, the conditions used in this study were based on another fermentable by-product from the sugar industry, namely sugarcane bagasse (Rabelo *et al.,* 2008). The pre-treatment factors analysed for the subsequent optimal enzymatic saccharification were lime load, temperature and time. The same factors were utilised in this study on SBP with the same limits for lime load and time however, the limits for temperature were changed to allow for a broader temperature range.

The lignin content of the two by-products differ; with SBP at 1-2% dry weight (Foster *et al.,* 2001) and sugarcane bagasse at 22% dry weight (Ouensanga and Picard, 1988). However, the rationale for this selection of conditions was supported by the conclusions drawn from the work of Rabelo *et al.* (2008) where the maximum results were observed at the highest levels of each of the three factors (lime load, time and temperature). Therefore, the optimal conditions for the pre-treatment of SBP were expected to fall within the range of the studied factors.

5.1 Preparation of SBP

The sugar beet (prior to the alkaline pre-treatment) underwent a physical pre-treatment to disrupt the crystalline structure and reduce the particle size, thus increasing the surface area (Hendriks and Zeeman, 2009). The initial process was cutting by hand as shown in Figure 5.1.

Figure 5.1 Sugar beet in the initial stages of preparing the SBP The sugar beets used were approximately 40 – 50 cm in length and 3.5 – 5 kg in weight.

The preparation steps included cutting the sugar beet by hand, shredding and blending to reduce the size of the fibrous beet. The excess surface sucrose was removed from the sugar beet by multiple washes and filtering with water and the filtrate tested with the dinitrosalicylic acid (DNS) method (section 3.7.1) to determine the successful removal of all sugars. The pH of the filtrate was also monitored until it was neutral to ensure that all the lime had been washed off. The sugar beet was now termed 'sugar beet pulp' (SBP).

Initial preparations of the SBP did not utilise the industrial vegetable shredder and the drying of the pulp was carried out at 40°C. The resulting pulp formed large solid fragments, thus the pulp preparation was optimised with a reduction in particle size to < 0.3 cm and drying at room temperature (section 3.11.1). The comparison of the two methods is displayed in Figure 5.2.

Figure 5.2 Dry SBP from two different preparations. The preparation steps were A: shredding and then drying at room temperature; B: no shredding and then drying at 40°C.

5.2 Pre-treatment Optimisation

5.2.1 Model Design and Results

A 2^3 factorial design was set up with central point replicates within each factor (lime load / time / temperature) as shown in Table 5.1.

Table 5.1 Operating levels of the 3 factor design with percentage weight loss

Pre-treatment				SBP
Run No.	Lime (g/g)	Time (h)	Temp (°C)	Percentage dry weight loss (%)
1	0.1	12	40	27.2
2	0.4	12	40	38.7
3	0.1	36	40	31.6
4	0.4	36	40	51.0
5	0.1	12	70	46.4
6	0.4	12	70	73.1
7	0.1	36	70	50.7
8	0.4	36	70	76.7
9	0.25	24	55	62.3
10	0.25	24	55	72.0
11	0.25	24	55	66.3

Each individual run was carried out on 4 g of dried SBP, several wash-and-filter steps were done to remove the lime. The pre-treated runs were dried at room temperature for 24 hours.

The highest values of percentage weight loss (Table 5.1) were noted from runs 6-11 with a range of 62.3% to 76.7%, except for run 7 with a low value of 50.7%. These results show a general increase in weight loss as the pre-treatment conditions became more

severe. It can also be noted that an increase in lime load increases the weight loss when the other factors of time and temperature are held constant. This correlates with the knowledge that lime can solubilise lignin as discussed in Chapter 1 (Table 1.2) and with the various wash-and-filter steps the lignin was removed from the pre-treated substrate, therefore decreasing the pulp weight. Some of this effect can also be attributed to a thermal pre-treatment that disrupted the cellulose fibres.

The response used in the factorial design was the release of reducing sugar from the saccharification of hemicellulose enzymatic degradation (Table 5.2). The total reducing sugars were determined by the DNS method. The enzyme treatments (in triplicate) were carried out with equal concentrations (5 μg/ml) of the hemicellulases on each of the pre-treatment runs (section 3.11.2).

Table 5.2 Reducing sugar yields from each factorial run

Pre-treatment				**Enzyme Assay**
Run No.	Lime (g/g)	Time (h)	Temp (°C)	Reducing sugar* (U)
1	0.1	12	40	0.9
2	0.4	12	40	8.7
3	0.1	36	40	3.8
4	0.4	36	40	**42.4**
5	0.1	12	70	13.9
6	0.4	12	70	15.1
7	0.1	36	70	16.4
8	0.4	36	70	6.3
9	0.25	24	55	11.0
10	0.25	24	55	18.7
11	0.25	24	55	30.6

Hemicellulase assays were performed with equal concentrations (5 μg/ml) of ArfA, ManA and XynA at 37°C and pH 5.5 (50 mM sodium citrate) for 24 hours.
*Average of triplicate values for each pre-treatment run.
U = μmol of reducing sugar per gram of SBP per 24 hours

The highest yield of reducing sugar is marked in bold at 42.4 U in Table 5.2 which resulted from run no. 4 with the highest conditions for lime (0.4 g / g SBP) and time (36 h) and yet the lowest condition of temperature (40°C). However, run no. 8 with

similar conditions for lime and time and the highest temperature (70°C) had a much lower yield of reducing sugar at 6.3 U. Temperature therefore had a major effect within the lime pre-treatment. A difference of 30°C was significant in decreasing the quantity of sugars released and thus it can be suggested that the runs at higher temperature resulted in a large fraction of hemicellulose removed along with the lignin in the wash steps.

5.2.2 Model Analysis

The one-way analysis of variance (ANOVA) with Design-Expert® 6.0.4 software was utilised to examine the pre-treatment data. The verification of the model and the effects of each factor are shown in Table 5.3.

Table 5.3 ANOVA for the yield of reducing sugars subsequent to hemicellulase saccharification of pre-treated SBP

Source	Sum of Squares	Degrees of Freedom	Mean Square	F-Value	Probability > F
Model	2774.25	7	396.32	8.61	< 0.0001
A – Lime	498.71	1	498.71	10.83	0.0033
B – Time	322.37	1	322.37	7.00	0.0148
C – Temperature	5.82	1	5.82	0.13	0.7255
AB	134.78	1	134.78	2.93	0.1012
AC	1079.40	1	1079.40	23.44	< 0.0001
BC	646.47	1	646.47	14.04	0.0011
ABC	627.04	1	627.04	13.62	0.0013
Curvature	358.53	1	358.53	7.79	0.0107

Firstly, it should be noted from Table 5.3 that the model F-value of 8.61 is significant. A confidence level of 95% was chosen and the terms A, B, AC, BC, ABC are therefore all significant as the probability values fall below 0.05. Term AC representing lime x temperature had the highest F-value at 23.44. However, temperature (C) had a high *p*-value of 0.7255 thus confirming the discrepancy of the temperature effect. A curvature F-value of 7.79 showed that there was significant curvature within the model. The triplicate central replicate points of the design matrix were included to give curvature and there is only a 1.07% chance that a Curvature F-value could be related to noise.

Two three dimensional perturbation plots were used to compare the concentration of lime load on the yield of reducing sugars as shown in Figure 5.3. Whilst the x axis (time) and y axis (temperature) were consistent with the pre-treatment conditions, the z axis (reducing sugars) represented the enzyme assays run subsequently to the pre-treatment. The highest yield of reducing sugar is 16.4 U in Graph A1 (lime load of 0.1 g / g SBP) and 42.4 U in Graph A2 (lime load of 0.4 g / g SBP). There is therefore a significant increase in yield as the concentration of lime is increased. It can be noted from Graph A1 that the fairly flat line at a low 0.1 g lime / g SBP load shows little overall effect; therefore changes in temperature or time had little effect in increasing the sugar yield. Although temperature had a relatively greater effect than time at this low concentration of lime, as at 40°C yields were 0.9 U (12 h) and 3.8 U (36 h) compared to 13.9 U (12 h) and 16.4 U (36 h) at 70°C. Graph A2 with a lime load of 0.4 g lime / g SBP displayed a 3 factor interaction with an increase in yield from 8.7 – 42.4 U (12 – 36 h) while the temperature was kept constant at 40°C. It is interesting to note that the highest reducing sugar yield of 42.4 U from Table 5.2 was at the highest lime load (0.4 g lime / g SBP) and time (36 h) but the lowest temperature of 40°C.

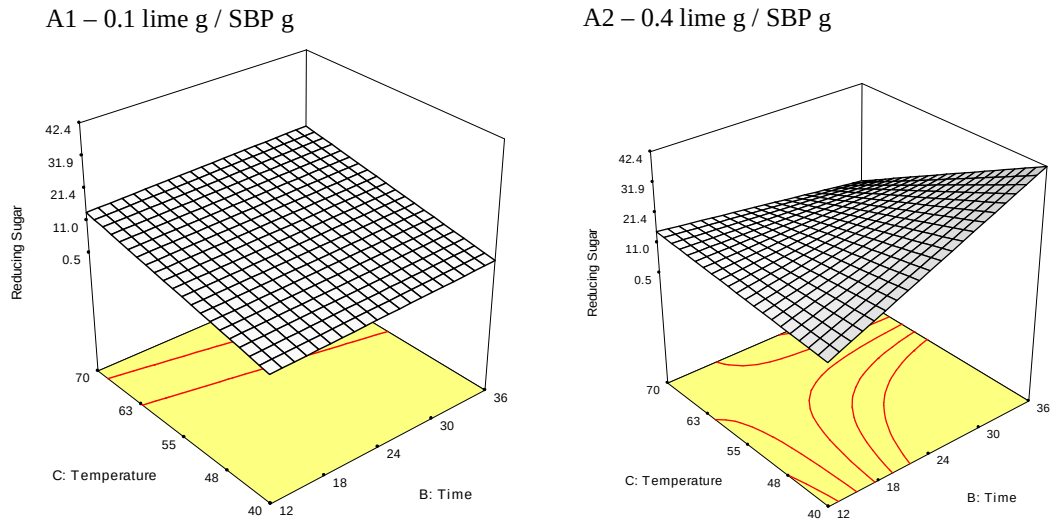

Figure 5.3 The effect of low and high lime load on the yield of sugars The released reducing sugars (U) is shown on the Z axis. An increased lime load gave a higher release of reducing sugar (A2).

The effect of pre-treatment time on the response of the release of reducing sugar was analyzed in Figure 5.4. A similar trend was seen in Figure 5.4 as between the low and high values of lime load from Figure 5.3. A longer pre-treatment time of 36 hours had the highest reducing sugar yield of 42.4 U (Graph B2) in comparison to 15.1 U (Graph B1) at 12 hours. Graph B1 shows that lime and temperature had little overall effect on the sugar yields, although lime had a relatively larger effect than temperature. At a low temperature (40°C), the increase in lime increased the released sugar from 0.9 – 8.7 U. However, at a high temperature (70°C) there was a smaller margin of increase from 13.9 - 15.1 U with the increase of lime. Graph B2 is significant, as it shows the optimal pre-treatment conditions. A significant response is observed at a low temperature of 40°C (36 h) between 3.8 U (0.1 g lime / g SBP) and 42.4 U (0.4 g lime / g SBP). At a high temperature of 70°C (36 h) there was a decrease in response from 16.4 U (0.1 g lime / g SBP) to 6.3 U (0.4 g lime / g SBP). This clearly shows the 3 factor interaction illustrated in Graph A2 in Figure 5.3.

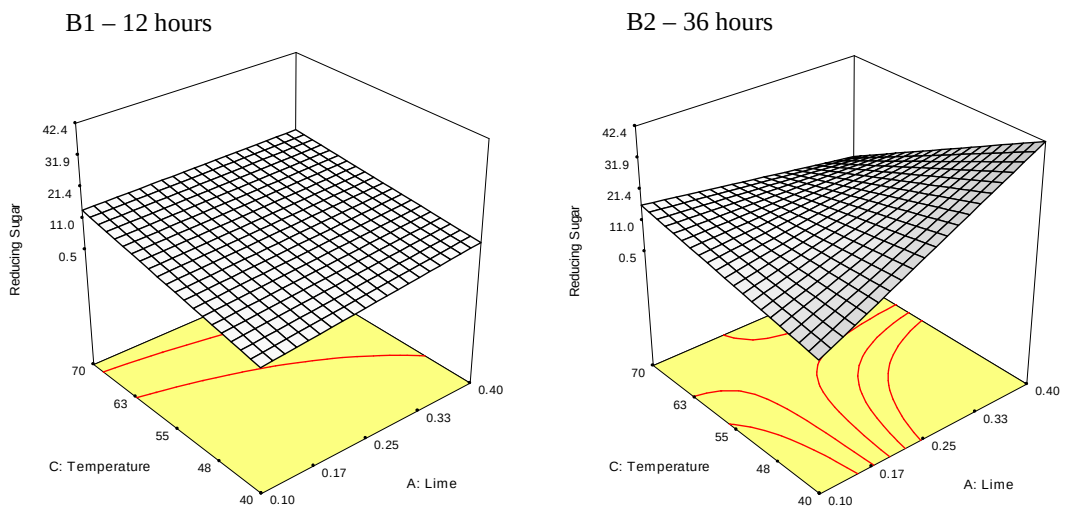

Figure 5.4 The effect of short and long time intervals on the yield of sugars The reducing sugar (U) released after hemicellulase activity is indicated on the Z axis and was noted to be higher in the second of the two graphs (B2) at 36 hours compared to 12 hours.

The temperature 3D perturbation plots are shown in Figure 5.5 with a clear indication that at a low temperature (Graph C1) both lime and time were influencing factors on the reducing sugar response, whilst at a high temperature (Graph C2) these two factors

showed little effect on changing the response. Graph C1 illustrates that at an incubation of 12 hours, the increase in lime load increased the response from 0.9 – 8.7 U. A 36 hour pre-treatment incubation with an increase in lime load increased the response from 3.8 – 42.4 U. As noted in Figure 5.3 (Graph A2) and Figure 5.4 (Graph B2) the most severe of pre-treatment conditions did not give the highest desired response; however, this is most clearly seen in Graph C2 (Figure 5.5). At a high temperature of 70°C the highest sugar yield was only 16.4 U.

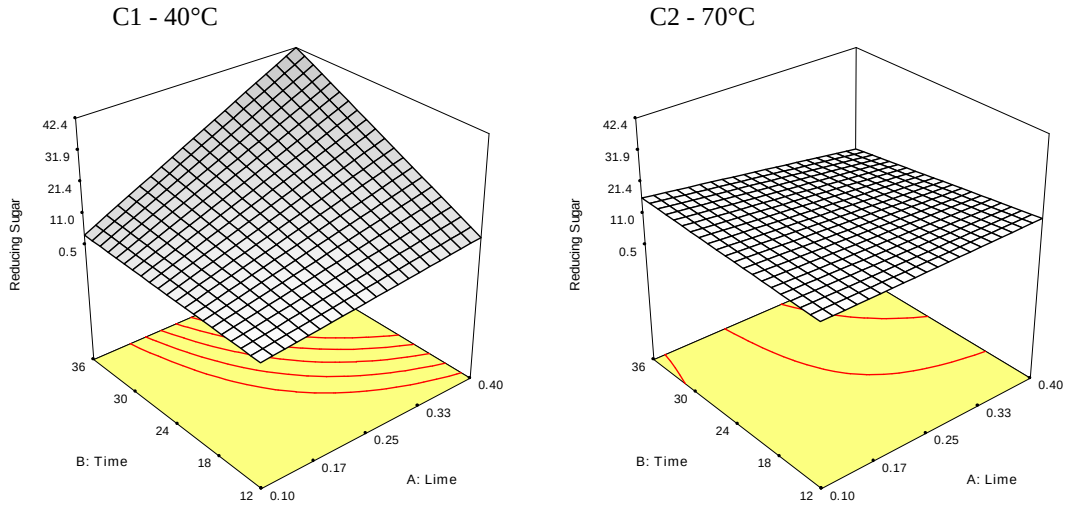

Figure 5.5 The effect of low and high temperatures on the yield of sugars The reducing sugar (µmol/ml/g) released after hemicellulase activity is indicated on the Z axis and was noted to be higher in the first of the two graphs (C1) at a lower temperature (40°C).

The model with an F-value of 8.61 was noted to be significant (Prob>F is < 0.0001); however, it should be pointed out that the temperature F-value of 0.13 is not significant with a Prob>F of 0.7255. Thus, it is possible that temperature did not have such a large influence on the desired response of the pre-treatment. In supporting this, Wyman *et al.* (2005) noted that a lime pre-treatment (regardless of the temperature) eliminated approximately 33% of the lignin and 100% of the acetyl groups. Industrially, a lower temperature is more favoured as there is a lower energy demand and with high temperatures a pressure vessel would be required (Wyman *et al.*, 2005). Thus, the optimal conditions from this study of 40°C, 0.4 g lime / g SBP and 36 hours will be used as the optimal pre-treatment conditions for all subsequent experiments.

5.3 Scanning Electron Microscopy of un-treated and lime pre-treated SBP

A comparison of the un-treated and pre-treated SBP was made with the scanning electron microscope (SEM) as shown in Figures 5.6 and 5.7, respectively.

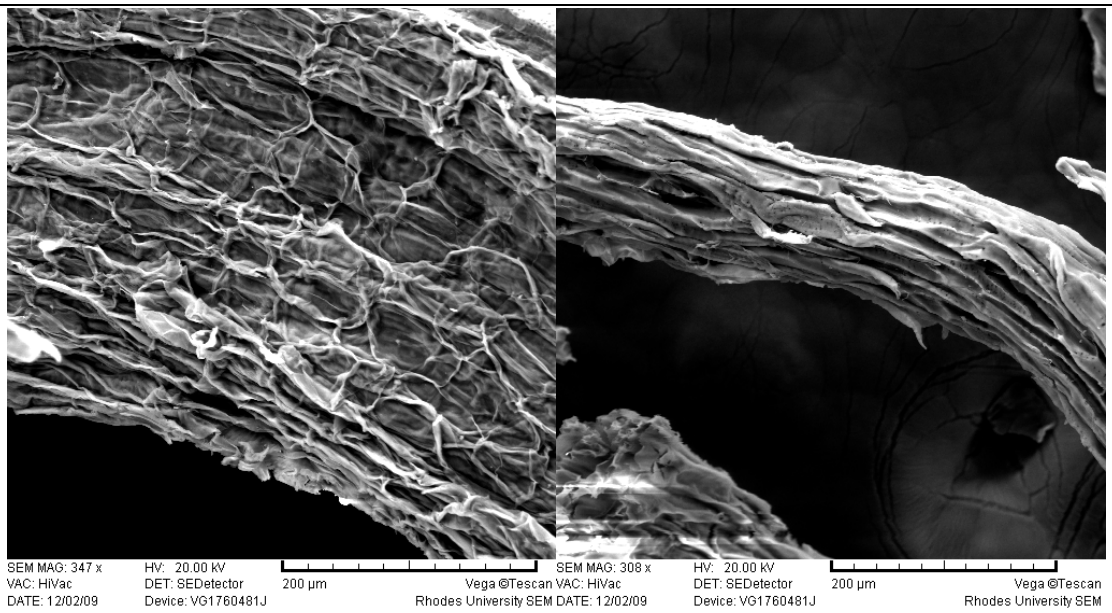

Figure 5.6 SEM of un-treated SBP The microscopic surface of the SBP prior to lime pre-treatment exhibits intact fibres.

The scanning electron micrographs displayed the significant difference of the lime pre-treatment on the SBP. The un-treated pulp fibres (Figure 5.6) had a structured architecture and each fibre was well defined. However, within the pre-treated micrograph (Figure 5.7) the fibres were indistinguishable and thus show the expected fibre disruption with chemical pre-treatment. The micrographs A and B represented the lime pre-treatment run 4 and 8 (See Table 5.2). The pre-treatment conditions were a lime load of 0.4 g lime / g SBP and 36 hour incubation; however run 4 was incubated at 40°C and run 8 at 70°C. The microscopic surfaces of the two pre-treatments (run 4 and 8) were similar.

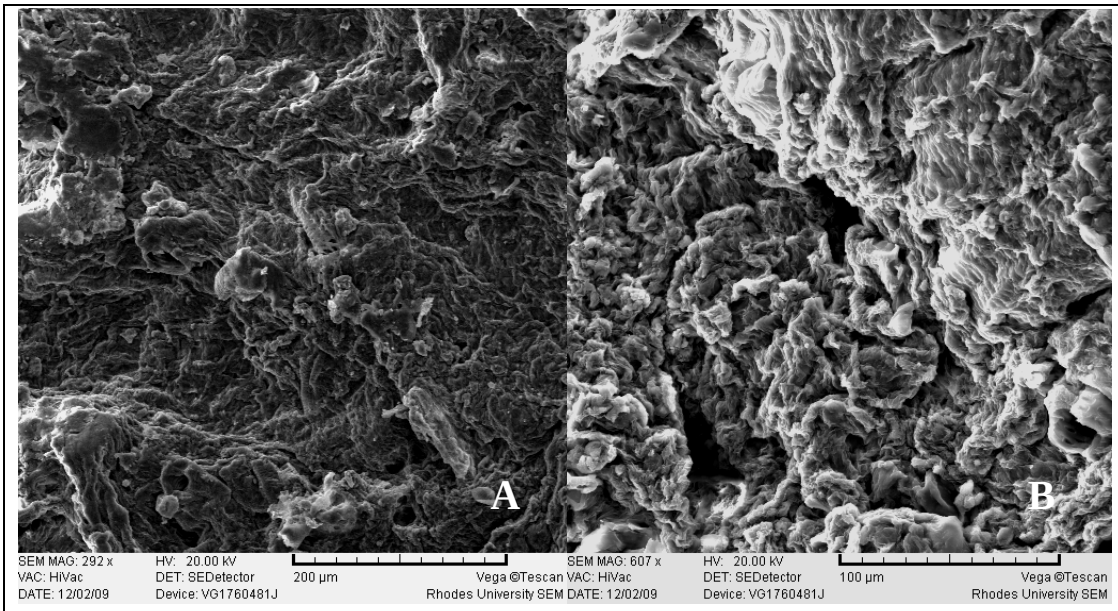

Figure 5.7 SEM of pre-treated SBP The lime pre-treatment has disrupted the fibrous surface of the SBP. The two scanning electron micrographs were taken from run no. 4 (A) and 8 (B).

The calcium hydroxide pre-treatment successfully disrupted the beet pulp fibres so that the pitted xylem vessels were observed in Figure 5.8.

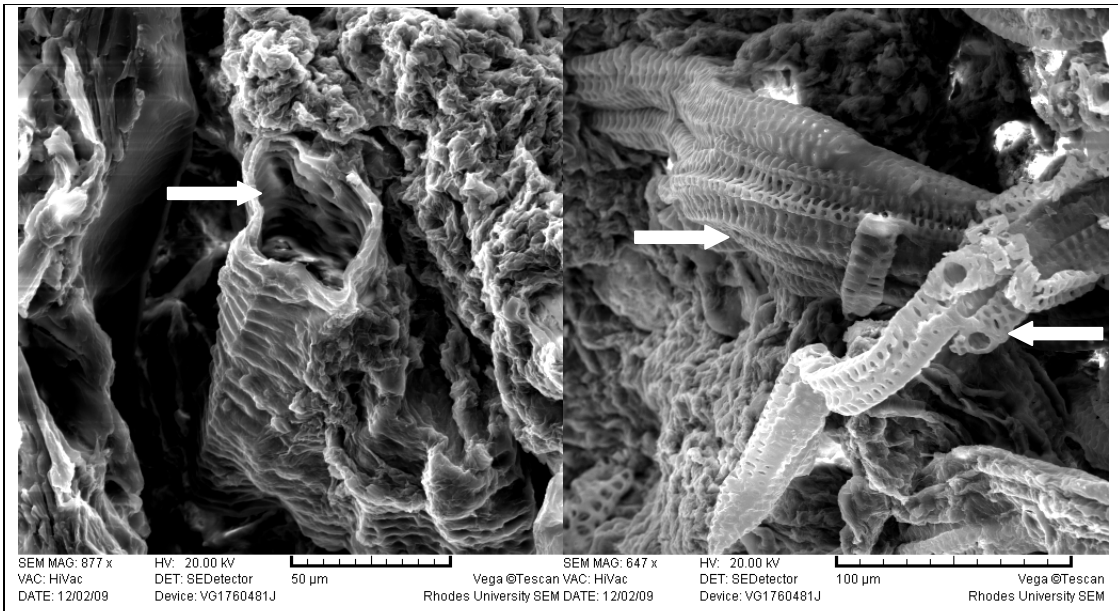

Figure 5.8 SEM of sieve plates from the pre-treated SBP The lime pre-treatment had stripped away the fibres for the sieve plates (see arrows) to be observed.

Conclusion

The alkaline (lime) pre-treatment was effective in disrupting the lignin and hemicellulose fibres as noted by the increased release of sugars in the hemicellulase assays. The SEM analysis supported the findings of fibre disruption.

The optimal pre-treatment conditions were determined within the 2^3 factorial experimental design, as expected. A lower temperature of 40°C was more effective at lignin removal without the accompanying removal of hemicellulose than a high temperature of 70°C, whilst the highest levels of the factors time and lime load were observed as optimal. Thus, lime pre-treatment at 0.4 g lime / g SBP load with 36 hour incubation at 40°C on SBP was shown to be optimal.

The research focus of this chapter was successful in producing novel pre-treatment data on SBP. The work presented here is significant for future studies relating to the production of bio-ethanol from SBP. The effective removal of hemicellulose from the SBP will allow for the hydrolysis of the glycosidic bonds, thus releasing glucose that will be fermented.

The experimental design in Chapter V utilised equal protein concentrations on the lime degraded SBP. However, a combination of various concentrations of each hemicellulase could show possible synergistic associations and will be explored in Chapter VI.

Chapter VI: Hemicellulase synergy

Introduction

In the search to understand how *C. cellulovorans* degrades biomass, several studies have been performed where synergistic associations have been confirmed between selected cellulases (Murashima *et al.,* 2002d), hemicellulases (Kosugi *et al.,* 2002c) as well as a combination of cellulases and hemicellulases (Beukes *et al.,* 2008; Han *et al.,* 2004; Jaeho *et al.,* 2007; Koukiekolo *et al.,* 2005 and Murashima *et al.,* 2003).

The synergistic degradation of sugarcane bagasse was previously studied with *C. cellulovorans* xylanase A (XynA), endoglucanase E (EngE) and mannanase A (ManA) and the highest degree of synergy occurred at an enzyme ratio of 25%XynA : 25%EngE : 50%ManA (Beukes *et al.,* 2008). The largest percentage of enzyme was ManA despite bagasse having a higher content of xylan compared to mannan (Sun *et al.,* 2004). It was therefore suggested by Beukes *et al*. (2008) that the mannan fibres were obstructing XynA and EngE from acting on the xylan and cellulose fibres.

The SBP, with a high arabinose content of 20.9% of dry weight (Micard *et al.,* 1996), presents a potentially novel target for the synergistic association of ManA, XynA and ArfA. Several studies have already shown that ArfA and XynA have a synergistic relationship on various substrates; the higher the arabinoxylan content, the higher the degree of synergy (Kosugi *et al.,* 2002c; Koukiekolo *et al.,* 2005).

The advantage of *C. cellulovorans* XynA lies in its ability to act as a xylanase as well as an acetyl xylan esterase, the latter via the NodB domain (Kosugi *et al.,* 2002b). Published research (Kosugi *et al.,* 2002b) has indicated a synergistic relationship between the xylanase catalytic domain and the acetyl xylan esterase domain and that the NodB domain has the ability to remove acetate from other substrates other than xylan.

Various combinations of ArfA, ManA and XynA were therefore tested on un-treated and pre-treated SBP in order to determine any synergistic associations. The degree of synergy was calculated as the actual activity of enzymes divided by the theoretical sum of the activities from various individual enzymes. Therefore, a degree of synergy of 1 or more is indicative of a synergistic relationship between the enzymes.

6.1 Enzymatic saccharification of un-treated SBP

The sugar beet, once treated with a mechanical pre-treatment, was termed SBP. The reaction assays were set up to determine any enzyme synergism on the un-treated SBP. The enzymes (ArfA; ManA ; XynA) were tested in every combination of 12.5%, 25%, 37.5%, 50%, 62.5%, 75% and 87.5% of the total enzyme protein concentration (40 μg/ml). The assays were carried out in triplicate at 40°C at pH 5.5 (50 mM sodium citrate) for 5 days. However, no released sugars were detected. These results indicated that a lime pre-treatment step was necessary for hemicellulase action.

6.2 Enzymatic saccharification of pre-treated SBP

Hemicellulase degradation was initially analysed with combinations of two and then with three hemicellulases. All hemicellulase reactions were carried out as explained on the un-treated SBP (section 6.1).

6.2.1 Activity of two hemicellulases on pre-treated beet fibres

The relationship between ArfA and XynA was studied at varying concentrations of each enzyme (Figure 6.1). The highest level of specific activity at 100%ArfA : 0%XynA can be directly related to the high arabinose composition of the SBP of 20.9% whilst there is only 1.7% of xylose present (Micard *et al.*, 1996). As noted previously, degree of synergy results of over 1.00 are deemed significant. In Figure 6.1 there are two such points, these are at 75%ArfA : 25%XynA and 25%ArfA : 75%XynA with degrees of synergy of 1.20 and 1.06, respectively.

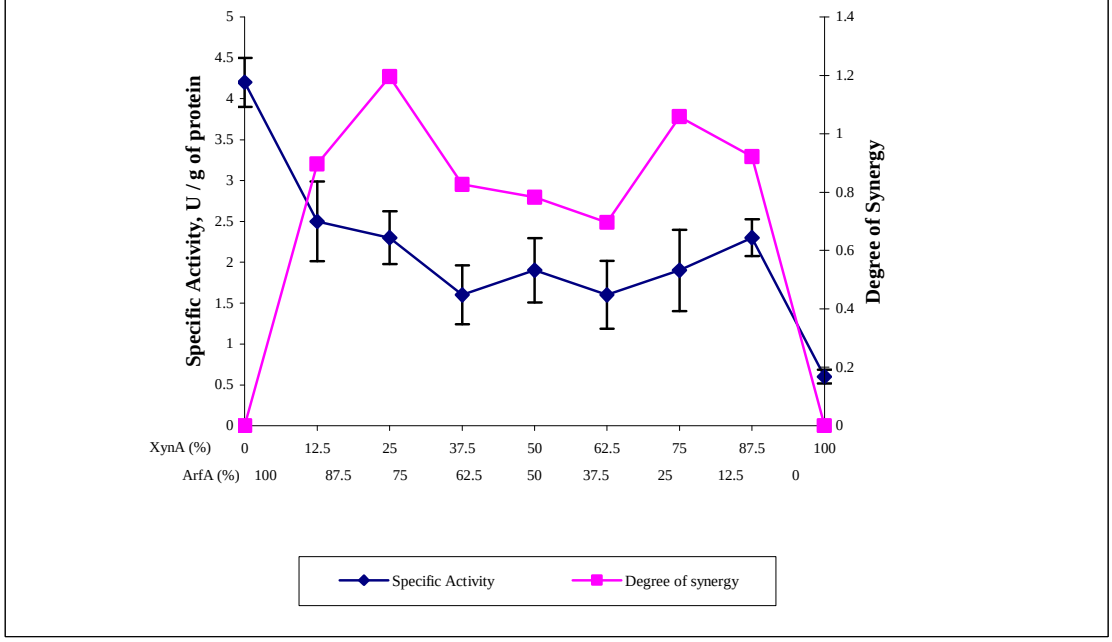

Figure 6.1 Specific activity and degree of synergy produced by combinations of ArfA and XynA The activities of various combinations of ArfA and XynA on pre-treated SBP. The total enzyme concentration was 40 µg/ml and the reaction conditions were 40°C, pH 5.5 (50 mM sodium citrate) for 5 days. Specific enzyme activity was expressed as the amount of enzyme required to release 1 µmol of reducing sugar per minute per g of protein. Values represent means ± SD, n=3.

While a 'smooth' trend was expected (since there was a gradual change in the percentages of each enzyme) it was to be noted that the reactions were performed on a complex substrate. Therefore, despite a homogeneous mixture of SBP, a gradual trend was not observed. An overall increase in specific activity with increasing ArfA enzyme

concentration correlated with the high arabinose content and low xylan content of the SBP.

Arabinose can form repeating α-1,5-linked L-arabinofuranosyl units known as arabinan, or as L-arabinofuranoside substitutions on β-1,3-linked galactose residues (arabinogalactan) and β-1,4-linked xylose resides (arabinoxylan) (Shallom and Shoham, 2003). These L-arabinofuranosyl residue-linked structures are depicted in Figure 6.2. The dry weight of the SBP has a high arabinose content of 20.9% and high galactose content of 5.1% (Micard *et al.*, 1996) thus, it is more likely that the arabinose is the form of arabinan and arabinogalactan. It is less likely that the arabinose is the form of arabinoxylan since there is a low xylan content of 1.7% SBP (dry weight).

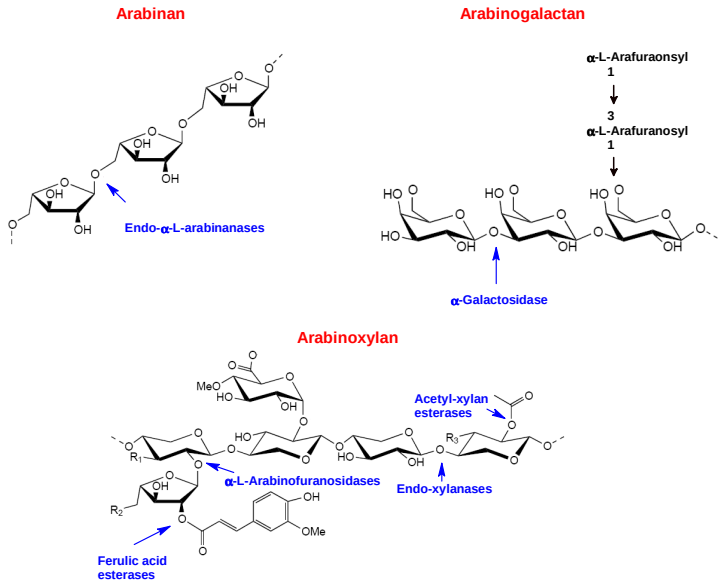

Figure 6.2 Arabinofuranosyl linked hemicellulose structures The α-1,5-linked L-arabinofuranosyl units are present in arabinan, arabinogalactan and arabinoxylan (Adapted from Figures 1.2 and 1.4).

The degradation of ArfA and ManA on SBP is illustrated in Figure 6.3. Again, it can be observed that the highest level of specific activity was at 100%ArfA. There was a gradual decrease until the point of 25%ArfA : 75%ManA where there was a gradual increase. The degree of synergy increases to 1.36 at 75%ArfA : 25%ManA and then slowly decreases. The mannose composition of the SBP is 1.1% dry weight (Micard *et al.,* 1996), which could explain the high specific activity and the highest degree of synergy occurring with only a small percentage of ManA. As shown by Beukes *et al.* (2008), these reactions demonstrated that there were probably mannan fibres hindering access of ArfA to the arabinofuranosyl residues. Therefore, the synergy between these two hemicellulases on SBP produced higher levels of reducing sugar.

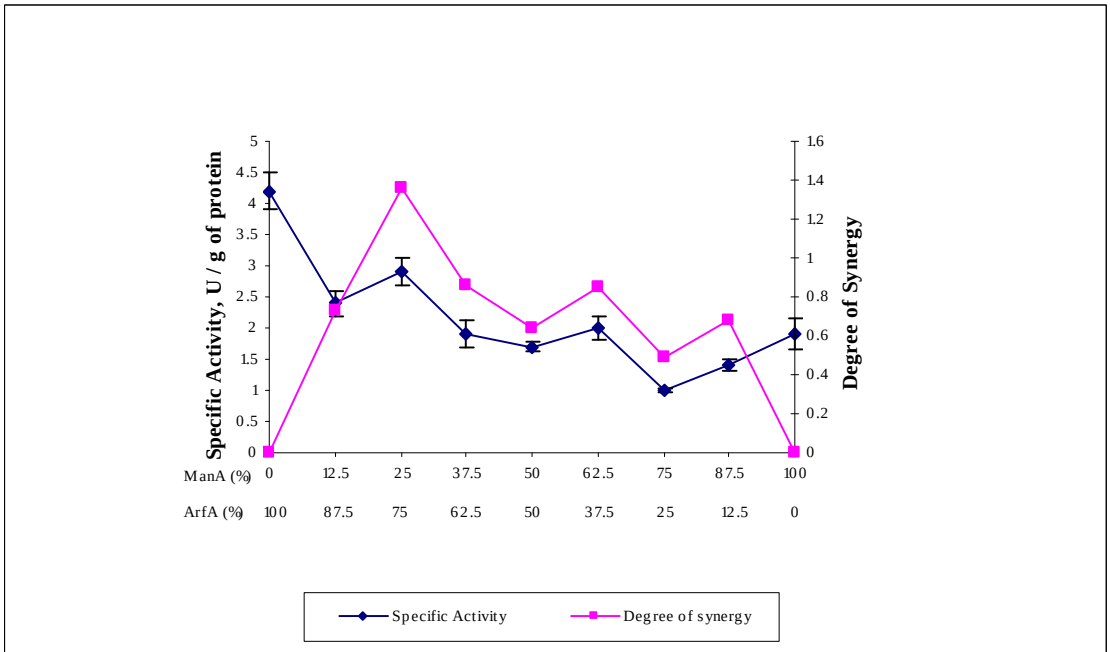

Figure 6.3 Specific activity and degree of synergy produced by combinations of ArfA and ManA The varying concentrations of ArfA and ManA on pre-treated SBP, measuring the specific activities and synergistic associations. The total enzyme concentration was 40 µg/ml and the reaction conditions were 40°C, pH 5.5 (50 mM sodium citrate) for 5 days. Specific enzyme activity was expressed as the amount of enzyme required to release 1 µmol of reducing sugar per minute per g of protein. Values represent means ± SD, n=3.

The mannose and xylose content of the SBP is similar at 1.1 and 1.7% dry weight, respectively (Micard *et al.,* 1996). Since these are such low percentages of the SBP,

Figure 6.4 shows an overall lower specific activity trend compared to Figure 6.1 and Figure 6.3. The only point of synergy was noted at 25%ManA : 75%XynA with a degree of synergy value of 1.10.

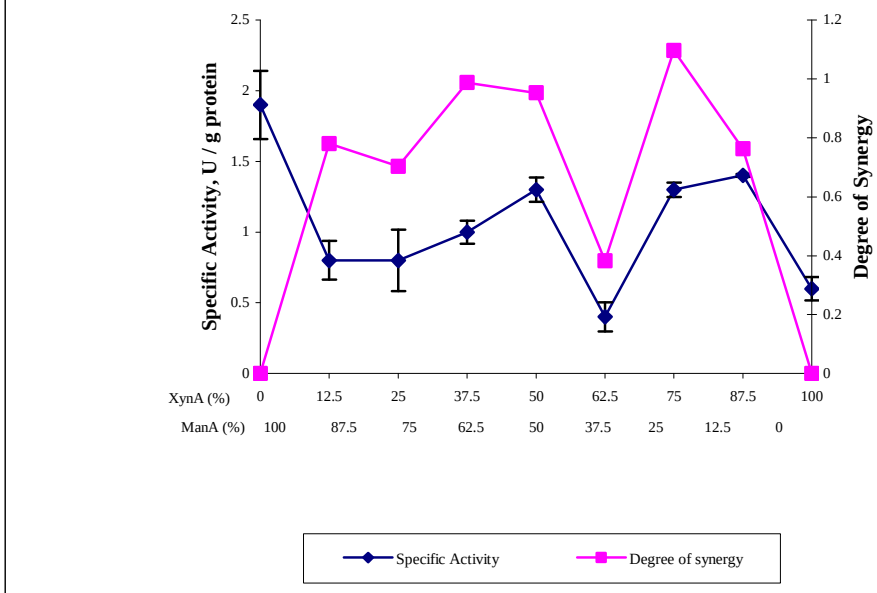

Figure 6.4 Specific activity and degree of synergy produced by combinations of ManA and XynA The hemicellulases degrading pre-treated SBP were analysed to show the highest specific activity at the point of 100%ManA : 0%XynA and highest synergy at 25%ManA : 75%XynA. The total enzyme concentration was 40 µg/ml and the reaction conditions were 40°C, pH 5.5 (50 mM sodium citrate) for 5 days. Specific enzyme activity was expressed as the amount of enzyme required to release 1 µmol of reducing sugar per minute per g of protein. Values represent means ± SD, n=3.

6.2.2 Activity of three hemicellulases on pre-treated beet fibres

The specific activities of various percentages of ArfA, ManA and XynA on pre-treated SBP were measured and depicted in a triangle format as shown in Figure 6.5. The legend of arrows explains the percentage of each enzyme at each point. At the point of highest specific activity between 3.3-4.2 U (green column), the arrows when followed to the three axis show 100%ArfA : 0%ManA : 0%XynA. This correlates with the higher arabinose percentage in the SBP compared to mannose and xylose.

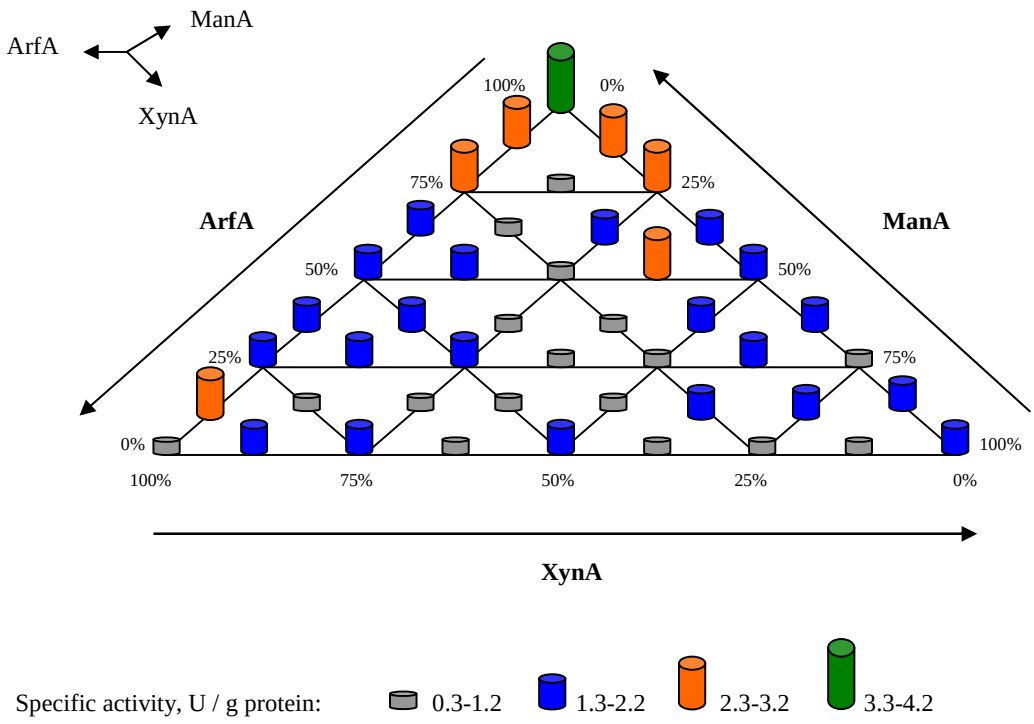

Figure 6.5 Triangle representation of specific activity on pre-treated SBP The increments of specific activity show the higher values to be at a high percentage of ArfA and low or zero percentage of ManA and XynA.

As noted previously with the reactions using two hemicellulases, the complex nature of the SBP is speculated to be the reason for the abrupt change rather than a gradual trend across the triangle design. In observing the points with 100% of an individual hemicellulase, the results show that the lowest specific activity occurred at 100%XynA. A speculated explanation is that xylose present in the SBP has many substitutions, thereby possibly hindering the action of the XynA.

Thermal stability studies at 40°C from Chapter IV showed a complete loss in enzyme activity after 11 hours for ManA and 84-96 hours for ArfA; however, XynA was noted to still be active after 120 hours. In light of these results, it is significant that although ManA has the lowest stability it still has a higher specific activity compared to XynA with the highest stability. The hemicellulase synergy experiments were run for 5 days, thus according to the thermal stability studies XynA would be the only enzyme to still show activity after this time. Therefore, together with the low specific activity results for

XynA this could further support the idea that the XynA was blocked by numerous substitutions on the xylan backbone.

Synergistic associations between the three hemicellulases were shown to be optimal (i.e. exhibit the highest degree of synergy) at 75%ArfA : 25%ManA (Figure 6.6). This correlates with the suggestion that the arabinose present in the SBP is in the form of arabinan and arabinogalactan rather than arabinoxylan. A high content of arabinoxylan in the SBP would have shown a high synergy degree between ArfA and XynA (Kosugi *et al.* 2002c).

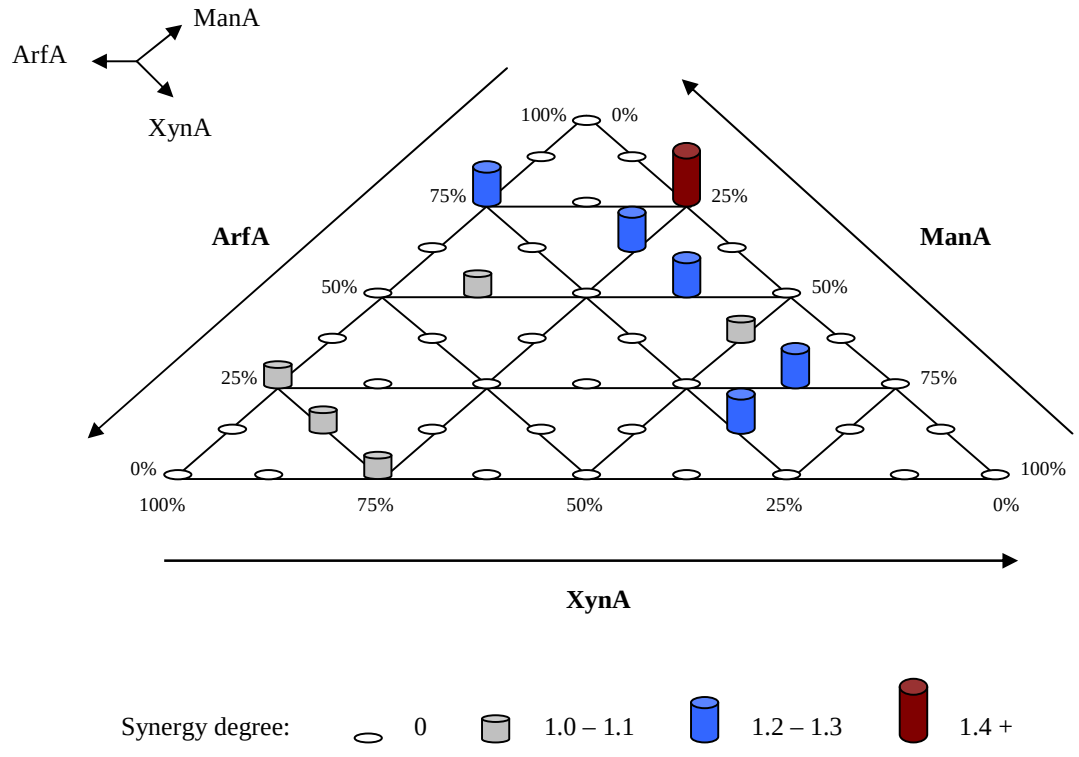

Figure 6.6 Triangle representation of the degree of synergy on pre-treated SBP The majority of synergistic associations were observed at low XynA concentrations of 0-25% and a range of ArfA and ManA concentrations.

A gradual trend was again not seen in the triangle design of degree of synergy (Figure 6.6). All points were repeated in triplicate and completed in one batch; therefore the only explanation for the lack of a gradual trend in the results is that they were obtained from a complex substrate.

High specific activity (Figure 6.5) and high degree of synergy (Figure 6.6) results were noted in a similar region of the triangle design, that is, a high percentage of ArfA and a low or zero percentage of either ManA or XynA. This correlates with the high arabinose content of the SBP mentioned previously.

Conclusion

The high arabinose content of the SBP resulted in the highest specific activity occurring with 100%ArfA at 4.2 U / g of protein. It was proposed that the arabinose was present in the form of arabinan or arabinogalactan compared to arabinoxylan. The two reasons to support this hypothesis include low xylan content and little to no synergistic associations between ArfA and XynA. This conclusion is further supported by the enzyme stability results reported in Chapter IV, which indicated that XynA was still active after 5 days.

Due to the fact that the content of mannose and xylose is lower than arabinose in the SBP, this study is significant in elucidating synergistic associations involving ArfA. The study does provide suggestions as to how the hemicellulose fibres are structured around the cellulose microfibrils and each other in the sugar beet cell walls. The activity of one hemicellulase may expose other hemicellulose structures for the effective degradation by another group of hemicellulase. Thus, the highest synergistic relationship was observed on optimised lime pre-treated SBP at 75%ArfA : 25%ManA .

Chapter VII: General Discussion and Conclusion

7.1 General Discussion and Conclusion

Enhancement of the saccharolytic phase of sugar beet pulp (SBP) was initiated with the successful isolation and partial characterisation of the *C. cellulovorans* hemicellulases arabinofuranosidase A (ArfA), mannanase A (ManA) and xylanase A (XynA) in Chapter IV. The optimal conditions for activity are summarised in Table 7.1. A set of conditions falling within the optimal range for the combined hemicellulase degradation was determined as pH 5.5 and 40°C. These conditions were utilised in the subsequent pre-treatment and hemicellulase synergy studies.

Table 7.1 Optimised conditions for the degradation of sugar beet pulp

Hemicellulase partial characterisation	**Optimal conditions**	
ArfA	pH 6.5 - 7.0	45°C
ManA	pH 5.5	40°C
XynA	pH 5.0 - 6.0	45°C
Lime pre-treatment		
Lime load	0.4 g lime / g SBP	
Time	36 hour	
Temperature	40°C	
Hemicellulase synergy		
Highest synergy	75%ArfA : 25%ManA	
Highest release of reducing sugars	4.2 U / g of protein at 100%ArfA	

U = μmol of reducing sugar per min

The optimal conditions were determined using simple substrates, thus with the move to the complex SBP substrate a chemical pre-treatment step was required. As there was no previously published data on optimised lime pre-treatment, conditions were determined using a similar substrate from the sugar industry was chosen, namely sugarcane bagasse. The factors of temperature, lime load and time were investigated in a 2^3 factorial design. The optimal chemical pre-treatment was established at a lime load of 0.4 g lime / g SBP

with 36 hour incubation at 40°C (Table 7.1). A lower temperature was significant in removing the lignin. With a higher temperature of 70°C some of the hemicellulose structures were disrupted and removed in the washes. It should, however, be borne in mind that these conditions may vary between different batches of sugar beet due to seasonal variations, growth conditions, age of the sugar beet, time of harvest and conditions of storage.

Use of SBP as a source of second generation biofuel removes the need by sugar beet sugar factories to dispose of the by-product. Currently, the pulp is utilised in paper mills and as fodder for the agricultural industry (Vaccari *et al.*, 2005). However, the SBP is not a readily accepted source for either the paper industry or agricultural industry. The paper mills require a cellulose-rich resource and therefore the SBP needs to be treated before it can be employed, whilst the fodder market is over-saturated. A benefit of using a lime pre-treatment prior to enzymatic saccharification is that the lime can be sourced from the beet factories, as lime is utilised in the processing of the sugar (Vaccari *et al.*, 2005). Additional benefits of using lime (calcium hydroxide) is that it is the least expensive hydroxide for use in an alkaline pre-treatment and that calcium hydroxide can be regenerated with a process that includes using carbon dioxide to neutralise and lime kiln technology (Kaar and Holtzapple, 2000).

The hemicellulase synergy studies revealed several synergistic associations between ArfA, ManA and XynA. The lime pre-treated SBP produced the highest synergistic relationship at a ratio of 75%ArfA : 25%ManA. These results correlated well with the approximate chemical composition of SBP of 20.9% arabinose, 1.1% mannose and 1.7% xylose (Micard *et al.*, 1996). *C. cellulovorans* hemicellulases can therefore act synergistically to degrade SBP. However, the highest release of reducing sugars was observed at 100% ArfA.

Results from Chapter VI suggested that the arabinose was present mainly in the form of arabinan and arabinogalactan (as opposed to arabinoxylan) as there was little to no synergistic relationship between ArfA and XynA. Thus, a comparison could not be

explored with the work on ArfA and XynA substrate specificity and synergy (Kosugi *et al.,* 2002c) or sequential synergistic reactions (Koukiekolo *et al.,* 2005).

Studies in literature have shown that an increased time of incubation with the substrate of interest increases the degree of synergy. However, the hemicellulase synergy studies were conducted for five days and the thermal stability studies from Chapter IV showed that ArfA and ManA lost complete enzymatic activity within this time. Therefore, further research to enhance enzymatic degradation on SBP will need to utilise arabinofuranosidases and mannanases with greater temperature stability, as well as exploring the synergistic associations from additional cellulase and hemicellulases.

When saccharification of the SBP is the primary objective, then the degree of synergy is the most significant factor. As a high value of this indicator will show that the reaction conditions (and substrate) were optimal for enzymatic degradation, in contrast to a reaction with a low or zero degree of synergy. However, when studying the potential use of SBP for bio-ethanol production then the total release of reducing sugars becomes the most significant factor. It is economically advantageous to have a higher concentration of sugars to be subsequently fermented to a higher concentration of bio-ethanol.

7.2 Future Recommendations

Reproducibility and enzyme stability are key features in the biotechnology industry. The thermal stability studies were performed at a temperature (40°C) that fell within the optimal temperature ranges of all three hemicellulases. However, further studies at a slightly lower temperature could show increased stability. Immobilised enzymes appear to have a greater stability. Thus, cellulosomal enzymes such as ManA and XynA could be bound to the scaffolding protein (CbpA) in order to form a designer (mini-) cellulosome. An additional benefit might be that the mini-cellulosome would hold the hemicellulases in closer proximity to the substrate, thus increasing activity (Fierobe *et al.*,

2005). Another advantage is that immobilised enzymes can readily be re-used and if need be, recovered.

A study with various concentrations of cellulases (endoglucanases and exoglucanases) on the hemicellulase degraded SBP could also be employed to determine further synergistic associations as well as a higher release of sugars.

The research hypothesis for this study stated that the SBP could be degraded synergistically for subsequent fermentation into bio-ethanol. Further research should include investigations into the degradation products to determine subsequent enzymatic pathways and possible inhibitory effects on enzymatic activity. In addition, fermentation studies would verify the value of this work. These studies include:

1. A fermentation with the sugars released from a reaction with 100% ArfA on lime pre-treated (0.4 g lime / g SBP; 36 hour; 40°C) SBP;
2. The quantity of sugars released from a cellulase degradation of the SBP after a lime pre-treatment and hemicellulase pre-treatment.

SBP has a high content of pectin to maintain cell wall integrity (Funami *et al.,* 2009; Micard *et al.,* 1996). Thus, *C. cellulovorans* pectate lyase A, pelA (Tamaru and Doi, 2001) may provide a potentially novel synergistic association with ArfA and ManA. However, due to time constraints, these synergistic relationships with pelA were not explored.

A comparison of the various cellulosomal enzymes from the *Clostridium* genus is required to establish the highest synergy and specific activity on pre-treated SBP. Designer cellulosomes could include a selection of the most effective enzymes (Fierobe *et al.,* 2002). The cohesion and dockerin binding between the scaffolding protein and enzymes of the cellulosome are species-specific, a characteristic that could be exploited in constructing the designer cellulosome (Mingardon *et al.,* 2007). Thus, the production of bio-ethanol could exploit a recombinant system within *Saccharomyces cerevisiae*

utilising the model of microbial cellulosomes (Bayer *et al.,* 2007; Ito *et al.,* 2009; Margeot *et al.,* 2009).

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Appendices

Appendix A: List of chemicals

Acrylamide	Sigma (Cat. No. A8887)
Agarose	Sigma (Cat. No. A9539)
Ammonium persulphate	Sigma Aldrich (Cat. No. A3678)
L-(+)-arabinose	Sigma (Cat. No. A-3256)
Bacteriological agar	Biolab (Cat. No. BX1)
Birchwood xylan	Fluka (Cat. No. 95588)
Bovine serum albumin (BSA)	Sigma (Cat. No. A7906)
Bradford reagent	Sigma (Cat. No. B6916)
Bromophenol blue	Sigma (Cat. No. B8026)
Calcium chloride	Saarchem (Cat. No. 1524900)
Coomassie Brilliant Blue R250	Merck (Cat. No. 1.12553)
3,5-Dinitrosalicylic acid	Sigma (Cat No. D0550)
Di-potassium hydrogen phoshate	Merck (Cat. No. 1.05104.1000)
Di-sodium hydrogen orthophosphate	Saarchem (Cat. No. 5822860)
Ethanol	Merck (Cat. No. 8.18700)
Glacial acetic acid	Merck (Cat. No. 1.00063)
D-Glucose	Saarchem (Cat. No. 2676020)
Glycerol	Saarchem (Cat. No. 2676520)
Glycine	Merck (Cat. No. 1.04169)
Imidazole	Merck (Cat. No. 1.04716)
IPTG	Calbiochem (Cat. No. 420322)
Kanamycin (Monosulphate)	Melford (Cat. No. K0126)
Locust bean gum	Fluka (Cat. No. 62631)
Lysozyme	Fluka (Cat No. 62971)
Magnesium chloride	Associated Chemical Enterprises (Cat. No. 7791-18-6)
Mannose	Sigma (Cat. No. M2069)

Methanol	Merck (Cat. No. 8.22283)
N,N-methylenebisacrylamide	Sigma (Cat. No.M7279)
2-mercaptoethanol	Fluka (Cat. No. 63700)
4-Nitrophenyl-α-L-arabinofuranoside	Sigma (Cat. No. N3641)
p-Nitrophenol	Sigma (Cat. No. 42,575-3)
PeqGold protein marker II	peqLab (Cat. No. 27-2010)
Phenol	Sigma (Cat.No. P3653)
Sodium azide	Merck (Cat. No. 8.22335)
Sodium carbonate	Merck (Cat. No. 1.06392.0500)
Sodium chloride	Saarchem (Cat. No. 5822320)
Sodium dodecyl sulphate (SDS)	BDH biochemicals (Cat. No. 301754)
Sodium hydroxide	Saarchem (Cat. No. 5823200)
Sodium metabisulfite	Sigma-Aldrich (Cat. No.255556)
Sodium Potassium tartrate	Merck (Cat. No. 1.08087)
Tris (hydroxymethyl) aminomethane	Merck (Cat. No. 1.08382)
Tri-sodium citrate dehydrate	Merck (Cat. No. 1.06448)
Tryptone	Fluka (Cat. No. 70169)
D-(+)-Xylose	Sigma (Cat. No. X-3877)
Yeast extract	Biolab (Cat. No. BX6)

Appendix B: Map of expression vector

The expression vector, pET-29a(+) as shown in Figure B.1 was utilised to over-express the *C. cellulovorans* genes.

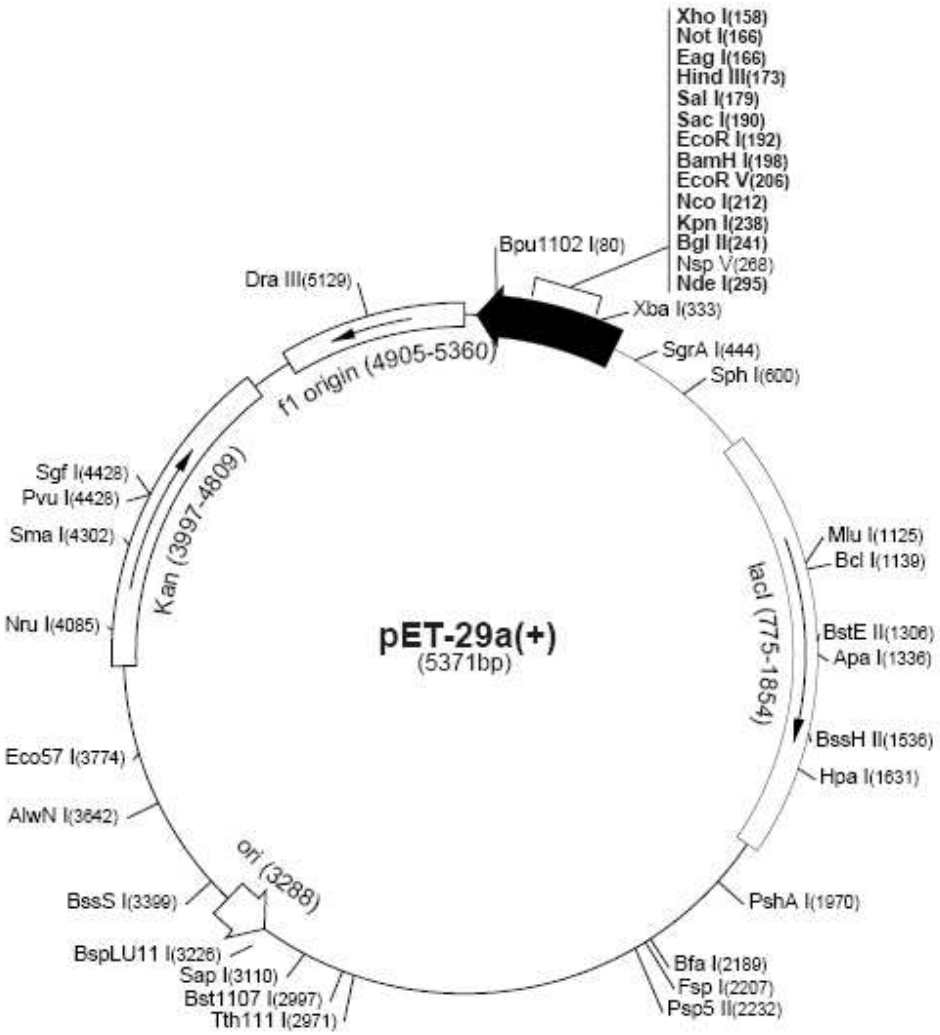

Figure B.1 Expression vector, pET-29a(+) The *C. cellulovorans* ArfA gene was inserted between XbaI and XhoI; and ManA and XynA genes were inserted between EcoRI and BamH1.

Appendix C: Standard curves for protein and enzymatic activity determination

C.1 Protein standard curve

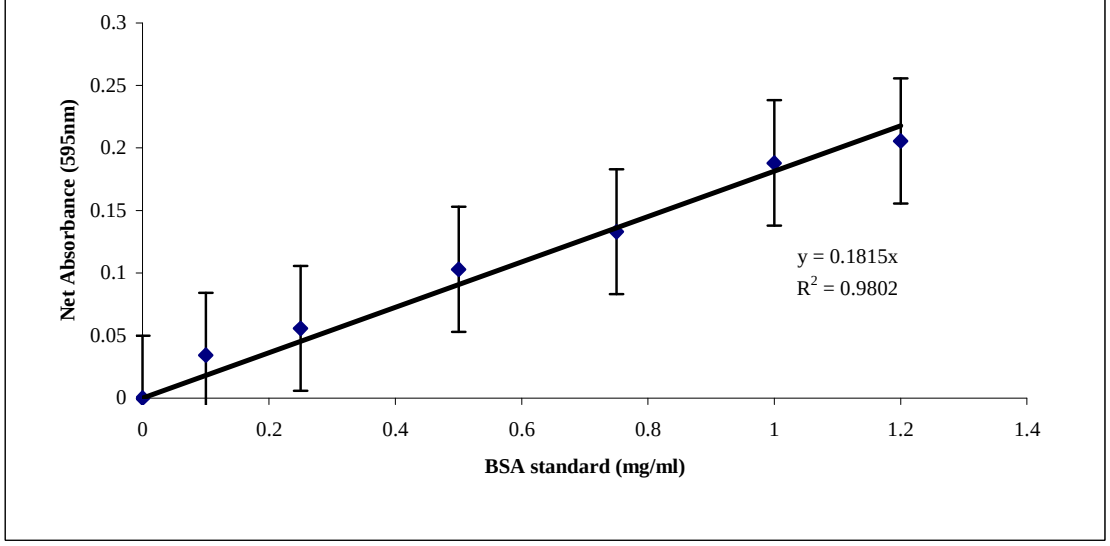

Figure C.1 Bradford standard curve to determine protein concentration BSA was utilised as the protein standard. Values represent means ± SD, n=3.

C.2 Enzymatic activity determination

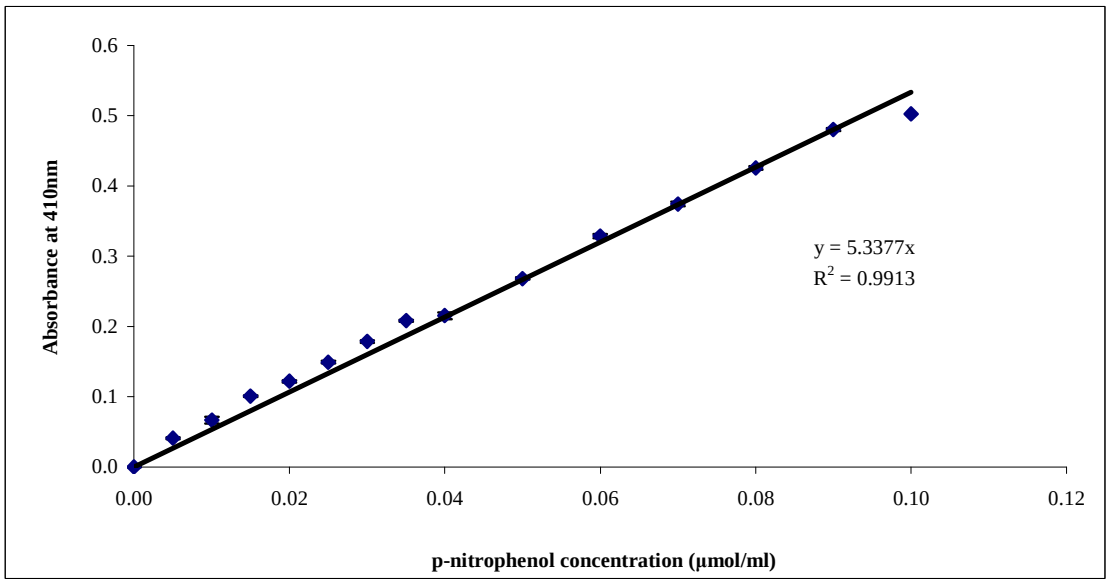

Figure C.2 *p*-Nitrophenol standard curve using the Arabinofuranosidase assay Values represent means ± SD, n=3.

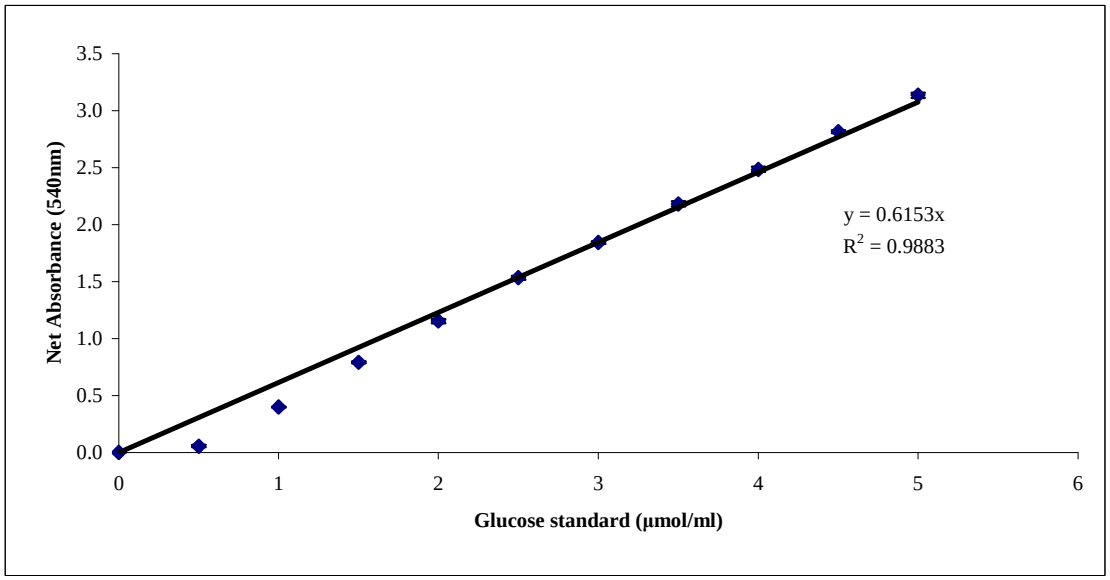

Figure C.3 Glucose standard curve using the DNS assay Values represent means ± SD, n=3.

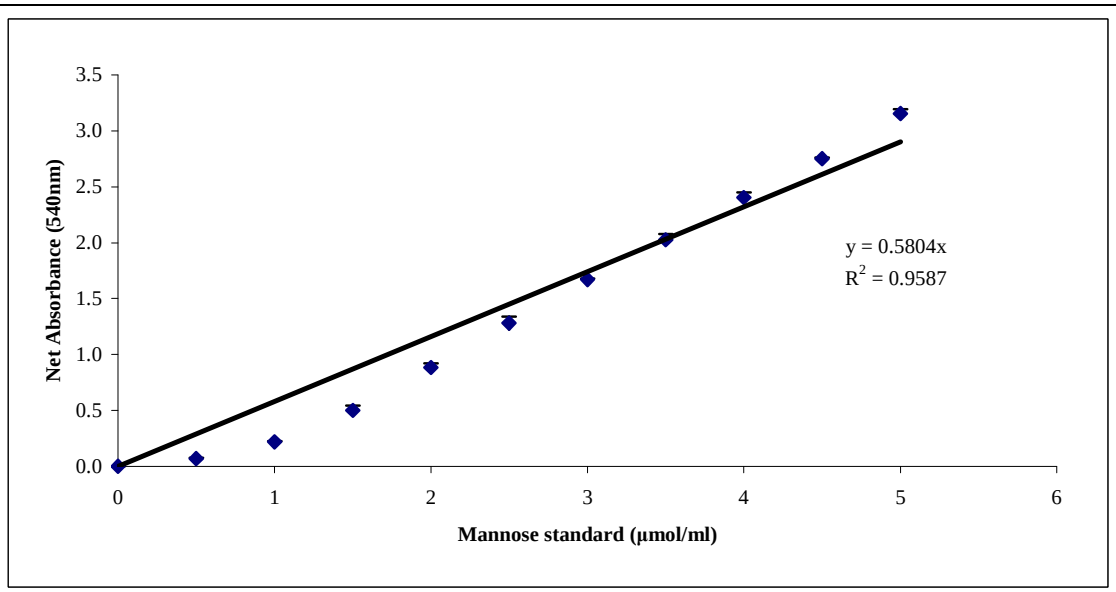

Figure C.4 Mannose standard curve using the DNS assay Values represent means ± SD, n=3.

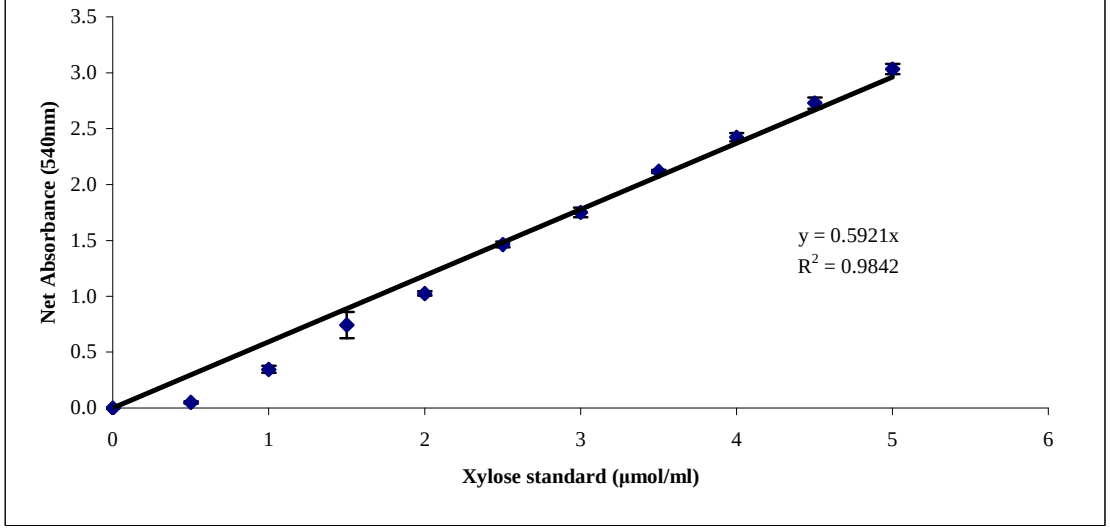

Figure C.5 Xylose standard curve using the DNS assay Values represent means ± SD, n=3.s

Appendix D: Synergy studies

The reaction mixtures for the synergy assays were made up to final volume of 500 µl with 50 mM sodium citrate (pH 5.5), as shown in Table D.1. Total protein concentration was 40 µg/ml. The assays were run in triplicate at 40°C for 5 days.

Table D.1 Combination of enzymes for synergy assays

Combination Number	Combination	ArfA (µl)	ManA (µl)	XynA (µl)	Buffer Volume (µl)
1A	ArfA 12.5	5			495
2A	ArfA 25	10			490
3A	ArfA 37.5	15			485
4A	ArfA 50	20			480
5A	ArfA 62.5	25			475
6A	ArfA 75	30			470
7A	ArfA 87.5	35			465
8A	ArfA 100	40			460
1M	ManA 12.5		5		495
2M	ManA 25		10		490
3M	ManA 37.5		15		485
4M	ManA 50		20		480
5M	ManA 62.5		25		475
6M	ManA 75		30		470
7M	ManA 87.5		35		465
8M	ManA 100		40		460
1X	XynA 12.5			5	495
2X	XynA 25			10	490
3X	XynA 37.5			15	485
4X	XynA 50			20	480
5X	XynA 62.5			25	475
6X	XynA 75			30	470
7X	XynA 87.5			35	465
8X	XynA 100			40	460
9	ArfA 87.5 ManA 12.5	35	5		460
10	ArfA 75 ManA 25	30	10		460
11	ArfA 62.5 ManA 37.5	25	15		460
12	ArfA 50 ManA 50	20	20		460
13	ArfA 37.5 ManA 62.5	15	25		460

14	ArfA 25 ManA 75	10	30		460
15	ArfA 12.5 ManA 87.5	5	35		460
16	ManA 87.5 XynA 12.5		35	5	460
17	ManA 75 XynA 25		30	10	460
18	ManA 62.5 XynA 37.5		25	15	460
19	ManA 50 XynA 50		20	20	460
20	ManA 37.5 XynA 62.5		15	25	460
21	ManA 25 XynA 75		10	30	460
22	ManA 12.5 XynA 87.5		5	35	460
23	XynA 87.5 ArfA 12.5	5		35	460
24	XynA 75 ArfA 25	10		30	460
25	XynA 62.5 ArfA 37.5	15		25	460
26	XynA 50 ArfA 50	20		20	460
27	XynA 37.5 ArfA 62.5	25		15	460
28	XynA 25 ArfA 75	30		10	460
29	XynA 12.5 ArfA 87.5	35		5	460
30	ArfA 75 ManA 12.5 XynA 12.5	30	5	5	460
31	ArfA 62.5 ManA 25 XynA 12.5	25	10	5	460
32	ArfA 50 ManA 37.5 XynA 12.5	20	15	5	460
33	ArfA 37.5 ManA 50 XynA 12.5	15	20	5	460
34	ArfA 25 ManA 62.5 XynA 12.5	10	25	5	460
35	ArfA 12.5 ManA 75 XynA 12.5	5	30	5	460
36	ArfA 12.5 ManA 62.5 XynA 25	5	25	10	460
37	ArfA 12.5 ManA 50 XynA 37.5	5	20	15	460
38	ArfA 12.5 ManA 37.5 XynA 50	5	15	20	460
39	ArfA 12.5 ManA 25 XynA 62.5	5	10	25	460
40	ArfA 12.5 ManA 12.5 XynA 75	5	5	30	460
41	ArfA 25 ManA 12.5 XynA 62.5	10	5	25	460
42	ArfA 37.5 ManA 12.5 XynA 50	15	5	20	460
43	ArfA 50 ManA 12.5 XynA 37.5	20	5	15	460
44	ArfA 62.5 ManA 12.5 XynA 25	25	5	10	460
45	ArfA 50 ManA 25 XynA 25	20	10	10	460
46	ArfA 37.5 ManA 37.5 XynA 25	15	15	10	460
47	ArfA 25 ManA 50 XynA 25	10	20	10	460
48	ArfA 25 ManA 37.5 XynA 37.5	10	15	15	460
49	ArfA 25 ManA 25 XynA 50	10	10	20	460
50	ArfA 37.5 ManA 25 XynA 37.5	15	10	15	460