CHARACTERISATION OF THE CELLULOLYTIC AND HEMICELLULOLYTIC SYSTEM OF *BACILLUS LICHENIFORMIS* SVD1 AND THE ISOLATION AND CHARACTERISATION OF A MULTI-ENZYME COMPLEX

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by

Jacoba Susanna van Dyk

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Abstract

The biological degradation of lignocellulose into fermentable sugars for the production of liquid transportation fuels is feasible and sustainable, but requires a variety of enzymes working in synergy as lignocellulose is a complex and recalcitrant substrate. The cellulosome is a multi-enzyme complex (MEC) with a variety of cellulolytic and hemi-cellulolytic enzymes that appears to facilitate an enhanced synergy and efficiency, as compared to free enzymes, for the degradation of recalcitrant substrates such as lignocellulose and plant cell walls.

Most of the studies on cellulosomes have focused on a few organisms; *C. thermocellum*, *C. cellulovorans* and *C. cellulolyticum*, and there is only limited knowledge available on similar complexes in other organisms. Some MECs have been identified in aerobic bacteria such as *Bacillus circulans* and *Paenibacillus curdlanolyticus*, but the nature of these MECs have not been fully elucidated.

This study investigated the cellulolytic and hemi-cellulolytic system of *Bacillus licheniformis* SVD1 with specific reference to the presence of a MEC, which has never been reported in the literature for *B. licheniformis*. A MEC of approximately 2,000 kDa in size, based on size exclusion chromatography using Sepharose 4B, was purified from a culture of *B. licheniformis*. When investigating the presence of enzyme activity in the total crude fraction as well as the MEC of a birchwood xylan culture, *B. licheniformis* was found to display a variety of enzyme activities on a range of substrates, although xylanases were by far the predominant enzyme activity present in both the crude and MEC fractions. Based on zymogram analysis there were three CMCases, seven xylanases, three mannanases and two pectinases in the crude fraction, while the MEC had two CMCases, seven xylanases, two mannanases and one pectinase. The pectinases in the crude could be identified as a pectin methyl esterase and a lyase, while the methyl esterase was absent in the MEC. Seventeen protein species could be detected in the MEC but only nine of these displayed activity on the substrates tested. The possible presence of a β-xylosidase in the crude fraction was deduced from thin layer chromatography (TLC) which demonstrated the production of xylose by the

crude fraction. It was furthermore established that *B. licheniformis* SVD1 was able to regulate levels of enzyme expression based on the substrate the organism was cultured on.

It was found that complexed xylanase activity had a pH optimum of between pH 6.0 and 7.0 and a temperature optimum of 55°C. Complexed xylanase activity was found to be slightly inhibited by $CaCl_2$ and inhibited to a greater extent by EDTA. Complexed xylanase activity was further shown to be activated in the presence of xylose and xylobiose, both compounds which are products of enzymatic degradation. Ethanol was found to inhibit complexed xylanase activity. The kinetic parameters for complexed xylanase activity were measured and the K_m value was calculated as 2.84 mg/ml while the maximal velocity (V_{max}) was calculated as 0.146 U (µmol/min/ml).

Binding studies, transmission electron microscopy (TEM) and a bioinformatic analysis was conducted to investigate whether the MEC in *B. licheniformis* SVD1 was a putative cellulosome. The MEC was found to be unable to bind to Avicel, but was able to bind to insoluble birchwood xylan, indicating the absence of a CBM3a domain common to cellulosomal scaffoldin proteins. TEM micrographs revealed the presence of cell surface structures on cells of *B. licheniformis* SVD1 cultured on cellobiose and birchwood xylan. However, it could not be established whether these cell surface structures could be ascribed to the presence of the MECs on the cell surface. Bioinformatic analysis was conducted on the available genome sequence of a different strain of *B. licheniformis*, namely DSM 13 and ATCC 14580. No sequence homology was found with cohesin and dockerin sequences from various cellulosomal species, indicating that these strains most likely do not encode for a cellulosome.

This study described and characterised a MEC that was a functional enzyme complex and did not appear to be a mere aggregation of proteins. It displayed a variety of hemi-cellulolytic activities and the available evidence suggests that it is not a cellulosome, but should rather be termed a xylanosome. Further investigation should be carried out to determine the structural basis of this MEC.

Declaration

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

____

Signed: Jacoba Susanna van Dyk

On this ________________ of _________________ 20___

Dedication

This thesis is dedicated to Morgan who always supported me and believed in me.

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I would like to acknowledge my supervisor, Dr Brett Pletschke for facilitating this research.

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List of outputs

Publications

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CHAPTER 1 – GENERAL INTRODUCTION

1.1 Biofuel Production

1.1.1 Introduction

For decades the world has relied on fossil fuels for the production of transportation fuels. However, the oil crisis in 1975 and again in 1979, which saw dramatic increases in the crude oil price, sparked initiatives by governments for large scale research and production of alternative liquid transportation fuels (United Nations, 2008). This was mainly driven by the high price of oil and the burden it placed on economies to import oil. Governments were driven to reduce their reliance on external oil supplies under circumstances of fluctuating prices and supply. One of the countries that are most advanced in terms of biofuel production is Brazil where 40% of the country's petroleum requirements are supplied by bioethanol from sugarcane (Goldemberg, 2008).

The second driving force behind government policies for renewable energy is the impact of fossil fuel usage on the global climate (Lynd *et al.*, 1991). It is recognized that bioenergy as a renewable energy source could have a potential impact on greenhouse gas emissions (Champagne, 2007; Goldemberg, 2008; United Nations, 2008).

At present, the US Department of Energy Office of the Biomass Program has set a target for biofuel production in the US to supply 60 billion gallons per year by 2030. This amounts to 30% of liquid fuel consumption for vehicles (at 2004 levels) (Himmel *et al.*, 2007). The European Union has also set a target for biofuel production by 2030 which amounts to 25% of transportation fuel requirements (Himmel *et al.*, 2007).

1.1.2 Liquid transportation fuel alternatives

While fossil fuels supply energy for heat, electricity and liquid transportation fuels, this review only focuses on biofuel as a replacement for liquid transportation fuels. About 97%

of current world requirements for liquid transportation fuel are derived from petroleum (Mielenz, 2001). The main biofuels with the potential to replace petroleum are biodiesel, bioethanol or biobutanol. Biodiesel is the production of fuel diesel from plant oils and this is done via a chemical transesterification process although some enzymatic processes are also used (Antczak *et al.*, 2009; Basha *et al.*, 2009; Sharma & Singh, 2009). Bioethanol refers to the bioconversion of sugars from plant sources to ethanol via fermentation processes. Biobutanol has been put forward as an alternative to bioethanol and has several advantages over bioethanol (Ezeji *et al.*, 2007; Wackett, 2008). It can be fermented by naturally occurring solventogenic clostridia such as *C. acetobutylicum* and *C. beijerinckii* (Montoya *et al.*, 2000; Qureshi *et al.*, 2006).

1.1.3 First generation biofuel, food security and environmental concerns

First generation biofuels refer to the production of biofuel from crops that are also used as food, e.g. sugar cane, sugar beet, maize, palm oil, oilseeds (United Nations, 2008). For bioethanol, the sugar is extracted and directly fermented into ethanol, or the starch in maize/corn is converted into sugars and then fermented into ethanol. The technology for this process, as well as for biodiesel production, is well-established and successful on a large scale. However, the use of food crops for this purpose has become an issue of great concern as it threatens food security worldwide.

In the USA the use of corn on a large scale in bioethanol production has had a significant impact on the price of corn and thus the price of food (Gomez *et al.*, 2008). According to the United Nations, food prices are currently the highest since the 1970's (United Nations, 2008). Although many factors influence prices, one of the direct causes is recognised as the use of food crops for biofuel production (United Nations, 2008).

Demands for the utilisation of maize for biofuel production have also been a hotly debated issue in the South African context (http://www.engineeringnews.co.za/article/maize-exclusion-039defies-logic039-rethink-possible-2008-01-18, date of access 31/08/2009, see Appendix 6b). Although the South African government approved a biofuel strategy for the

country at the end of 2007, maize was excluded from the first phase (2008-2013) (Statement by Minister of Agriculture on World Food Day, 16 October 2008).

Apart from food security issues, it is also questioned whether producing bioethanol from maize would in fact reduce carbon emissions as crops such as maize require a high input of fertilizers derived from fossil fuels (Gomez *et al.*, 2008). In fact, it is becoming clear that the production of biofuel per se does not necessarily result in an environmental benefit (Cockerill & Martin, 2008). For example, if forest areas are cleared to grow palm oil as happens in Malaysia, any benefit derived from greenhouse gas savings may be offset by the increased emissions due to the change in land use (United Nations, 2008).

Thus it is clear that first generation biofuel technologies can be problematic and is likely to be unsustainable for large scale production of biofuel as world population grows and food demands increase. In this respect there is an ongoing debate regarding the benefits of first generation versus second generation technologies (Cockerill & Martin, 2008; Moore, 2008).

1.1.4 Second generation biofuels

Second generation biofuels refer to the production of bioethanol from plant biomass and can provide a solution to many of the problems currently facing first generation biofuel technologies.

Since lignocellulose contains about 75% polysaccharide sugars it can be a valuable feedstock for production of bioethanol (Gomez *et al.*, 2008; Lynd *et al.*, 1991). Lignocellulose in plant biomass can be obtained from various sources, such as agricultural waste, wood, grass and even dedicated crops such as miscanthus or switchgrass (Champagne, 2007; Duff & Murray, 1996; Gomez *et al.*, 2008; Jasinskas *et al.*, 2008; United Nations, 2008). These are not food crops and thus will not threaten food security while at the same time dedicated crops such as switchgrass can be cultured on marginal soils with limited fertilizer. As lignocellulose is the most abundant biological material on earth, it can increase the volume of available feedstock for biofuel production. According to Schwarz (2001), net biomass production worldwide is estimated to be 60 x 10^{12} tons per year in terrestrial and 53 x 10^{12} tons per year in marine

ecosystems. Thus Bayer *et al.* (2007), states that "biomass is the only domestic, sustainable and renewable primary energy resource that can provide liquid transportation fuels". It is estimated that the United States could potentially produce 1.4 billion tons of biomass waste per annum (Bayer *et al.*, 2007). Biomass could also be obtained at a fraction of the cost of existing crops such as maize for biofuel production (Demain *et al.*, 2005).

Although production of biofuel from cellulosic biomass has not been achieved on a commercially sustainable basis, research in this regard is ongoing. The main bottleneck in the use of plant biomass for biofuel remains the saccharification step. Lignocellulose is highly recalcitrant to microbial degradation (Himmel *et al.*, 2007). This relates to the natural defences of the plant against attack, but at a molecular level is based on the crystallinity and insolubility of cellulose which are in turn sheathed in hemicellulose and covalently linked to lignin (Himmel *et al.*, 2007). This structural complexity necessitates a consortium of enzymes required to hydrolyse all the various bonds in the structure.

Himmel *et al.* (2007) identified the slow enzyme kinetics in the hydrolysis of cellulose to sugars, low yields of sugars from other polysaccharides and removal of lignin as three main areas requiring improvement. If these obstacles could be overcome through research, biomass is able to provide the solution for provision of an alternative source of liquid transportation fuel. The enzymatic hydrolysis of biomass is the focus of this work and will be addressed in further detail. Removal of lignin is generally achieved through pretreatment of biomass via mechanical or chemical means such as acid hydrolysis, steam treatment or alkaline treatment which renders the biomass more susceptible to enzymatic degradation (Galbe & Zacchi, 2007; Himmel *et al.*, 2007; Lynd *et al.*, 2002; Mielenz, 2001). Delignification can also be achieved by using enzymes such as lignin peroxidase, manganese peroxidase and laccase which can be derived from fungi. Pretreatment may also be done to increase the specific surface area and pore volume, thus improving the accessibility of the material for enzymes (Walker & Wilson, 1991).

A further aspect is the utilisation of all sugars, both hexose and pentose, in fermentation as the main organism utilised for ethanol production, *Saccharomyces cerevisiae*(bakers yeast), is only able to utilise glucose for fermentation (Himmel *et al.*, 2007). While the main component of plant biomass is cellulose, consisting of glucose monomers, a large component

is hemicellulose with a variety of pentose sugars such as xylose and arabinose, and hexose sugars such as mannose and galactose. The ability to utilize all these sugars for fermentation would greatly enhance the productivity and efficiency of the conversion process. One line of research has been genetic modifications of *S. cerevisiae* strains to express and degrade cellulose and hemicellulose and subsequent utilisation of the products for fermentation (Becker & Boles, 2003; Ho *et al.*, 1999; Jeffries, 2006; Katahira *et al.*, 2004, Kuyper *et al.*, 2005). An alternative approach is the utilisation of Gram-negative bacteria such as *Escherichia coli*, *Klebsiella oxytoca* and *Zymomonas mobilis* (Dien *et al.*, 2003; Fortman *et al.*, 2008). *E. coli* and *K. oxytoca* are able to use a variety of sugars and research has focused on engineering these organisms to produce ethanol. On the other hand, *Z. mobilis* can produce ethanol, but only from glucose or fructose. Research has thus focused on engineering this organism to utilise arabinose and xylose as well (See reviews in Dien *et al.*, 2003; Fortman *et al.*, 2008).

A noteworthy observation in biofuel production, is that depolymerisation of cellulose is not the only rate limiting step, as downstream metabolism of soluble sugars by microorganisms into bioethanol has also been found to become a limitation under certain conditions (Desvaux, 2006).

1.2 The structure of lignocellulosic biomass and the enzymes required for its degradation

1.2.1 Introduction

In order to overcome the obstacles in degradation of lignocellulosic biomass, the chemical structure of lignocellulose has to be understood and the reasons for the difficulty experienced in achieving the hydrolysis thereof.

Plant cell walls consist mainly of polysaccharides with cellulose microfibrils embedded in a matrix of hemicellulose and pectin (Raven *et al.*, 1999). The principal framework of the cell is cellulose, consisting of chains of glucose in microfibrils. Hemicellulose is more varied in

structure and composition than cellulose and includes xylan, mannan, galactan and arabinan polymers (Beg *et al.*, 2001). The specific classification of hemicellulose is dependent on the sugar moieties present within a hemicellulose fraction such as D-xylose, D-mannose, D-galactose and L-arabinose (Beg *et al.*, 2001).

Pectin, a hydrophilic polysaccharide, forms part of the intracellular network and imparts pliability to the cell through its association with water (Raven *et al.*, 1999). Older and more woody plants also contain high levels of lignin deposited in cell walls to give rigidity and strength, and makes cell walls waterproof and provides effective protection against pathogens (Raven *et al.*, 1999). Our interest is focused on the polysaccharide component of plant cell walls and the extent to which they can be utilised for saccharification and fermentation into bioethanol. The importance of other structural components such as lignin lies in the degree to which they prevent access to enzymes and therefore degradation of plant cell wall polysaccharides.

As second generation biofuels rely on obtaining sugars from complex biomass substrates or waste, it is important to understand the composition of some of these substrates. Table 1.1 below gives the composition of some lignocellulose materials that are considered for saccharification.

Table 1.1. Approximate composition (as a percentage) of various biomass materials or agricultural waste products that could potentially be used for saccharification for biofuel production.

Biomass	**Cellulose %**	**Hemicellulose %**	**Lignin %**	**Reference**
Bermuda grass	47.8	13.3	19.4	Li *et al.*, 2009a
Reed	39.5	29.8	24	Li *et al.*, 2009a
Rapeseed stover	27.6	20.2	18.3	Li *et al.*, 2009a
Willow	49.3	14.1	20	Bridgeman *et al.*, 2008
Wheat straw	41.3	30.8	7.7	Bridgeman *et al.*, 2008
Reed canary straw	42.6	29.7	7.6	Bridgeman *et al.*, 2008
Corn cobs	35-39	38-42	4.5-6.6	Okeke and Obi, 1994
Rice hulls	24-29	12-14	11-13	Okeke and Obi, 1994
Melon shells	35	19	30	Okeke and Obi, 1994
Corn stover	39	19.1	15.1	Lee, 1997
Wheat straw	36.6	24.8	14.5	Lee, 1997
Rice straw	41	21.5	9.9	Lee, 1997
Rice hulls	36.1	19.7	19.4	Lee, 1997
Bagasse	38.1	26.9	18.4	Lee, 1997
Newsprint	64.4	21.7	21	Lee, 1997
Populus tristis	40	23	20	Lee, 1997
Douglas fir	50	17.8	28.3	Lee, 1997

The percentage composition of a substrate may vary depending on the exact plant species used and the time of harvest. Furthermore, the higher the lignin content, the more pretreatment will most likely be required in order to make the material accessible to bioconversion. As the lignin itself cannot be used for saccharification, high lignin content also lowers the saccharification potential of the material.

1.2.2 The composition of cellulose

Cellulose has a simple chemical composition and consists of D-glucose residues linked by β-1,4-glycosidic bonds to form linear polymer chains (Carpita, 1996; Raven *et al.*, 1999; Teeri,

1997). The structural subunit of cellulose is cellobiose, formed by two adjacent glucose residues as shown in figure 1.1 below (Schwarz, 2001). Due to the glucose residues being in a chair conformation, hydroxyl groups are forced into radial orientation while aliphatic hydrogen atoms are found in axial positions, causing strong hydrogen bonding to take place between adjacent cellulose chains (Himmel *et al.*, 2007). Further, weaker, hydrophobic interactions take place between cellulose sheets (Himmel *et al.*, 2007).

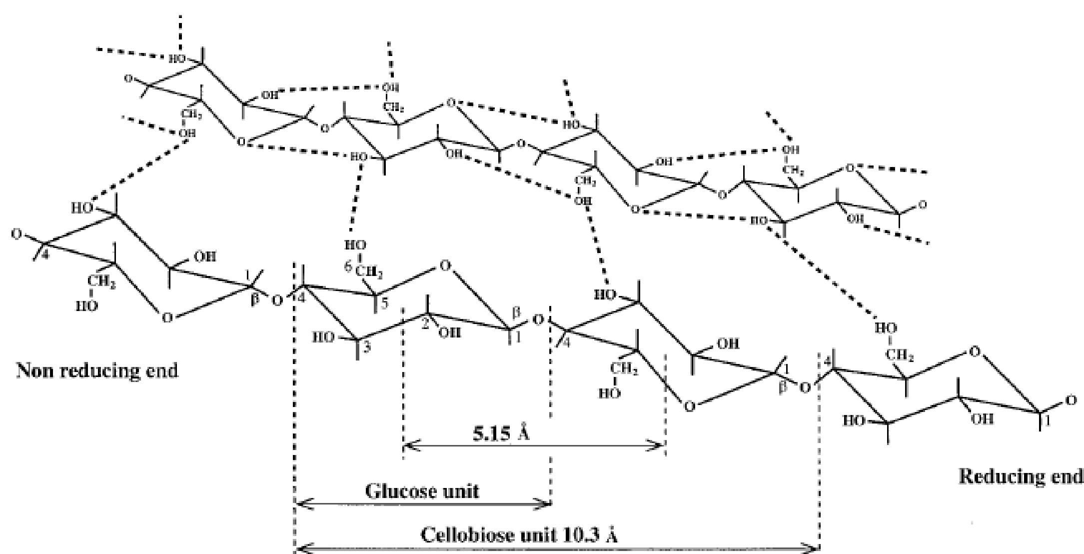


Figure 1.1 Chains of cellulose demonstrating the subunits and the potential hydrogen bonding as well as Van der Waals forces that connect adjacent chains (From Hilden & Johansen, 2004)

In flowering plants, about 36 cellulose chains are joined together to form microfibrils that coil around cells. Chains consist of several thousand glucose residues but may have endings at various places within a microfibril and microfibrils themselves overlap within the larger structure (Carpita, 1996). Thus, although chemically homogenous, the structure of cellulose is not uniform. The structure of these chains is generally highly crystalline, but also form amorphous or paracrystalline regions and chains overlap to form chain ends in any part of the crystalline region. Chains may also be twisted and disordered, presenting as imperfections in the crystalline structure (Teeri, 1997). Cellulose crystal structure itself is described as having two distinct allomorphs, I_α and I_β phases, where I_α represents the less dense region that is more reactive to chemical or enzymatic degradation and I_β the more dense region (Bayer *et al.*, 1998a, Boisset *et al.*, 1999). Every source of crystalline cellulose will contain a distinct ratio of I_α and I_β phases which will affect its susceptibility to degradation (Boisset *et al.*, 1999).

To study cellulose hydrolysis, commercial cellulose is often used, for example Avicel, a crystalline cellulose, or carboxymethyl cellulose (CMC), a low viscosity soluble cellulose. Zhang *et al.* (2006) provided an an overview of different commercially available preparations that can be used for detection of cellulase activity (See also Teeri, 1997).

1.2.3 Enzymes required to degrade cellulose

The enzymes required to degrade cellulose are generally termed cellulases. It has generally been considered that three enzymes are required to hydrolyse cellulose into glucose monomers, namely exo-glucanases (also termed cellobiohydrolyases, exo-1,4-β-glucanases, EC 3.2.1.91), endo-glucanases (also termed endo-1,4-β-glucanases, EC 3.2.1.4) and cellobiases (also termed β-glucosidases, EC 3.2.1.21). However, this model is perhaps over-simplified as it assumes that all exo-glucanases and endo-glucanases are identical. This is not the case and organisms that degrade cellulose effectively actually produce several exo-glucanases and endo-glucanases with different specificities that are required to act in synergy before degradation is achieved (Schwarz, 2001). Hydrolysis of crystalline substrates also requires the presence of specialised carbohydrate binding modules (CBM) for significant enzyme activity (Schwarz, 2001). And not all organisms with cellulolytic capability will possess all three of these enzymes. The cellulase system is thus far more complex than originally proposed.

Synergy between enzymes is important and the main forms of synergy that has been identified in cellulase systems have been between different exo-glucanases, between endo and exo-glucanases, between exo-glucanases and β-glucosidases and lastly, the synergy observed internally between the CBM and the catalytic site (Lynd *et al.*, 2002).

Cellulases are all classified as glycosyl hydrolases according to the classification of Henrissat (Henrissat, 1991; Henrissat & Bairoch, 1993). The mechanism by which glycosyl hydrolases function is a general acid catalysis mechanism in which “two amino acid residues participate in a single-displacement or double-displacement reaction resulting in inversion or retention of configuration at the anomeric carbon atom of the hydrolysed glycoside” (Henrissat, 1991;

Davies & Henrissat, 1995). Apart from the IUB enzyme nomenclature (EC classification), cellulases are therefore also grouped into families according to their amino acid sequence similarity which can be accessed on the Carbohydrate Active Enzyme database (CAZY) (http://www.cazy.org/fam/acc_GH.html).

1.2.3.1 Exo-1,4-β-glucanases (EC 3.2.1.91)

Exo-glucanases have generally been considered the key component in degradation of crystalline cellulose and are said to be the only enzymes that are able to effectively degrade crystalline cellulose (Klyosov, 1990; Schulein, 2000). Exo-glucanases act in a processive manner to attack the cellulose chain from the ends of each chain, produce cellobiose as main product and decrease the DP of the substrate very slowly (Teeri, 1997). Because these enzymes are able to attack cellulose chains from the ends, they are not hampered by the crystallinity of the substrate. However, the exact mechanism by which crystalline cellulose is degraded is not completely elucidated (Rabinovich *et al.*, 2002). In general, cellulases with high activity on crystalline cellulose have been classified as exo-glucanases. However, the classification may not always be that simple as some endo-glucanases may display activity on crystalline cellulose.

Two different types of exo-glucanases or cellobiohydrolases are found that appear to have specificity for opposite ends of the cellulose chain, i.e. reducing and non-reducing ends. These can work in synergy to achieve overall degradation of crystalline cellulose (Teeri, 1997). Figure 1.2 illustrates the action of cellobiohydrolase I and II, together with endo-glucanases as a proposed model of degradation.

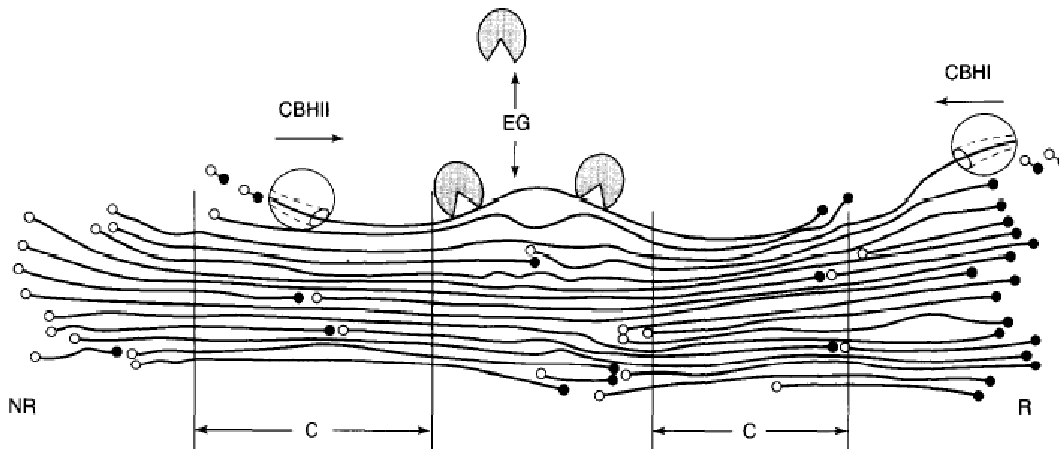


Figure 1.2. Representation of crystalline cellulose demonstrating the mode of attack by endo- and exo-glucanases. CBHI and CBHII represent the exo-glucanases while EG represents the endo-glucanases. CBHI attacks from the reducing end(R) while CBHII attacks from the non-reducing end (NR). Reducing ends are denoted as (•) while non-reducing ends are denoted as (◦). The crystalline region (C) is preferentially attacked by the exo-glucanases while the endo-glucanase attacks the amorphous region (Figure taken from Teeri, 1997).

Cellobiohydrolases have been isolated from many bacteria and fungi, such as *Trichoderma reesei* and *Cellulomonas fimi*. *T. reesei* produce both types of cellobiohydrolase, while *C. fimi* only produces one type (Teeri, 1997).

The active sites of cellobiohydrolases that attack the chain ends of cellulose, have been shown to form a tunnel shape enclosed by two or four loops (Teeri, 1997). This shape is different from the endo-glucanases that have a more open active site, allowing it to bind to the middle of cellulose chains (Teeri, 1997). Teeri indicates that it is thought that the cellobiohydrolase binds to a cellulose chain with six to ten binding sites within the tunnel. The one type of cellobiohydrolase has a shorter active site tunnel than the other. Cellobiose is cleaved off and released but the cellulose chain remains within the active site and is processively cleaved and shortened (Teeri, 1997). Through retaining the cellulose chain within the active site tunnel, hydrogen bonding with the remaining crystal structure is disrupted and the cellulose chain is prevented from readhering to the crystalline cellulose. Cellobiohydrolases with a shorter tunnel may exhibit endo-glucanase activity. Some endo-glucanases may also display a processive mode of attack (Teeri, 1997).

1.2.3.2 Endo-1,4-β-glucanases (3.2.1.4)

Endo-glucanases hydrolyse the β-1,4-glycosidic bonds in cellulose chains through cleaving bonds along the length of the chain, thereby decreasing the degree of polymerisation (DP) of the substrate. In soluble cellulose preparations, this will also lead to a decrease in viscosity (Teeri, 1997). In crystalline cellulose, the cellulose chains can remain associated with the rest of the crystalline cellulose after cleavage and therefore few soluble products may be observed (Teeri, 1997). Endo-glucanase activity is often measured as CMCase activity as CMC is a soluble, amorphous cellulose. Endo-glucanases do not specifically form cellobiose as a product. They cooperate with exo-glucanases in crystalline cellulose degradation as they are thought to cleave cellulose chains at random, often in amorphous regions, thereby creating new chain ends for the action of exo-glucanases (Teeri, 1997).

1.2.3.3 β-glucosidases (EC 3.2.1.21)

β-glucosidases are significant enzymes in a cellulase system as they hydrolyse the short cellooligosaccharides and cellobiose into glucose monomers to achieve the complete saccharification of cellulose (Wood & Bhat, 1988). These enzymes have been found in a variety of sources such as human tissue, plants, fungi and bacteria (Li & Lee, 1999). They are generally divided into two families, 1 and 3 (Henrissat, 1991; Henrissat & Bairoch, 1993), based on amino acid sequence comparison. β-glucosidases also have transferase activity and can form dimers, trimers and higher oligosaccharides from glucose (Hakulinen *et al.*, 2000; Wood & Bhat, 1988). They play an important role to prevent inhibition of other cellulases by cellobiose (Wood & Bhat, 1988). While it is generally considered that β-glucosidases do not have activity on cellulose, Gundlapalli *et al.* (2007) demonstrated that the joining of a cellulose binding domain to a β-glucosidase increased its activity on crystalline and soluble cellulose. β-glucosidases may have activity on a wide range of substrates, such as cellobiose, 4-nitrophenol-beta-D-glucopyranoside, 4-nitrophenol-beta-D-fucoside, 4-nitrophenyl-beta-D-galactoside (Dion *et al.*, 1999), cellotriose, cellotetraose, gentiobiose (McCleary & Harrington, 1988), lactose (Park *et al.*, 2005), laminarin and lichenan (Yang *et al.*, 2008).

1.2.4 The composition of xylan

The most abundant hemicellulose in nature is xylan, containing mainly β-D-xylopyranosyl residues linked by 1,4-glycosidic bonds (Beg *et al.* 2001; Koukiekolo *et al.* 2005). In plants, the xylan forms an overlying layer through hydrogen bonding with the cellulose, while covalently linked with lignin which forms an outside sheath to protect the plant (Beg *et al.* 2001). Xylan forms an important part of plant cell walls, forming 30-35% of total dry weight, although the exact abundance of the xylan may differ between plants (Beg *et al.* 2001). Homoxylans, comprised solely from xylose residues, are not common in nature and have only been isolated from a few plants. The most abundant form is heteroxylan, which is comprised of xylose residues in the backbone with acetyl, arabinosyl and glucuronosyl residues as substituents (Sunna & Antranikian, 1997).

In hardwood plants, the xylan differs from that in softwood plants and occurs as *0*-acetyl-4-*0*-methylglucuronoxylan and is highly acetylated (Sunna & Antranikian, 1997). They have at least 70 xylopyranosyl residues with an average degree of polymerisation (DP) of 150 to 200 (Sunna & Antranikian, 1997). The acetyl groups are responsible for xylan solubility in water, but are removed through alkali extraction (Beg *et al.,* 2001). Softwood xylans are not acetylated and contain α-L-arabinofuranose linked by α-1,3-glycosidic bonds at the C-3 position (Sunna & Antranikian, 1997). Softwood xylan chains are shorter than hardwood chains with an average DP of 70 to 130 and are also less branched (Sunna & Antranikian, 1997). Xylan does not only occur in woody plants, but also in annuals such as maize and sugar cane where it can constitute up to 30% (Prade, 1995).

Based on an analysis of the carbohydrate composition of different commercially available xylans it is apparent that the term “xylan” does not refer to a homogeneous substrate. Li *et al.* (2000) reported that birchwood xylan, for instance, contained 94.1% xylose residues, while oat spelt xylan only contained 52.5% xylose residues with 22.3% arabinose and 15.7% glucose. Larchwood xylan, on the other hand, contained 47.5% xylose, 26.5% glucose and 26% mannose residues (Li *et al.,* 2000). They were able to show that different xylanases had different activities against the commercial xylans, based on the chain length and the degree of substitution (Li *et al.,* 2000). The substituent groups can be a limiting factor in achieving

complete and efficient hydrolysis of a substrate as these groups often present a steric hindrance which affects the access of enzymes to cleavage sites (Sunna & Antranikian, 1997). However, if enzymes capable of removing these substituents are present, the synergy between the enzymes can result in effective degradation of the substrate (De Vries *et al.*, 2000).

1.2.5 Enzymes required to degrade xylan

Due to the complexity of xylan, several enzymes are required to work in synergy in order to degrade this carbohydrate into monomeric sugars. Figure 1.3 illustrates the various chemical bonds that require specialised enzymes to hydrolyse and the main enzymes that are involved. However, degradation of these substrates may be more complex than this model suggests. For example, some endo-xylanases can possess an additional domain for cleaving acetyl groups from the main backbone (Fernandes *et al.*, 1999).

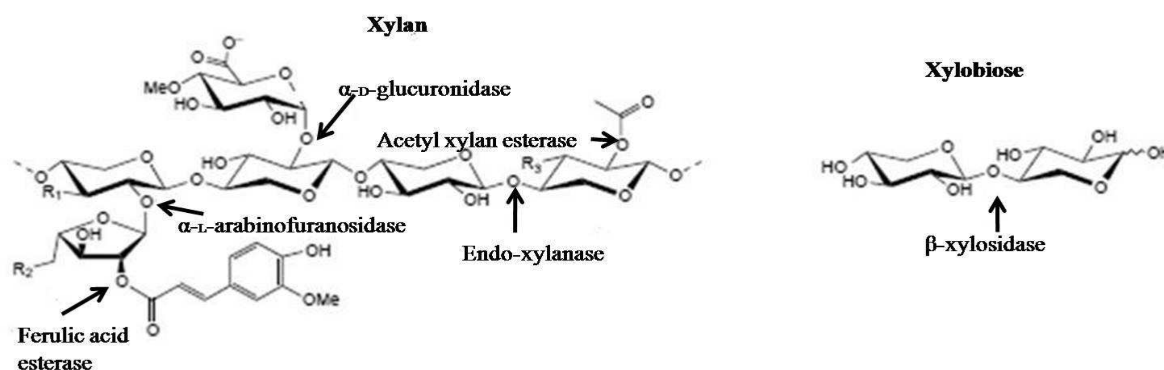


Figure 1.3. Model structure of xylan, illustrating some of the main bonds and the enzymes required to cleave them (Figure taken from Shallom & Shoham, 2003).

1.2.5.1 Endo-1,4-β-xylanases (EC 3.2.1.8)

Xylanases occur very widely and have been reported for a range of organisms such as bacteria, fungi, algae, protozoa, snails, crustaceans, insects and seeds of terrestrial plants (Sunna & Antranikian, 1997). Both β-1,3 and β-1,4-endo-xylanases are found but β-1,4-endo-xylanase is the main enzyme required to cleave the backbone in most xylans (Sunna & Antranikian, 1997). Endo-xylanases are also classified as glycosyl hydrolases and use an acid-base catalysis mechanism in cleaving substrates.

Most xylanases are found in two families, namely family 10 and 11, however, some xylanases also fall within families 5, 8 and 43 (Kolenova *et al.*, 2006). Family 10 and 11 xylanases show distinct differences in properties such as molecular mass, isoelectric point, three-dimensional structure and catalytic activity (Kolenova *et al.*, 2006). Family 10 xylanases are high molecular weight proteins with a low pI and form oligosaccharides with a low degree of polymerisation (Jeffries, 1996). Family 10 xylanases consist of a carbohydrate binding domain and a catalytic domain with a flexible linker region in between (Torronen & Rouvinen, 1997). They appear to be more versatile with lower substrate specificity than family 11 xylanases (Biely *et al.*, 1997). According to Biely *et al.* (1997), family 10 endo-xylanases are capable of cleaving glycosidic linkages in the main chain closer to the substituents and are also capable of releasing smaller products. In some cases the family 10 endo-xylanases also display β-D-xylosidase activity (Biely *et al.*, 1997). The differences in catalytic activity between the two families can most likely be ascribed to their tertiary structure (Biely *et al.*, 1997). Biely *et al.* (1997) describes the differences as a shallower substrate binding site and greater conformational flexibility in family 10 endo-xylanases. Family 10 endo-xylanases are able to hydrolyse β-1,4-glucosidic linkages which family 11 xylanases are unable to do. The catalytic efficiency of these enzymes is, however, much higher against the β-D-xylobioside linkages, for example in *Cellulomonas fimi* where the k_{cat}/K_m was 50 times higher (Biely *et al.*, 1997).

Family 11 xylanases are generally low molecular weight proteins with high pI (Jeffries, 1996). Their active site is situated in a deeper cleft than family 10 xylanases and they have higher substrate specificity. According to Subramaniyan and Prema (2002), family 11 xylanases require three unsubstituted xylopyranose residues next to a branching point for activity.

Many xylanases are reported to form xylobiose, even as the exclusive product of hydrolysis (Jeffries, 1996). A xylanase from *Aeromonas* has, however, been reported to only form xylotetraose (Jeffries, 1996).

According to Jeffries (1996), xylanases possess three to five subsites for binding the xylopyranose ring which are situated near the catalytic site. These sites are characterised by the presence of tyrosine which present a hydrophobic surface that interacts with the aromatic

chains (Jeffries, 1996). This is compared to the tryptophan residue found in substrate binding domains in other glycosidases.

Not all xylanases possess a substrate-specific xylan binding domain (XBD) (Jeffries, 1996). A few xylanases, however, have specific XBDs as well as cellulose binding domains (CBDs), such as found in xylanases in *Thermonospora fusca, Cellulomonas fimi* and *Streptomyces thermoviolaceus* and arabinofuranosidase from *Streptomyces lividans* (Ratanakhanokchai *et al.*, 1999). Some xylanases have only cellulose binding domains, such as XynA from *Clostridium stercorarium* (Araki *et al.*, 2004; Sun *et al.*, 1998).

Many microorganisms produce several xylanases that appear to act in synergy to degrade substrates (Beg *et al.*, 2001; Wong *et al.*, 1988). The presence of different xylanases within an organism suggests different functions that allow the enzymes to improve degradation of the substrate through synergy. As many of these xylanases have different pH and temperature optima, their functionality may also be related to their being able to degrade substrates under differing conditions (Wong *et al.*, 1988).

Cross-specificity occurs where an enzyme has activity on two distinct substrates. This has been reported for a cellulase from *Trichoderma viride* which has activity on both carboxymethylcellulose and xylan (Wong *et al.*, 1988). Some β-glycosidases have wide specificity and display β-xylosidase activity (Wong *et al.*, 1988).

1.2.5.2 β -D-xylosidase (EC 3.2.1.37)

These are enzymes that hydrolyse short xylo-oligosaccharides and xylobiose from the nonreducing end to produce xylose (Sunna & Antranikian, 1997). True β-xylosidases should have activity on artificial substrates such as ρ-nitrophenyl β-D-xyloside from which it is able to release xylose (Sunna & Antranikian, 1997). β-D-xylosidases are enzymes with high molecular weights between 60 and 360 kDa and may occur as mono- or dimeric proteins (Sunna & Antranikian, 1997). β-xylosidase has been reported to occur both in the cell-associated fraction and extracellularly (Sunna & Antranikian, 1997). Very few β-xylosidases are able to produce xylose from xylan and the affinity of the enzyme towards

xylooligosaccharides decreases with increasing DP (Sunna & Antranikian, 1997). Most β-xylosidases are inhibited by xylose. Some β-xylosidases display transferase activity and may form products of higher molecular weight than the original substrate while others are reported to also have α-arabinosidase activity, β-glucosidase activity and even activity on laminarin (Saxena *et al.*, 1995; Shao & Wiegel, 1992).

1.2.5.3 Arabinases and arabinofuranosidases

Arabinans are composed of a backbone of α-1,5-arabinofuranosyl residues with further arabinofuranoside residues substituted on this backbone (Shallom & Shoham, 2003). Arabinofuranoside residues also occur as substitutions in some types of xylans, such as oatspelt xylan. There are two types of arabinase enzymes, namely the exo-acting α-L-arabinofuranosidase (EC 3.2.1.55) and the endo-1,5-α-L-arabinase (EC 3.2.1.99). The latter is only active towards linear arabinans while the former has activity towards branched arabinans and synthetic substrates such as ρ-nitrophenyl-α-L-arabinofuranoside (Sunna & Antranikian, 1997). Arabinofuranosidases are important enzymes that exhibit wide substrate specificity and act in synergy with other glycosyl hydrolases to degrade arabinose containing polysaccharides (Numan & Bhosle, 2006).

Native arabinofuranosidases may occur in mono-, di-, tetra-, hexa- and even octameric forms and can have molecular weights of 53 kDa (a monomeric enzyme from *Aspergillus niger*) up to 495 kDa (an octameric enzyme from *Streptomyces purpurascens*) (Sunna & Antranikian, 1997).

Both types of enzymes form arabinose as products upon hydrolysis of arabinose-containing polysaccharides. Some arabinosidase enzymes are very specific for arabinoxylans and release arabinose from the substrate without degrading the xylan backbone (Sunna & Antranikian, 1997).

1.2.5.4 Other

Several esterases and glucuronidases have been identified as contributing to xylan degradation. Acetyl xylan esterases (EC 3.1.1.6) remove the *0*-acetyl substituents at the C-2 and C-3 positions of xylose residues in acetylxylan (Sunna & Antranikian, 1997). Although xylan is highly acetylated, the acetyl groups are generally removed through alkali extraction and these enzymes have not been well-studied. As these acetyl groups prevent the access of xylanases to the backbone through steric hindrance, these enzymes play an important synergistic role with xylanases to promote effective hydrolysis of the substrate (Sunna & Antranikian, 1997).

The ferulic acid esterase cleaves ester linkages between arabinose sidechains and ferulic acids in xylan, while the ρ-coumaric acid esterases cleave the ester linkage between arabinose and ρ-coumaric acid (Sunna & Antranikian, 1997). Their presence contributes to removal of lignin from hemicelluloses by cleaving of ester linkages and plays an important role in disruption of the cell wall structure (Subramaniyan & Prema, 2002).

Another enzyme, α-glucuronidase (EC 3.2.1.1), cleaves glucuronic acid directly from glucuronoxylan to release 4-*O*-methyl-D-glucuronic acid, which exists as side chains on xylan, although several only have activity on substituted xylooligosaccharides, resulting from the activity of endo-xylanases on glucuronoxylan (Biely *et al.,* 2000; Nagy *et al.*, 2002; Sunna & Antranikian, 1997).

1.2.6 The composition of mannan and the major enzymes involved in its degradation

Mannan generally consists of a backbone of 1,4-β-D-mannopyranose residues. Unsubstituted mannans occur in leguminous and non-leguminous seeds and act as a carbohydrate reserve (Hrmova *et al.*, 2006). Other types of mannan are galactomannans, glucomannans and galactoglucomannans. Galactomannans have α-D-galactosyl residues substituted on the main mannan backbone, with the degree of substitution varying between plant species. Glucomannans contain both mannose residues and glucose residues in the backbone at various ratios depending on the plant source. Galactoglucomannans are glucomannans with

galactose substituents on the backbone (Hrmova *et al.*, 2006). Gluco- and galactoglucomannans occur mostly in lignified secondary walls of coniferous gymnosperms (Hrmova *et al.*, 2006). Galactoglucomannan can make up to 25% of the polysaccharide composition of softwood (Hägglund, 2002). Even where mannan is only a minor component in cell walls, it is tightly bound to cellulose microfibrils and thus affects the accessibility of cellulases to cellulose (Carpita, 1996). Several enzymes are involved in the degradation of mannan as illustrated in Figure 1.4.

The main enzyme involved in mannan hydrolysis is β-mannanase (EC 3.2.1.78) which liberates short oligomers, including mannobiose. The short oligomers are further hydrolysed into mannose monomers by β-mannosidases (EC 3.2.1.25) (Shallom & Shoham, 2003). β-mannosidases can also release mannose monomers from mannan polysaccharides. β-mannosidases are also found in animals such as ruminants and humans where they play a role in lysosomal degradation of glycoproteins. Deficiency of this enzyme can lead to skeletal abnormalities and abnormal mental development (Shallom & Shoham, 2003). Other enzymes involved in mannan degradation are α-galactosidase to remove galactose substituents, acetyl-mannan esterase to remove acetyl groups and β-glucosidase to remove glucose from the backbone (Shallom & Shoham, 2003).

Mannanases may possess cellulose binding domains or specific mannan binding domains (Cann *et al.*, 1999; Halstead *et al.*, 1999; Ximenes *et al*., 2004).

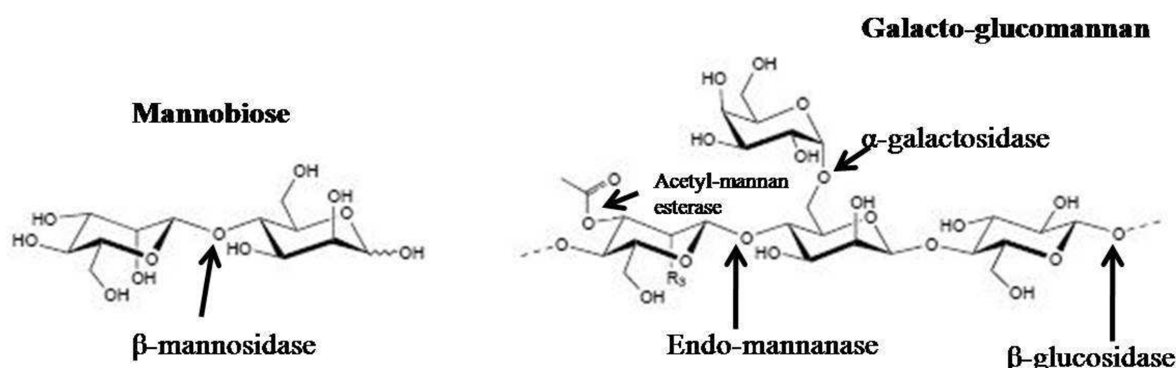


Figure 1.4. Galacto-glucomannan and mannobiose and the main enzymes required to cleave major bonds (Figure taken from Shallom & Shoham, 2003).

1.2.7 The composition of pectin and the main enzymes involved in its degradation

Pectins are highly hydrophilic polysaccharides and form a separate component of plant cells. The "smooth region" consists of homogalacturonan which is a backbone of D-galacturonic acid residues with α-1,4-linkages and can be methylated at the O-6 position. The "hairy region" consists of rhamnogalacturonan which occur as two types. Type I rhamnogalacturonan has the galacturonic acid backbone with substitutions of α-1,2 linked rhamnopyranose residues and galactose and arabinose sidechains. Type II rhamnogalacturonan has more complex sidechains on the galacturonic acid backbone, consisting of L-rhamnose, D-xylose, L-fucose and D-galactose (Yadav *et al.*, 2009).

Many enzymes are involved in degrading pectin. The classification according to Jayani *et al.* (2005) reveal enzymes with eight different EC classifications in two main groups, the pectin methyl esterases and the depolymerising enzymes. The pectin methyl esterases play an important role to remove methyl groups from pectin so that the depolymerising enzymes can act upon the resulting product. The depolymerising enzymes are divided into two groups based on their mechanism of reaction, namely hydrolases or lyases, both groups which effect the cleavage of large pectin molecules into oligo-, di- or monogalacturonates (Jayani *et al.*, 2005). Polygalacturonase, pectate lyase or pectinase are all terms that refer to enzymes able to depolymerise pectin or pectic acid and are often used interchangeably unless the specific mechanism of the enzyme is known. Polygalacturonases catalyse the hydrolytic cleavage between two galacturonic acid residues by the introduction of water. The lyase mechanism cleaves linkages between two galacturonic acids through a β-elimination reaction (Jayani *et al.*, 2005). A further distinction is drawn between pectin and pectate lyases. Pectin lyases are more specific on methyl esterified substrates such as pectin while pectate lyases refer to specificity on unesterified substrates, namely pectate or polygalacturonic acid (Soriano *et al.*, 2005). It is generally accepted that pectate lyase requires Ca^{2+} for activity while pectin lyase does not, which enables differentiation between these enzymes (Michaud *et al.*, 2003; Soriano *et al.*, 2005). Pectin lyases generally occur in fungi while pectate lyases are predominant in bacteria (Michaud *et al.*, 2003). Further rhamnogalacturonan lyases are responsible for degradation of the "hairy regions" in the pectin (Michaud *et al.*, 2003).

1.2.8 The role of carbohydrate binding modules (CBMs)

There are generally two sites in the enzyme involved in binding: the active, catalytic site and the CBM which are linked through a PTS (proline, threonine, serine) linker region (Schwarz, 2001). CBMs, previously termed cellulose binding domains, are amino acid sequences involved in recognition of and binding to polysaccharides. Such modules are generally always found as part of polysaccharide-degrading enzymes, with the exception of the scaffoldin protein in the cellulosome. CBMs are classified into families based on amino acid sequence similarity and Boraston reported that 39 families of CBM existed (Boraston *et al.,* 2004). However, the website on carbohydrate active enzymes (CAZY) reported 53 families at the time of submission of this work (http://www.cazy.org/fam/acc_CBM.html). CBMs generally show specificity for binding certain substrates.

Within families, CBMs are subclassified where type A CBMs are also termed surface-binding CBMs and bind to insoluble, crystalline substrates such as cellulose or chitin (Boraston *et al.,* 2004). Type B CBMs bind to soluble polysaccharide chains and type C binds to small sugars (Boraston *et al.* 2004; Shoseyov *et al.* 2006). Differentiation and classification of these three types is based on their amino acid sequence as well as their three-dimensional conformation. Although aromatic amino acids are prevalent in CBMs, the type A CBM has a very distinct, planar hydrophobic face formed by these aromatic residues which are able to bind to the planar crystalline surface of crystalline substrates such as cellulose and chitin. The scaffoldin protein CBM is a type 3a CBM with the ideal surface to bind crystalline cellulose (Tormo *et al.*, 1996). Type B CBMs form a groove or cleft which is accessible by a glycan chain (Boraston *et al.,* 2004). The binding affinity for type B CBMs change depending on the degree of polymerisation of the glycan chain and virtually no binding will take place where the DP is three or less (Boraston *et al.,* 2004). The type C CBMs do not have extended grooves to accommodate glycan chains as they bind to mono-, di- or tri-saccharides (Boraston *et al.,* 2004). It is generally considered that hydrogen bonding may play an important role in the binding affinity of type B and C CBMs, but not in type A (Boraston *et al.,* 2004). In some CBMs, metal ions have also been shown to play a role in ligand binding affinity (Boraston *et al.,* 2004).

Boraston has identified three general functions of CBMs (Boraston *et al.,* 2004). The CBM concentrates the enzyme on the surface of the substrate and this is thought to lead to improved degradation of the substrate which has been shown experimentally (Bolam *et al.,* 1998; Boraston *et al.,* 2004). However, it has been shown in some instances that removal of the CBM from an enzyme only affected activity on the substrate where such substrate was insoluble (Boraston *et al.,* 2004). The CBM is also able to cause disruption of cellulose fibres without any corresponding hydrolytic activity (Boraston *et al.,* 2004; Shoseyov *et al.* 2006). However, the ability of a CBM to disrupt a substrate such as crystalline cellulose has only been shown in a few cases and does not apply in general to all CBMs (Boraston *et al.,* 2004; Din *et al.*, 1994; Hilden & Johansson, 2004).

The CBM plays a very important role in substrate binding and targets the enzyme towards specific substrates, conferring selectivity in binding. It was illustrated that where the CBM of an enzyme was changed, the substrate specificity of the enzyme was changed (Araki *et al.*, 2004; Boraston *et al.,* 2004). CBMs have also been shown to selectively bind to certain areas of a polysaccharide, such as the reducing end, allowing the enzyme access to sections that may be preferentially degraded. By targeting different regions of a substrate through different CBMs, the same enzyme may be able to hydrolyse a substrate more efficiently (Boraston *et al.,* 2004).

It has also been shown that some thermophilic organisms have enzymes with more than one CBM. This is thought to allow improved binding at higher temperatures (Boraston *et al.,* 2004). The role of CBMs and their interaction with catalytic domains are still not completely understood. Ali *et al.* (2001) reported a xylanase (Xyn10B) from *Clostridium stercorarium* with three CBMs, two family 22 CBMs and one family 9 CBM as well as two surface layer homology (SLH) domains. This protein, while having xylanase activity, acts to bind both to cell walls and to cellulose, thus anchoring the cells to insoluble substrate (Ali *et al.*, 2001). The family 22 CBMs were shown to give the enzyme activity on β-1,3-1,4-glucan, even though the catalytic domain classified this enzyme as a xylanase (Araki *et al.*, 2004).

1.3 Cellulolytic and hemi-cellulolytic multi-enzyme complexes

1.3.1 The cellulosome

1.3.1.1 Introduction

The cellulosome was first observed in the early 1980s in *Clostridium thermocellum* by Lamed and Bayer (Lamed *et al.*, 1983). Subsequently, such structures have also been described in other bacteria namely *Clostridium cellulovorans* (Doi & Tamaru, 2000), *Clostridium cellulolyticum* (Belaich *et al.*, 1997; Desvaux, 2005), *Clostridium acetobutylicum* (Sabathe *et al.*, 2002), *Clostridium josui* (Kakiuchi *et al.*, 1998), *Clostridium papyrosolvens* (Pohlschroder *et al.*, 1994), *Butyrivibrio fibrisolvens* (Lin & Thomson, 1991), *Acetovibrio cellulolyticus* (Xu *et al.*, 2003), *Bacteroides cellulosolvens* (Xu *et al.*, 2004a), *Ruminococcus albus* (Ohara *et al.*, 2000) and *Ruminococcus flavefaciens* (Kirby *et al.*, 1997) (Bayer *et al.*, 1998a, Doi, 2008). Evidence for cellulosome production has also been found in aerobic bacteria (*Vibrio*) and anaerobic fungi such as *Neocallimastix patriciarum* (Fanutti *et al.*, 1995), *Orpinomyces* (Li *et al.*, 1997) and *Piromyces* sp. strain E2 (Fanutti *et al.*, 1995) (Bayer *et al.*, 1998a; Doi, 2008; Shoham *et al.*, 1999).

The cellulosome is a large extracellular enzyme complex with various enzyme subunits such as cellulases and hemicellulases attached through dockerin amino acid sequences to a non-enzymatic scaffolding protein (Bayer *et al.*, 1998b; Doi *et al.,* 2003). Cohesin domains are present on the scaffolding protein and are the site where the dockerin sequences bind. The complex contains a cellulose binding domain (CBD) or carbohydrate binding module (CBM), present on the scaffolding protein, which binds very strongly to the cellulose substrate, thus facilitating the localized degradation of the substrate by the enzymatic subunits. The entire structure is attached to the cell wall through an anchoring protein, such as in *C. thermocellum* (Bayer *et al.*, 1998b), or through hydrophilic domains present on the scaffoldin as found in *C. cellulovorans* (Doi & Tamaru, 2000). Analysis of cellulosomes found in various organisms revealed flexibility in structure which does not always conform to this model. *A. cellulolyticus*, for instance, contains three different scaffolding proteins which allow

formation of a complex structure with as many as 96 enzymes (Xu *et al.*, 2003). The scaffolding protein in this organism also contains a catalytic subunit (Ding *et al.*, 1999).

1.3.1.2 Efficiency of the cellulosome vs free extracellular enzymes

Cellulosomal complexes have evoked great scientific and commercial interest after it was ascertained that the cellulosome from *C. thermocellum* had 50 times the specific activity on cotton compared to free cellulases from *Trichoderma reesei* (Johnson *et al.*, 1982). Thus it appeared as though the presence of enzymes within the cellulosome complex provided an advantage to the organism.

Schwarz proposed four reasons why the cellulosome complex conferred an advantage to an organism in degradation of cellulose (Schwarz, 2001):

- Synergy can take place because enzymes are present in the correct ratio;
- “Non-productive adsorption “is avoided as enzyme components are spaced in an optimal fashion.
- Competitive binding is avoided as the non-enzymatic component contains a strong binding domain and not the individual enzymes.
- The presence of different enzymes ensures that complex substrates can be degraded (Schwarz, 2001).

The combination of enzymes as well as the role of the non-catalytic scaffolding protein with specialised carbohydrate binding modules ensures that the cellulosome operates in a different manner which makes it more efficient and highly effective, particularly when acting on complex or crystalline substrates. Boisset *et al.* (1999) was able to demonstrate that the cellulosome degraded crystalline cellulose in a completely different manner than free enzymes of the same type.

Having enzymes within a complex imparts a further advantage to microorganisms, specifically in anaerobic environments. When microorganisms grow on large substrates such as polysaccharides, they are forced to secrete extracellular enzymes into the medium as they

are unable to take large substrates up in the cell. Where cells are present at low concentrations, such enzymes could diffuse away from the cell, resulting in product formation that is inaccessible to the cell, thus failing to support growth. By having the extracellular enzymes in a complex at the cell interface, such as the cellulosome, cells can overcome this problem (Shoham *et al.*, 1999).

1.3.1.3 Cohesin domains

The cohesin domains are the hydrophobic amino acid sequences present on the scaffolding proteins of cellulosomes and are the sites to which cellulosomal enzymes bind via their dockerin sequences. As the cohesins are the binding sites for cellulosomal enzymes, information on these domains will greatly contribute towards an understanding of cellulosome assembly and its regulation.

The number of cohesins present on a scaffolding protein vary from organism to organism, with the smallest number of cohesins found in *C. acetobutylicum* (5) and the largest number found in *Bacteroides cellulosolvens* (11) (Doi *et al.*, 2003). Cohesin domains on a single scaffolding protein in one organism are not identical, although there is a high level of homology and conservation (Desvaux, 2005). Cohesins from *C. thermocellum* and *C. cellulolyticum* have a higher level of homology between cohesins than in *C. cellulovorans* (Park *et al.*, 2001). Cohesin domains between scaffolding proteins from two organisms demonstrate some conservation but with a lower identity. According to Doi *et al.* (1998), the cohesins of CbpA (*C. cellulovorans*) and CipA (*C. thermocellum*) have only 25-36% identity. Although there is conservation between cohesins from different species, the binding is highly specific inter-species and cohesins from one organism do not interact with dockerins from another species (Desvaux, 2005; Schwarz, 2001).

There are three, perhaps four types of cohesins that have been identified. Type I cohesins are found in mesophilic clostridia, *C. thermocellum* and *A. cellulolyticus* (ScaA and ScaD). Type II cohesins are found in the *C. thermocellum* anchoring proteins, *B. cellulosolvens*, and *A. cellulolyticus* (ScaB and ScaD), while type III are found only in *R. flavefaciens*. A possible type IV was found in *A. cellulolyticus* ScaC (Xu *et al.*, 2003). The crystal structure of a Type

I cohesin domain from *C. thermocellum* has been solved and consists of a nine-stranded β-sandwich with jelly-roll topology (Bayer *et al.*, 1998; Shimon *et al.*, 1997; Tavares *et al.*, 1997).

It appears that in the case of *C. thermocellum* and *C. cellulolyticum*, binding of cellulosomal enzymes may occur equally well with all the cohesins within that organism (Pagès *et al.*, 1999; Yaron *et al.*, 1995). However, this is not the case for all cellulosomes. It was demonstrated that cohesins from *C. cellulovorans* do not all have the same affinity for dockerins of cellulosomal enzymes (Doi and Tamaru, 2000; Park *et al.*, 2001). It was also found that in *C. cellulovorans* cohesins could bind each other and thus it is suspected that cellulosomes could bind to each other through the scaffolding proteins in order to form large multi-cellulosomes (Doi and Tamaru, 2000). Jindou *et al.* (2004) also found differences in binding affinity of cohesins for different dockerins in *C. josui*.

Although the presence of cohesins is considered to be key evidence for the presence of cellulosomes in an organism, such evidence should be considered against the background of additional information. Cohesins domains were, for instance found in the genome of an archaea, *Archaeoglobus fulgidus*, although this organism does not possess a cellulosome or even identifiable coding sequences for glycosyl hydrolases (Bayer *et al.*, 1999).

1.3.1.4 Dockerin domains

An enzyme is generally classified as cellulosomal when it has a dockerin domain which allows it to bind to the scaffolding protein via a cohesin domain (Bayer *et al.*, 1998). The dockerin domain in bacteria is a sequence of about 70 amino acids within which is found a highly conserved 22 amino acid sequence displaying a characteristic calcium-binding EF-hand motif (Bayer *et al.*, 2004). Dockerin sequences have been identified in fungi which are only 40 amino acid residues but also display a calcium-binding motif (Fanutti *et al.*, 1995). It has been shown that both subdomains of the dockerin were required for binding to the cohesin (Lytle & Wu, 1998).

It has been demonstrated that dockerin binding to cohesins was calcium dependent in *C. thermocellum* (Bayer *et al.*, 1998; Choi & Ljungdahl, 1996) and *C. celluloyticum* (Fierobe *et al.*, 1999; Schwarz, 2001). In fact, calcium also enhanced the activity of the cellulosome in these cases. However, Doi *et al.* (1998) indicated that binding ability of the major exoglucanase (ExgS) in the *C. cellulovorans* cellulosome was not affected by calcium ions or ethylenediaminetetraacetic acid (EDTA).

Not all dockerin domains within a cellulosomal organism are identical. In *R. flavefaciens*, it was demonstrated that dockerins have different sequences in different enzymes and, as a result, displayed differing binding specificities with cohesins present on the scaffolding proteins in this organism (Rincon *et al.*, 2003). Some dockerin-containing enzymes in this organism were unable to bind the cohesins in the main scaffolding protein (ScaA) (Rincon *et al.*, 2003).

1.3.1.5 Cohesin and dockerin interactions /Cellulosome assembly

The manner in which the cellulosome is assembled outside the cellular environment is not clearly understood. It has also not been established whether assembly of catalytic components within the complex is random or regulated. The presence of varied binding specificities between dockerins and cohesins indicate that some enzymes will bind preferentially to cohesins and will thus be more prevalent in the cellulosome, pointing to a regulated assembly. If assembly simply occurred in a random fashion, Bayer *et al.* (1998) speculated that one would expect a hetereogeneous population of cellulosomes to be produced. However, in *C. thermocellum*, where binding affinities of cohesins appear to be similar, the organism produced a homogeneous batch of cellulosomes, contrary to expectations (Bayer *et al.,* 1998).

In *C. cellulovorans*, however, the organism formed four different cellulosomal complexes for each substrate it was cultured on even though binding affinities of cohesins are different (Han *et al.*, 2005). At the same time, some components, such as the scaffolding protein (CbpA), as well as the major endoglucanase (EngE) and exoglucanase (ExgS) were constitutively expressed (Han *et al.*, 2005). It can therefore be questioned whether organisms are able to

regulate this process so that assembly occurs in an organised fashion and how this is accomplished.

Since the scaffolding protein contains a limited number of cohesin sites for enzymes to bind it is of great interest how cellulosome assembly is regulated under circumstances where more enzymes are produced than are able to be incorporated into a complex. In *C. thermocellum*, for instance, the number of enzymes bearing dockerin sequences is double the number of cohesins present on the scaffolding (Shoham *et al.,* 1999).

The question regarding assembly relates to the need for synergy in order to achieve optimal degradation of substrates. Biomass has great complexity and variability and it has been shown that synergy between different enzymes, with different catalytic abilities, as well as non-catalytic modules such as CBMs are required for efficient degradation of such substrates. If an organism is able to degrade different types of biomass, it should be able to express these enzymes in optimal ratios to achieve the required synergy. How it is able to accomplish this, is still a matter under investigation.

1.3.1.6 Scaffolding proteins

The scaffolding protein forms the basis of the cellulosome and the general structure is of a large, glycosylated protein with a CBM domain and several cohesin domains (Bayer *et al.*, 1998b; Doi *et al.*, 2003; Doi, 2008). With the study of different cellulosomes from various organisms, this model had to be reviewed. The *C. thermocellum* scaffolding protein contains, in addition, a dockerin domain which allows it to bind to surface anchoring proteins (Bayer *et al.*, 1998b). The *A. cellulolyticus* scaffolding protein furthermore contains a catalytic domain for a family 9 glycoside hydrolase (Xu *et al.*, 2004a). And some cellulosomal organisms also have more than one scaffolding protein. *A. cellulolyticus*, for instance, possesses four different scaffolding proteins termed ScaA, ScaB, ScaC and ScaD (Xu *et al.*, 2003, Xu *et al.*, 2004b). All these scaffolding proteins can bind together through cohesin-dockerin interactions and to the cell surface to form a superstructure capable of binding up to 96 enzymes (Xu *et al.*, 2003). *R. flavefaciens* also possesses more than one scaffolding protein, ScaA and ScaB, with ScaA acting as the surface anchoring protein (Rincon *et al.*, 2003).

ScaA in *R. flavefaciens*, although the main scaffolding for binding dockerin-containing enzymes, appear to lack a CBM domain, which is unusual in cellulosomal scaffolding proteins (Rincon *et al.*, 2003).

Scaffolding proteins vary quite considerably in size, for example, *C. thermocellum* (CipA) is 196 kDa (Bayer *et al.*, 1998), *C. cellulovorans* (CbpA) is 189 kDa (Shoseyov *et al.*, 1992), *C. cellulolyticum* (CipC) is 158 kDa (Pages *et al.*, 1996) and *C. josui* (CipA) is 120 kDa (Kakiuchi *et al.*, 1998). As the scaffolding proteins are generally highly glycosylated, their electrophoretic mobility falsely project a larger molecular weight as adjudged by SDS-PAGE analysis (Schwarz, 2001). Accurate sizes are therefore based on the gene sequence, rather than the biochemical characterisation. Almost all cellulosomal scaffolding proteins to date have been found with a specific CBMIIIa domain (Shimon *et al.*, 2000; Tormo *et al.*, 1996).

1.3.1.7 Evidence for cellulosome production

In order to accurately identify or classify a multi-enzyme complex as a cellulosome, it is useful to look at the evidence that has been used to prove that a cellulosome exists in an organism. The main structural features of the cellulosome, as indicated above, are the scaffolding protein containing cohesin domains, and the presence of dockerin domains on cellulosomal enzymes. Where genomic or genetic information for an organism exists, it is relatively simple using a bioinformatic analysis to identify the presence of the dockerin and cohesin domains, and concomitantly, the presence of a scaffolding protein. This is ultimately the only accurate way in which the presence of a cellulosome can be determined. However, this evidence alone should also be assessed with caution, as cohesin domains and a dockerin domain were detected in *Archaeoglobus fulgidus* even though the presence of a cellulosome in this organism was excluded (Bayer *et al.*, 1999).

However, where such genomic or genetic information does not exist, it becomes more difficult to determine the presence of a cellulosome. Other evidence that has been considered is the presence of a high molecular weight protein as demonstrated through size exclusion chromatography, reaction with antibodies for CipA from *C. thermocellum*, and the presence of cell protuberances using electron microscopy (Bayer *et al.*, 1998; Schwarz, 2001).

1.3.1.8 Comparison of various cellulosomes

To demonstrate the variability in cellulosomes between different organisms, a brief summary of different cellulosomes are described and discussed below.

(a) C. thermocellum

C. thermocellum is a moderately thermophilic bacterium with an optimum growth temperature of 55- 65°C (Schwarz, 2001). It forms a cellulosome on all cellulose and hemicellulose substrates. The *C. thermocellum* cellulosome may differ in size between strains, varying from 2 MDa to 6.5 MDa (Schwarz, 2001). Aggregation of cellulosomes into polycellulosomes can also take place, forming large structures of up to 100 MDa.

The *C. thermocellum* cellulosome has a large scaffolding protein with 9 homologous cohesin domains. Unlike some of the other scaffolding proteins, the CBMIIIa domain is situated internally and it also contains a type II dockerin domain which serves to anchor the scaffolding protein to the cell surface (Bayer *et al.*, 1998b; Bayer *et al.*, 2004). The anchoring function is mediated by three proteins, SdbA, Orf2P and OlpB that each contains three surface layer homology (SLH) domains at their C-terminus (Bayer *et al.*, 1998b). SLH modules have also been identified in proteins from Gram-positive bacteria where they mediate the noncovalent binding of proteins to the peptidoglycan or cell-surface components (Bayer *et al.*, 1998b). Another protein, OlpA, is similar to the cell-anchoring proteins but contains a Type I cohesin which can bind individual enzymes to the cell wall (Bayer *et al.*, 1998b; Schwarz, 2001).

The *C. thermocellum* cellulosome has two types of dockerin and cohesin domains. Type I dockerin and cohesin domains mediate the binding of the enzyme subunits while Type II cohesin and dockerins mediate the interaction between the scaffolding and the cell surface. Scaffolding proteins from *C. cellulovorans*, *C. cellulolyticum* and *C. josui* do not possess a Type II dockerin domain and while they are also found to be cell-associated, mediation with the cell surface takes place through a different interaction (Bayer *et al.*, 1998b; Doi & Tamaru, 2000).

Unlike *C. cellulovorans*, the cellulosome in *C. thermocellum* also forms when the organism is cultured on cellobiose, although they occur in a resting state on the surface of the cells (as seen by electron micrographs) (Bayer *et al.*, 1998b). When cultured on cellulose, the cellulosomes form protracted structures between the cells and the cellulose (Bayer *et al.*, 1998b).

Many genes for cellulosomal enzymes have been identified in *C. thermocellum*, including five exo-glucanases, nine endo-glucanases, one lichenase, one chitinase, one mannanase and five xylanases (Bayer *et al.*, 1998b; Schwarz, 2001). Xylanases XynY and XynZ also possess modules with feruroyl esterase activity (Schwarz, 2001). The cellulosome is thus able to produce a host of different enzymes to attack complex substrates. Interestingly, *C. thermocellum* is able to degrade xylan and other hemicellulose substrates, but is not able to utilise sugars such as xylose for growth (Shoham *et al.*, 1999).

The cellulosomal complex of *C. thermocellum*, when separated on an SDS-PAGE gel has shown up to 50 protein species although only 18 cellulosomal genes have been described (Bayer *et al.*, 1998b; Schwarz, 2001). However, most characterisation has been achieved by the cloning and expression of the coding sequences of the scaffolding protein and catalytic subunits.

Expression of some of the cellulosomal genes appear to be constitutive, although growth on different substrates appears to change the expression of enzymes and thus the composition of the cellulosome (Shoham *et al.*, 1999).

(b) C. cellulovorans

C. cellulovorans is an anaerobic spore-forming, mesophilic bacterium that was isolated from a wood-chip pile (Doi, 2008). It produces a cellulosome when cultured on cellulose, but not when cultured on glucose or cellobiose (Doi, 2008). Individual subunits are secreted into the extracellular medium when the organism was cultured on cellobiose, but they do not appear to assemble into cellulosomes until cellulose is present in the medium (Matano *et al.*, 1994). The cellulosome is approximately 1,000 kDa in size (Doi, *et al.*, 1998).

The cellulosome has a large non-enzymatic scaffolding protein (CbpA) which contains 9 cohesin domains, capable of binding dockerin domains on the enzyme subunits, and a CBMIII. The scaffolding protein also contains 4 hydrophilic domains (surface layer homology domains) which probably serve to bind the scaffolding to the cell surface (Doi, 2008). The scaffolding protein is 189 kDa in molecular weight and has no catalytic activity (Shoseyov *et al.*, 1992). One of the endoglucanases, EngE, contains three tandem repeat SLH domains at the N-terminus and thus appears to be able to bind to both the cell wall and the scaffolding protein (Doi and Tamaru, 2000).

The genes encoding the cellulosome of *C. cellulovorans* is found in a large gene cluster encoding the CbpA as well as various enzyme subunits (ExgS, EngH, EngK, HbpA, EngL, ManA, EngM and EngN). Genes encoding other cellulosomal enzymes have been identified throughout the genome, including EngB, EngE, pectate lyase A, XynA and XynB (Doi, 2008). Doi proposed that *C. cellulovorans* most likely encodes for 50-60 cellulosomal enzymes (Doi, 2008). With this versatile range of enzymes, this organism is able to degrade cellulose, xylan, mannan and pectin. Expression of genes have been shown to be related to the substrate the organism was grown on and this indicates that expression is modified by the organism to obtain optimal levels of the enzymes required to degrade the substrate (Han *et al.*, 2003; Han *et al.*, 2004). It was also shown that subpopulations of cellulosomes were produced when grown on any substrate indicating that the cellulosome was not homogenous in its composition. These subpopulations also varied from substrate to substrate (Han *et al.,* 2005).

(c) C. cellulolyticum

The cellulosome of *C. cellulolyticum* has a large scaffolding protein (CipC) which contains 8 cohesin domains, a CBM and two hydrophilic domains (X2 and S2) (Desvaux, 2005). The cellulosome is relatively small and forms a structure of 600 kDa (Desvaux, 2005). The *C. cellulolyticum* genome possesses a large gene cluster encoding for cellulosomal genes such as the CipC and various enzymes (Cel48F, Cel8C, Cel9G, Cel9E, orfX, Cel9H, Cel9J, Man5K, Cel9M, Rgl11Y and Cel5N) (Desvaux, 2005). However, many other cellulosomal genes that are not part of the gene cluster were also found on the genome, such as Cel5A, Cel5D and Cel44O. To date, 22 proteins containing dockerin sequences have been found in this genome

(Doi, 2008). Interestingly, some of the cellulosomal enzymes also possess a CBM, namely, Cel5D, Cel9E, Cel9H, Cel9J and Cel9G (Desvaux, 2005). This is unusual as the cellulosomal enzymes generally bind to the cellulose substrate via the CBM on the scaffolding protein. Cel9G, when expressed, displays high activity against crystalline cellulose, which is absent when its CBM is removed. However, its CBM (a CBM3c) has no ability to bind to crystalline cellulose (Desvaux, 2005). Although a putative cellulosomal mannanase has been identified in the genome, it has not been characterised. While xylanase activity has been demonstrated, this has not been extensively characterised and identified as part of the cellulosome. A β-xylosidase has been purified from *C. cellulolyticum*, but it has not been identified as part of the cellulosome (Desvaux, 2005). A cellulosomal rhamnogalacturonase, Rgl11Y, has been characterised and is a family 11 polysaccharide lyase (EC 4.2.2) (Desvaux, 2005).

(d) C. acetobutylicum

C. acetobutylicum has been shown to produce inactive cellulosomes (Sabathe *et al.,* 2002). It contains a gene cluster encoding for a scaffolding protein (CipA) with a CBM, 5 cohesin domains and 6 hydrophilic domains. The gene cluster also encodes for 8 glycosyl hydrolases (Sabathe *et al.,* 2002).

(e) C. papyrosolvens

Pohlschroder *et al.* (1995) demonstrated that *C. papyrosolvens* produced seven, extracellular multi-enzyme complexes with approximate sizes of 500-660 kDa. Some of these complexes had predominant cellulase activity while others had xylanase activity. It is not clear whether these complexes were cellulosomes. It was demonstrated that this organism produced seven extracellular multi-enzyme complexes of approximately 700 kDa, some with cellulolytic activity but others with mainly xylanolytic activity (Pohlschroder *et al.*, 1995). The presence of a 125 kDa non-catalytic glycoprotein in all of the complexes which appeared to be similar to the scaffoldin protein, may have indicated that these complexes were cellulosomes (Pohlschroder *et al.*, 1995). However, further data on the presence of cohesins and dockerins

has not been reported in the literature and thus it is not established whether this organism produced cellulosomes or xylanosomes.

(f) C. josui

The cellulosome in *C. josui* has a similar structure to that of *C. cellulovorans* and *C. cellulolyticum* with a large, non-enzymatic scaffolding protein (CipA) (Kakiuchi *et al.*, 1998). The scaffolding protein contains a CBM and 6 cohesin domains. The main genes encoding for the cellulosome are again present in a large cluster in the genome, containing the scaffolding protein (CipA) and several enzymes with dockerin domains (Cel48A, Cel8A, Cel9E, Cel9F, orfX, Cel9G, Cel9H and Man5A) (Kakiuchi *et al.*, 1998). Other cellulosomal genes are dispersed on the genome, including a gene encoding for an α-galactosidase (Aga27) with activity on galactomannan. The molecular weight of the cellulosome is approximately 700 kDa (Schwarz, 2001).

(g) Acetovibrio cellulolyticus

To date, four scaffolding proteins have been identified in *A. cellulolyticus*, namely ScaA, ScaB, ScaC and ScaD (89 kDa) (Xu *et al.*, 2003; Xu *et al.*, 2004). ScaA is able to bind to ScaB, which in turn binds to ScaC. ScaA is the main scaffolding protein and contains a CBM3b domain as well as a catalytic site for a family 9 glycoside hydrolase. It has seven cohesins and a C-terminal dockerin domain. ScaB is 100 kDa and has four cohesins and a C-terminal dockerin domain. ScaC is 124 kDa and has three cohesins and a C-terminal SLH module (Xu *et al.*, 2003). ScaD also contains an SLH module and therefore appears to be a cell-anchoring protein together with ScaC. ScaD also has three cohesin domains capable of binding dockerin-bearing enzymes, two Type II and one Type I cohesin (Xu *et al.*, 2004).

(h) Ruminococcus flavefaciens

The cellulosome of *R. flavefaciens* have at least two scaffolding proteins, ScaA and ScaB, but the existence of a further scaffoldin is suspected (Rincon *et al.*, 2003). ScaA is 90 kDa with three cohesins, a C-terminal dockerin and a unique N-terminal domain of unknown function, but lacks an identifiable CBM (Rincon *et al.*, 2003). ScaB has seven cohesins which have al been shown to interact with the ScaA protein (Rincon *et al.*, 2003).

(i) Ruminococcus albus

R. albus produces a cellulosome larger than 1,500 kDa (Ohara *et al.*, 2000). SDS-PAGE of the cellulosome shows 15 bands with eleven having endoglucanase and/or xylanase activity (Ohara *et al.*, 2000).

1.3.1.9 Synergy

The synergy between enzymes in the cellulosome has been suggested as one of the main reasons why the cellulosome is so efficient at degradation of complex substrates. A synergistic association is said to exist when the combined activity of two or more enzymes together is greater than the theoretical sum of the individual activities of the enzymes on the same substrate. Much research has been conducted to investigate the synergistic effects of combinations of individual enzymes on the catalytic efficiency with respect to various substrates. This has been done for cellulosomal and other enzymes. If optimal ratios are known, this could be utilised to ensure tailor-made enzyme cocktails in bioprocessing, or used to construct designer cellulosomes for the same purpose.

Various studies have investigated synergy in the degradation of cellulose. It has been demonstrated that synergistic relationships exist between different exo-glucanases (Hoshino *et al.*, 1997; Teeri, 1997). Synergy also takes place when endo- and exo-glucanases are combined (Murashima *et al.*, 2002; Teeri, 1997; Valjamae *et al.*, 1999). While some reports suggest that no synergy takes place between endo-glucanases, such synergy has been reported in some instances (Liu *et al.*, 2004; Zhou & Ingram, 2000).

The role of the CBM cannot be underestimated in the activity of an enzyme on its substrate, which is termed an "intramolecular synergism" by Din *et al.* (1994). This plays a particular role in synergy studies involving designer mini-cellulosomes where enzymes are immobilised onto a mini-scaffolding protein containing a CBM. Such immobilisation leads to enhanced synergy whether one, two or three enzymes are immobilised in this manner (Doi, 2008; Fierobe *et al.*, 2001, 2002, 2005; Fukumura *et al.*, 1997; Koukiekolo *et al.*, 2005; Murashima *et al.*, 2002, 2003). It has generally been found that enhanced synergy takes place on crystalline or other recalcitrant substrates rather than soluble substrates (Fierobe *et al.*, 2001, 2002; Murashima *et al.*, 2002).

Other studies have concentrated on the degradation of hemicelluloses where it has been reported that degradation of arabinoxylan is enhanced in the presence of an endo-xylanase combined with α-L-arabinofuranosidase and arabinoxylan arabinofuranohydrolase (De Vries *et al.*, 2000; Sorensen *et al.*, 2002) or combined with a feruroyl esterase (Vardakou *et al.*, 2004).

Synergistic effects may vary when enzymes are used simultaneously or sequentially and the exact ratios of enzymes used have an impact (Doi, 2008). It was also shown that synergy occurred between cellulosomal and non-cellulosomal enzymes. In fact, optimal degradation was found when cellulosomes were used in conjunction with non-cellulosomal enzymes, for example a β-glucan glucohydrolase (BglA) (Doi, 2008; Kosugi *et al.*, 2006). In a similar manner, it was demonstrated that using whole microorganisms for degradation had an enhancing effect when compared to purified enzymes without the microbes (Lu *et al.*, 2006). It thus appears as though the presence of the microbe itself was able to enhance the hydrolysis rate which has important implications for bioprocessing.

1.3.2 Other cellulolytic and hemi-cellulolytic multi-enzyme complexes

The cellulosome is a very specific and defined multi-enzyme complex with distinct structural features such as the scaffoldin protein, dockerins and cohesins. Another feature of the cellulosome has been its high activity on crystalline cellulose. There are some reports in

literature of multi-enzyme complexes from other microorganisms. In most cases, these complexes have exhibited mainly hemi-cellulolytic activity, although some have been reported with activity on crystalline cellulose. To distinguish these complexes from cellulosomes, the term xylanosome has been used. However, no specific structural features for a xylanosome have been identified to date.

One of the first organisms reported to possess a xylanosome was *C. papyrosolvens C7* (Cavedon *et al.*, 1990). Sunna & Antranikian (1997) classified these multi-enzyme complexes as xylanosomes, while Doi *et al.* (2003) and Schwarz (2001) classified them as cellulosomes. Another organism in which a multi-enzyme complex was isolated and identified as a xylanosome was *Butyrivibrio fibrisolvens* (Lin & Thomson, 1991). This complex was greater than 669 kDa and possessed endo-glucanase activity in three bands on a zymogram and xylanase activity in eleven bands. Doi *et al.* (2003) and Schwarz (2001) both identified this complex as a cellulosome even though no further evidence confirming the presence of structural features of a cellulosome was reported.

Two multi-enzyme complexes (MECs) with molecular masses of about 669 kDa and 443 kDa were also identified in *Bacillus circulans* F-2 (Kim & Kim, 1993). The larger complex had at least five protein species with endoglucanase activity and two with xylanase activity while the smaller complex had three protein species with endoglucanase and four with xylanase activity as shown by zymograms. Both complexes had cellobiohydrolase activity, but the smaller complex had high activity towards filter paper, a recalcitrant cellulose substrate. The organism did not display any cellulolytic activity when cultured on soluble oligosaccharides such as glucose or cellobiose but produced cellulolytic activity when cultured on Avicel, xylan or CMC (Kim & Kim, 1993). No further reports exist in the literature as to the true identity of these complexes. However, it is interesting to note that none of the literature on cellulosomes or xylanosomes make any reference to the presence of these complexes.

Paenibacillus curdlanolyticus B-6 is a facultative anaerobic bacterium that was isolated from an anaerobic digester (Pason *et al.*, 2006). It was found to produce two multi-enzyme complexes under aerobic conditions as demonstrated by gel filtration chromatography, one of 1,450 kDa and the other of 400 kDa in molecular weight. The first complex of 1,450 kDA was shown to have eight proteins by SDS-PAGE with seven protein species displaying

xylanase activity and five displaying endoglucanase activity. The proteins with endoglucanase activity also displayed xylanase activity. The second complex of 400 kDa displayed seven proteins by SDS-PAGE of which five had xylanase activity and three endoglucanase activity (Pason *et al.*, 2006).

Jiang *et al.* (2004) reported a large xylanosome in *Streptomyces olivaceoviridis* E-86 based on native PAGE gel electrophoresis. This complex had predominant xylanase activity with four bands on zymogram analysis having xylanase and one band having endoglucanase activity. N-terminal sequencing of bands after SDS-PAGE separation revealed that five of the bands were two xylanases and truncated versions of these xylanases, probably formed by proteolytic degradation (Jiang *et al.*, 2006). The other three bands present on SDS-PAGE could not be analysed due to low concentrations of protein. It is not clear whether these proteins had formed a complex or aggregate.

The presence of a multi-enzyme complex (MEC) was also reported for *Bacillus megaterium* (Beukes & Pletschke, 2006). Only one type of activity was present (Avicelase) and no further data was given to verify the nature or size of such complex. A cellulolytic multi-enzyme complex of more than 1400 kDa in size with 12 subunits was also reported for *Bacteroides* sp. strain P-1 (Ponpium *et al.*, 2000). The production of a large multi-enzyme complex by an aerobic fungus *Chaetomium* sp. nov. MS-017 was reported by Ohtsuki *et al.* (2005). The complex had five xylanolytic, four cellulolytic and eight pectinolytic components as deduced from zymogram analysis. The myxobacterium *Sorangium* was also reported to possess a cellulolytic complex of between 1000 and 2000 kDa in size (Hou *et al.*, 2006). Due to the industrial importance of bacilli and the occurrence of MECs in this genus, they are discussed in greater detail below.

1.4 *Bacillus* spp. and *B. licheniformis*

Bacilli are Gram positive, rod-shaped microorganisms with important industrial application. They have been used in the production of enzymes, recombinant proteins, antibiotics, insecticides and amino acids (Arbige *et al.*, 1993). They are attractive species for use in

industry as they are generally non-pathogenic, except species such as *B. anthracis*. They are also able to grow fast and secrete high amounts of protein into the extracellular medium (Schallmey *et al.*, 2004). They are generally simple to cultivate and relatively easy to manipulate genetically (Arbige *et al.*, 1993). Many strains of bacilli also produce enzymes that are tolerant of alkaline pHs and high temperatures, thus making them very useful in applications such as detergents (Schallmey *et al.*, 2004). Schallmey estimated that commercial enzymes from *Bacillus* spp. make up about 50% of the enzyme market (Schallmey *et al.*, 2004). They are therefore seen as preferred hosts for many commercial protein products.

Due to the commercial potential of the bacilli, many of their genomes have been sequenced. The genome sequences of *B. subtilis* 168, *B. cereus* ATCC 14579, *B. anthracis* A202, *B. thuringiensis* subsp. *israelensis* (Schallmey *et al.*, 2004) and *B. licheniformis* ATCC 14580/ DSM 13 is completed and available (Rey *et al.*, 2004; Veith *et al.*, 2004).

B. licheniformis specifically, is considered an important industrial organism and is used to produce many commercial enzymes. It has been classified as a GRAS (generally regarded as safe) organism by the US Food and Drug Administration (Schallmey *et al.*, 2004). It has been used to produce a commercial alkaline serine protease and an α-amylase that is able to operate at 95°C as well as withstand temperatures of 105-110°C for short periods (Schallmey *et al.*, 2004). Alkaline proteases from *B. licheniformis* RP1 have been shown to retain activity in commercial laundry detergents, indicating potential for use in these products (Sellami-Kamoun *et al.*, 2008). *B. licheniformis* is also used to produce commercial antibiotics such as bacitracin and surfactin, as well as poly-γ-glutamic acid (Birrer *et al.*, 1994; Schallmey *et al.*, 2004). *B. licheniformis* has also been shown to degrade deoxynivalenol, a toxic compound produced by *Fusarium* spp. (Cheng *et al.*, 2008). It has also been used in the construction of microbial fuel cells (Choi *et al.*, 2004). Emptage *et al.* (2009) also report the isolation of a nitroreductase from *B. licheniformis* with a potential use in anti-cancer treatment as a prodrug activator. *B licheniformis* has further been used for the synthesis of silver nanoparticles (Kalishwaralal *et al.*, 2008).

It is interesting to note that *B. licheniformis* has been found to produce an exopolysaccharide (EPS). Larpin *et al.* (2002) reported a strain that was able to produce this EPS when cultured

on glucose or other monomeric sugars. Such strains were identified through the production of distinctly viscous colonies that have a "ropy" character. The bacteria cause ropiness in beers, ciders and wines which is a contamination that does not affect the taste of the product, but causes an increase in viscosity. Upon analysis of the EPS, Larpin *et al.* (2002) found that it was composed of 80% mannose. It is interesting that EPS production was enhanced in the presence of ethanol (up to 7% v/v).

EPS production by a strain of *B. licheniformis* has been shown in other research to have potential use in bioremediation as it was shown to adsorb 90-95% lead(II) at concentrations of up to 100mg/l (Li *et al.*, 2008). Growth of this strain was enhanced at high concentrations of lead. An extracellular glycoprotein containing 95% polysaccharide produced by *B. licheniformis* X14 has been shown to have potential use as a bioflocculant (Li *et al.*, 2009c).

Another strain of *B. licheniformis*, YP1A, had an organic solvent-stable alkaline protease with potential use in biocatalysis (Li *et al.*, 2009b). The organism itself was highly tolerant to growth in organic solvents (Li *et al.*, 2009b).

The use of *Bacillus* spp. in biofuel research has been limited to first generation biofuel technology where a commercially available, thermostable α-amylase from *B.licheniformis* has been used for the liquefaction of wheat flour (Das Neves *et al.*, 2006) and corn meal (Mojovic *et al.*, 2006). The hydrolysates were then saccharified and fermented to ethanol in a further processing step using *Saccharomyces cerevisiae* (Das Neves *et al.*, 2006; Mojovic *et al.*, 2006).

There have, however, been numerous reports of carbohydrate degrading enzymes in *B licheniformis*. Table 1.2 gives a list of most of the important carbohydrate-degrading enzymes that have been identified in various strains of *B. licheniformis*. Information on some enzymes are reported based on biochemical characterisation, while the predicted function and molecular weight of others are based on the published genome sequence for strain DSM 13 on Genbank.

The main cellulose-degrading enzymes present in various strains of *B. licheniformis* have all been characterised as endo-glucanases which indicates that *B. licheniformis* should be able to

hydrolyse amorphous cellulose, but not crystalline cellulose. Although strain DSM 13/ATCC 14580 is reported to possess a gene for a cellulose 1,4-β-cellobiosidase (cellobiohydrolase), it does not appear to contain a signal peptide and therefore is not released extracellularly (Rey *et al.*, 2004). Endo-xylanases have been identified in many strains but no β-xylosidase, which would convert shorter xylooligosaccharides to xylose. Various pectate lyases and several enzymes for degradation of mannan have also been identified. The strain DSM 13/ATCC 14580 furthermore has gene sequences for several enzymes for degradation of arabinan and for removal of arabinose substituents from arabinoxylan. Table 1.2 below summarises the source and biochemical differences of these groups of enzymes isolated from different strains of *B. licheniformis*.

Table 1.2. Some of the main carbohydrate-degrading enzymes as reported in literature for *B. licheniformis*. (NR- not reported)

Class of enzyme	Accession no. UniProt/TrEMBL	Type of enzyme	Family	MW	Source	Reference
Cellulose degrading enzymes	Q65JI7	EC 3.2.1.4 Endo-1,4-b-glucanase	GH 5	63.578	Nucl seq	Rey et al., 2004, Veith *et al.*, 2004
	Q65JI8	EC 3.2.1.91 Cellulose 1,4-β-cellobiosidase	GH 48	79.464	Nucl seq	Rey et al., 2004, Veith *et al.*, 2004
	Q65JI9[a]	EC 3.2.1.4 Endo-1,4-b-glucanase	GH 9	73.621	Nucl seq	Rey et al., 2004, Veith *et al.*, 2004
	Q6SYB5[a]	EC 3.2.1.4 Cellulase Cel 9A	GH 9	71.242	Nucl seq Strain GXN 151	Liu *et al.*, 2004
	Q5QSM2[+]	EC 3.2.1.4 Endo-1,4- β-glucanase CelA	GH 5	56.799	Nucl seq Strain F11, F5	Waldeck *et al.*, 2006
	Q1EM84[+]	EC 3.2.1.4 Endo-1,4- β-glucanase	GH 5	56.861	Nucl seq Strain F11, F5	Waldeck *et al.*, 2006
	Q7X4S4	EC 3.2.1.4 Endo-1,4- β-glucanase Cel12A	GH 12	29.068	Nucl seq Strain GXN 151	Liu *et al.*, 2004
	P27051*	EC 3.2.1.73 Endo- β-1,3-1,4-glucanase	GH 16	27.435	Nucl seq	Lloberas *et al.*, 1991
		Endo-glucanase Cel 5A	GH 5	62	B lich B-41361	Bischoff *et al.*, 2007
		Endo- β-1,3-1,4-glucanase	GH 16	23.6	B lich EGW039 (CGMCC 0635)	Teng *et al.*, 2006
Xylan degrading enzymes	Q65MX1	Endo-1,4- β-xylanase	Polys Deac 1	51.11	Nucl seq	Rey et al., 2004, Veith *et al.*, 2004
	Q5K2M7	EC 3.2.1.8 Endo-1,4- β-xylanase	Polys	30.646	Nucl seq	Waldeck et al 2006
	Q45VU7[b]	EC 3.2.1.8 endo-xylanase xyl B	GH 11	23.255	Nucl seq	Lu *et al.* Genbank
	A5H0S3[b]	EC 3.2.1.8 endo-xylanase xyl 11	GH 11	23.402	Nucl seq Strain I5	Helianti *et al.*, 2008
		Cellulase-free xylanase	NR	45	B lich A99	Archana & Satyanarayana, 2003
		Xylanase	NR	NR	B lich	Liu & Liu, 2008
		Endo-xylanases	NR	17 & 40	B lich 77-2	Damiano *et al.*, 2006
		Xylanase (Xyn11)	NR	23.4	B lich MS5-14	Lee *et al.*, 2008
Pectin degrading enzymes	Q65KV6	Pectate lyase Pel	Family 1	48.694	Nucl seq	Rey et al., 2004, Veith *et al.*, 2004
	Q65G96	Pectate lyase PelB	Family 1	54.693	Nucl seq	Rey et al., 2004, Veith *et al.,* 2004
	Q65EF5	EC 4.2.2.2 Pectate lyase precursor PelC		23.618	Nucl seq	Rey et al., 2004, Veith *et al.,* 2004
	Q65DC2[c]	EC 2.1.3.3 Pectate lyase	Family 1	37.365	Nucl seq	Rey et al., 2004, Veith *et al.,* 2004
	Q8GCB2[c]	EC 4.2.2.2 Pectate lyase Pel	Family 1	33.451	Nucl seq	Berensmeier *et al.*, 2004.
		EC 4.2.2.9 Exopolygalacturonate lyase	NR	38	B lich	Singh *et al.*, 1999
Mannan-degrading enzymes	Q65JI6	Endo-1,4- β-mannosidase	GH 5	45.46		Rey et al., 2004, Veith *et al.*, 2004
		EC 3.2.1.25 Endo-1,4- β-mannosidase	NR	NR		Araujo & Ward, 1990
	Q5K2L9	Endo-1,4- β-mannosidase ydhT (fragment)	GH 5	33.907	Nucl seq	Waldeck *et al.*, 2006
		β-mannanase	NR	26	B lich	Zhang *et al.*, 2000
		β-mannanase	NR	NR	B lich NK-27	Feng *et al.*, 2003
Arabinan and arabinose degrading enzymes	Q65GC7	EC 3.2.1.55 α-L-arabinofuranosidase	GH 51	57.05	Nucl seq	Rey et al., 2004, Veith *et al.*, 2004
	Q65D31	EC 3.2.1.99 arabinan-endo-1,5- α-L-arabinosidase	GH 43	53.064	Nucl seq	Rey et al., 2004, Veith *et al.*, 2004
	Q65L63	EC 3.2.1.55 Endo- α-L-arabinosidase	GH 43	36.358	Nucl seq	Rey et al., 2004, Veith *et al.*, 2004

1.5 Problem statement

Lignocellulose is the most abundant biological material on earth and can provide a sustainable and renewable resource for production of liquid transportation fuels. The biological degradation of lignocellulose into fermentable sugars is feasible and sustainable, but requires a variety of enzymes working in synergy as lignocellulose is a complex and recalcitrant substrate. The cellulosome is a cellulolytic MEC that has been discovered in several anaerobic bacteria such as the clostridia. The cellulosome appears to facilitate an enhanced synergy and efficiency of its enzymes, as compared to free enzymes, for the degradation of recalcitrant substrates such as cellulose and plant cell walls.

Most of the studies on cellulosomes have focused on a few organisms, *C. thermocellum*, *C. cellulovorans* and *C. cellulolyticum* and there is only limited knowledge available on complexes in other organisms. Some MECs have been identified in aerobic bacteria such as *Bacillus circulans* and *Paenibacillus curdlanolyticus*, but it has not been established whether these MECs have a structure similar to cellulosomes. Very little is also known about the enzyme composition of these MECs and whether the organism regulates the composition based on the environment.

There is scope for the isolation, purification and characterisation of MECs from different organisms in order to understand their composition and behaviour. By expanding our knowledge and understanding of cellulolytic and hemi-cellulolytic systems of microorganisms, and specifically the formation of MECs, we may be better equipped to improve biotechnological processes such as biofuel production.

1.6 Aims and objectives

The general aims and objectives of this study were as follows:

(i) To isolate and identify a *Bacillus* sp. with cellulolytic or hemi-cellulolytic activity that produces a MEC;

(ii) To purify the MEC from this organism;

(iii)To characterise the MEC by investigating enzyme composition and other features;

(iv) To perform a preliminary investigation into the regulation of enzyme composition when the growth substrate changes; and

(v) To compare the features of the MEC in this study with characteristics of cellulosomes.

CHAPTER 2 – CULTURING, SCREENING AND IDENTIFICATION OF *BACILLUS LICHENIFORMIS* SVD1

2.1 Introduction

Microorganisms that derive their nutrients from complex plant material produce a variety of cellulolytic and hemi-cellulolytic enzymes. The carbohydrates in plant material are generally large molecules, and therefore such enzymes have to be secreted into the extracellular environment. Cellulolytic systems of bacteria are quite complex. While some organisms produce only free extracellular enzymes in order to degrade carbohydrate substrates, others assemble enzymes in a multi-enzyme complex (MEC) such as the cellulosomes found in *Clostridium thermocellum*, *C. cellulovorans* and *C. cellulolyticum*, as well as in other anaerobic organisms such as *Ruminococcus albus* and *R. flavefaciens* (Doi *et al.*, 2003).

Cellulolytic and hemi-cellulolytic MECs have also been discovered in aerobic microorganisms which may differ from cellulosomes. Kim & Kim (199) reported the presence of two cellulolytic MECs in *B. circulans*, while a MEC was also reported for *B. megaterium* (Beukes & Pletschke, 2006) and *Paenibacillus curdlanolyticus* (Pason *et al.*, 2006). The structures of these MECs have not been elucidated in detail, but many of them contain predominantly xylanase activity rather than the prominent cellulase activity found in cellulosomes. MECs with predominant xylanase activity are generally termed xylanosomes. The importance of MECs in lignocellulose degradation has been described in detail in Section 1.3.1. Through a greater understanding of MECs we may be able to harness them for a potential commercial purpose.

A distinctive feature of MECs is the fact that they are large, high molecular mass protein complexes. The molecular weight of MECs based on size exclusion chromatography has been reported as ranging from 650 kDa to 2.5 MDa in cellulosomes (Doi *et al.*, 2003) and 400 kDa and 1450 kDa in the xylanolytic MEC in *P. curdlanolyticus* (Pason *et al.*, 2006). Sepharose 4B is a size exclusion resin commonly used in MEC research as it has a separation range of 60-20,000 kDa for globular proteins and is thus able to separate very large proteins and complexes.

Screening for cellulolytic and hemi-cellulolytic organisms is often performed by utilising Congo Red dye which binds only to carbohydrates of five residues or longer (Carroll & Van Dyk, 1952). Where enzymes cleave the carbohydrates into moieties shorter than five residues, the dye is unable to bind. Thus a clear zone is displayed around plated colonies with cellulolytic or hemi-cellulolytic activity.

Bacilli are ubiquitous in nature. They are Gram positive and rod-shaped and a distinctive feature of bacilli is the formation of endospores. Endospores are formed within the cells and can be seen as refractile structures under a light microscope (Todar, 2005). Some bacilli are strictly aerobic although a few are also facultative anaerobes (Priest, 1993). Although the presence of an MEC has been identified in two bacilli, little characterisation of the MEC was reported (Beukes & Pletschke, 2006; Kim & Kim, 1993).

It is of great interest and importance to isolate organisms that produce MECs and to study and characterise such MECs. This may lead to a greater understanding of the cellulolytic and hemicellulolytic systems of such organisms, the manner in which they degrade carbohydrate substrates and the role of the MEC in this process.

2.2 Objectives

- To isolate an organism with a MEC containing cellulolytic and hemi-cellulolytic activity

2.3 Methods and materials

2.3.1 Isolation of organism

B. licheniformis SVD1 was isolated from a co-culture with *Clostridium beijerinckii* sLM01 from a biosulphidogenic bioreactor (Mayende, 2006). Initially, cultures were grown anaerobically at 37°C in a liquid medium containing 1 g NH_4Cl, 5 g yeast extract, 1 g $MgSO_4.7H_2O$, 0.5 g KCl, 4 g tryptone, 0.5 g K_2HPO_4, 5 g birchwood xylan, 0.01 g $MnSO_4.4H_2O$, 0.01 g $FeSO_4.7H_2O$, 8 mg *p*-aminobenzoic acid, 0.04 mg biotin and 1.0 mg resazurin (in 1 l). A 1 ml volume of a 15% (w/v) solution of cysteine-HCl was added to the

medium after autoclaving. Flasks were inoculated from glycerol stocks that had been heated at 80°C for 10 min. The medium was flushed with N_2 until it turned pale yellow. Flasks were then sealed with rubber stoppers and connected to a gas trap containing zinc acetate. Cultures were incubated at 37°C for 14 days before purification. Cultures were also grown on agar plates containing the same medium as above, but with 12 g of bacteriological agar (per l). Agar plates were incubated at 37°C in anaerobic jars using the Anaerocult® system to obtain an anaerobic environment.

B. licheniformis SVD1 appeared as mucoid colonies on agar plates that were distinct from the *C. beijerinckii* colonies. The two distinct colony types were separated through standard microbiological procedures such as picking of isolated colonies and streaking until pure cultures were obtained.

2.3.2 Screening

Screening was performed on the pure cultures which had been grown on the anaerobic medium as described in section 2.3.1.

Screening of isolates was carried out using an assay for enzyme activity. Enzyme activity was measured by the reducing sugars formed in a modified dinitrosalicylic acid (DNS) method (Miller, 1959) using xylose as the standard (see Appendix 4b). The composition of DNS reagent was as follows: 2 g sodium hydroxide, 2 g 3,5 dinitrosalicylic acid, 40 g potassium sodium tartrate, 0.4 g phenol and 0.1 g sodium metabisulfite in 200 ml dH_2O. The assay was performed by mixing 100 µl enzyme preparation with 50 µl of a 2% (w/v) solution of the desired substrate and 250 µl buffer (50 mM potassium phosphate at pH 6.5). Assays were performed at 50°C for 30 min (unless otherwise stated). After incubation, the assay mixture was centrifuged for 1 min at 16,000 *x g* to remove any insoluble substrate or cells. The colour development was performed by adding 150 µl of the supernatant of the assay to 300 µl of DNS reagent after which it was heated at 100°C for 5 min, cooled on ice for 5 min and readings measured at 540 nm. One unit of enzyme activity (U) was defined as the amount of enzyme within the assay that released 1 µmol of reducing sugar per min under the conditions indicated.

B. licheniformis SVD1 was screened for endoglucanase and xylanase activity through culturing on agar plates. Incubation at 37°C was carried out until colonies became visible, and plates were then stained with 0.3% Congo Red and destained with 1 M NaCl. Activity was observed as clear zones around colonies.

Screening for the presence of a multi-enzyme complex was carried out by centrifuging a culture of *B. licheniformis* SVD1 for 10 min at 12,000 *x g* to remove the cells. A 50 ml volume of the supernatant was concentrated by placing it in dialysis tubing (10 kDa cut-off) on a bed of polyethylene glycol (PEG) 20,000. A sample of concentrated supernatant protein was then loaded onto a Sepharose 4B column (50 x 2.5 cm) and eluted with 50 mM Tris-HCl buffer at pH 7, containing 0.03% (w/v) sodium azide. Fractions of 3 ml were collected and the absorbance of fractions determined at 280 nm. Blue dextran (2,000 kDa), thyroglobulin from bovine thyroid (670 kDa) and laccase from *Trichoderma reesei* (70 kDa) were used as standards to determine the approximate size of peaks.

2.3.3 DNA Isolation, PCR amplification of 16s rDNA region and sequencing

Genomic DNA was prepared according to the method of Ausubel *et al.* (2002). PCR on genomic DNA of *B. licheniformis* SVD1 was performed using the following primers: 9F (5' - GATTTGATCCTGGCTCAG – 3') and 1541R (5' - AAGGAGGTGATCCAGCC – 3') from Inqaba Biotech and using a KAPA HiFi PCR kit (KAPA Biosystems). The PCR reaction was performed in a Sprint thermal cycler using the following cyclical parameters: 95°C for 2 min, 98°C for 30 s, 61°C for 30 s, 68°C for 1 min (cycle repeated 25 times), 68°C for 5 min, end at 4°C. The presence of a PCR product and its molecular weight was confirmed using a agarose gel electrophoresis with 1% agarose. The PCR product was sequenced using the same primers and an ABI 3130 XL Genetic analyser (Applied Biosystems, Foster City, CA), incorporating the ABI Big Dye Terminator Cycle Sequencing kit version 3.1 (Applied Biosystems, Foster City, CA). Sequencing was performed by Inqaba Biotechnical Industries Pty. Ltd., South Africa. Electropherograms of the sequences generated were inspected with FinchTV software (Geospiza). Forward and reverse sequences were aligned using a ClustalW alignment tool (http://www.ebi.ac.uk/Tools/clustalw2) and the combined sequence was used for a nucleotide BLAST search (http://www.ncbi.nlm.nih.gov/blast).

2.3.4 Culture conditions and storage

Upon identification of *B. licheniformis* SVD1 from its 16S rDNA sequence, culture conditions were changed to aerobic conditions as it was established from literature that *B. licheniformis* preferred aerobic conditions although it was a facultative anaerobe. A modified culture medium from Choudbury *et al.* (2006) for production of xylanase activity was used containing 5 g yeast extract, 4 g tryptone, 1 g K_2HPO_4, 0.2 g $MgSO_4$ and 5 g birchwood xylan.

For routine maintenance of cultures, the organism was grown on nutrient agar or in nutrient broth. Cultures were routinely checked for purity by spreading on nutrient agar plates and examining colonies. Cultures were stored as glycerol stocks at -20°C.

2.3.5 Morphology and characteristics

Colony characteristics on nutrient agar plates were noted, as well as on agar plates containing cellulolytic medium. Wet mounts were made by picking single colonies, resuspending them in buffer on a microscope slide and placing under a light microscope to determine morphology and motility. A Gram stain was performed to determine if the organism was Gram positive or negative.

The presence of endospores was detected using the Schaeffer-Fulton method (http://www2.muw.edu/~lbrandon/Micro/endospore.doc, see Appendix 6a). After fixing cells from a colony onto a slide, the cells were flooded with malachite green stain and kept over a boiling waterbath for five minutes with the stain refreshed periodically. After five minutes, the stain was washed off with distilled water and counterstained with safranin for two minutes. Endospores were stained green while vegetative cells were stained red.

2.4 Results

2.4.1 Isolation of organism

B. licheniformis SVD1 was isolated from an anaerobic culture that was co-culturing with *C. beijerinckii* SLM01. *B. licheniformis* colonies had a mucoid appearance (see Figure 2.3A) and were separated from *C. beijerinckii* through picking and streaking. As the co-culture had displayed xylanase activity and appeared to form a multi-enzyme complex, the pure cultures were separately screened to determine whether these attributes were present in both cultures.

2.4.2 Screening

Cultures of *C. beijerinckii* and *B. licheniformis* were tested for xylanase activity using the DNS method. *C. beijerinckii* displayed almost no xylanase activity while *B. licheniformis* displayed high xylanase activity (data not shown). Screening of *B. licheniformis* colonies using agar plates containing a CMC or birchwood xylan substrate displayed clear activity after staining with Congo Red. Figure 2.1 shows a typical example of an agar plate with enzyme activity shown as clear zones with a red background.

Figure 2.1 A typical photo of an agar plate containing CMC on which *B. licheniformis* SVD1 was streaked prior to incubation at 37°C. After incubation the plate was washed with 0.3% Congo Red for 15 min and then destained with 1 M NaCl. Clear zones are an indication of enzyme activity.

Screening of both the *B. licheniformis* and *C. beijerinckii* culture supernatants for formation of a multi-enzyme complex revealed the elution of a protein peak at approximately 2,000 kDa on a Sepharose 4B column for the *B. licheniformis* culture, but not the *C. beijerinckii* culture.

A typical chromatogram of such an elution is shown in Figure 2.2, indicating the presence of a possible multi-enzyme complex in *B. licheniformis* SVD1. Further characterisation of this possible complex is discussed in further chapters.

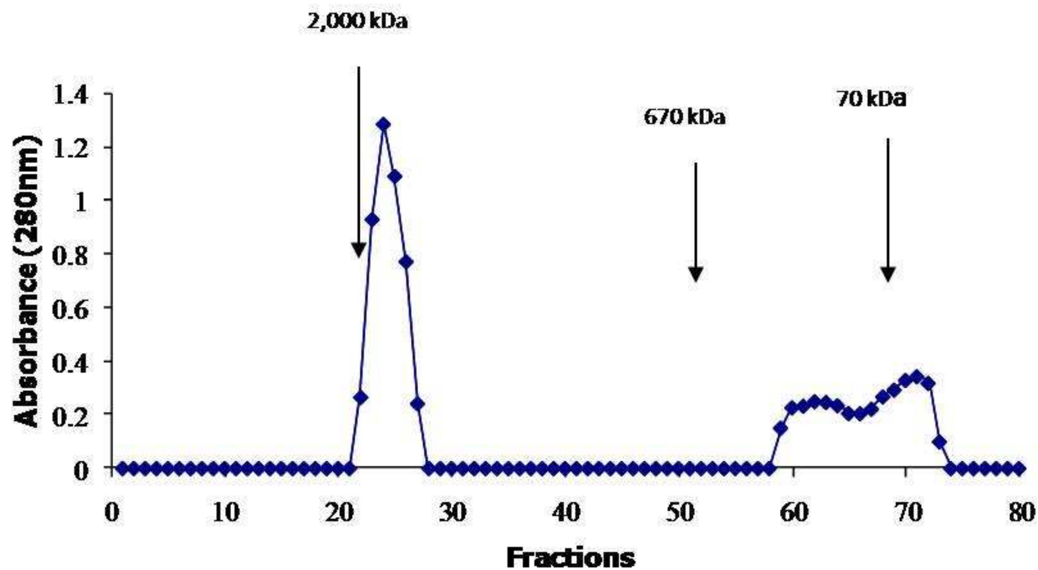


Figure 2.2. Sepharose 4B gel filtration chromatogram to illustrate the possible presence of a multi-enzyme complex. Obtained using a 50 x 2.5 cm column with a flow rate of 40 ml per h. Fractions of 3 ml fractions were collected and the absorbance at 280 nm measured. Blue dextran (2,000 kDa), thyroglobulin from bovine thyroid (670 kDa) and laccase from *Trichoderma reesei* (70 kDa) were used as standards.

Based on the high xylanase activity measured from this isolate and the presence of a peak using size exclusion chromatography indicating formation of a multi-enzyme complex, it was decided to continue further investigation of *B. licheniformis* SVD1.

2.4.3 DNA Isolation, PCR amplification of 16s rDNA region and sequencing

Based on the nucleotide BLAST search performed, the organism had the highest sequence homology and score with a number of strains of *B. licheniformis* (See Appendix 2 for Blast results). The full sequence of 1430 base pairs is reproduced in Appendix 1 and was lodged with Genbank with accession number EU 770587.

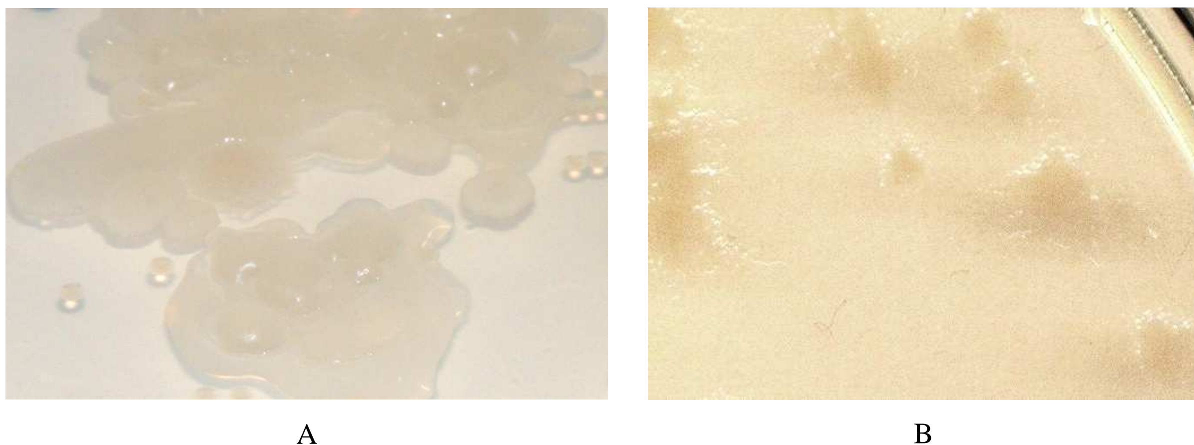
2.4.4 Culture conditions

A comparison of aerobic and anaerobic growth indicated that the organism was a facultative anaerobe, but growth under anaerobic conditions was markedly reduced (data not shown). It

was therefore decided to continue culturing under aerobic conditions. This confirmed reports from literature that *B. licheniformis* was only able to grow weakly under anaerobic conditions (Priest, 1983).

2.4.5 Morphology and characteristics

The organism was identified as Gram positive, motile and rod-shaped with subterminal endospores. Colonies on agar plates exhibited irregular margins and often had a distinctive mucoid appearance (see Figure 2.3). This is reported to be indicative of exopolysaccharide formation (Todar, 2005). PCR amplification and sequencing of the 16s rDNA region for both these types of colonies were carried out and shown to possess the identical 16s rDNA sequence as compared to other individual colonies.



A B

Figure 2.3. Photos of colonies of *B. licheniformis* SVD1 on agar plates containing CMC. Image A displays the distinctly mucoid character of colonies under certain conditions while Image B displays the other type of colony with irregular margins.

2.5 Discussion

The presence of *B. licheniformis* in a co-culture with *C. beijerinckii* could have been the result of an effective symbiotic relationship between these two organisms. Priest indicates that Group II bacilli are able to grow in the absence of oxygen, particularly if nitrate is present (Priest, 1993). The method used to establish anaerobic conditions included the flushing of the medium with nitrogen. *C. beijerinckii* is one of the clostridia capable of fixing nitrogen and would thus be able to provide the nitrates required for *B. licheniformis* growth by fixation of nitrogen (Chen, 2005). Concurrently, *C. beijerinckii* did not display significant

xylanase activity but appeared to be able to grow on birchwood xylan. Therefore it is possible that *B. licheniformis* was able to degrade the birchwood xylan substrate and supply a carbon source for growth of the *C. beijerinckii* under these conditions.

B. licheniformis was screened and seemed to require a very simple medium for growth and production of extracellular endoglucanase and xylanase activity. A large peak at approximately 2,000 kDa indicated the formation of a possible MEC. As a complex was not shown to be present in *C. beijerinckii*, further work was continued on *B. licheniformis*.

Identification of *B. licheniformis* took place through 16s rDNA sequencing. *B. licheniformis* falls within Group II of bacilli which are facultative anaerobes but do not grow well under anaerobic conditions (Priest, 1993). It is an industrially important bacterium as it produces many commercial enzymes, antibiotics and chemicals and it plays an important role in nature in nutrient cycling (Rey *et al.*, 2004). *B. licheniformis* is used to produce a commercial alkaline serine protease and an α-amylase. The amylase has been shown to be able to operate at 95°C as well as withstand temperatures of 105-110°C for short periods (Schallmey *et al.*, 2004). It is also used to produce commercially available antibiotics such as bacitracin and surfactin, as well as poly-γ-glutamic acid (Schallmey *et al.*, 2004). The genome sequence for one strain of *B. licheniformis* has been completed, namely DSM 13 /ATCC 14580 (Rey *et al.*, 2004, Veith *et al.*, 2004). A whole range of cellulolytic and hemicellulolytic enzymes have been identified and isolated in various strains of *B. licheniformis*. They are listed in greater detail in section 1.4, Table 2. As *B. licheniformis* has furthermore been classified as a GRAS organism by the US Food and Drug Administration, it seemed a suitable organism for further study (Schallmey *et al.*, 2004). To date there are no reports in literature on MEC production in *B. licheniformis* and thus the study and characterisation of an MEC in this organism was regarded as sufficiently novel for this study.

Two different types of colony morphologies were identified and the one type had distinctive mucoid characteristics. This may indicate that two strains of the same organism was present, although the 16s rDNA regions of these colonies had identical nucleotide sequences. Even where a mucoid colony was picked and inoculated into a culture, the cells from such a culture did not all display the same mucoid phenotype when spread on agar plates (data not shown). Mucoid colonies, however, did appear to have higher activity than other colonies on substrates such as CMC and birchwood xylan based on Congo Red staining of agar plates

(data not shown). This was found even though mucoid colonies had a lower cell density than other colonies based on observation under a light microscope of wet mounts (data not shown). It is possible that the mucoid colony may represent a different strain that becomes degenerate during culturing conditions, mutating into a non-mucoid colony. It is also possible that exopolysaccharide formation only occurs under certain culturing conditions which have not been established. The literature seems to indicate that exopolysaccharide formation can occur under various culture conditions (Costerton, 1999).

Formation of exopolysaccharide can be affected by factors such as divalent cation concentration, carbon-nitrogen ratio, specific substrate and the physical nature of the medium (solid vs. liquid) (Costerton, 1999). According to Costerton, starvation can lead to a decrease in exopolysaccharide formation which can be restored by the provision of suitable nutrients (Costerton, 1999). Uhlinger and White (1982) found that exopolysaccharide formation was affected by the carbon source and the presence of sand in the medium that increased the surface area for attachment of microorganisms.

It is not clear what role the exopolysaccharide formation in *B. licheniformis* may play. Exopolysaccharides (EPS), also referred to as the glycocalyx or capsule, is formed in many bacteria as a carbohydrate layer on the cell surface. It is reported to be involved in adhesion of cells to insoluble substrates and is also the substance involved in biofilm formation (Costerton, 1992; Costerton, 1999). EPS, however, does not have a uniform chemical composition and may differ substantially from organism to organism (Erlandsen *et al.*, 2004). EPS has been linked with cellulolytic activity and biomass degradation in very few instances. The EPS produced by *Cellulomonas flavigena* KU has been shown to form part of an extracellular glycocalyx that is involved in cellulose degradation (Kenyon *et al.*, 2005). *Ruminococcus albus*, which forms a cellulosome, is said to bind to cellulose through various mechanisms, including the production of EPS (Weimer *et al.*, 2006).

At present, any hypothesis on the role of EPS formation in carbohydrate metabolism, perhaps by trapping enzymes within this viscous matrix, relies purely on anecdotal evidence and requires further substantiation. It can further be questioned whether the EPS plays any role in formation of the MEC and how this could take place. These questions fall outside the scope of this study, but could be the subject of future work based on this study.

2.6 Conclusion

A strain of *B. licheniformis* was isolated from a coculture with *C. beijerinckii*. Upon examination of the pure cultures, *B. licheniformis* displayed high xylanase activity and appeared to form a large MEC of 2,000 kDa. These characteristics were not found in pure cultures of *C. beijerinckii*. *B. licheniformis* was characterised as having Gram positive, motile, rod-shaped cells that formed subterminal endospores. The organism was identified through PCR and sequencing of its 16S rDNA region. *B. licheniformis* was able to grow under anaerobic conditions although growth under aerobic conditions was superior. Under certain conditions, colonies of *B. licheniformis* had a mucoid appearance and appeared to form an exopolysaccharide layer around cells. Future work could involve an investigation of the exopolysaccharide layer and the conditions under which it was formed, as well as the role it plays in degradation of substrates.

B. licheniformis is an important industrial bacterium and is reported to contain many cellulolytic and hemicellulolytic enzymes, although there are currently no reports in the literature of the presence of a cellulolytic or hemicellulolytic MEC in this organism. Further investigation of the cellulolytic and hemicellulolytic system of strain SVD1 will be performed to identify the presence of various enzymes and their levels of activity when the organism is cultured on various substrates.

CHAPTER 3 – COMPARISON OF THE CELLULOLYTIC AND HEMICELLULOLYTIC SYSTEM OF *BACILLUS LICHENIFORMIS* SVD1 WHEN CULTURED ON VARIOUS CARBOHYDRATE SUBSTRATES

3.1 Introduction

In order to effectively degrade complex plant material for utilisation as a carbon source, microorganisms have to produce a whole range of cellulolytic and hemicellulolytic enzymes which were discussed in detail in Section 1.2.

The genome sequence for strain SVD1 is not available, but the genome sequence for another strain, DSM 13 /ATCC 14580, is available (Rey *et al.*, 2004, Veith *et al.*, 2004). This information, together with other reports in literature, describe a plethora of cellulolytic and hemicellulolytic enzymes in various strains of *B. licheniformis* (See section 1.4, Table 2). Even where the genomic data exists indicating the presence of enzymes in an organism, it is important to study the conditions under which these enzymes are expressed. Han *et al.* (2003) stated that: "It is important to understand how bacteria regulate expression of the various hydrolytic enzymes in order to produce optimal enzyme mixtures for the degradation of different plant materials". Due to the complexity of plant materials, the combination of enzymes and the ratios required in order to degrade it is not completely understood and thus remains a fundamental problem in biotechnology applications. One way in which researchers hope to understand this process better is by studying the way in which an organism itself regulates this process. Fundamental questions that have arisen in this regard are: How does an organism respond to changes in the environment such as the carbon source? And which enzymes are expressed for the degradation of a specific substrate and in what ratios?

This chapter involves a preliminary investigation of the range of cellulolytic and hemicellulolytic enzyme activities that are present in *B. licheniformis* SVD1 when it is cultured on different carbon substrates. This work aims to further the knowledge on the enzymes expressed by an organism such as *B. licheniformis* SVD1 and how this changes when the carbon substrate is altered. This study also aims to gain insight into the ability of some substrates to induce the expression of certain enzymes. Protein induction is a very

complex process and takes place in response to the surrounding environment. It is generally accepted that an organism would only express key and essential enzymes at a constitutive level in order that unnecessary resources are not wasted on synthesis of enzymes when a more readily utilisable carbon source is available (Madigan *et al.*, 2003). Inducers are generally small molecules that are able to enter the cell and react with other proteins or DNA to induce expression (Madigan *et al.*, 2003). They are often the substrate or the product of enzyme activity or may be an analog of such substances (Madigan *et al.*, 2003). Generally, some enzymes are constitutively expressed by the organism, but these are often key enzymes required for growth under all conditions (Madigan *et al.*, 2003). Other enzymes are selectively expressed based on the environment the organism finds itself in.

3.2 Objectives

- To culture *B. licheniformis* on different carbohydrate substrates and compare the cellulolytic system in each case by:
    - Determining whether the MEC is formed under all conditions
    - Investigating changes in enzyme activity of total extracellular protein
    - Viewing changes in composition of the crude extract and the purified MEC by utilising sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)
    - Investigating and comparing the zymogram profile for the crude extract and MEC for each culture

3.3 Methods and materials

3.3.1 Culturing of the organism on different substrates

Culture medium was prepared with 5 g yeast extract, 4 g tryptone, 1 g K_2HPO_4, 0.2 g $MgSO_4$ and 5 g per l of substrate (Avicel® PH101, cellobiose, birchwood xylan, bagasse and locust bean gum). Cultures of 100 ml each were inoculated from equal volumes of a glycerol stock of *B. licheniformis* and incubated at 37°C for 48 h with shaking at 200 rpm under aerobic conditions.

Sugarcane bagasse was kindly donated by I. Ramluken from Ushukela Milling (Pty) Ltd. The bagasse was washed several times with milli-Q H_2O to remove all residual sugars, after which it was centrifuged and the pellet dried and ground in a blender to a fine powder.

3.3.2 Enzyme assays

Enzyme assays were performed as described in section 2.3.2.

In order to determine the activity of cultures on various substrates, the standard assay was performed using the concentrated supernatant (prepared as per section 3.3.3). Avicel® PH-101 was used to determine exoglucanase activity, birchwood xylan to determine endo-xylanase activity, oatspelt xylan to determine endo-xylanase and arabinofuranosidase activity, locust bean gum to determine mannanase activity, carboxymethyl cellulose (low viscosity) to determine endoglucanase activity and polygalacturonic acid to determine pectate lyase activity.

3.3.3 Protein purification

Cultures of 100 ml were centrifuged at 12,000 *x g* for 10 min and the supernatant separated from the pellet. The supernatant was concentrated to about 15 ml by applying it to an Amicon 8200 ultrafiltration cell using a PBGC filter with a nominal molecular weight cutoff of 10 kDa (Millipore). This fraction was designated the concentrated supernatant and was used for enzyme assays and SDS-PAGE analysis. The concentrated supernatant protein (8 ml) from each culture was loaded onto a Sepharose 4B column (50 x 2.5 cm) and eluted with 50 mM Tris-HCl buffer at pH 7.5, containing 0.03% (w/v) sodium azide. Fractions of 3 ml were collected and the absorbance of fractions determined at 280 nm. The peak eluting at approximately 2,000 kDa was designated the MEC and fractions representing the MEC were pooled and concentrated using polyethylene glycol (PEG) 20,000 and used for further experiments.

3.3.4 Electrophoresis and zymograms

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 10% resolving and 4% stacking gels by the method developed by Laemmli (1970) (see Appendix 5). Samples were concentrated where necessary using acetone precipitation. Excess ice cold acetone was added to samples followed by vortexing and incubation at -20°C for 10 min after which it was centrifuged at maximum speed (16,000 *x g*) for 5 min. The supernatant was removed and the pellet dried until no acetone was detected. Pellets were then redissolved in sample buffer and boiled for 5 min prior to loading onto gels. Approximately 10 µg of protein was loaded per well. Electrophoresis was carried out at 180 V for 45 min and gels stained with PageBlue^{TM} Protein Staining Solution according to the manufacturers' guidelines. Gels were photographed using a Uviprochemi geldoc imaging system (Whitehead Scientific) and protein bands were analysed to determine their molecular weights using Uviband software (v. 11.9).

Activity of separated bands was detected by incorporation of 0.1% (w/v) substrate (CMC or birchwood xylan) prior to polymerisation. After electrophoresis, the gels were renatured for 1 hour in 2.5% (v/v) Triton X-100 in 50 mM potassium phosphate buffer (pH 6.5). The gels were then incubated in 50 mM phosphate buffer at 37°C for 3-12 h. After removal of the buffer, gels were stained with 0.3% Congo Red for 15 min and then destained with 1 M NaCl until bands appeared. Gels were then counterstained with 5% (v/v) acetic acid. Zymogram gels were photographed using a Uviprochemi geldoc imaging system.

3.4 Results

3.4.1 Culturing of the organism on different substrates

Growth on Avicel® and bagasse was generally poor compared to the cultures grown on other substrates.

3.4.2 Enzyme assays

The enzyme activities present in the concentrated supernatants of each culture was measured by using different substrates that are able to detect different types of enzyme activities. The results of these enzyme assays are summarised in Table 3.1.

Table 3.1 Activity of the supernatants from *B. licheniformis* cultured on Avicel®, cellobiose, birchwood xylan, bagasse and locust bean gum. Activity was measured on a variety of carbohydrate substrates as reducing sugars produced. Activities are shown as U/min/mg protein. Values are shown as mean values ± SD (n=3).

	CULTURE SUBSTRATE				
Enzyme activity measured (substrate)	**Avicel® culture**	**Cellobiose culture**	**Birchwood xylan culture**	**Bagasse culture**	**Locust bean gum culture**
Exoglucanase (Avicel®)	0	0.023±0.001	0.016±0	0	0.026±0.001
Endoglucanase (CMC)	0.004±0.001	0.056±0.002	0.020±0.002	0.005±0	0.032±0.001
Endoxylanase (birchwood xylan)	0.054±0.006	0.166±0.006	0.203±0.021	0.058±0.004	0.190±0.024
Endoxylanase and arabinofuranosidase (oatspelt xylan)	0.046±0.004	0.121±0.006	0.200±0.021	0.054±0.003	0.158±0.023
Pectate lyase (Polygalacturonic acid – PGA)	0.034±0.003	0.047±0.002	0.025±0.001	0.077±0.006	0.107±0.005
Endomannanase (Locust bean gum - LBG)	0.006±0	0.044±0.001	0.021±0.002	0.030±0.003	0.202±0.011
Bagasse	0	0.126±0.006	0.022±0.001	0	0.128±0.006

All the crude cultures exhibited the highest activity on xylan substrates except for the locust bean gum culture which had slightly higher mannanase activity than xylanase activity. The activity on birchwood xylan and oatspelt xylan was virtually identical in the birchwood xylan culture, whereas the activity on oatspelt xylan was generally lower than the activity on birchwood xylan in the other cultures. Only the cellobiose, birchwood xylan and locust bean gum cultures displayed any activity on Avicel®.

Pectate lyase activity was generally low in all the cultures, although by comparison slightly elevated in the Avicel®, cellobiose and bagasse cultures. However, in the locust bean gum culture the pectate lyase activity was much higher than in other cultures indicating that some induction of pectate lyase took place. The locust bean gum culture also produced the highest mannanase activity. The substrate, locust bean gum, which the organism was cultured on, appeared to induce the production of high levels of mannanase activity.

There was generally low enzyme activity in the Avicel® and bagasse cultures compared to other cultures.

3.4.3 Purification of MEC

The chromatograms obtained from the purification of the MEC from different cultures (Figure 3.1) displayed a peak at approximately 2,000 kDa and then a much larger peak at around 70 kDa where free extracellular enzymes should elute. The size of the first peak differed substantially between the birchwood xylan culture and the other cultures and was much larger in the birchwood xylan culture. The first peak for every culture was designated the MEC and was pooled and concentrated for use in SDS-PAGE and zymograms.

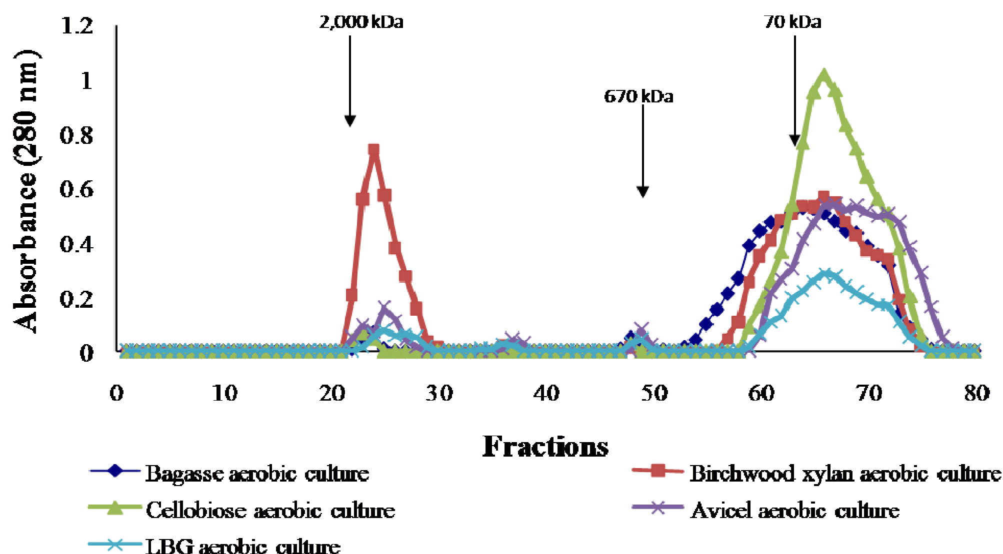


Figure 3.1 Sepharose 4B chromatograms of concentrated supernatant from *B. licheniformis* cultured on different substrates. Chromatograms were obtained using a 50 x 2.5 cm column and collecting fractions of 3 ml using 50mM Tris-HCl buffer at pH 7.5. Blue dextran (2,000 kDa), thyroglobulin from bovine thyroid (670 kDa) and laccase from *Trichoderma reesei* (70 kDa) were used as standards.

3.4.4 Electrophoresis and zymograms

The SDS-PAGE and zymogram profiles for each of the crude cultures are displayed in Figure 3.2 below. There were discrete differences between the SDS-PAGE profiles for each culture with some bands being more prominent than others in comparison. The largest visible protein in the crude supernatants was approximately 129 kDa in size.

Zymograms were done using birchwood xylan and CMC as substrates incorporated into the gel. These two substrates were chosen for zymogram analysis as they were the only substrates for which successful zymograms could be obtained, even though activity on CMC was relatively low.

The zymogram profiles displayed relatively few bands with xylanase and endoglucanase activity. There were two to four xylanase active bands present in all the cultures. The most prominent xylanase active bands were at 21 kDa and 45 kDa, while additional bands at 40 kDa and 18 kDa were present in some instances. The most prominent endoglucanase active band was measured to be at 30 kDa, while additional and less active bands were present at 25

kDa and 55 kDa. The most endoglucanase active bands were found in the cellobiose culture where additional bands at 37 kDa and 45 kDa were also present.

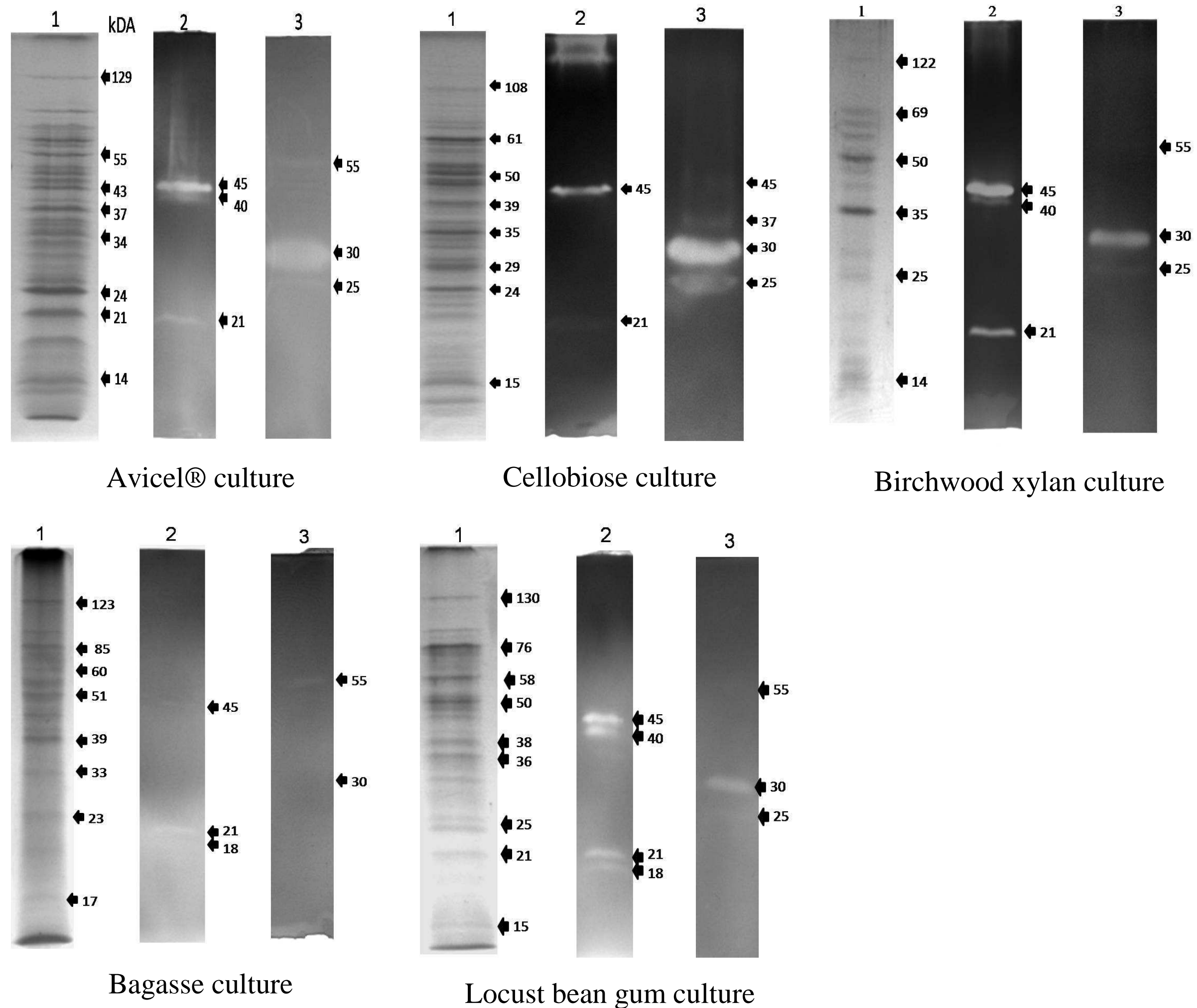


Figure 3.2. Analysis of protein species and zymogram profiles for the crude fractions from various cultures. Stained SDS-PAGE gels (Lane 1) and zymograms on 0.1% (w/v) birchwood xylan (Lane 2) and 0.1% (w/v) CMC (Lane 3) are shown for different cultures. Approximately 10 µg of protein was loaded in each lane.

The SDS-PAGE profile of the MECs from the different cultures in Figure 3.3 displayed multiple protein bands. The largest protein observed was approximately 70-77 kDa. There were differences between the numbers of bands observed for each MEC as well as differences in the prominence of some bands compared to others.

Zymogram profiles for xylanase activity displayed active protein bands at 21 kDa and 45 kDa. The active band at 40 kDa was now only observed in the bagasse MEC and not in any of the other MECs. Endoglucanase active bands were observed at 30 kDa in all MECs with the

cellobiose, birchwood xylan and locust bean gum cultures also displaying an endoglucanase active band at 25 kDa. The cellobiose culture also displayed a band at approximately 28 kDa. The 55 kDa band that was present in the crude cultures was not present in any of the MECs.

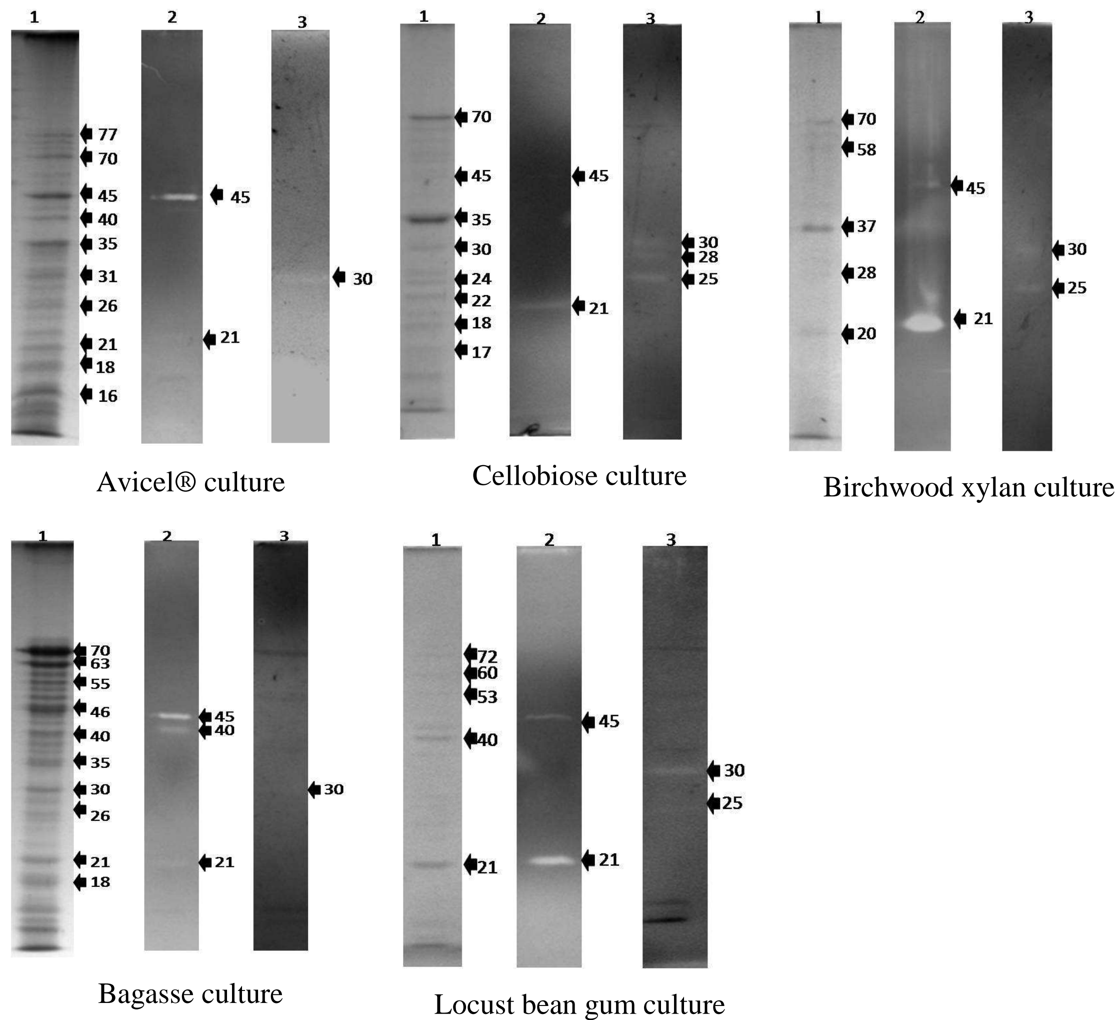


Figure 3.3. Analysis of protein species and zymogram profiles for the MEC fractions from various cultures. Stained SDS-PAGE gels (Lane 1) and zymograms on 0.1% (w/v) birchwood xylan (Lane 2) and 0.1% (w/v) CMC (Lane 3) are shown for different cultures. Approximately 10 μg of protein was loaded in each lane.

3.5 Discussion

The crude supernatants of the various cultures were assayed for cellulolytic and hemicellulolytic activities. It is clear that *B licheniformis* SVD1 is an organism with predominant hemicellulolytic activity as only moderate levels of cellulolytic activity was observed. Even when the organism was cultured on cellulose (Avicel®) and cellobiose, which are known to induce cellulases, only limited cellulolytic activity was observed. It thus appears as though *B. licheniformis* occupies a niche in the environment where hemicellulose in plants will be preferentially utilised above cellulose.

From the enzyme assay results for the crude cultures, it appears that hemicellulases, namely xylanases, mannanases and pectate lyases, are constitutively expressed in all cultures investigated. Low levels of endoglucanase activity are also observed in all cultures, and perhaps are also constitutively expressed. When differences in cultures are compared, it appears that induction of enzymes occurred when the carbon source in the medium was changed. Xylanase activity was induced by cellobiose, birchwood xylan and locust bean gum, while higher levels of endo- and exoglucanase activity was induced only by cellobiose and locust bean gum. The highest induction of pectate lyase activity was observed in cultures using locust bean gum as substrate, but moderate induction was also observed when cellobiose and bagasse was used in the culture medium. Mannanase activity was induced to high levels in the locust bean gum culture and slightly induced in the cellobiose culture.

Xylanase activity was measured on two different xylanase substrates, namely birchwood xylan and oatspelt xylan, as different sets of auxiliary enzymes are required with endo-xylanases to degrade these substrates. Birchwood xylan requires endo-xylanases and α-glucuronidase for degradation while oatspelt xylan requires endo-xylanases, α-arabinofuranosidases and acetyl xylan esterases. The chemical structure of these substrates differs substantially. Birchwood xylan is composed of 94.1% xylose residues in chains with very few substituents and is thus a good substrate for endoxylanase activity (Li *et al.*, 2000). Oat spelt xylan, on the other hand, contains 52.5% xylose residues with 22.3% arabinose and 15.7% glucose and activity of endoxylanases are hampered by the steric hindrance of arabinose substituents (Li *et al.*, 2000). Arabinofuranosidase, which cleaves arabinose

substituents from the main chain, is therefore required to ensure high activity on oatspelt xylan. It can be noted that, whereas activity on birchwood and oatspelt xylan was at almost the same level in the birchwood xylan culture, in the other cultures the activity on oatspelt xylan was lower than the activity on birchwood xylan. This appears to indicate that perhaps arabinofuranosidase was not expressed at the same level in the other cultures.

Some exoglucanase activity was observed in the cellobiose, birchwood xylan and locust bean gum cultures, indicating induction of enzyme activity. However, one should be cautious in interpretation of the results for exoglucanase activity as this may not be true exoglucanase activity. Endoglucanases may have limited activity on crystalline substrates, especially when using an enzyme assay such as the DNS assay which measures reducing ends of sugars. Cleavage of amorphous regions within the crystalline cellulose could have resulted in such reducing ends being formed within the cellulose chains. This could have resulted in apparent exoglucanase activity. It was most likely to be the case in this instance as true exoglucanase activity would probably have shown higher activity. It was proposed that limited exoglucanase activity could be observed because the corresponding endoglucanase activity within the same culture was at a higher level. In the Avicel® and bagasse cultures very low endoglucanase activity could be observed and no exoglucanase was present and this appeared to support the hypothesis.

In the cellobiose culture, there appeared to be upregulation of exo- and endoglucanase activity which, in turn, led to higher activity on bagasse. Bagasse contains a large percentage of cellulose (40-50%, Sun *et al.*, 2004a) and would require higher exo- and endoglucanase activity for its degradation. Bagasse also contains 25-35% hemicellulose (Sun *et al.*, 2004b), and thus the combined synergistic effect of all the expressed enzymes in the cellobiose and locust bean gum led to higher activity on bagasse.

Inducers are generally small molecules that are able to enter the cell. However, in the context of degradation of cellulose and hemicellulose, it must be borne in mind that these substrates are generally large and unable to enter the cell. Sophorose (β-1,2-glucobiose) has been identified as an inducer of cellulases in *Trichoderma reesei* although it is not clear whether this compound was a natural inducer of cellulases (Lynd *et al.*, 2002). Other inducers have been hypothesised to be cellobiose, δ-cellobiose-1,5-lactone and xylobiose (Lynd *et al.*,

2002). It could be argued that, by constitutively expressing low levels of enzymes, such enzymes are, in the presence of substrate, able to cleave the substrate to produce small sugars that are able to act as an inducer to elevate the levels of expression of enzymes (Lynd *et al.*, 2002). Depending on the location of genes on a genome, an inducer may activate higher levels of expression of several enzymes at the same time if such enzymes are situated within an operon. Thus an inducer such as cellobiose could often result in upregulated expression of both cellulases and hemicellulases (Lynd *et al.*, 2002).

Cellobiose has been demonstrated to act as an inducer of cellulolytic and some hemicellulolytic enzymes (Han *et al.*, 2003). This was also found to be the case in this study as the presence of cellobiose in the culture medium induced cellulases and hemicellulases to a limited extent. In the same manner, the cleavage of xylobiose from birchwood xylan could act as a mechanism of induction to elevate xylanase activity. In the locust bean gum culture, induction could take place through the production of low levels of mannobiose from cleavage of the mannan. This serves to activate expression of not only mannanase, but also xylanase and pectate lyase. Induction of enzymatic activity did not appear to take place in the presence of insoluble substrates such as Avicel® and bagasse. In the absence of true exoglucanase activity, cellobiose is unlikely to be released from these substrates in order to act as an inducer, hence the possible reason for activity remaining low.

From this data it can be concluded that *B. licheniformis* SVD1 was able to regulate enzyme expression based on the substrate it was cultured on. While many of the enzymes were constitutively expressed, even at low levels, the organism was able to upregulate such enzymes under certain substrate conditions. The constitutive expression of these enzymes suggests that the main carbon source of this organism is probably hemicellulose substrates, and to a limited extent cellulose substrates.

Based on size exclusion chromatography on Sepharose 4B, a MEC was formed in all the cultures investigated. The MEC purified from cultures of *B. licheniformis* was approximately 2,000 kDa which is quite large compared to many MECs. The molecular weight of MECs based on size exclusion chromatography has been reported as ranging from 650 kDa to 2.5 MDa in cellulosomes (Doi *et al.*, 2003) and 400 kDa and 1450 kDa in the xylanolytic MEC in *P. curdlanolyticus* (Pason *et al.*, 2006).

Based on readings at 280 nm which is generally accepted to be a reflection of protein content, it appeared as though a larger percentage of the total protein in the birchwood xylan culture was present as an MEC. In the other cultures, the MEC peak at 2,000 kDa was relatively small and the majority of protein was present as free extracellular protein. The reason for the observed difference is unclear at this stage. It was attempted to determine protein content in the fractions, but the standard Bradford's procedure was unable to give reliable results for protein in fractions and it appeared that protein content was generally low and outside the detection range. It is not clear why absorbance measurements at 280 nm were comparatively much higher. Based on the chromatography results for absorbance at 280 nm, it was decided to continue culturing the organism on birchwood xylan for purification and further characterisation of the MEC.

It can be noted that, in the literature on MECs and cellulosomes, it was not reported what percentage of extracellular protein was present as a complex compared to other free extracellular protein. It is therefore not possible to comment on whether the low levels of MEC produced are comparable to other organisms.

From the SDS-PAGE results for both the crude and MEC fractions for all the cultures, differences could be observed which seemed to further indicate that the organism was able to change its protein expression based on its environment. The composition of the crude and the MEC fractions seemed to vary based on changes in the culture medium, which can be seen in the differences in the protein bands observed and the relative intensity of these bands.

Zymogram analysis indicates the number of proteins that have activity on a specific substrate incorporated within the gel. However, there are some constraints associated with using this method. Protease activity within a culture could result in degradation of proteins. Should cleavage of a protein take place in such a manner that the active site of the enzyme is unaffected; the smaller degraded protein containing the active site could still display activity on the substrate. This would result in the appearance of two individual bands on a zymogram, the original full-size protein and the smaller degraded protein. Thus one could erroneously conclude that two separate enzymes were expressed by the organism, while it was in fact only one enzyme in two forms. This can only be established if individual proteins on a gel were identified through methods such as N-terminal sequencing. Since this data was not available,

zymogram data was interpreted on the basis that individual active bands represented individual proteins.

A further problem associated with zymogram analysis was the fact that protein samples were heated at 100°C prior to electrophoresis in order to achieve full separation of proteins. In the case of SDS-PAGE analysis, samples were denatured by heating at 100°C in the presence of 2-mercaptoethanol and SDS. In the present study it was apparent that, even after five minutes of boiling in the presence of SDS and 2-mercaptoethanol, it was possible to renature enzymes to display activity on the zymogram. This indicated that enzymes were able to withstand high temperatures and denaturation conditions for a short period of time. However, it is possible that activity was affected by the denaturation process, either being reduced or totally abolished. So while some active bands were observed on the zymogram, it is possible that some of the enzymes had lost activity after the denaturation process. Unfortunately, separation of proteins within the complex could not be achieved without the denaturation step (data not shown). Thus zymograms can be a useful tool for identifying enzymes through their activity, but the limitations of this method has to be taken into account.

Zymograms indicated the presence of active proteins but the intensity of the bands could not be used as a quantitative measurement of enzyme activity in comparison to other cultures as different incubation times were required to achieve observable bands in all cases. While zymogram analysis is a sensitive method, the length of incubation is critical and enzymes present at very low levels may not be observed at all should the incubation period be too short.

Based on zymogram analysis of xylanase activity, between two and four xylanases could be observed in crude cultures. All the crude cultures displayed at least two xylanases at 21 kDa and 45 kDa. The Avicel®, birchwood xylan and locust bean gum cultures were shown to express a further xylanase active band at 40 kDa, while the bagasse and locust bean gum cultures had a further xylanase active band at 18 kDa. What could be observed in the cellobiose and birchwood xylan cultures was a clearing zone at the top of the resolving gel, indicating xylanase activity in this region. However, no proteins were visible in this region and it is most likely that not all of the MEC or specific components of the MEC did not sufficiently separate under the denaturing and separation conditions noted above. It is

possible that for some reason, the proteins in these two cultures did not separate clearly and remained aggregated at the top of the gel when entering the resolving portion. It is, however, not clear why such protein would not be observed after staining.
The xylanase activity in the crude Avicel and bagasse cultures was found to be very low and it was postulated that this indicated low levels of constitutively expressed xylanase activity. From the zymogram analysis, the Avicel® culture had three xylanase active bands at 21 kDa, 40 kDa and 45 kDa, while the bagasse culture had active bands at 18 kDa, 21 kDa and 45 kDa. It is not clear why all four enzymes were not detectable in both cultures. The activity levels for xylanase activity were virtually identical and thus an identical pattern was expected on the zymogram. From the enzyme assays it did not appear as though induction of xylanases took place in these cultures. Thus it appears as though all four these xylanases were expressed constitutively in *B. licheniformis*. The observed increase in activity in other cultures appeared to represent an upregulation of the same enzymes as no additional bands were observed.

Where induction of xylanase activity clearly seemed to take place, such as in the cellobiose and birchwood xylan cultures, relatively few bands were observed. Only two bands were found in the cellobiose culture and only three in the birchwood xylan culture, although these have the same molecular weights as in the Avicel and bagasse cultures. The locust bean gum culture was the only culture where all four xylanase active bands at 18, 21, 40 and 45 kDa could be observed.

Xylanase activity in the MEC was limited to two active bands at 21 kDa and 45 kDa. Surprisingly, the bagasse culture had an additional band at 40 kDa with xylanase activity. Thus it appears as though only two xylanases were generally present in the MEC and that not all the xylanases found in the crude was incorporated into the MEC. There is also a possibility that the composition of the MEC may differ between cultures with a different carbon source in the medium. However, in this case the xylanase active bands were similar between all the MECs.

CMCases or endoglucanases were present in the crude as a very prominent band at 30 kDa with additional active bands at 25 kDa and in some cultures at 55 kDa. As expected, the cellobiose culture, with the highest activity, displayed the most active bands with additional

bands at 37 kDa and 45 kDa that were not present in any other culture. It appeared as though endoglucanases at 25 kDa, 30 kDa and 55 kDa were constitutively expressed in all the cultures under the conditions listed above. Upregulation of endoglucanase activity, that seemed to take place in the cellobiose culture, lead to additional endoglucanase active bands at 37 kDa and 45 kDa.

In the MEC, no endoglucanase active band was visible at 55 kDa in any of the cultures. All cultures displayed a band at 30 kDa and the cellobiose, birchwood xylan and locust bean gum cultures also had an active band at 25 kDa. The cellobiose culture, in addition, also displayed an active band at 28 kDa. Thus it appears as though the 55 kDa endoglucanase did not form part of the MEC and that the composition of the MEC with respect to cellulases was different between different cultures.

The SDS-PAGE patterns of the MECs from the different cultures displayed some differences, although the resolution and staining in the birchwood xylan and locust bean gum cultures was poor and made comparison difficult. Perhaps a different staining method would have been able to display the protein species more effectively. The largest observable band in the MEC was in the region of 70 – 77 kDa. The largest band in SDS-PAGE patterns from cellulosomal studies was often found to represent the scaffoldin protein. The smallest scaffoldin found to date was 90 kDa in *Ruminococcus flavefaciens* (Rincon *et al.*, 2003). Generally the scaffoldin is a large protein and the main scaffoldin in *Clostridium thermocellum* has a molecular weigth of 196 kDa (Bayer *et al.*, 1998). See section 1.3.1.6 for more detail on scaffoldin proteins.

The MEC contained up to twenty different observable protein bands based on the SDS-PAGE data obtained. This will be further characterised in later chapters. It is not clear why the MEC, with so many proteins, seemed to possess so few with xylanase activity as this is the predominant enzyme activity found in all cultures.

3.6 Conclusions and future work

It can be concluded that *B. licheniformis* was able to regulate enzyme expression based on the substrate it was cultured on. The organism expressed mainly hemicellulolytic enzymes, with xylanase activity being predominant, while high levels of mannanase and pectate lyase were achieved in some cultures. Endoglucanase activity remained low even where some induction took place. Exoglucanase activity was only observed in the cellobiose and locust bean gum cultures. While the enzymes appeared to be constitutively expressed at low levels, the organism was able to upregulate such enzymes under certain substrate conditions.

Biochemical methods were used in this chapter to determine the extent to which changes in enzyme activity was induced by different carbon substrates. These methods have certain limitations, however, as enzyme activity could also be affected by methods of purification, storage of the protein and the assay conditions itself. A more accurate way to examine regulation of enzyme expression would be analyse mRNA levels of various enzymes within the cell. However, this is only possible where the genes of various enzymes are known. When this work was carried out, such information was not available and therefore biochemical methods were the only possible means to look at enzyme expression and induction. Perhaps one should have also cultured the organism on monomeric sugars to determine the effect of this on the expression of enzymes and whether catabolite repression took place under certain circumstances. One could furthermore culture the organism in the absence of a carbohydrate substrate to determine the exact levels of constitutive expression of the cellulolytic and hemi-cellulolytic enzymes.

Comparison of SDS-PAGE and zymogram patterns for crude cultures and MECs was carried out. Between two and four xylanase active bands were observed in crude cultures while the MEC mainly contained two xylanase active bands at 21 kDa and 45 kDa. Between two and four endoglucanase active bands were observed in the crude with the greatest number being present in the cellobiose culture. The MECs exhibited mainly one or two endoglucanase active bands at 25 kDa and 30 kDa.

In order to characterise the MEC in greater detail, it will be isolated and purified from a birchwood xylan culture for further study. This study forms the basis of the work covered in the following chapters.

CHAPTER 4 – PURIFICATION OF A MULTI-ENZYME COMPLEX FROM *BACILLUS LICHENIFORMIS* SVD1

4.1 Introduction

When assessing the approaches used in the literature for purification of MECs, it must be born in mind that most of the literature on multi-enzyme complexes focuses on the cellulosome. This is important as one of the distinct structural features of the cellulosome is used in its purification. The general model of a cellulosome is that it contains a noncatalytic scaffoldin protein to which all the catalytic subunits bind. The scaffoldin protein possesses a CBM3a domain which binds strongly to cellulose (See section 1.3.1.6 for more detail.). It is this characteristic that has most frequently been used in purification strategies by utilising the binding of the cellulosome to cellulose to isolate it from contaminating proteins. However, it should be noted that this model of the scaffoldin is not always applicable as the scaffoldin in the cellulosome of *R. flavefaciens* did not possess an identifiable CBM (Rincon *et al.*, 2003), and the scaffoldin in *A. cellulolyticus* had a CBM3b which was not specific for crystalline cellulose (Xu *et al.*, 2003) (see section 1.2.8 on CBMs and their classification).

The majority of purification methods in the literature on MECs commence with the concentration of the culture supernatant, followed by an affinity binding step in which the concentrated protein interacts with cellulose, either crystalline or amorphous. Through its CBM3a domain, the whole cellulosome binds to the cellulose and this allows its separation from other proteins. The bound protein is then eluted from the cellulose using 1% (v/v) triethylamine (Bayer *et al.*, 1985, Kosugi *et al.*, 2001, Murashima *et al.*, 2002, Pason *et al.*, 2006). A cellulosome is not the only protein that will bind to cellulose, as free cellulases could also possess a CBM domain specific for binding cellulose. The bound protein is therefore likely to be a mixture of complexed and free protein and has to be separated using a further purification step.

The affinity binding is followed by size exclusion chromatography using a resin with an appropriate range such as Sepharose 4B, Sephacryl S-200 or Sephacryl S-300. Sepharose 4B has the widest separation range from 60-20,000 kDa. This particular resin was mainly used

in the purification of *C. thermocellum* that possesses the largest cellulosome of between 2,000-6,500 kDa. Sephacryl S-200 and S-300 have narrower separation ranges and a complex of 2,000 kDa would elute in the void volume of columns based on this resin. The separation range for Sephacryl S-200 is 5-250 kDa while that of Sephacryl S-300 is 10-1500 kDa. These resins were used in purification of smaller MECs such as the cellulosome in *C. cellulovorans* which is about 1,000 kDa (Kosugi *et al.*, 2001), or the MECs found in *C. papyrosolvens* that was between 500 and 660 kDa (Cavedon *et al.*, 1990). The choice of resin in each instance appears to be based on knowledge of the size of the specific MEC.
In some cases, the purification method was mainly based on isolating a protein complex with a large size. This was used in the case of *C. papyrosolvens* (Cavedon *et al.*, 1990) and *R. albus* (Ohara *et al.*, 2000) where it was not known whether the MEC had a CBM3a domain.

The hypothesis that cellulosomes may not be homogeneous in composition has led to the use of anion exchange chromatography in cellulosome purification. After purification of the cellulosome through size exclusion chromatography, different cellulosomal subpopulations were separated using anion exhange chromatography (Han *et al.*, 2005, Murashima *et al.*, 2002).

An MEC was purified in one instance using anion exchange as an initial step, which was then followed by size exclusion chromatography (Kim & Kim, 1993). This method allowed for the purification of two different MECs from *Bacillus circulans* (Kim & Kim, 1993).

Generally, it is recommended that a purification strategy should use the fewest steps possible in order to obtain the desired protein as this will most likely give the highest yield (Walker, 2000). The number of purification steps should be based on considerations such as the degree of purity and the purpose for which the protein is required.

Purification of proteins forms the basis of their biochemical characterisation. Protein purification is generally focused on isolating one protein from a large number of proteins such as those found in the cell or secreted extracellularly. In studying a multi-enzyme complex, a group of enzymes are purified, rather than only one protein. Traditional methods of monitoring the purification process of an enzyme measures the enzyme activity in

fractions and uses a purification table to measure the success of the purification in terms of yield and fold purification. When purifying a multi-enzyme complex, these methods can still be used, but the information gained from it has to be assessed from a different perspective. Generally, in purifying an MEC, the enzyme activity of a single enzyme is measured, such as cellulase or xylanase activity. Should this enzyme occur both in the MEC fraction and other fractions, the resulting yield of MEC may be low, although the objective of the purification process was achieved by isolating the MEC. Furthermore, as only one enzyme activity is measured against a background of a group of proteins, the specific activity and therefore the fold purification will generally be low which explains why most reported literature on MEC purification does not contain any form of purification table.

4.2 Objectives

- To purify an MEC from a birchwood xylan culture of *B. licheniformis* SVD1

4.3 Methods and materials

4.3.1 Culturing of organism

B. licheniformis was cultured in the medium as described in section 2.3.4. Cultures were grown to stationary phase (3-4 days) before purification of the MEC.

4.3.2 Protein purification

4.3.2.1 Purification protocol 1

The initial purification method used was based on an affinity purification method as used in the cellulosome field. The basis of the method assumes the presence of a CBM3a on the scaffoldin protein which is able to bind strongly to cellulose.

Cultures were centrifuged at 12,000 *x g* for 10 min. The supernatant was precipitated with 50% saturation with ammonium sulfate, followed by further centrifugation at 12,000 x *g* for 10 min. The pellet was suspended in 50 mM Tris-HCl buffer at pH 7.5 and dialysed against the same buffer. Phosphoric acid-swollen amorphous Avicel was added to the dialysed pellet and then incubated for 3 hours at 4°C. The amorphous Avicel and protein suspension was then centrifuged at 12,000 *x g* for 10 min and the supernatant stored as the free enzymes that were unable to bind to the amorphous Avicel. The pellet was washed with 1% (v/v) triethylamine and kept at 4°C for 20 min after which it was centrifuged at 12,000 *x g* for 10 min and the supernatant dialysed against distilled water. The dialysed extract was concentrated with PEG 20,000 and then loaded onto a Sepharose 4B column (27 x 1.5 cm). The protein was eluted with 50 mM Tris-HCl buffer at pH 7.5, containing 0.03% (w/v) sodium azide. Fractions of 1 ml were collected and the absorbance of fractions determined at 280 nm.

4.3.2.2 Purification protocol 2

This purification method was based on the principle that an MEC will be large in size and relies on size exclusion chromatography to purify the MEC from the total extracellular protein.

Stationary phase cultures were centrifuged at 12,000 *x g* for 10 min to remove the cells. The supernatant was concentrated using two different methods which were then compared. The first method of concentration used was 80% ammonium sulphate precipitation followed by centrifugation for 10 min at 12,000 *x g*. The second concentration method used was

ultrafiltration using an Amicon 8200 ultrafiltration cell (Millipore, South Africa) with a PBGC filter with a nominal molecular weight cut-off of 10 kDa.

The concentrated protein in each case was loaded onto a Sepharose 4B column (50 x 2.5 cm) and eluted with 50 mM Tris-HCl buffer at pH 7.5, containing 0.03% (w/v) sodium azide. Fractions of 3 ml were collected and the absorbance of fractions determined at 280 nm. The fractions from the first peak at 2,000 kDa which represented the MEC, were pooled and concentrated using polyethylene glycol (PEG) 20,000 and used for further experiments.

These two concentrating steps were compared by evaluating the final yield of MEC obtained through each method.

4.3.2.3 Purification protocol 3

Although the methods listed in 4.3.2.2 produced an MEC, a further purification method was employed for the following reasons: (i) possible aggregation of proteins due to the concentrating step was suspected. If this was the case, the purified MEC might in fact be an aggregate; (ii) it was established that there was protease activity within the MEC; and (iii) previous purification methods commenced with concentration of the total extracellular protein while we were mainly interested in the characterisation of the MEC.

The purification method was adjusted to include an ion exchange step prior to size exclusion chromatography.

A culture at stationary phase was centrifuged at 12,000 *x g* for 10 minutes. The supernatant was then filtered using an Amicon 8200 ultrafiltration cell with a filter with a nominal molecular weight cut-off of 50 kDa (not 10 kDa as previously used). The concentrated ultrafiltration pellet was loaded onto an ion exchange column. Both cation exchange and anion exchange methods were tested as the pI of the MEC was not known. For cation exchange, Tosohaas Tsk gel CM 650M resin was used and Toyopearl DEAE 650M (Separations, South Africa) for anion exchange. Anion exchange was tested at pH 7.5, pH 8 and pH 9, while cation exchange was tested at pH 5. For anion exchange, a 20 mM Tris-HCl

buffer was used while a 20 mM sodium acetate buffer was used for cation exchange. Sample volumes of 10 ml were loaded onto the column and elution of protein was carried out with a stepwise gradient of increased NaCl concentrations from 0 mM NaCl to 1 M NaCl. Fractions were collected (5 ml each) and the absorbance of each fraction was measured at 280 nm. Protein concentration was measured in each fraction using a modified Bradford method (see section 4.3.4 below) (25 µl of sample with 230 µl of Bradford's reagent) and xylanase activity in each fraction was measured using the DNS method (as described in section 2.3.2) but conducting the assay for 3 hours.

The fractions from each peak from the ion exchange chromatography which contained xylanase activity were pooled and concentrated using PEG 20,000. Samples from each peak were loaded on a Sepharose 4B column. Each peak was also tested for protease activity. The fractions from the final purification method were used to calculate a purification table. Fractions from all the purification steps were also subjected to electrophoresis on an SDS-PAGE gel.

4.3.3 Enzyme assays

Enzyme assays were conducted as described in section 2.3.2.

4.3.4 Protein determination

Protein was measured according to the Bradford method (Bradford, 1976). The method was modified to be able to accurately determine protein concentration in samples with low protein. Sample volumes of 5 µl, 10 µl or 25 µl were used with Bradford's reagent in the ratios of 5:250 µl, 10:250 µl or 25:230 µl. Standard curves for each ratio was prepared (see Appendix 4a). The modified method with larger sample volumes allowed greater sensitivity, accuracy and consistency in measurements.

4.3.5 Measurement of protease activity

Protease activity was measured using a modified assay by Najafi *et al.* (2005). A 1% (w/v) protein solution was prepared by mixing 10 ml of fat free milk and 20 ml of Tris-HCl buffer (50 mM, pH 7.5). The substrate mixture (450 µl) was pre-incubated at 37°C after which 100 µl of enzyme mixture was added and the assay mixture incubated for 2 hours at 37°C. The assays were removed from incubation and 750 µl of a 5% (w/v) trichloroacetic acid solution added. The mixture was briefly vortexed and incubated at room temperature for 30 min. Thereafter it was centrifuged at 16,000 *x g* in a benchtop centrifuge for 5 min and the supernatant removed. Protein was measured in the supernatant by taking measurements at 280 nm. Each enzyme mixture (100 µl) together with Tris-HCl buffer was used as a negative control.

4.3.6 Electrophoresis

Electrophoresis was carried out as described in section 3.3.4 except that only 2 µg protein was loaded per lane and gels were stained with PageSilver^{TM} Silver Staining kit (Fermentas) according to the manufacturer guidelines.

4.4 Results

4.4.2 Protein purification

4.4.2.1 Purification protocol 1

When following this purification method, the protein fraction that was bound to the Avicel was eluted with triethylamine and loaded on a Sepharose 4B column. The chromatogram from a typical size exclusion step is depicted in Figure 4.1 and displayed a peak on the Sepharose 4B size exclusion chromatogram in the same place as the MEC purified using other methods (data not shown). This peak had protein and xylanase activity and no other peaks appeared in the chromatogram for the duration of the elution (40 ml).

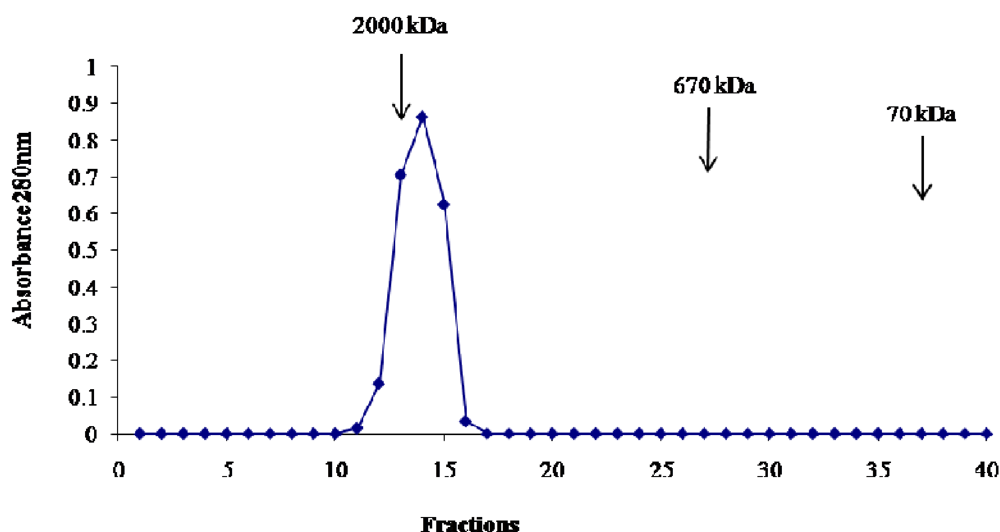


Figure 4.1. A typical Sepharose 4B chromatogram of the protein fraction eluted from amorphous Avicel with 1% triethylamine (v/v). A column of 28 x 1.5 cm was used and fractions of 1 ml collected. Blue dextran (2,000 kDa) and thyroglobulin from bovine thyroid (670 kDa) and laccase from *Trichoderma reesei* (70 kDa) were used as standards.

4.4.2.2 Purification protocol 2

The comparison of the two types of concentration steps, namely ammonium sulphate precipitation and ultrafiltration, demonstrated that the highest yield was achieved through using ultrafiltration as a concentration step. The MEC was purified through ultrafiltration to a yield of 54% while purification through ammonium sulphate only achieved a yield of 21%. Figure 4.2 represents a typical chromatogram of the purification of the MEC through size exclusion chromatography using Sepharose 4B resin. This result was obtained by ultrafiltration using a 10 kDa molecular weight cut-off filter. A large peak was observed at approximately 2,000 kDa using both the absorbance at 280 nm and measurement of protein content using the Bradford’s method. A second protein peak was observed eluting between fractions 45 and 60. This peak did not coincide with the absorbance peak measured at 280 nm which eluted between fractions 50 and 75 and was most likely due to the xylan components in the medium which absorbs at 280 nm.

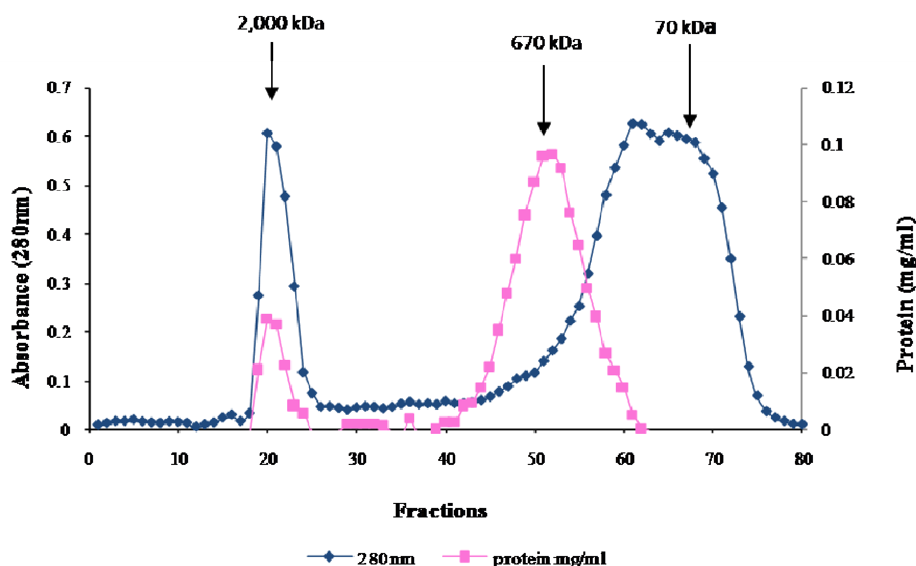


Figure 4.2. A typical Sepharose 4B chromatogram of concentrated supernatant from a birchwood xylan culture. Obtained using a 50 x 2.5 cm column and collecting fractions of 3 ml. Blue dextran (2,000 kDa), thyroglobulin from bovine thyroid (670 kDa) and laccase from *Trichoderma reesei* (70 kDa) were used as standards.

4.4.2.3 Purification protocol 3

This purification method used an ion exchange step prior to the size exclusion step during purification. The first ion exchange step used was anion exchange at pH 8. A large peak, based on protein measurement and absorbance at 280 nm, was observed at the start of the elution with 20 mM Tris-HCl and no NaCl (data not shown). Other significant peaks eluted with 200 mM NaCl and 300 mM NaCl (data not shown). Xylanase activity was present in all the peaks (data not shown). When samples from these peaks were loaded on a Sepharose 4B column, peak 1 eluted with one protein peak at 2,000 kDa. Peak 2 and 3 displayed an elution profile with an insignificant amount of protein at 2,000 kDa and the majority of protein eluting as small extracellular protein. Thus it appeared as though the MEC did not bind to the resin.

As a result, the procedure was repeated but using 20 mM Tris-HCl at pH 9 to achieve binding. However, the same pattern of elution was found, with the MEC eluting with the 20 mM Tris-

HCl before any salt gradient was applied (data not shown). As a result, cation exchange at pH 5 was employed in an attempt to find a working pH below the pI of the MEC protein. Again, a similar pattern of elution was observed as the MEC eluted at the start of the chromatogram before the salt gradient commenced and thus no binding to the resin took place (data not shown). Therefore it was decided to use this characteristic of the MEC, the inability to bind to the anion or cation exchange resin, as a means to purify it.

As the anion exchange was followed by size exclusion chromatography with 50 mM Tris-HCl buffer at pH 7.5, it was decided to test the anion exchange at this buffer ionic strength and pH. If the same pattern was observed, then these buffer conditions would remove the need for an additional dialysis step between anion exchange and size exclusion chromatography which could result in the loss of protein.

A typical anion exchange chromatogram using 50 mM Tris-HCl buffer at pH 7.5 is shown in Figure 4.3 and comprises of four distinct peaks using readings at a wavelength of 280 nm as well as protein measurement using the Bradford's method. The activity for each fraction was tested for xylanase activity over three hours as the protein concentration was very low in each fraction. Each peak displayed xylanase activity, with peak one having the highest specific activity. Fractions in each peak were pooled and concentrated. Samples from peak 1, 2 and 3 were loaded on a Sepharose 4B size exclusion column to determine the presence of an MEC within that peak. Peak 4 contained a negligible protein concentration and the sample was too small for further analysis. The chromatograms of the size exclusion step are shown in Figures 4.4, 4.5 and 4.6. From Figure 4.3, it is clear that peak 1 from the anion exchange step consisted solely of protein in an MEC as a single peak eluting at approximately 2,000 kDa.

Peak 2 from the anion exchange displayed two peaks in the size exclusion step (Figure 4.5). The first peak had very low protein, although the measurement of absorbance at 280 nm indicated a large peak. However, due to the absorbance of birchwood xylan at 280 nm, these readings should be interpreted with caution. The protein measurement using the Bradford's method is the only reliable method of determining the presence of protein. Although the first peak eluted at approximately 2,000 kDa, the protein present in this peak appeared to be insignificant. The vast majority of protein eluted as free extracellular protein.

Peak 3 from the anion exchange step displayed mainly one protein peak in the size exclusion step (Figure 4.6). While a large peak is observed at approximately 2,000 kDa with the absorbance readings at 280 nm, the protein measurements show that there was virtually no protein present in this particular fraction. The majority of the protein eluted as free extracellular protein.

Protease activity was only measured in the MEC when purified using size exclusion chromatography as performed in purification method 2 in section 4.4.2.2 and it was observed that protease activity was present (data not shown). On the basis that no protease activity had been reported previously in MECs, it was considered that the proteases were perhaps aggregating with the MEC. For this particular reason ion exchange was included as a purification step. Following anion exchange, protease activity was measured in the pooled and concentrated peaks 1-3 as shown in Figure 4.3. It was found that peak 1 exhibited no protease activity, while peak 2 and 3 had protease activity (data not shown).

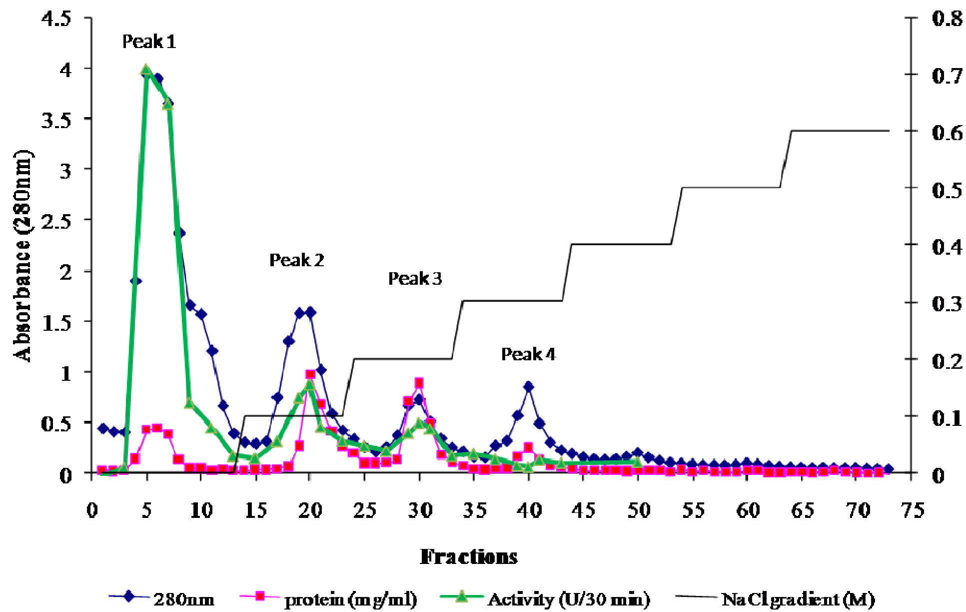


Figure 4.3. A typical chromatogram of the anion exchange step from purification method 3. Toyopearl DEAE 650M anion exchange resin was used with 50 mM Tris-HCl buffer at pH 7.5 with a stepwise gradient of NaCl. (column 2.5 x 6.5 cm, flowrate 11 ml/min). The secondary y-axis represents protein concentration (mg/ml), xylanase activity (U/30 min) and NaCl concentration (M).

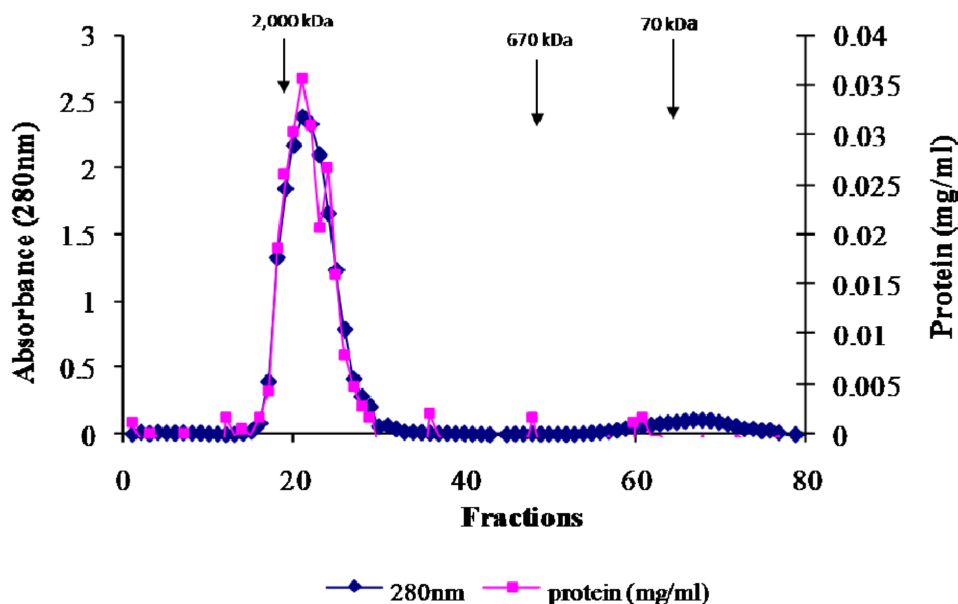


Figure 4.4 Size exclusion chromatography of the pooled fractions from Peak 1 in Figure 4.3. A Sepharose 4B column was used (50 x 2.5 cm) and fractions of 3 ml collected (flow rate 40 ml/h). Blue dextran (2,000 kDa), thyroglobulin from bovine thyroid (670 kDa) and laccase from *Trichoderma reesei* (70 kDa) were used as standards.

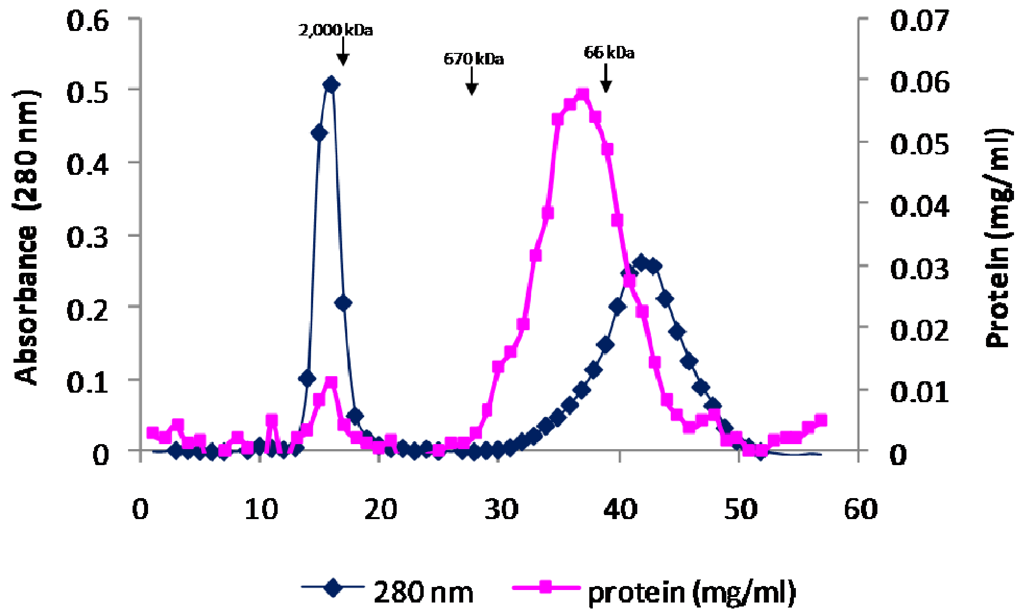


Figure 4.5 Size exclusion chromatography of Peak 2 of the anion exchange purification step from Figure 4.3. A Sepharose 4B column was used (27 x 1.5 cm) and fractions of 1 ml collected (flow rate 25 ml/h). Blue dextran (2,000 kDa), thyroglobulin from bovine thyroid (670 kDa) and bovine serum albumin (BSA) (66 kDa) were used as standards.

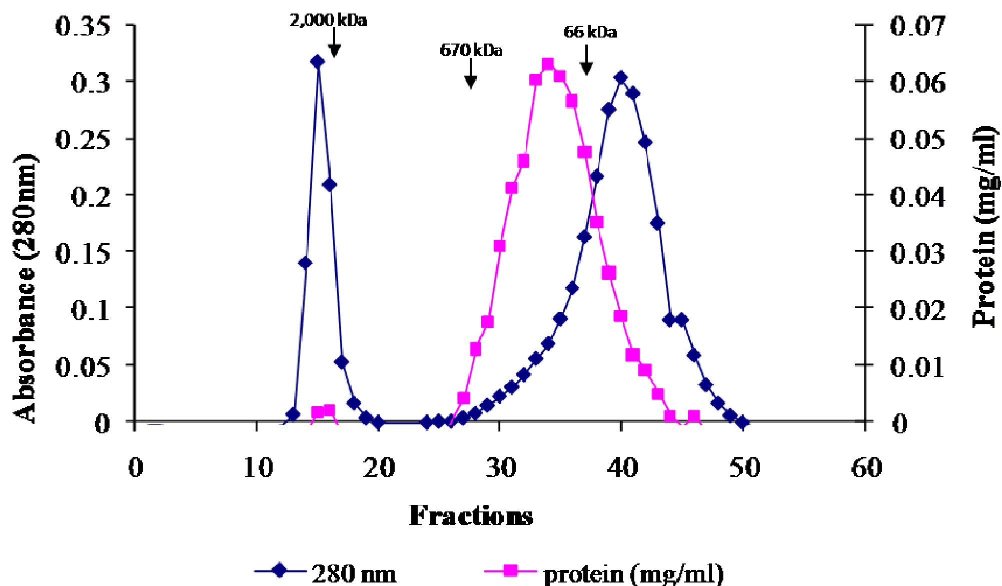


Figure 4.6 Size exclusion chromatography of Peak 3 of the anion exchange purification step from Figure 4.3. A Sepharose 4B column was used (27 x 1.5 cm) and fractions of 1 ml collected (flow rate 25 ml/h). Blue dextran (2,000 kDa), thyroglobulin from bovine thyroid (670 kDa) and bovine serum albumin (BSA) (66 kDa) were used as standards.

Table 4.1 summarises the progressive purification through the different steps used. This purification table is based on method 3, using anion exchange with 50 mM Tris-HCl buffer at pH 7.5 followed by size exclusion chromatography with Sepharose 4B. Firstly, some activity was present in the pellet, although the majority of activity was present in the supernatant. During the ultrafiltration process using a 50 kDa filter, activity appeared to be lost as a high level of activity was present in the ultrafiltration filtrate. Free extracellular xylanases present in the supernatant fluid that are smaller than 50 kDa would appear in the filtrate, while larger extracellular xylanases or xylanases present in the MEC would be retained. While the purpose of the purification was the isolation of an MEC, it would be inevitable that free xylanases would be lost during the purification process. In this respect, conventional assessment of the purification table would not be accurate.

From the purification table, it can be observed that the yield after anion exchange was 17% while the yield after both anion exchange and size exclusion chromatography was only 11%. This is a low yield compared to purification methods using only size exclusion chromatography where yields of up to 50% were achieved. However, the purpose of the purification was not to achieve a high yield, but simply obtaining enough material to characterise the MEC.

Table 4.1 Purification table showing the different steps during purification method 3 using anion exchange with 50 mM Tris-HCl at pH 7.5 and thereafter size exclusion using Sepharose 4B. Ultrafiltration was carried out using a 50 kDa nominal molecular weight cut-off filter. Protein was measured using the Bradford's method while activity is represented as xylanase activity (with birchwood xylan used as substrate).

	Protein (mg/ml)	**Enzyme activity (U/ml)**	**Yield (%)**	**Specific activity (U/mg)**	**Fold purification**
Crude	0.71	0.05	100	0.07	1.00
Pellet	8.20	0.05	6	0.01	0.09
Supernatant	0.11	0.04	89	0.41	6.05
Ultrafiltration pellet	0.48	0.14	21	0.29	4.31
Ultrafiltration filtrate	0.05	0.02	46	0.41	6.09
Anion exchange Peak 1	0.12	0.10	17	0.79	11.83
MEC after size exclusion step	0.07	0.05	11	0.78	11.57

A 10% SDS-PAGE gel analysis of the purification steps is displayed in Figure 4.7. It is apparent from Lane 5 that several proteins larger than 50 kDa appeared in the filtrate. It is generally considered that, to retain a protein of interest, it should be 30-50% larger than the cut-off molecular weight of the filter (Bollag *et al.*, 1996). Thus it is probably possible that proteins of up to 75 kDa may not be retained by the filter. What should also be considered is that native, folded proteins may behave similar to smaller proteins during filtration compared to their molecular weight when denatured. The band at approximately 120 kDa is, however, unexpected even when taking all these considerations into account.

Lane 6 of Figure 4.7 displays the protein in the anion exchange peak 1, while Lane 7 displays the MEC after size exclusion chromatography. These two lanes were very similar in terms of the proteins that were present. Some proteins only differed with respect to their intensity after staining, indicating that some protein was lost during the size exclusion step. The most prominent difference was the protein at approximately 30 kDa in Lane 6 which did not appear to be present in Lane 7.

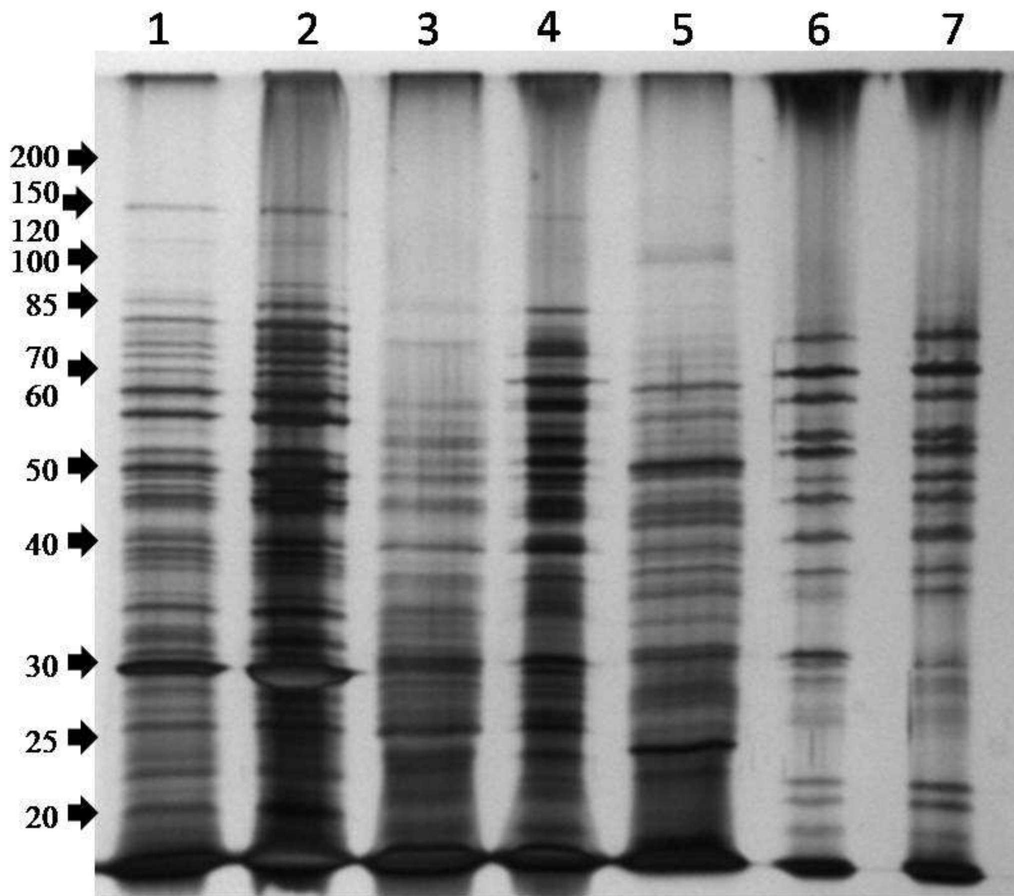


Figure 4.7. A 10% SDS-PAGE gel displaying purification steps (Purification method 3). Each lane was loaded with approximately 2 µg of protein. Lanes: 1-Crude, 2-pellet, 3-Supernatant, 4-Ultrafiltration pellet, 5-Ultrafiltration filtrate, 6-Anion exchange peak 1, and 7-MEC after size exclusion chromatography.

4.5 Discussion

Purification method 1 was based on an assumption that all MECs would possess the feature of a CBM3a domain which would allow the entire complex to bind to cellulose, thereby facilitating purification. However, in retrospect this was based on a further assumption that the MEC in *B. licheniformis* was a cellulosome and therefore had the same structural features as other cellulosomes. As more cellulosomes are discovered, it is also becoming apparent that not all cellulosomes are structurally identical and not all scaffoldin proteins have a binding domain for cellulose. Unless this structural feature has been shown to exist in the MEC from *B. licheniformis*, this method would thus be an inappropriate choice and was not

explored further in this work. Another characteristic of MECs, namely its large size, was used in further purification methods.

It is not clear why size exclusion chromatography of the triethylamine eluent (proteins able to bind to cellulose) produced a peak at 2,000 kDa as further binding study characterisation demonstrated that the MEC was unable to bind to crystalline cellulose (see section 7.4.2). It could be possible that binding in this purification method did not take place through a binding domain such as a CBM3a, but rather through interaction between the cellulose and polysaccharides that co-purified with the MEC. It has been reported that xylan interacts through hydrogen bonding with cellulose and becomes deposited on the cellulose surface in solution (Hon, 1996).

Purification method 2 was mainly based on using size exclusion chromatography to purify and MEC. However, the initial concentration step was assessed to determine the optimal method for purification. On the basis of the difference in purification yield from purification method 2, it was decided to continue using ultrafiltration as a concentrating step rather than ammonium sulphate precipitation. It appeared as though the ammonium sulphate possibly denatured the protein in the supernatant. The effectiveness of ammonium sulphate precipitation also depends on the starting protein concentration and Bollag *et al*. (1996) recommends a starting protein concentration of at least 1 mg/ml. The protein in the supernatant was generally much lower than 1 mg/ml as can be seen from the purification Table 4.1 where the supernatant protein concentration was only 0.11 mg/ml. This could explain the low yield achieved through ammonium sulphate precipitation.

Purification of the MEC through size exclusion chromatography revealed a large peak at approximately 2,000 kDa. However, the size exclusion chromatogram from purification method 2 displayed a second protein peak. This was expected as the concentrated protein contained both MEC and other extracellular protein. Based on the elution profile of the second peak, it can be questioned whether this peak did not also contain an MEC as it eluted at an approximate size between 70 kDa and 600 kDa. However, this peak was very broad and probably the result of an overloaded column, thus giving an overestimation of the size of the proteins in the peak. Furthermore, the Sepharose 4B resin does not separate proteins in

smaller size ranges very well. Virtually no difference in elution was observed between protein standards as varied as 70 kDa and 300 kDa (data not shown). The protein in this second peak was therefore not further characterised.

It must be noted that during this purification, it was found that birchwood xylan absorbed strongly at 280 nm. This was determined through a spectral scan of the substrate (data not shown). Thus readings at this wavelength, normally seen as a reflection of the protein content of a fraction, were not reliable unless protein was also measured in each fraction. This was made possible by the adaptation of the normal Bradford's method to allow for measurement of samples at lower concentrations.

Furthermore, it was also found that the birchwood xylan substrate itself displayed two peaks on a Sepharose 4B size exclusion column based on absorbance at 280 nm (data not shown). The first peak eluted at 2,000 kDa, at the same position as the MEC. This appeared to represent the high molecular weight fraction of the birchwood xylan. However, no protein was detected in this peak when running the birchwood xylan only. When the MEC was purified through size exclusion chromatography, the peak at 2,000 kDa contained protein and had xylanase activity. The discrepancy between the absorbance at 280 nm and the protein measurement of fractions appears to be as a result of the presence of birchwood xylan in this peak which seemed to co-elute with the MEC. It was not clear whether the protein was bound to the birchwood xylan or whether it simply co-purified. It was also not clear why, with such high xylanase activity in the medium, the higher molecular weight portions of the xylan substrate appeared to remain intact after several days of culturing. Further characterisation of the MEC confirmed the presence of polysaccharides in the purified protein which is presumed to be birchwood xylan. This was detected through the high background sugar reading of the purified MEC.

The birchwood xylan substrate also displayed a second peak in the size exclusion chromatogram, measured as absorbance at 280 nm. This peak was detected at a position where smaller proteins would elute. During purification of the MEC, absorbance measurements at 280 nm displayed a peak in the same position (Figure 4.2 peak 2). However, when protein readings were taken for all fractions, the position of the protein peak was quite

different from the peak at 280 nm. Again, it was found that absorbance readings measured at 280 nm were deceptive and not an accurate reflection of the elution of protein from the column as it was influenced by absorbance of the substrate at the same wavelength.

In the previous chapter at section 3.4.3 and Figure 3.1, the MEC peak in the birchwood xylan culture was much larger than the MEC peaks from other culture media. The chromatograms in Figure 3.1 were solely based on absorbance readings at 280 nm. The fact that the birchwood xylan absorbed at this wavelength and eluted a peak in the same position, possibly explains the difference in the size of the peaks observed. The other soluble substrates used in that instance, namely cellobiose and locust bean gum, showed only weak absorbance at 280 nm and did not display a peak at 2,000 kDa (data not shown). Thus, the peaks found at 2,000 kDa for these substrates, or for the insoluble substrate such as Avicel and bagasse, did not show interference from the media components.

The third purification method was based on the introduction of an ion exchange step prior to size exclusion chromatography. From the results of this purification method it was apparent that most of the protein in the MEC did not bind to either the cation or anion exchange resin at any of the pH values tested. Therefore it was decided to exploit this characteristic in the purification. As a result, an anion exchange step at the most convenient pH, 7.5, was used in further purifications as it achieved sufficient separation as seen in Figure 4.3.

It is not clear why the MEC was unable to bind to the anion or cation exchange resin. The general principle of ion exchange is that proteins would be negatively charged at a pH above its pI which would allow it to bind to the anion exchange resin. At a pH below the pI, proteins would be positively charged, which would allow it to bind to cation exchange resin. The pI of the MEC in this case was not known, therefore various pHs were tested with different resins. Although pH 7.5, 8 and 9 was tested, the MEC did not bind to the anion exchange resin. This could indicate that the MEC was positively charged at those pHs, and that the pI of the MEC was very high, at pH 9 or above. However, this would imply that the MEC would also be positively charged at pH 5, with the result that it should have displayed a strong interaction with the cation exchange resin, but this did not take place.

The most likely explanation for the MEC behaviour during ion exchange chromatography is the exclusion limits of the ion exchange resin. According the manufacturers' guidelines, the exclusion limit for both the cation and anion exchange resin used is 1×10^6 Daltons (http://www.grom.de/phases_bulk/toyopearl.htm). As the MEC is approximately 2,000 kDa in size, it is possible that the MEC was not able to interact with the resin as it could not enter the pores. As a result, the ion exchange resin acted in a similar manner to size exclusion resin and the MEC eluted with the initial application of buffer. Contaminating proteins that were smaller than the exclusion limit were able to interact with the resin. Thus they were retained and eluted with the application of the salt gradient.

If this is the case, it must be questioned whether purification method 3 and the inclusion of an ion exchange step was in fact a valid and effective method of purification. The purpose of the purification was the isolation of an MEC for further study and characterisation. While the combination of anion exchange and size exclusion chromatography did achieve this purpose, the yield of MEC was quite low. However, the two-step process in method 3 resulted in faster purification times as the anion exchange column has faster flow rates and removed all contaminating material. Therefore fewer runs of the size exclusion step were required for the final purification step. It has also been demonstrated that protease activity was removed during the anion exchange step. This was one of the initial aims of the third purification method. This was based on an assumption that, as the MEC was hemi/cellulolytic in nature, that proteases should not form part of such a complex. However, it should be noted that a cellulosomal serine protease gene has been identified in *Clostridium thermocellum* based on its nucleotide sequence which displayed a dockerin domain (See the UniProtKB/TrEMBL database at http://www.uniprot.org where the sequence is lodged as Q2HPT9_CLOTM) (Zverlov, VV & Schwarz, WH). While it is therefore possible that the proteases had formed an aggregate with the MEC, it is equally possible that a protease may form part of the MEC. However, based on the protease assay conducted, the anion exchange process removed protease activity from the MEC and protease activity appeared in other peaks in the anion exchange which contained either no MEC or an insignificant amount of MEC. It may therefore be more likely that the protease activity in the MEC was caused as a result of aggregation.

A further reason why the anion exchange step represents an improved purification protocol is provided in the results obtained from native PAGE gels of the MEC. Native PAGE data of the MEC (not shown), revealed that the MEC was unable to migrate out of the wells in the gel. When the MEC was purified using only size exclusion chromatography, two lower molecular weight protein species appeared on native gels and did not appear to be part of the MEC, which clearly remained in the wells. This indicated that the MEC perhaps had some free proteins aggregated to it. However, after purification using both anion exchange and size exclusion chromatography, these proteins were no longer detected; only the MEC was visible in the wells. Thus it appears as though the MEC purified through both anion exchange and size exclusion chromatography had a higher degree of purity than the MEC purified through size exclusion only.

One further aspect that has to be considered is the presence of xylan substrate in the purified MEC. It is not known if the proteins are bound to the xylan or how the proteins are bound, but the chemical composition of the xylan and its presence with the MEC will probably affect the behaviour of the proteins. It is therefore possible that cultures grown on substrates other than xylan may require additional optimisation of the purification procedure.

4.6 Conclusions

An MEC was successfully purified from a culture of *B. licheniformis* SVD1. Through size exclusion chromatography on Sepharose 4B the apparent size of the complex was found to be approximately 2,000 kDa. Various purification methods were used to purify the MEC. Using a single size exclusion chromatography step in the purification resulted in a reasonably high yield of the MEC, although this MEC may not be very pure and appeared to contain possible contaminating proteins. However, an MEC purified in this manner may possibly be used for certain applications where a high degree of purity is not required.

Using an anion exchange step prior to size exclusion chromatography resulted in an MEC with an apparently higher purity. The yield of the MEC from this purification was, however, very low. While contaminating proteins appeared to be removed, some MEC protein was also lost in the process. The behaviour of the MEC in failing to bind to the anion exchange

resin raised some questions with respect to the effectiveness of this method. Further optimisation such as other types of ion exchange resins should perhaps be explored in any future attempts to purify an MEC from this organism.

In conclusion, it appears as though both purification methods, using size exclusion chromatography or anion exchange in conjunction with size exclusion chromatography could be used for the successful purification of a MEC from *B. licheniformis* SVD1.

CHAPTER 5 – CHARACTERISATION OF THE CELLULOLYTIC AND HEMI-CELLULOLYTIC SYSTEM OF *B. LICHENIFORMIS* SVD1 WHEN CULTURED ON BIRCHWOOD XYLAN

5.1 Introduction

Chapter 3 introduced data of a preliminary investigation of the cellulolytic and hemicellulolytic system of *B. licheniformis* when cultured on various substrates. In this chapter the focus is on studying the cellulolytic/hemi-cellulolytic system in greater detail when cultured on birchwood xylan only. Where the presence of endoglucanases and xylanases were investigated in Chapter 3, the presence of mannanases and pectinases within the crude and MEC fractions are now also examined.

To study individual enzymes within a complex, one should ideally purify and isolate these from other enzymes. However, components of MECs have generally been found to be very tightly bound together and difficult to dissociate and study individually. Complete dissociation could only take place in the presence of a detergent such as sodium dodecyl sulfate (SDS) and at high temperatures (Morag *et al.*, 1990). These conditions precede electrophoresis which allows the MEC to be dissociated into its individual components. Morag *et al.* (1996) was able to dissociate the cellulosome of *C. thermocellum* under mild conditions using ethylenediaminetetraacetic acid (EDTA) and cellulose. However, this was possible due to the requirement of calcium for cohesin-dockerin binding. With the chelation of calcium in the presence of EDTA, the structural integrity of the cellulosome was undermined, allowing the dissociation of the components. The presence of cellulose separates the scaffoldin protein from the enzymatic subunits as the scaffoldin contains a CBM that allows it to bind to cellulose. As the enzymatic subunits do not contain CBMs, they can be separated from the scaffoldin protein and studied individually (Morag *et al.*, 1996). However, this method will not be effective on an MEC that has a different structural basis. For instance, Doi *et al.* (1998) indicated that the binding ability of the major exoglucanase (ExgS) in the *C. cellulovorans* cellulosome was not affected by calcium ions or EDTA. Furthermore, not all scaffoldin proteins have a CBM, such as ScaA in *R. flavefaciens*

(Rincon *et al.*, 2003) and in some cases cellulosomal enzymes have been found with their own CBM such as in *C. cellulolyticum* (Desvaux, 2005).

Characterisation of MECs based on studies such as SDS-PAGE and zymograms can be used very effectively to identify enzymes present within the complex. However, these processes have some limitations which should be taken into account as many enzymes will not retain activity after the complete denaturation from heat and denaturing agents used in traditional SDS-PAGE. This was also found by other researchers, for example, Morag *et al.* (1990) reported that enzymes such as exoglucanase and β-xylosidase in the *C. thermocellum* cellulosome did not exhibit activity after boiling and thus a different procedure had to be utilised to obtain zymogram patterns for these enzymes. This involved heating the protein sample at 70°C rather than at 100°C.

A more prevalent method in research on MECs for identification of components of the complex has been cloning and sequencing of genes and, in some cases, whole genome sequencing. In the cellulosome field, identification of cellulosomal enzymes is possible due to the presence of dockerin sequences within the enzyme as all cellulosomal enzymes contain a dockerin sequence (See section 1.3.1.4). However, should a complex have a different structural basis than dockerin-cohesin interaction, identification of enzyme components of the complex may prove challenging.

Another method that was used successfully by Zverlov *et al.* (2005) to identify components of the *C. thermocellum* cellulosome was two-dimensional electrophoresis followed by MALDI-TOF/TOF. Identification was aided by the availability of the genome sequence for *C. thermocellum*. The advantage of analysing the composition of a purified complex, as opposed to a gene sequence, is that it gives information about the actual enzymes that the organism has harnessed for the degradation of a specific substrate. Thus it can provide valuable insight into the enzymes present and their ratios for the synergistic degradation of complex substrates. Identification of enzymes from a genome or gene sequence only provides the theoretical possibility that they may occur within the MEC, but does not indicate under which circumstances they will be present within the MEC. Although cloning and studying of such enzymes can provide valuable information about the MEC characteristics, their role within the MEC may not be understood, hence the rationale for studying the

complex itself. A method which was effectively used by Han *et al.* (2005) was the utilisation of antibodies against known components of the *C. cellulovorans* cellulosome to identify their presence within purified complexes cultured on a variety of substrates. Cloned genes were utilised to purify proteins for the main components of the cellulosome which were, in turn, used to produce antibodies.

In this study our focus will be on techniques such as enzyme assays, SDS-PAGE and zymograms to identify and characterise enzyme activities within the MEC. Even with the shortcomings of these methods, it will provide valuable information on enzymes present in the crude and MEC fractions, which can form the basis of further studies such as cloning and sequencing of genes within this organism and the MEC. It should be clear that certain methods and techniques such as cloning of genes for components of an MEC can only be used effectively if more information is available about the structural basis of the MEC present in *B. licheniformis* SVD1.

5.2 Objectives

- To compare the enzyme activities of the crude fraction of a birchwood xylan culture with the MEC fraction from the same culture
- To determine the ability of *B. licheniformis* to utilise different monomeric and dimeric sugars
- To determine the parameters of a growth curve of a *B. licheniformis* culture
- To determine the products formed from insoluble birchwood xylan by the crude fraction and MEC based on thin layer chromatography characterisation
- To determine the pH profile of various enzyme activities within the crude and MEC fractions in order to perform zymograms at optimal pH
- To characterise the crude extract and MEC of a birchwood xylan culture of *B. licheniformis* using SDS-PAGE and zymograms to determine endoglucanase, xylanase, pectinase and mannanase active proteins

5.3 Methods and materials

5.3.1 Culturing of organism

B. licheniformis was cultured in the medium as described in section 2.3.4 and using 5% (w/v) birchwood xylan as substrate.

5.3.2 Protein purification

The crude fraction utilised in this chapter refers to the supernatant from a culture where the cells were removed through centrifugation at 12,000 *x g* for 10 min. The supernatant was then concentrated by applying it to an Amicon 8200 ultrafiltration cell (Millipore) using a PBGC filter with a nominal molecular weight cut-off of 10 kDa. The concentrated crude fraction was stored at 4°C and used for analysis. The MEC fraction was prepared as described in section 4.3.2.3.

5.3.3 Enzyme assays

Enzyme assays were conducted as described in section 2.3.2 and 3.3.2.

5.3.4 Utilisation of sugars

To determine the ability of *B. licheniformis* SVD1 to utilise various sugars, the organism was cultured in the same medium as described in 2.3.4 but without the birchwood xylan and with 5% (w/v) glucose, galactose, xylose, arabinose, mannose or cellobiose incorporated into the medium. Cultures (10 ml) were grown in triplicate aerobically at 37°C on a rotational shaker. After 24 hours incubation, the sugar content of the controls and experiments was measured using the DNS assay and compared. Utilisation of the sugars in the medium was calculated based on the reduction in sugar compared to an uninoculated control.

5.3.5 Growth curve

The organism was cultured as described in section 2.3.4 using 0.5% (w/v) birchwood xylan in a 400 ml volume. The culture was inoculated with 1 ml of a glycerol stock of *B licheniformis* SVD1. Samples (4 ml) were taken approximately every 6 h over 5 days. Samples were immediately centrifuged to separate the cells from the supernatant. The cells were resuspended in 4 ml PBS buffer and sodium azide added to both the cell suspension and the supernatant to a final concentration of 0.03% (w/v). Pelleted cells of *B. licheniformis* SVD1, resuspended in PBS buffer, was used to measure the whole cell density at an absorbance of 600 nm (blanked against 1 x PBS). Supernatants were analysed for pH, protein concentration, activity on birchwood xylan and concentration of reducing sugars. Cell pellets, resuspended in PBS buffer, were used to measure growth as the optical density at 600 nm, and were assayed for activity on birchwood xylan. All readings and assays were done in duplicate.

5.3.6 Thin Layer Chromatography (TLC)

Products of hydrolysis were analysed using TLC. Insoluble birchwood xylan (10-20 mg) was pre-equilibrated in a volume of distilled water for an hour. The water was then removed by centrifugation and 500 µl of purified MEC added. The reaction mixtures were incubated at 37°C on a rotary shaker for between 24 h and 120 h and samples removed at intervals for analysis. Samples were centrifuged to remove the insoluble xylan. This was followed by an acetone precipitation of the supernatant in which 750 µl of ice cold acetone was added, the mixture vortexed and incubated at -20°C for 10 minutes. The precipitated protein and xylan were removed through centrifugation at maximum speed for 5 minutes. Identical volumes of the supernatant were applied to Silica Gel 60 F254 HPTLC plates (Merck, Darmstadt, Germany). Plates were developed twice with acetone:ethyl acetate:acetic acid (2:1:1, v/v/v). To detect carbohydrates, plates were covered with a 1:1 (v/v) mixture of 0.2% (w/v) methanolic orcinol and 20% (v/v) sulfuric acid, air dried and then heated at 110°C (Kosugi *et al.*, 2001).

5.3.7 pH optimum determination

The optimal pH of the crude extract as well as the MEC was determined for xylanase, endoglucanase, pectinase and mannanase activity using birchwood xylan, CMC, pectin and locust bean gum as substrates. Enzyme assays were carried out in duplicate at pH values ranging from pH 3.0-10.0 in citrate buffer (citrate-NaOH) (pH 3.0-5.0), potassium phosphate buffer (pH 6.0-8.0) and glycine buffer (glycine-NaOH) (pH 9.0-11.0) at 50°C under standard conditions.

5.3.8 Electrophoresis

SDS-PAGE was performed as described in section 3.3.4 except that 2 µg of protein was used per lane and samples were only boiled for 45 s prior to loading onto gels. Electrophoresis was performed at 180 V and gels stained with PageSilver^{TM} Silver Staining kit (Fermentas) according to the manufacturers' instructions. Gels were digitally imaged using a Uviprochemi geldoc system (Whitehead Scientific, (Pty) Ltd) and protein bands were analysed to determine their molecular weights using Uviband software (v. 11.9).

Non-denaturing polyacrylamide gel electrophoresis (native PAGE) was performed using a continuous buffer system (1 M Tris-HCl pH 8.8) and 6% acrylamide. Samples were prepared at a concentration of 1 µg per sample after which it was suspended in native sample buffer before loading on the gel. Electrophoresis was conducted at 100 V for one and a half hours before staining the gel with PageSilver^{TM} Silver Staining kit according to the manufacturers' instructions. A duplicate gel, which was treated in an identical manner, was used for zymogram analysis using the overlay method.

5.3.9 Zymograms

For purposes of determining the activity of protein bands in SDS-PAGE gels, 0.1% (w/v) substrate was incorporated into the gel prior to polymerisation, except pectin where 0.3% (w/v) was used. Substrates used for zymograms were birchwood xylan, CMC, locust bean gum, apple pectin and polygalacturonic acid (PGA).

Activity of separated bands was detected after electrophoresis by renaturing the gel for 1 hour in 2.5% (v/v) Triton X-100 in a buffer at the required pH. Buffers at different pHs were used for zymograms based on the optimal pH range as determined for the crude and MEC fractions. For pH 5.5 a 50 mM sodium acetate buffer was used while a 50 mM potassium phosphate buffer was used for pH 6.5. For pH 7.0-9.0, a 50 mM Tris-HCl buffer was used while a glycine buffer was used for pH 10.0. The gel was then incubated in the same buffer at 37°C for 12-48 h. Samples of the crude and MEC fractions were loaded in duplicate or triplicate on gels. In most cases, duplicate zymograms were conducted but with varying incubation times. After removal of the buffer, the gels incorporating birchwood xylan, CMC or mannan were stained with 0.3% (w/v) Congo Red for 15-30 min and then destained with 1 M NaCl until bands appeared. Gels were then counterstained with 5% (v/v) acetic acid. Where pectin had been incorporated into the gel, staining was done for 1 hour with 0.05% (w/v) Ruthenium Red and destained with distilled water. In some cases, pectin zymograms were conducted in a 50 mM Tris-HCl buffer at pH 7.5 containing 0.2 mM or 5 mM $CaCl_2$. Zymogram gels were digitally imaged using a Uviprochemi geldoc system.

The molecular weight of protein bands with activity was determined by excising the section of the gel containing the molecular marker and staining this separately with Coomassie stain. After destaining, the marker portion of the gel was rehydrated and placed next to the zymogram to determine the sizes of active bands.

For the non-denaturing gels, the substrate was not incorporated into the gel but zymogram analysis was carried out with an overlay method. The overlay was prepared with 0.1% (w/v) birchwood xylan substrate and 10% acrylamide in a 50 mM potassium phosphate buffer at pH 6.5. The overlay was poured onto a glass plate and after electrophoresis, the nondenaturing gel was placed on top of the overlay and kept in contact with the overlay by placing the gel sandwich between two glass plates. Incubation took place at 37°C with the gel and overlay sandwich suspended over buffer within a plastic container. After incubation, the overlay was removed and stained with 0.3% (w/v) Congo Red as described above.

5.3.10 Protease activity

Protease activity of crude cultures was determined using agar plates containing 10% (v/v) fat free milk. Wells of approximately 4 mm diameter were made in the agar using a sterilised Pasteur pipette and 20 µl of each crude culture was pipetted in triplicate into wells. Plates were incubated at 37°C for 24 h and then observed for clearing zones around wells.

5.3.11 Protein determination

Protein in fractions was determined according to the Bradford's method as described in section 4.3.4.

5.4 Results

5.4.1 Enzyme activity present in the crude and MEC fractions

Table 5.1 displays the cellulolytic and hemi-cellulolytic activity that was found in the crude fraction of a birchwood xylan culture as compared to the activity of the purified MEC.

Table 5.1. Activity of the crude supernatant compared to activity of the MEC when cultured on birchwood xylan. Activities are expressed as U/mg/h protein. Values are shown as mean values ± SD (n=3).

Substrate on which activity was tested	Crude	MEC
Avicel	0.93 ± 0.028	0
CMC	1.22 ± 0.037	1.00± 0.18
Birchwood xylan	12.2 ± 0.366	24.84± 0.47
Oatspelt xylan	12 ± 0.36	13.47± 0.29
Locust bean gum	1.5 ± 0.045	2.02± 0.08
Pectin	1.42±0.08	9.55±0.35
Polygalacturonic acid	1.24 ± 0.037	6.50± 0.31
Bagasse	1.33 ± 0.04	1.87± 0.05

The crude fraction displayed the highest activity on both birchwood and oatspelt xylan and, in addition, displayed Avicelase, endoglucanase, mannanase and pectinase activity, as well as activity on bagasse. The MEC had its highest activity on birchwood xylan and oatspelt xylan, as well as pectinase, mannanase, endoglucanase activity and activity on bagasse. However, activity on Avicel disappeared in the MEC. The activity on birchwood xylan and oatspelt xylan was virtually identical in the crude fraction, but in the MEC the activity on oatspelt xylan is only about 50% of the activity on birchwood xylan in the crude fraction.

5.4.2 Utilisation of sugars

Table 5.2 displays the percentage sugar that had been utilised by *B. licheniformis* SVD1 after 24 hours incubation. Percentage utilisation was calculated as the reduction in the sugar content compared to an uninoculated control.

Table 5.2. Percentage of sugars utilised by *B. licheniformis* SVD1 after 24 hours incubation at 37°C. Percentage utilisation was calculated compared to an uninoculated control. Values represent the mean ± SD (n=3).

Sugar	% utilised
Glucose	82 ± 2
Galactose	18 ± 2
Xylose	0 ± 8
Arabinose	33 ± 4
Mannose	80 ± 7
Cellobiose	49 ± 2

Based on the results it is apparent that *B. licheniformis* SVD1 was able to utilise glucose and mannose effectively as these cultures displayed an 82% and 80% reduction in sugars after the incubation period. The organism was able to utilise cellobiose, arabinose and galactose to some extent as the reduction in sugars for these experiments were 49%, 33% and 18% respectively. Notably, *B. licheniformis* SVD1 was not able to utilise xylose as a carbon source as no reduction in sugar was observed.

5.4.3 Growth curve

The growth curve displaying various parameters is displayed in Figure 5.1.

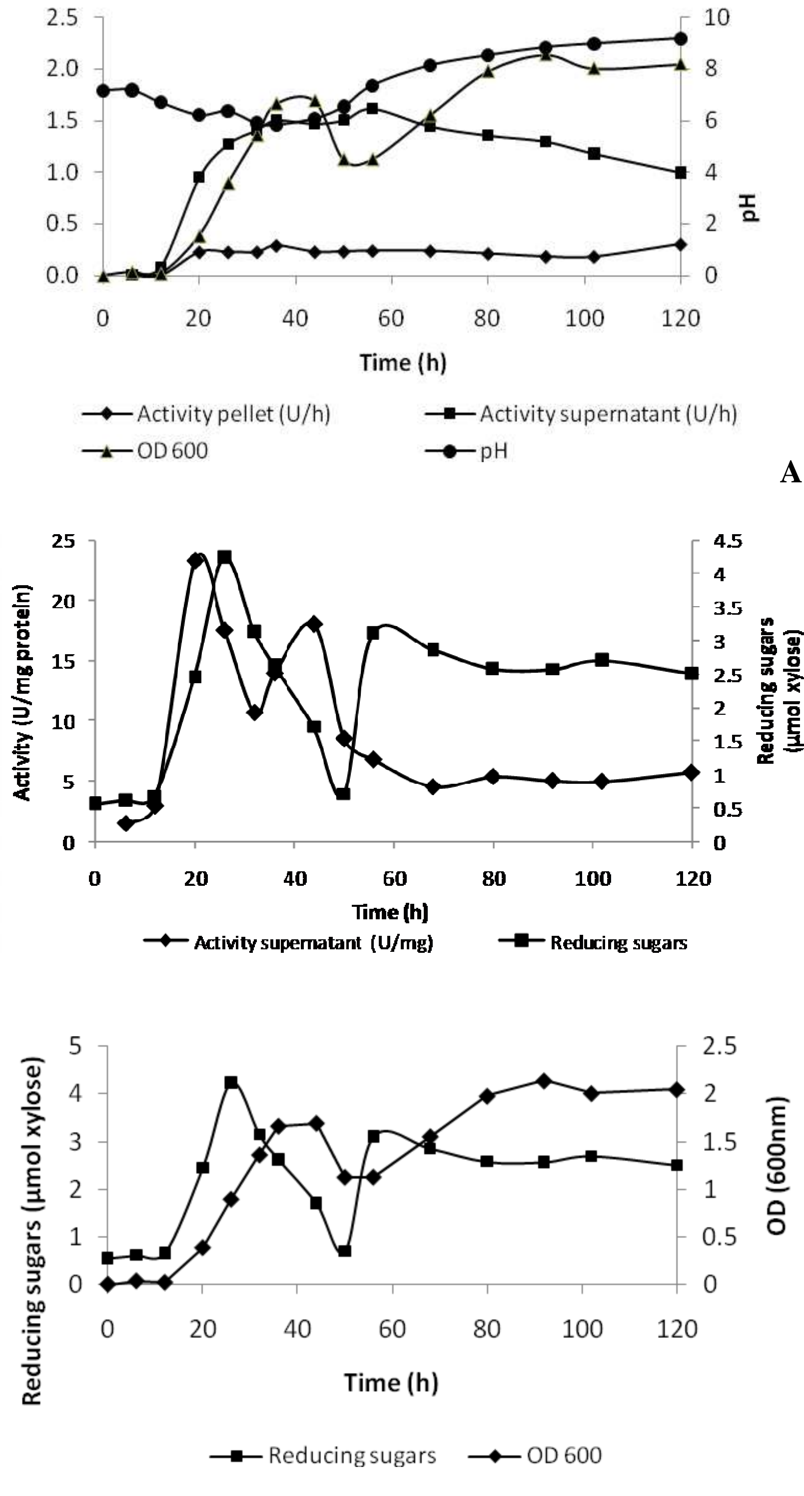


Figure 5.1. Growth curve of a birchwood xylan culture over time. Culture medium (400 ml) was incubated over 5 days and 4 ml samples taken periodically. Pelleted cells of *B. licheniformis* SVD1 were resuspended in PBS buffer and the whole cell density measured at an absorbance of 600 nm (blanked against 1 x PBS). Pellets and supernatants from each sample were measured for activity on birchwood xylan, while the supernatant was measured for pH, protein content and reducing sugars (as xylose equivalents). Plots of these parameters and their relationships are shown as A, B and C.

The growth curve in Figure 5.1A indicated the presence of xylanase activity in both the supernatant and the cell pellet after 20 h of incubation. The activity in the cell pellet remained low and reached a maximum at 20 h after which it remained relatively constant. The xylanase activity in the supernatant continued to increase until it reached a maximum between 38 and 60 h, after which it steadily declined during further incubation. The absorbance at 600 nm, which indicated cell growth, exhibited a pattern of diauxic growth, reaching two peaks over the duration of the incubation period. Initial growth peaked at approximately 40 h, followed by a dramatic reduction in optical density and then a further increase with a peak at approximately 90 h. The pH changed quite dramatically over the course of incubation. Starting at pH 7, it dropped below pH 6 after 30 h of growth, followed by an increase in pH to above 9 after 120 h.

Other parameters measured during incubation were the reducing sugars observed in the supernatant during growth as seen in Figure 5.1B. This was plotted against specific xylanase activity present in the supernatant. The initial peak of specific activity after 20 h was immediately followed by a sharp increase in reducing sugars in the supernatant. A further peak of specific activity after 40 h was again followed by a sharp increase in detection of reducing sugars at 60 h. The concentration of reducing sugars decreased slightly but then remained constant for the remainder of the incubation period. Figure 5.1C illustrates the relationship between cell growth and reducing sugars present in the medium. An increase in reducing sugars was followed by an increase in cell growth. Both cell growth and reducing sugars show two distinct peaks over the course of the incubation.

5.4.4 TLC Results

The TLC results are displayed in Figure 5.2 with Figure 5.2A representing the result from incubation of the crude extract with insoluble birchwood xylan while Figure 5.2B represents the results from incubation of the MEC with insoluble birchwood xylan.

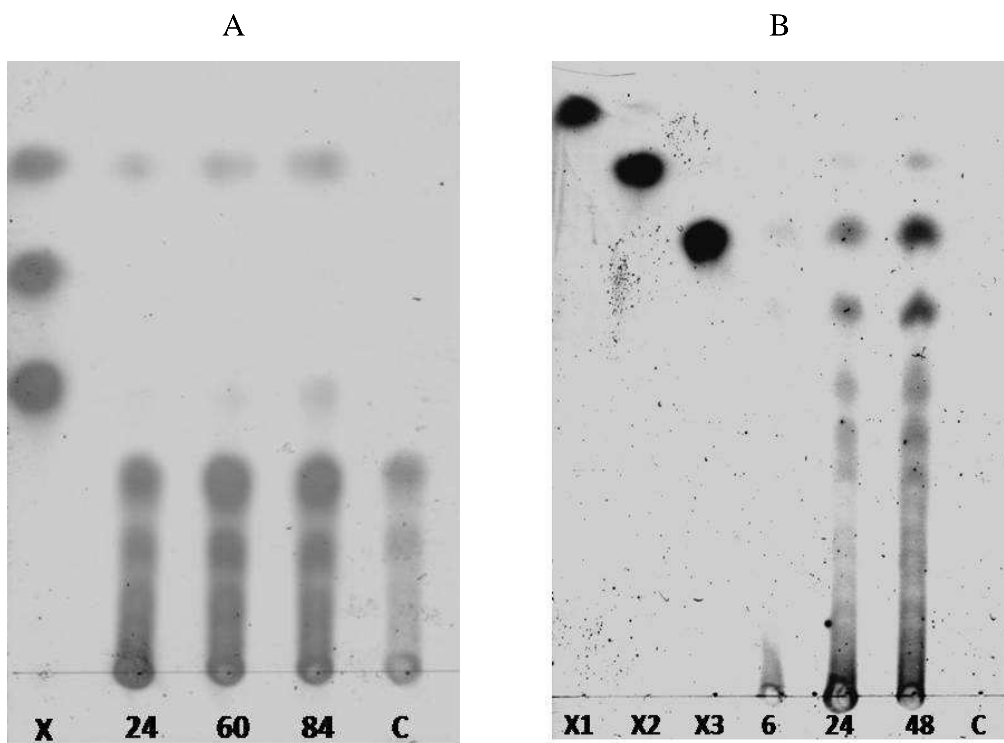


Figure 5.2. Thin layer chromatography of the products formed from the hydrolysis of insoluble birchwood xylan when incubated with (A) crude supernatant and (B) purified MEC. Lanes in A : X (xylose, xylobiose, xylotriose), 24, 60, 84 (number of h incubation), and C (control with crude supernatant only). Lanes in B: X1 (xylose), X2 (xylobiose), X3 (xylotriose), 6, 24, 48 (number of h incubation), and C (control with insoluble xylan only).

From the TLC results in Figure 5.2A, it can be observed that the crude fraction was able to degrade insoluble birchwood xylan to form smaller products as compared to the products present in the crude control. At 24 h incubation, the formation of xylose becomes apparent. From the TLC results where only the purified MEC was incubated with insoluble birchwood xylan in Figure 5.2B, smaller products were progressively formed during the incubation. Initially, after 6 h, very small amounts of xylotriose could be seen which increased with increased incubation time. At 24 and 48 h, xylobiose was present, as well as xylotriose and other products. No xylose was formed by the MEC, even after prolonged incubation periods of up to 120 h (data not shown).

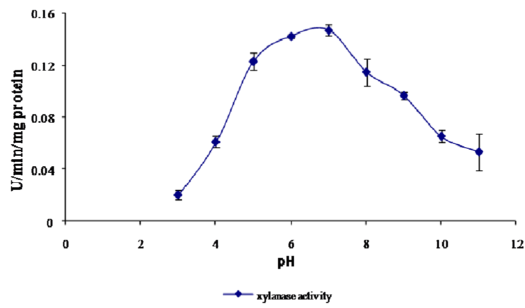
5.4.5 pH optima of various enzymes in the crude and MEC fractions

The pH optima for various enzymes in the crude fraction are displayed in Figure 5.3A-D and the pH optima for the MEC are displayed in Figure 5.3E-H. The pH optima were performed to determine the pH at which zymograms could be conducted to identify different enzymes present in those fractions.

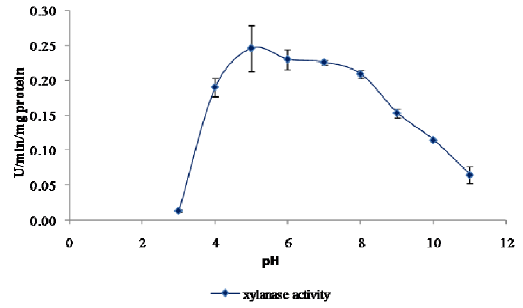
The pH optimum for xylanase activity in the crude fraction (5.3A) and MEC (5.3E) are very similar. From Figure 5.3 it can be observed that xylanase activity remained constant between pH 5.0 and pH 7.0, with the optimal pH between 6.0 and 7.0 in the crude. The MEC fraction displayed a slightly higher activity at pH 5.0. However, the standard deviation for this data point was quite large. The complexed xylanase activity displayed a higher level of residual activity at alkaline pH than at acidic pH with approximately 50% of the optimal activity still present at pH 10.0 and about 30% still present at pH 11.0. At pH 3.0, the activity virtually disappears. It should be noted that at low pHs in the citrate buffer, precipitation of the MEC was observed which could account for the marked reduction in activity.

The graphs of pH optima for pectinase activity in Figure 5.3B and F were characterised by large standard deviations which could not be decreased even with repeated attempts to measure these optima. The reasons for the varying data are thought to be as a result of the behaviour of the substrate which has a high background absorbance and behaves differently at the different pHs. However, this data only served as a guideline for preparation of zymograms. The crude fraction displayed a pH optimum at pH 8.0 and what appears to be another peak at pH 10.0-11.0. In contrast, the pectinase activity in the MEC fractions had a pH optimum at pH 7.0 and then again at pH 10.0-11.0.

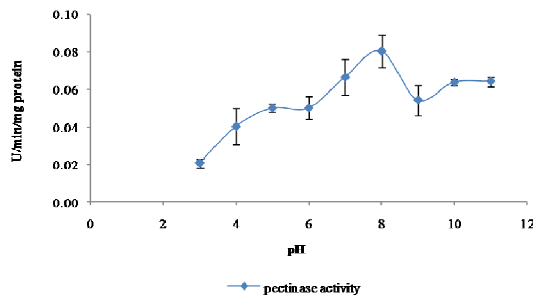
The pH optima for mannanase activity in the crude and MEC fractions are displayed in Figure 5.3C and G. The two graphs show substantial differences, with the crude fraction displaying a pH optimum at pH 8.0 with another smaller peak at pH 5.0. The MEC fraction, on the other hand, displayed optima at pH 6.0-7.0 and again at pH 9.0.



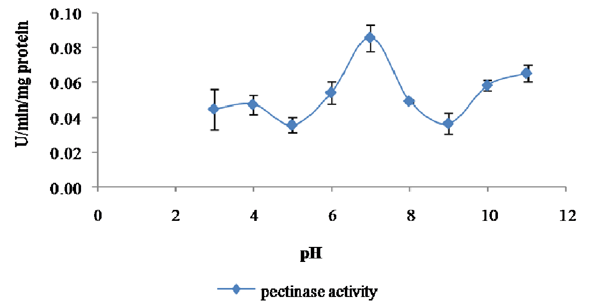
A



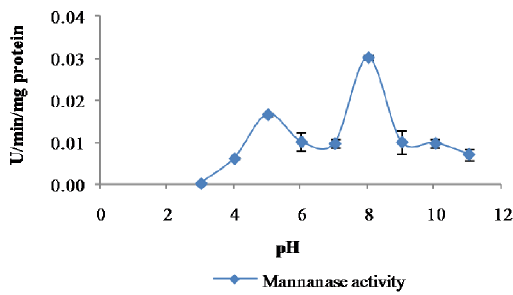
E



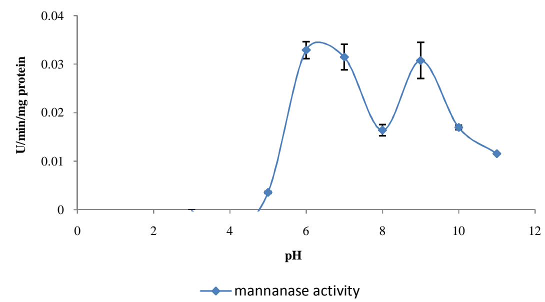
B



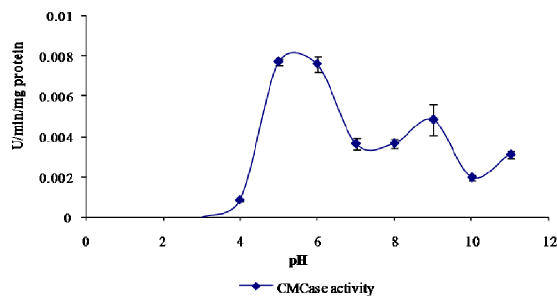
F



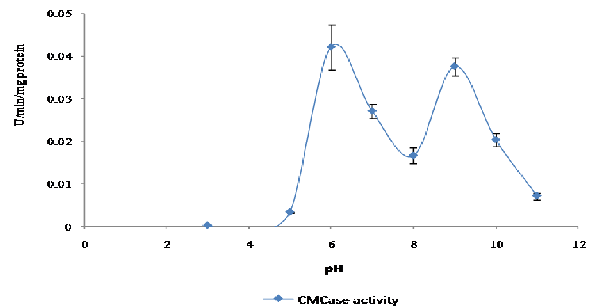
C



G



D



H

Figure 5.3. The pH optima of xylanase, pectinase, mannanase and endoglucanase activity in the crude fraction (A-D) and the MEC fraction (E-H). Values are presented as mean values ± SD (n=2)

The optimum pH for endoglucanase activity is displayed in Figure 5.3D and H. In the crude fraction the optimum was between pH 5.0-6.0 and there appeared to be another small peak of activity at pH 9.0. The MEC fraction displayed pH optima at pH 6.0 and pH 9.0.

5.4.6 Electrophoresis and zymogram patterns for the crude and MEC fractions of a birchwood xylan culture

SDS-PAGE was conducted on the crude and MEC fractions, followed by zymogram analysis for xylanase, endoglucanase, mannanase and pectinase activity at various pHs based on the pH optima data in Figure 5.3. The SDS-PAGE and zymogram patterns for the crude fraction are displayed in Figure 5.4 while Table 5.3 is a summary of these results.

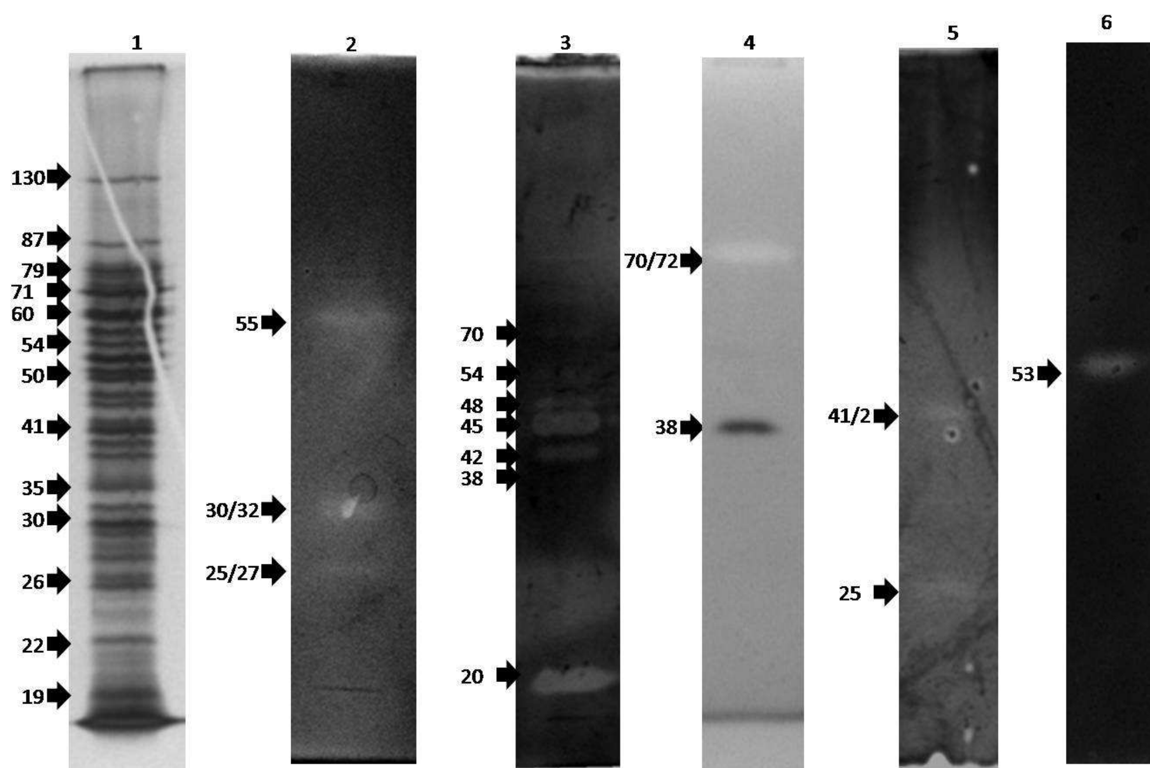


Figure 5.4. SDS-PAGE and zymograms of the crude fraction of a birchwood xylan culture. Lane 1 represents the separation of proteins from a crude birchwood xylan extract on a 10% SDS-PAGE gel and stained with PageSilver™ from Fermentas. Lane 2 - zymogram with 0.1% (w/v) CMC at pH 6.5; Lane 3 - zymogram with 0.1% (w/v) birchwood xylan at pH 6.5; Lane 4 - zymogram with 0.3% (w/v) apple pectin at pH 7.5 with 5 mM $CaCl_2$; Lane 5 - zymogram with 0.1% (w/v) locust bean gum at pH 6.5; Lane 6 – zymogram with 0.1% (w/v) locust bean gum at pH 8.5.

More than twenty-three protein species were visible after silver staining (Figure 5.4, lane 1) and their approximate molecular weights were summarised in Table 5.3. Some fainter bands were also visible while some proteins appear as doublets. The CMC zymogram patterns in Figure 5.4 (lane 2) displayed three bands with activity at approximately 25-27 kDa, at 30-32 kDa and at 55 kDa. At 30-32 kDa it appears as though two active bands could be present on top of each other. This zymogram was carried out at pH 6.5, but a zymogram carried out at

pH 5.5 displayed the same result. A zymogram at pH 9 did not show any clear bands with activity. This may be due to the concentration of protein, active at that pH, being too low or not being stable enough for the long incubation period required.

The zymogram of xylanase activity (Figure 5.4, lane 3) resulted in the detection of seven bands exhibiting activity on birchwood xylan at approximately 20 kDa, 38 kDa, 42 kDa, 45 kDa, 48 kDa, 54 kDa and 70 kDa.

The zymogram on the pectin substrate (Figure 5.4, lane 4) exhibited a red-purple band at approximately 38 kDa while a clear band was visible at approximately 70-72 kDa.

The proteins active on locust bean gum are displayed in Figure 5.4 (lane 5 and 6). The zymogram in lane 5 was carried out in 50 mM phosphate buffer at pH 6.5 and displays two bands with activity at about 25 kDa and 40-42 kDa. The zymogram in lane 6 was performed in 50 mM Tris-HCl at pH 8.5 and displays an active band at about 53 kDa.

Table 5.3 provides a summary of the molecular weights for the crude fraction as identified from the silver stained gel, as well as the activities identified for each band. As the clear bands on the zymograms are often broad and diffuse, values for active bands are approximate. In a few cases, certain proteins appear to have two different enzyme activities.

Table 5.3. The proteins in the crude fraction and the identified enzyme activity present for each protein as identified through zymogram analysis. Xy = xylanase, C=endoglucanase, M=mannanase, PL=pectin/pectate lyase, PME=pectin methyl esterase

<table>
<tr><th>Crude MW</th><th>Xy</th><th>C</th><th>M</th><th>PL</th><th>PME</th></tr>
<tr><td>131.9</td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>87.5</td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>79.2</td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>71.2</td><td>X</td><td></td><td></td><td>X</td><td></td></tr>
<tr><td>59.5</td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>56.6</td><td></td><td>X</td><td></td><td></td><td></td></tr>
<tr><td>53.8</td><td>X</td><td></td><td>X</td><td></td><td></td></tr>
<tr><td>51.2</td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>49</td><td>X</td><td></td><td></td><td></td><td></td></tr>
<tr><td>45.8</td><td rowspan="2">X</td><td></td><td></td><td></td><td></td></tr>
<tr><td>44.2</td><td></td><td></td><td></td><td></td></tr>
<tr><td>41</td><td>X</td><td></td><td>X</td><td></td><td></td></tr>
<tr><td>39.7</td><td></td><td></td><td></td><td></td><td>X</td></tr>
<tr><td>38.1</td><td>X</td><td></td><td></td><td></td><td></td></tr>
<tr><td>35</td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>32.3</td><td></td><td rowspan="2">X</td><td></td><td></td><td></td></tr>
<tr><td>30.8</td><td></td><td></td><td></td><td></td></tr>
<tr><td>28.2</td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>26.7</td><td></td><td>X</td><td></td><td></td><td></td></tr>
<tr><td>24.4</td><td></td><td></td><td>X</td><td></td><td></td></tr>
<tr><td>22.6</td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>21</td><td rowspan="2">X</td><td></td><td></td><td></td><td></td></tr>
<tr><td>19</td><td></td><td></td><td></td><td></td></tr>
</table>

SDS-PAGE analysis of the MEC fraction was conducted and zymograms to detect xylanase, endoglucanase, pectinase and mannanase activity were carried out. The SDS-PAGE results displaying the protein bands and zymograms for the MEC are shown in Figure 5.5. Seventeen protein species are visible after silverstaining (Figure 5.5, lane 1) and their respective molecular weights are summarised in Table 5.4.

Zymograms for the MEC fraction were done under the same conditions as for the crude fraction. The zymogram on CMC (Figure 5.5, lane 2) displays two bands at 25-27 kDa and 30-32 kDa. The band at 55 kDa that was present in the crude fraction was no longer present in the MEC. The zymogram on birchwood xylan (Figure 5.5, lane 3) exhibited the same

number of clear bands as the crude fraction with active bands at approximately 20 kDa, 38 kDa, 42 kDa, 45 kDa, 48 kDa, 54 kDa and 70 kDa. The zymogram on pectin (Figure 5.5, lane 4) exhibited a clear band at 70-72 kDa, similar to that found in the crude. However, the dark purple band was no longer present in the MEC. The zymogram with locust bean gum in the gel (Figure 5.5, lane 5) displayed two bands at pH 6.5, at 25 kDa and 41/42 kDa. These two bands were also present in the zymogram of the crude fraction. The mannanase-active band at approximately 53 kDa that was found at pH 8.5 was no longer present in the MEC.

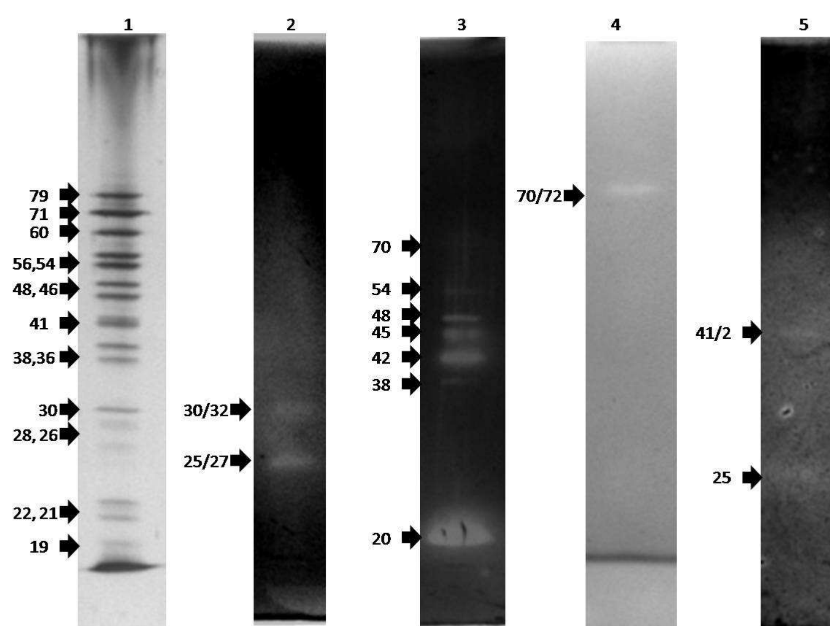


Figure 5.5. SDS-PAGE and zymograms of the MEC from a birchwood xylan culture. Lane 1 represents the separation of proteins from the MEC from a birchwood xylan extract on a 10% SDS-PAGE gel and stained with PageSilver^{TM} silverstaining kit. Lane 2 - zymogram with 0.1% (w/v) CMC at pH 6.5; Lane 3 - zymogram with 0.1% (w/v) birchwood xylan at pH 6.5; Lane 4 - zymogram with 0.3% (w/v) apple pectin at pH 7.5 with 5 mM $CaCl_2$; Lane 5 - zymogram with 0.1% (w/v) locust bean gum at pH 6.5.

Table 5.4 provides a summary of the molecular weights for proteins found in the MEC fraction and the corresponding activity found for each band. It appears as though the mannanase and xylanase active bands at approximately 42 kDa could be the same protein displaying different activities. However, from the silverstained gel, it could be possible that two proteins co-eluted at that position. It appears, furthermore, as though one protein at approximately 27 kDa displays both mannanase and endoglucanase activity. However, it is again possible that these could be two different proteins as the precise sizing of zymogram bands can become difficult where clear zones are slightly broader.

Table 5.4. The proteins in the MEC fraction and the identified enzyme activity present for each protein as identified through zymogram analysis. Xy = xylanase, C=endoglucanase, M=mannanase, PL=pectin/pectate lyase, PME=pectin methyl esterase

<table>
<tr><th>MEC MW</th><th>Xy</th><th>C</th><th>M</th><th>PL</th><th>PME</th></tr>
<tr><td>79.8</td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>71.2</td><td>X</td><td></td><td></td><td>X</td><td></td></tr>
<tr><td>59.5</td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>56.6</td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>53.8</td><td>X</td><td></td><td></td><td></td><td></td></tr>
<tr><td>51.6</td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>48</td><td>X</td><td></td><td></td><td></td><td></td></tr>
<tr><td>45.8</td><td>X</td><td></td><td></td><td></td><td></td></tr>
<tr><td>41.5</td><td>X</td><td></td><td>X</td><td></td><td></td></tr>
<tr><td>38.4</td><td>X</td><td></td><td></td><td></td><td></td></tr>
<tr><td>36.3</td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>30.2</td><td></td><td>X</td><td></td><td></td><td></td></tr>
<tr><td>28.7</td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>26.8</td><td></td><td>X</td><td>X</td><td></td><td></td></tr>
<tr><td>22.4</td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>21.2</td><td rowspan="2">X</td><td></td><td></td><td></td><td></td></tr>
<tr><td>19</td><td></td><td></td><td></td><td></td></tr>
</table>

From the summary in Table 5.4 it becomes apparent that there are several proteins present in the MEC fraction that did not display any activity on the substrates and under the conditions examined. These could be enzymes with a different activity that was not tested, or could be inactive enzymes or non-catalytic proteins.

Non-denaturing polyacrylamide gel electrophoresis (native PAGE) was conducted using the MEC. However, the complex was so large that under the conditions utilised, it did not migrate from the wells into the gel (data not shown). The zymogram analysis using birchwood xylan in an overlay revealed that all the xylanase activity had remained in the loading wells and was thus associated with the MEC (data not shown).

5.4.7 Protease activity

The crude culture was found to display some protease activity as small clearing zones were observed around wells after the incubation period, indicating degradation of the protein substrate within the agar.

5.5 Discussion

Enzyme assays demonstrated that xylanase activity was the most prominent enzyme activity present in both the crude and MEC fractions. For the enzyme assays, both birchwood xylan and oatspelt xylan were used as substrates for the reason that the compositions of these two substrates are very different. Birchwood xylan consists of 94.1% xylose residues, 1.4% glucose and 4.5% galactose whereas oatspelt xylan consists of 52.5% xylose, 22.3% arabinose, 15.7% glucose and 9.5% galactose (Li *et al.*, 2000). High activity on oatspelt xylan would generally require the presence of α-L-arabinofuranosidase and perhaps α-glucuronidase and α- or β-galactosidase enzymes to remove the high concentration of arabinose, glucose and galactose substituents in order to give access to endo-xylanases. These substituents may pose a steric hindrance to endo-xylanases, although differences would exist between the ability of family 10 and family 11 xylanases to overcome this hindrance (Biely *et al.*, 1997). The high enzyme activity on oatspelt xylan found in the crude could indicate the presence of an α-L-arabinofuranosidase in this fraction. The dramatic drop in activity on oatspelt xylan in the MEC probably indicates that the α-L-arabinofuranosidase is no longer present in the MEC fraction. Based on the pattern of growth on birchwood xylan as displayed in the growth curve, the crude fraction potentially contains a glucuronidase and/or a galactosidase which allowed the organism to access substituent sugars from the birchwood xylan as it was unable to utilise the xylose. Release of sugars such as glucose or galactose could account for the one peak observed in the growth curve when reducing sugars were measured. The other sugar peak is most likely as a result of the release of xylose from the birchwood xylan. However, *B. licheniformis* was unable to grow on the xylose. The diauxic growth pattern could therefore be as a result of growth on the glucose and galactose sugars initially, and thereafter the second growth peak observed could be as a result of growth on the other medium components such as the yeast extract and peptone. It was found from

observation that *B. licheniformis* could be cultured on the medium without additional carbohydrates or sugars (data not shown).

The crude and MEC fractions also had activity on both pectin and polygalacturonic acid and thus both these fractions contain pectinases. Polygalacturonic acid consists of long chains of galacturonic acid residues, while pectin has the same structure, except that carboxyl groups on the galacturonic acid becomes methylated (See section 1.2.7). Polygalacturonases (EC. 3.2.1.15) and pectate lyases (EC. 4.2.2.2) generally display a preference for polygalacturonic acid or pectate, while pectin lyases (EC. 4.2.2.10) display a preference for pectin. However, many pectin and pectate lyases show activity on either substrate. In addition, pectin methyl esterases, which cleave methyl groups from pectin, could be present in either fraction. From the enzyme assays alone it is difficult to speculate which type of enzymes may be present although zymogram results were performed in order to provide more information on the suite of active enzymes in the crude and MEC fractions.

Although the crude fraction had some activity on Avicel, the TLC studies that were conducted using crude supernatant and Avicel as substrate, showed that no cellobiose or glucose was formed after even lengthy incubation times (data not shown). This indicated that no true Avicelase activity was present, as Avicelase activity generally refers to cellobiohydrolase enzymes that are able to release cellobiose as a product. Another type of enzyme such as an endoglucanase was possibly able to create reducing ends in the Avicel that allowed a reaction with the DNS assay, thus giving a low activity reading on Avicel.

Based on the presence of high xylanase activity in the organism, the inability of *B. licheniformis* SVD1 to utilise xylose was unexpected. It should be noted that the inability of an organism to utilise a sugar such as xylose even though it produces high xylanase activity is not uncommon. *C. thermocellum* was found to possess various cellulosomal and non-cellulosomal xylanases, as well as a β-xylosidase, yet was reported as unable to grow on this monomer sugar (Morag *et al.*, 1990). Five xylanases have been identified in the cellulosome of *C. thermocellum* alone (Bayer *et al.*, 1998b). As Morag *et al.* (1990) stated: "It is difficult to comprehend why a bacterium which fails to grow on (or utilize) xylose would be energetically encumbered with a complex set of xylanolytic enzymes capped with a seemingly superfluous β-xylosidase. There may still be a cryptic ecological advantage in

retaining the xylanase system in *C. thermocellum*, and the loss of a key enzyme (e.g., xylose isomerase) may have prevented the utilization of xylose in this organism."

One could hypothesise that the complex nature of plant structure necessitates the removal of hemicellulose such as xylan before an organism could access the cellulose, its "true" carbon source, thus explaining the presence of xylanases. However, in the case of *B. licheniformis* SVD1, it does not appear to have enough cellulase activity to indicate that cellulose would be a main carbon source.

The ability of *B. licheniformis* SVD1 to utilise glucose and cellobiose raises further questions regarding the preferred carbon substrate of this organism. Utilisation of cellobiose indicates that the organism most likely possesses a β-glucosidase in order to cleave the cellobiose into glucose subunits. However, it has been demonstrated that the organism expressed only low levels of endoglucanase activity in various media and only with certain substrates was any exoglucanase activity induced (See section 3.4.2). It thus appears that the organism, while able to utilise glucose, would be unable to access it from substrates such as cellulose as the enzymatic degradation of such substrates was very inefficient.

However, it is ill-advised to study an environmental isolate such as *B. licheniformis* without considering the array of complex interactions that may exist between this isolate and other microorganisms in the natural environment. It is likely that some symbiotic relationships may exist to allow one organism to utilise the products of degradation from another organism and vice versa.

It is interesting to note that mannose was the only hemicellulose sugar that was utilised at high levels by *B. licheniformis* SVD1. Mannanase activity was found in all culture media and the highest activity was present when the organism was cultured on locust bean gum (See chapter 3). It therefore appears that *B. licheniformis* SVD1 was able to degrade mannan effectively based on the high enzyme activities on locust bean gum and also utilise it as a carbon source. Since mannan does not occur as commonly in nature as substrates such as cellulose or xylan, this may indicate that the organism occupies a specific niche in the environment (See section 1.2.6).

B. licheniformis SVD1 had the ability to utilise arabinose and galactose to some extent. Arabinose and galactose are generally sugars that occur as substituents on other polysaccharides such as mannan or xylan. In the case of arabinose, an enzyme such as arabinofuranosidase would be required to release this sugar from the main backbone while a galactosidase would be required in the case of galactose substituents (see sections 1.2.5 and 1.2.6).

When observing the growth curve, most of the xylanase activity was found in the supernatant at an early stage of growth and only a small percentage was associated with the cells. This is not unlike the expression of xylanase in *C. cellulovorans* where the large majority of xylanase was expressed in the supernatant (Kosugi *et al.*, 2001). However, the growth curve observed in this instance was very unusual, showing a pattern of diauxic growth which is normally only observed where two energy sources are present in the medium. This pattern of growth is often present under conditions of catabolite repression, but it is not clear whether it was the case in this study. Only one carbon source was used in the medium, namely birchwood xylan. However, birchwood xylan also contains approximately 1.4% glucose and 4.5% galactose subunits (Li *et al.*, 2000). Based on the results as reported in Section 5.4.2, the organism was unable to utilise the xylose generated from enzymatic degradation of the birchwood xylan. Thus it is possible that the initial, brief growth peak observed was as a result of utilisation of glucose and galactose which was cleaved from the birchwood xylan. However, since such a limited amount of these sugars were available, growth ceased and therefore an initial growth peak was observed.

When the formation of reducing sugars during cell growth (Figure 5.1C) was observed, it was apparent that both increases in growth were preceded by a sharp increase in reducing sugars in the medium, suggesting that the growth pattern was caused by the release of sugars from the xylan substrate. However, after the second increase in reducing sugars no reduction took place. Although enzymatic activity continued to produce sugars in the medium, most likely xylose, the organism was unable to utilise the xylose. Another peak of growth was observed at this stage, but this did not coincide with a reduction in sugars in the supernatant as initially observed. In fact, the reducing sugars in the medium remained relatively constant even after prolonged incubation. If the organism was unable to grow on the xylose, it must have been able to grow on other components of the medium, without the addition of carbohydrates and

sugars, which has been observed (data not shown). This may explain the pattern of growth observed, namely that the medium components such as the yeast extract and peptone were able to sustain growth after the glucose and galactose had been used.

From the TLC results, the formation of xylose after incubation with the crude fraction indicated that β-xylosidase was probably present in the crude extracellular supernatant as this enzyme was required to cleave xylobiose into xylose subunits. Thus, β-xylosidase was probably not present in the MEC fraction as no xylose was formed.

pH optima studies were conducted for endoglucanase, xylanase, pectinase and mannanase activity in order to optimise zymogram conditions. The pH profiles for xylanase activity in the crude fraction and MEC were virtually identical while the endoglucanase pH profiles also display substantial similarities. However, the pectinase and mannanase pH profiles differed substantially between the two fractions. This indicated that the enzyme composition between the crude and MEC fractions differed substantially. This was in fact found specifically with respect to pectinase and mannanase activity as can be seen from zymogram analysis.

The pH optima for various enzymes ranged from pH 5.0 to pH 11.0, indicating that the carbohydrate degrading system of *B. licheniformis* SVD1 can operate at many different pHs. Xylanase activity was very stable over this entire pH range. From the growth curve it was apparent that the pH changed dramatically during growth over the incubation period. However, xylanases would be able to operate over the whole range of pH found in the growth medium.

When the silver stained protein pattern in Figure 5.4 is compared to the protein patterns from Figure 3.2 from the same culture, it is noticeable that far more protein bands are observed in Figure 5.4. This can be ascribed to two factors, namely the amount of protein loaded on the SDS-PAGE gel and the staining method. In Figure 3.2, approximately 10 µg of protein was loaded per lane and PageBlue® staining used, which is a Coomassie-based stain. In Figure 5.4, approximately 2 µg protein was loaded per lane and Pagesilver® stain (Fermentas) was used, which is a silver staining method with higher sensitivity. The amount of protein that was initially used (10 µg) was based on various considerations. In the literature on MECs, it was common to load large amounts of protein on SDS-PAGE gels (Cavedon *et al.*, 1990-15

μg; Jiang *et al.*, 2006-25 μg; Morag *et al.*, 1990-up to 15 μg; Pason *et al.*, 2006-100 μg). Han *et al.* (2005) used low amounts of protein (1 μg) but was able to achieve staining with colloidal Coomassie dye. With this work it was found that with protein levels below 6-8 μg per lane it was impossible to achieve staining with Coomassie-based stains. However, with a large amount of protein loaded in a lane, separation was difficult and often aggregated protein was visible at the top of the resolving gel. Thus it appears possible that, with high protein levels, some of the proteins remained aggregated and was not able to resolve adequately, accounting for the variability in activity observed in these experiments.

When observing zymogram patterns for the crude fraction, it becomes clear that the result for zymograms on birchwood xylan was very different from the zymogram pattern of the crude birchwood xylan culture in Figure 3.2 where only three bands at about 20-21 kDa, 40- 42 kDa and at 45 kDa were visible. Although these bands were still present, additional bands had now become visible. This can be ascribed to using lower levels of protein for the zymogram (2 μg), as well as much longer incubation times to develop the zymogram. Previously incubation took place for 2 or 3 h, while incubation times of up to 18 h were used in this case. Furthermore, boiling of samples was reduced from 5 min to 45 s after it was determined that sufficient denaturation was achieved for proteins to separate adequately. Below 45 s boiling time proteins did not separate on a 10% denaturing gel during electrophoresis. It is therefore possible that some of the xylanases were more susceptible to inactivation or denaturation when exposed to high temperatures for a longer period of time and this could account for the additional bands now visible.

The zymogram on pectin for the crude fractions displays an interesting result with one red-purple band and one clear band visible. When staining with ruthenium red, pectin methyl esterases (PME) would display a red-purple band where activity was present, whereas pectin or pectate lyases (PL) would display a clear band (Kobayashi *et al.*, 2003). A purple band representing a PME was visible at approximately 38 kDa, while a clear band indicating lyase activity was visible at approximately 70-72 kDa. The PME band was first observed when the zymogram was performed at pH 6.5. At this pH, the clear band with lyase activity was absent. However, when the renaturation and incubation buffers were changed to pH 7.5 with the addition of 5 mM $CaCl_2$, a clear band was observed. It should be noted that the change of pH to 7.5 and the addition of 5 mM $CaCl_2$ appeared to affect the activity of the PME as the

purple band at 38 kDa as it was lower in intensity. At 0.2 mM $CaCl_2$, the lyase band was very faint and increased in intensity with an increase in calcium concentration (data not shown). It is generally accepted that pectate lyases have a requirement for calcium, but that pectin lyases do not (Soriano *et al.*, 2005) (See also section 1.2.7). Thus it appears that the enzyme at 70-72 kDa could be a pectate lyase which also has activity on pectin. However, although it was reported by Soriano *et al.* (2005) that pectin lyase is not activated by calcium; several reports claim activation of pectin lyases by calcium (Adejuwon & Olutiola, 2007; Kester & Visser, 1994; Wijesundera *et al.*, 1984). It is thus not clear whether activation by calcium provides a clear distinction between pectin and pectate lyases. It should be noted that the clear band at 70-72 kDa was also present when the zymogram was performed using 0.1% (w/v) PGA instead of pectin in the gel (data not shown). While it is generally correct that pectate lyases have activity on pectate (PGA) whereas pectin lyases have activity on pectin, this distinction is not always this defined and many pectate lyases may have activity on pectin and vice versa, although lower than on its preferred substrate (Schnitzhofer *et al.*, 2007).

It is apparent that the same protein band at approximately 70-72 kDa has both pectin/pectate lyase activity as well as xylanase activity (Table 5.3). While the literature reports, in one instance, a xylanase with activity on amylopectin (Ghosh *et al.*, 1980), to our knowledge no reports have been published regarding a pectin/pectate lyase with cross-activity on xylan. It is possible in this case, that there are in fact two different proteins of similar size present within the crude culture.

The bands at 40-42 kDa as well as 53 kDa in the crude fraction, appear to display both xylanase and mannanase activity. So many proteins are present in the crude fraction that it is conceivable that these are two different proteins of similar size. However, there are reports in literature of xylanases having activity on glucomannan (Fournier *et al.*, 1985; Lappalainen, 1986; Mansour *et al.*, 2003) and a mannanase having activity on xylan (Jiang *et al.*, 2006b). Thus it is possible that one enzyme may display two different activities. While the main catalytic activity of an enzyme may be used for its classification, enzymes often have cross-reactivity on other substrates.

Based on the summary in Table 5.3, there were many bands that did not display any activity on the substrates tested. However, considering that this was a crude fraction, it was expected that other proteins would also be present in this fraction, not only cellulolytic or hemicellulolytic enzymes. Furthermore, enzymes such as β-xylosidase were not tested when TLC results have indicated that this enzyme is most likely present in the crude fraction.

The MEC fraction exhibited approximately 17 proteins species. Some of the large proteins that were present in the crude fraction were absent from the MEC fraction. Again, the proteins that were visible differed from the SDS-PAGE gel displayed in Chapter 3 where only about 5 protein bands were visible, but the reasons for this has been discussed. When looking at other MECs in the literature, *C. thermocellum* has displayed up to 50 protein bands on SDS-PAGE gels (Schwarz, 2001) although Zverlov *et al.* (2005) reports 71 potential cellulosomal components from the genome sequence of *C. thermocellum*. The cellulosome in *R. albus* displayed 15 bands on an SDS-PAGE gel with 11 having endoglucanase or xylanase activity (Ohara *et al.*, 2000). The two MECs in *P. curdlanolyticus* displayed 8 and 7 proteins, respectively (Pason *et al.*, 2006).

From the summary in Table 5.4 it becomes apparent that there are several proteins in the MEC fraction that didn't display any activity on the substrates tested. With the substrates used, 9 out of the 17 proteins displayed activity. It is possible that they were not tested under the right conditions and as a result did not display activity. Alternatively, some of the smaller proteins that didn't display activity could have been fragments of enzymes that were no longer catalytically active as a result of cleavage by proteases. However, the larger proteins could also be non-catalytic, such as a possible scaffoldin protein analogous to that found in the cellulosome. These proteins generally do not display any activity and serve as a structural protein for the complex, although ScaA in *A. cellulolyticus* is one of the few examples of a scaffoldin with a catalytic site (Xu *et al.*, 2004). The scaffoldin protein has generally been considered to be a large protein and, until a few years ago, the smallest reported scaffoldin was that in *C. josui* at 120 kDa (Kakiuchi *et al.*, 1998). However, analysis of cellulosomes in *A. cellulolyticus* and in *R. flavefaciens* has revealed scaffoldin proteins as small as 89 kDa (ScaD) (Xu *et al.*, 2003; Xu *et al.*, 2004) and 90 kDa respectively (Rincon *et al.*, 2003). A technique such as N-terminal sequencing would have to be used in order to properly identify these proteins. It was an aim of this study to perform N-terminal sequencing on proteins to

accurately identify them. However, too little total protein was electrophoresed to detect individual species after electroblotting.

The presence of protease activity in the crude cultures could indicate that proteins found within the supernatant are potentially degraded after being secreted into the culture medium, resulting in fragments of proteins being observed in SDS-PAGE gels. The SDS-PAGE results have shown numerous protein bands, although many of them did not have enzyme activity. However, many of these bands could be formed as a result of proteolytic cleavage which has been found in other organisms (Jiang *et al.*, 2006a).

These results can be compared with reports in literature on various strains of *B. licheniformis* to determine similarities with strain SVD1. Rey *et al.* (2004) reported on the completed genome sequence of *B. licheniformis* ATCC 14580 and indicated that this strain had two putative endoglucanases belonging to glycoside hydrolase families GH9 and GH5, a probable cellulose-1,4-β-cellobiosidase of family GH48 and two genes for β-glucosidases. They concluded, therefore, that this strain had all the required enzymes for utilisation of cellulose and its conversion into cellobiose and glucose. This group furthermore confirmed that they found this particular strain capable of growing on CMC as a sole carbon source (Rey *et al.*, 2004). While the zymogram data from this study on strain SVD1 indicated the presence of three enzymes active on CMC, activity on this substrate in enzyme assays were found to be low. Growth on CMC was also found to be poor (data not shown). Although strain ATCC 14580 is reported to possess a gene for a cellulose 1,4-β-cellobiosidase (cellobiohydrolase), it does not appear to contain a signal peptide and therefore is not released extracellularly (Rey *et al.*, 2004).

With respect to cellulases and cellulose utilisation, Veith *et al.* (2004) reported on the genome sequence of *B. licheniformis* DSM 13. This strain, similarly, also contains three endo-glucanases, a cellulose 1,4-β-cellobiosidase and some glucosidases, two α-glucosidases and one β-glucosidase (Veith *et al.*, 2004). Numerous other reports exist in literature for the isolation of endo-glucanases from various strains of *B. licheniformis* (See Table 2 in section 1.4 for a summary). Liu *et al.* (2004) even reports on a *B. licheniformis* strain GXN 151 that was able to degrade Avicel efficiently. This directly contradicts the results found in this study and thus it appears as though large differences exist between various strains of this

organism. In fact, when conducting TLC with the crude fraction from *B. licheniformis* SVD1 and using Avicel as a substrate, no cellobiose or glucose was found to be released (data not shown).

Only one xylanase was reported to be present in the genome of *B. licheniformis* DSM 13 or ATCC 14580 although many genes appear to be present in these genomes for the degradation of arabinan substrates (arabinan endo-1,5-L-arabinase, arabinan endo-1,5- α -L-arabinosidase) and for cleavage of arabinose from substrates (α-L-arabinofuranosidase) (Rey *et al.*, 2004; Veith *et al.*, 2004). There are also many reports in literature of endo-xylanases isolated from various strains of *B. licheniformis* (see Table 1.2 of section 1.4). When compared to the results in this study, there appears to be up to seven xylanases present in *B. licheniformis* SVD1, while the presence of α-L-arabinofuranosidase was predicted from the activity on oatspelt xylan. Again it appears as though many differences exist in this regard between strains.

In this study, we furthermore found three different mannanases and high activity on locust bean gum as well as the ability to utilise mannose to the same extent as glucose. However, literature reports indicate the presence of only one mannosidase in the genome of *B. licheniformis* DSM 13 or ATCC 14580 although Rey *et al.* (2004) reported the presence of a β-mannanase (Rey *et al.*, 2004; Veith *et al.*, 2004) (see section 1.2.6).

B. licheniformis SVD1 appeared to possess a pectin methyl esterase as well as a pectin or pectate lyase from zymogram data. Veith *et al.* (2004) reported on the presence of four pectate lyase genes in *B. licheniformis* DSM 13, as well as a gene encoding for a pectin methyl esterase. Other reports in literature all indicate the presence of pectate lyase in other strains of *B. licheniformis* but no reports of a pectin lyase are available (see Table 1.2 of section 1.4).

It is also reported in literature that *B. licheniformis* has a *xyl* regulon encoding for xylose utilisation (Scheler *et al.*, 1991) and the genome sequences for *B. licheniformis* strains DSM 13 and ATCC 14580 both contain a gene for xylose isomerise, the key enzyme required for xylose utilisation (Rey *et al.*, 2004; Veith *et al.*, 2004). Thus it appears as though *B.*

licheniformis SVD1 may have a mutation that prevents expression of this enzyme or renders this enzyme inactive. This will have to be further investigated.

It must therefore be clear that, while comparison with reports in literature can provide valuable information, numerous differences exist between various strains of *B. licheniformis* and thus the characteristics of each strain have to be examined individually.

5.6 Conclusions

The cellulolytic system of *B. licheniformis* displayed a variety of enzyme activities on a range of substrates and included endoglucanase, xylanase, mannanase and pectinase activity. All these activities were also found in the MEC although xylanases were by far the predominant enzyme activity present in both the crude and MEC fractions. *B. licheniformis* SVD1 therefore seems to occupy a niche in the environment for the utilisation of hemicellulases.

Based on zymogram analysis there were three endoglucanases, seven xylanases, three mannanases and two pectinases in the crude fraction, while the MEC had two endoglucanases, seven xylanases, two mannanases and one pectinase. The pectinases in the crude could be identified as a pectin methyl esterase and a lyase, while the methyl esterase was absent in the MEC.

B. licheniformis was unable to utilise xylose sugars, but was able to utilise glucose and mannose; and, to a lesser extent, cellobiose, arabinose and galactose. This characteristic was apparent when the unusual pattern of growth in the growth curve was analysed as sugars were produced but not utilised. It seems curious that an organism would produce high levels of xylanases, and most likely a β-xylosidase based on TLC results, yet be unable to utilise the products of enzymatic degradation. However, this may have some practical application in biotechnological processes such as bioethanol production. Utilisation of sugars by the organism itself would reduce the amount of sugars available for bioethanol production and by choosing an organism with these characteristics, higher levels of bioethanol could be obtained.

As a cellulolytic and hemi-cellulolytic system is generally very complex, future work could include the investigation of other enzymes that may be present within the cellulolytic system of this organism such as glucuronidase, galactosidase, arabinofuranosidase, etc. Cloning of genes for various enzymes could also be undertaken to identify and characterise various enzymes.

In the following chapter the xylanase activity within the MEC is further investigated as this is the most prominent enzyme activity found within this organism.

CHAPTER 6 – THE EFFECT OF pH, TEMPERATURE AND CHEMICALS ON COMPLEXED XYLANASE ACTIVITY IN *BACILLUS LICHENIFORMIS* SVD1

6.1 Introduction

An investigation into the characteristics of an enzyme gives valuable information about the preferred environment the enzyme requires for optimal catalytic activity. When the enzyme can potentially be used in biotechnological applications, this information becomes vital to ensure the design of an optimal environment for maximal efficiency.

Standard characterisation includes the determination of optimal pH, temperature and temperature stability. The inhibitory or activatory effect of possible compounds expected to be present within a bioreactor environment on enzyme activity can also be determined.

The effect of the following compounds on complexed xylanase activity was measured in this study: calcium, EDTA, xylose, xylobiose and ethanol. An important reason for measuring the effect of calcium is the role it has been shown to play in dockerin-cohesin interaction in the cellulosome. Choi and Ljungdahl (1996) demonstrated that calcium was essential for dockerin-cohesin interaction in *C. thermocellum* and this was also shown for *C. celluloyticum* (Fierobe *et al.*, 1999). Calcium in *C. thermocellum* was shown to enhance activity of the cellulosome (Johnson *et al.*, 1982), but Demain *et al.* (2005) reported that the enhancement was only with respect to the exoglucanase activity within the cellulosome even though the major endoglucanase in the cellulolosome had three binding sites for calcium.

EDTA, probably due to its ability to chelate the calcium in the cellulosome, has been shown to have an inhibitory effect on cellulosomal activity (Demain *et al.*, 2005; Johnson *et al.*, 1982). EDTA has, in fact, been shown to allow dissociation of the cellulosome of *C. thermocellum* under mild conditions, allowing for the separation of components (Morag *et al.*, 1996). It is interesting to note that, after treatment with EDTA, the protein species in the *C. thermocellum* cellulosome showed a variation in molecular weight, with the formation of truncated proteins (Demain *et al.*, 2005).

The products of enzyme activity are often found to inhibit the enzyme at high concentrations through a negative feedback mechanism. For this reason, the effect of xylose and xylobiose on complexed xylanase activity was measured.

The broader purpose for studying MECs in microorganisms is to determine the possible role these complexes may play in biofuel production. While *B. licheniformis* SVD1 does not produce bioethanol itself, it may possibly be used in a co-culture with another organism such as *S. cerevisiae* for production of ethanol. In bioethanol production the tolerance of the organism itself for ethanol is an important factor to consider, as is the impact that ethanol has on enzyme activity. Dien *et al.* (2003) established that organisms producing bioethanol (or present in a co-culture for ethanol production) should have a tolerance for ethanol greater than 40 g/l. Research has been done on *C. thermocellum* and the tolerance of this organism for ethanol. Some ethanol-resistant strains have been isolated that show 50% inhibition at 27 g/l (Demain *et al.*, 2005). A *C. thermosaccharolyticum* strain has even been shown to tolerate ethanol concentrations of 50 g/l and at this concentration was only 50% inhibited (Demain *et al.*, 2005). A comprehensive review of the basis of ethanol tolerance in microorganisms was reported by Jeffries and Jin (2000). However, few reports exist in literature regarding the effect of ethanol on enzyme activity in a bioreactor environment.

6.2 Objectives

- To determine the pH optimum of complexed xylanase activity within the MEC fraction of a birchwood xylan culture
- To determine the temperature optimum and temperature stability of xylanase activity in the MEC
- To determine the effect of EDTA, $CaCl_2$, xylose, xylobiose and ethanol on xylanase activity in the MEC

6.3 Methods and materials

6.3.1 Enzyme assays

Enzyme assays were carried out as described in Section 2.3.2.

6.3.2 pH optimum of complexed xylanase activity

The optimal pH of complexed xylanase activity was measured using birchwood xylan as a substrate. Enzyme assays were carried out in triplicate at pH values ranging from pH 3.0-10.0 in 50 mM citrate buffer (citrate-NaOH) (pH 3.0-6.0), 50 mM potassium phosphate buffer (pH 6.0-8.0) and 50 mM glycine buffer (glycine-NaOH) (pH 9.0-11.0) at 50°C under standard conditions.

6.3.3 The temperature optimum and temperature stability of complexed xylanase activity

The optimal temperature of complexed xylanase activity was carried out using birchwood xylan as a substrate and was determined by performing the standard assay in triplicate at the optimum pH (6.5) over a range of temperatures from 20°C to 100°C. An Arrhenius plot was constructed for complexed xylanase activity using the above temperature optimum data and the apparent activation energy was calculated from the slope.

The temperature stability of complexed xylanase activity was determined by heating 500 µl aliquots of the complex at 55°C over a period of 2 hours. Aliquots were removed at various time intervals over this period and stored on ice. At the end of this period, the standard assay was conducted in triplicate for each aliquot and xylanase activity determined.

6.3.4 The effect of various compounds on complexed enzyme activity

The effect of EDTA and $CaCl_2$ was measured on complexed xylanase activity with concentrations ranging from 0-7 mM. The standard assay was conducted in the presence of

either EDTA or $CaCl_2$. Controls consisted of each concentration of EDTA or $CaCl_2$ with the enzyme, as well as a separate negative control with substrate and EDTA or $CaCl_2$.

The effect of xylose and xylobiose on complexed xylanase activity was measured at concentrations ranging from 0-1.5 mM. The standard assay was used with the xylose or xylobiose included in the assay. The effector at different concentrations was included with the substrate as a negative control.

The effect of ethanol on complexed xylanase activity was measured in the presence of ethanol concentrations ranging from 0-50 g/l using the standard assay conditions. An additional control was incorporated with the enzyme and the ethanol at various concentrations in order to exclude the possibility that the protein is precipitated by the ethanol, thus causing an inhibition.

6.3.5 Effect of varying substrate concentrations on complexed xylanase activity

To determine the effect of varying substrate concentrations on complexed xylanase activity, assays were conducted under standard condtions using birchwood xylan substrate concentrations between 3-10 mg/ml. The data was used to prepare a Hanes-Woolf plot from which the Michaelis constant (K_m) and the maximum velocity (V_{max}) values for complexed xylanase activity were determined (Garrett & Grisham, 1999).

6.4 Results

6.4.1 pH optimum of complexed xylanase activity

The pH profile for complexed xylanase activity as seen in Figure 6.1 indicated that activity was very similar between pH 5.0 and pH 7.0, with the optimal pH between 6.0 and 7.0. The complexed xylanase activity retained a higher degree of activity at alkaline pH than at acidic pH with approximately 50% of the optimal activity still present at pH 10.0 and about 30% still present at pH 11.0. At pH 3.0, the activity was negligible.

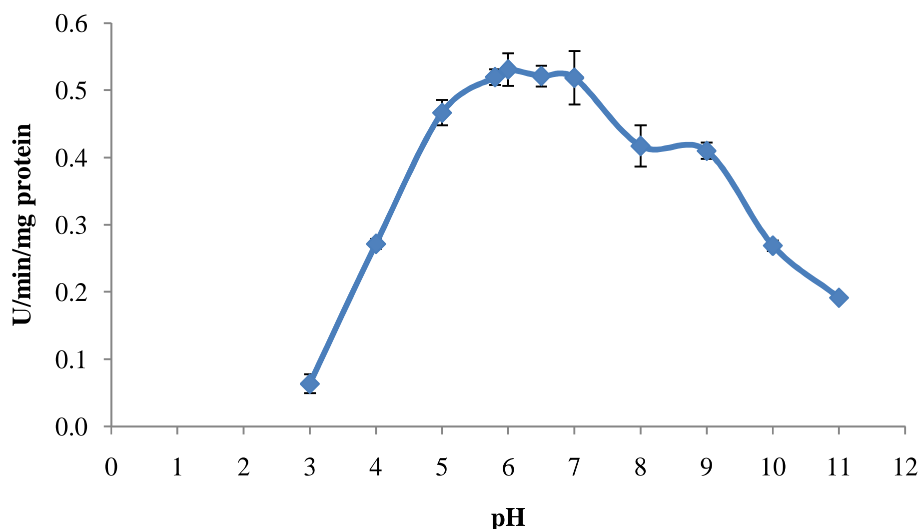


Figure 6.1. pH optimum of complexed xylanase activity. Activity was measured on birchwood xylan using citrate-NaOH buffer from pH 3.0-5.8, potassium phosphate buffer from pH 5.8-8.0 and glycine-NaOH buffer from pH 8.0-11.0. Values are presented as mean values ± SD (n=3).

6.4.2 The temperature optimum and temperature stability of complexed xylanase activity

The temperature profile for complexed xylanase activity can be seen in Figure 6.2. The optimal temperature appears to be 55°C although the activity between 50°C and 60°C was very similar.

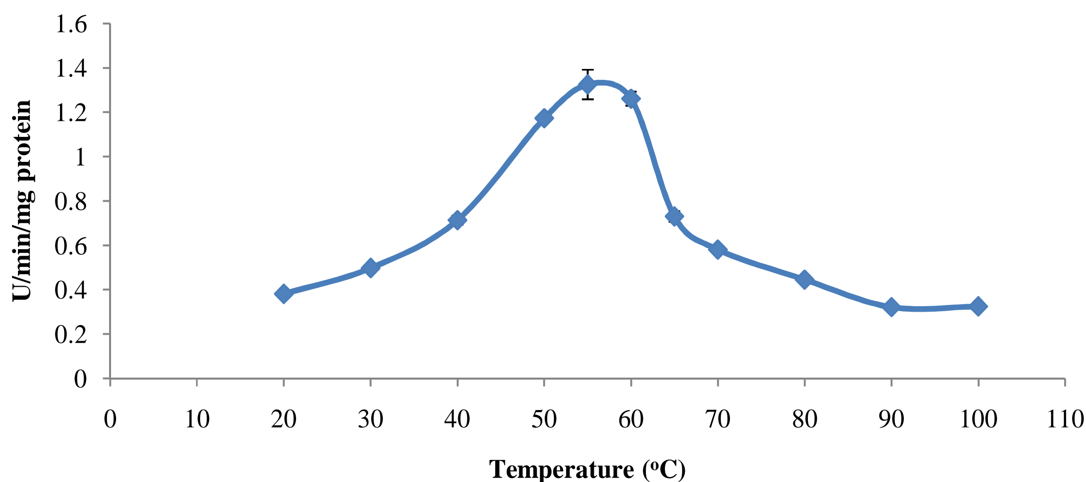


Figure 6.2. Temperature optimum of complexed xylanase activity. Activity was measured using birchwood xylan as a substrate under standard conditions from 20°C to 100°C. Values are presented as mean values ± SD (n=3).

An Arrhenius plot (Figure 6.3) was constructed for complexed xylanase activity using the temperature optimum data as displayed in Figure 6.2. The apparent activation energy (E_a, app) or the transition-state energy was calculated from the slope according to the following relationship: $-2.303E_a/R$ and was determined to be 1331.8 J/mol.

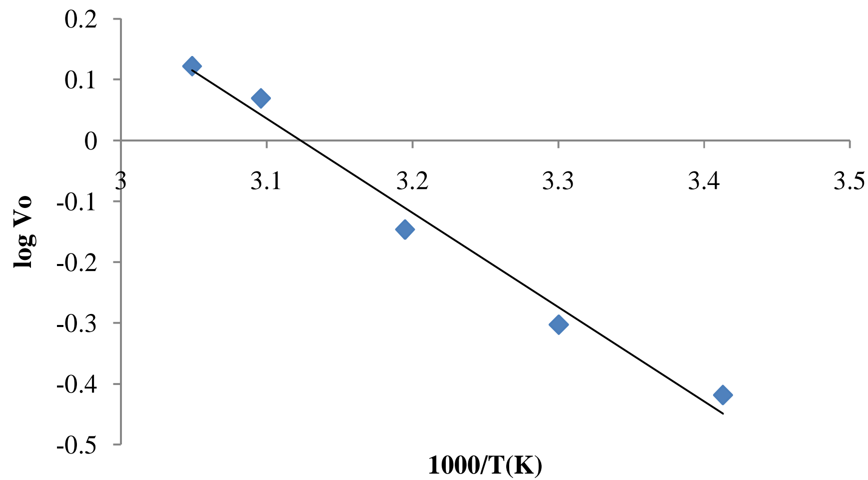


Figure 6.3. Arrhenius plot for complexed xylanase activity. The equation of the trendline y=-1.5491x + 4.8383, was used to calculated the apparent activation energy.

The temperature stability of complexed xylanase activity was observed over a two-hour incubation period (Figure 6.4).

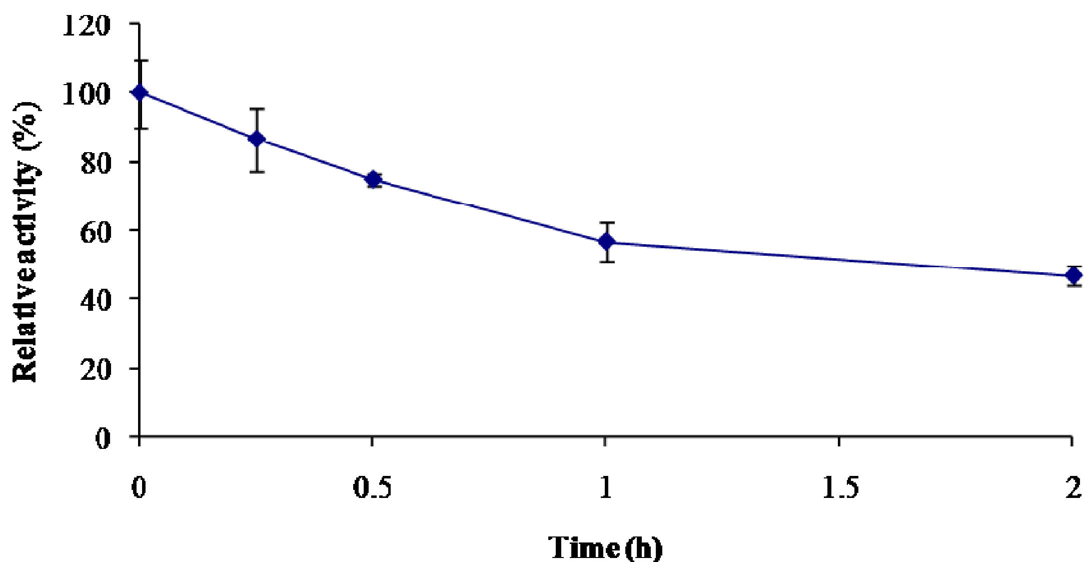


Figure 6.4. Stability of complexed xylanase activity at 55^oC. Aliquots (500 µl) of purified MEC was incubated at 55^oC and removed after each time interval. Aliquots were kept at 4^oC and then assayed for activity using birchwood xylan as a substrate under standard conditions. Values are presented as mean values ± SD (n=3).

Within 30 minutes, the activity decreased to 75% and after 2 hours incubation at 55^oC, 46% of activity was still present.

6.4.3 Effect of EDTA and $CaCl_2$ on complexed xylanase activity

The effect of EDTA and $CaCl_2$ on complexed xylanase activity was measured (Figure 6.5). It was found that EDTA inhibited complexed xylanase activity at concentrations as low as 1 mM where activity decreased to 73% respectively. Further inhibition was not observed with increased concentrations of EDTA and relative activity remained around 70% for all the concentrations tested. $CaCl_2$ had a very limited impact on complexed xylanase activity and only low levels of inhibition was found at concentrations of 3 mM and 7 mM where activity decreased to 88% and 89% respectively. Neither EDTA nor $CaCl_2$ had any effect on the substrate control which remained constant, but both compounds had an effect on the enzyme control which was observed to decrease with increased effector concentration.

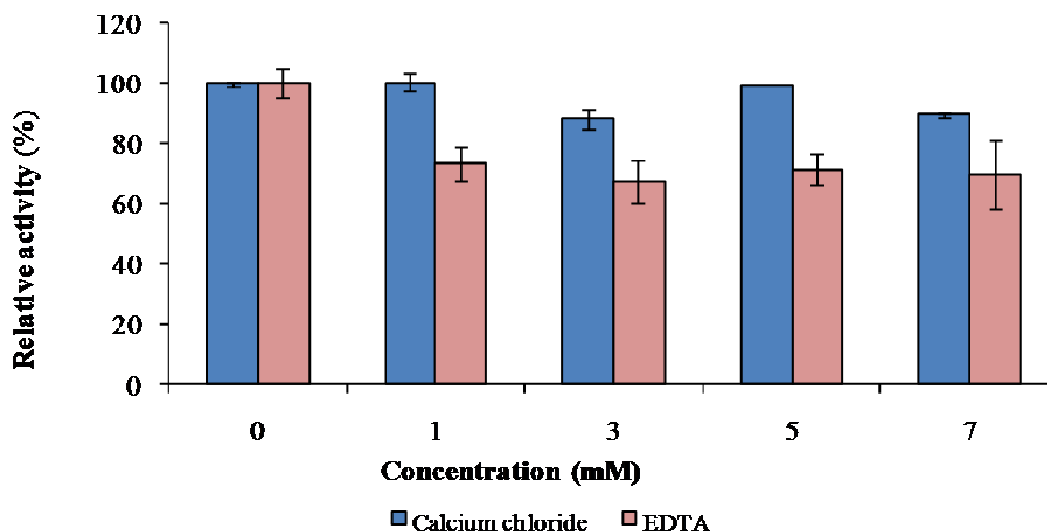


Figure 6.5. Effect of $CaCl_2$ and EDTA on complexed xylanase activity. Assays were done using birchwood xylan as a substrate under standard conditions. Values are presented as mean values ± SD (n=3).

6.4.4 The effect of xylose, xylobiose and ethanol on complexed xylanase activity

The effect of xylose and xylobiose on complexed xylanase activity was measured and the data suggested an enhancement of activity in the presence of these compounds (Figure 6.6). At its highest levels, activity appeared to be at 150% of the activity in the absence of any xylose or xylobiose, even when taking the contribution of the controls into account.

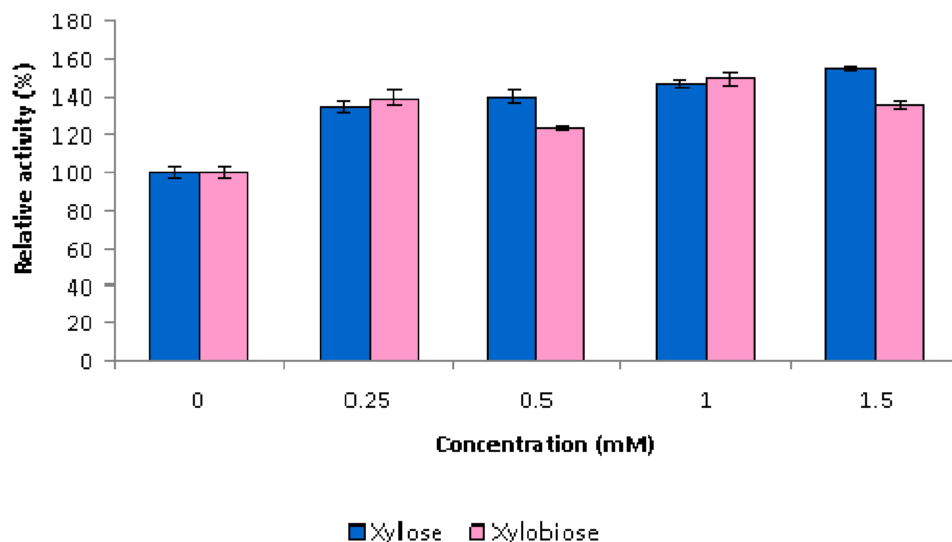


Figure 6.6. Effect of xylose and xylobiose on complexed xylanase activity. Assays were carried out in the presence of xylose and xylobiose using birchwood xylan as a substrate under standard conditions. Values are presented as mean values ± SD (n=3)

The effect of ethanol on complexed xylanase activity (Figure 6.7) indicated that inhibition took place at all the concentrations of ethanol tested which gradually increased with increased ethanol concentration. At 10 g/l 87% of activity was still present while at 50 g/l of ethanol the activity decreased to 58%.

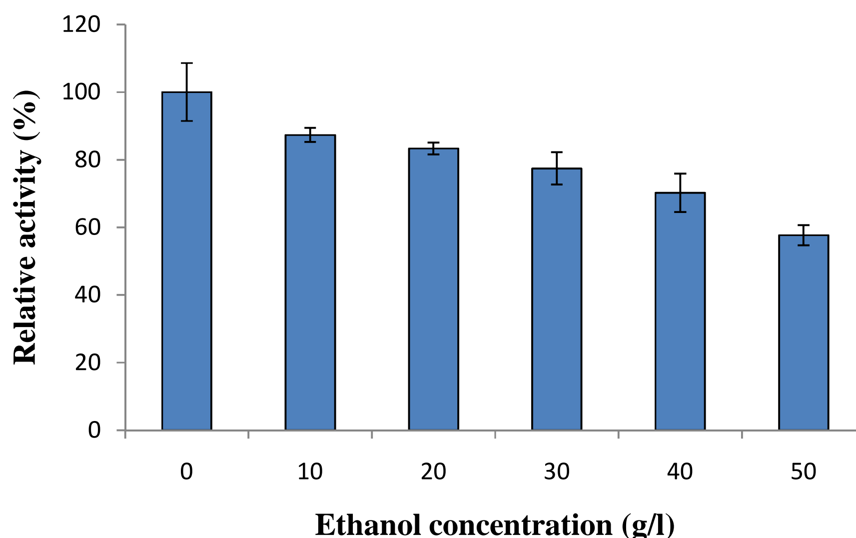


Figure 6.7. Effect of ethanol on complexed xylanase activity. Assays were carried out in the presence of ethanol using birchwood xylan as a substrate under standard conditions. Values are presented as mean values ± SD (n=3).

6.4.5 Effect of varying substrate concentrations on complexed xylanase activity

The rate of complexed xylanase activity for each concentration of birchwood xylan substrate was used to prepare a Hanes-Woolf plot (Figure 6.8). From this plot, the Michaelis constant (K_m) value was calculated as 2.84 mg/ml while the maximal velocity (V_{max}) was calculated as 0.146 U (µmol/ml/min).

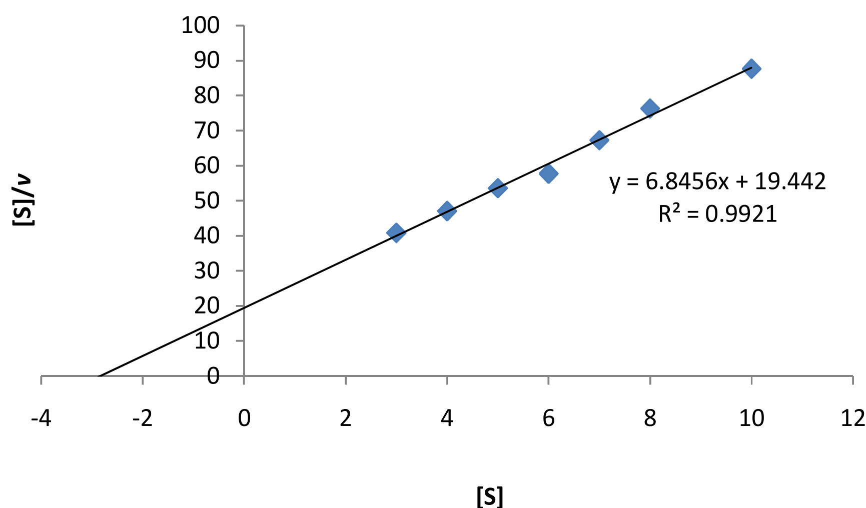


Figure 6.8. Hanes-Woolf plot of initial velocity data for complexed xylanase activity. Enzyme activity was measured at standard conditions. Values represent the mean of triplicate experiments. V is in $\mu mol.ml^{-1}.min^{-1}$. [S] is the concentration of birchwood xylan in $mg.ml^{-1}$.

6.5 Discussion

The pH profile for complexed xylanase activity indicated that xylanase activity had a pH optimum between 6.0 and 7.0, yet also exhibited a high level of stability over a wide pH range. At pH 9, 77% of maximal activity was still present. Although the pH profile of the multiple xylanases within the MEC was measured, it could be useful to compare this result with free xylanases. An optimum in this range is quite a common feature for xylanases in bacilli as well as stability over a wide range of pH (Beg *et al.*, 2001; Sunna and Antranikian, 1997).

The temperature profile indicated a clear optimum between 50°C and 60°C. Some activity (25%) was still present at 100°C. According to Sunna and Antranikian (1997) most characterised endoxylanases have optimal activity between 45°C and 75°C which correlates with the data in this study. Beg *et al.* (2001) lists the temperature optima of various xylanases from bacilli which indicates that the majority have optima between 50°C and 70°C. At its optimal temperature of 55°C, complexed xylanase activity was not stable for a lengthy period of time as it had lost 43% of its activity after an hour. However, when stored at 4°C xylanase activity was stable for months (data not shown).

The fact that complexed xylanase activity was inhibited by EDTA indicated that it required a metal co-factor for activity. However, no activation was observed in the presence of $CaCl_2$ and activity was slightly inhibited in the presence of calcium. Sunna and Antranikian (1997) reported that various bacterial xylanases were inhibited by calcium, including two xylanases from *Cellulomonas fimi* and two from *Clostridium stercorarium*. Very few reports in the literature indicate inhibition of xylanase activity by EDTA, although it has been found in *Thermotoga thermarum* (bacterium) and *Aspergillus nidulans* and *Neocallimastix frontalis* (both fungi) (Sunna & Antranikian, 1997). Lee *et al.* (2008) found that a xylanase from *B. licheniformis* displayed activation in the presence of $CaCl_2$.

Some reports in literature propose that inhibition by EDTA indicates a requirement by an enzyme for calcium (Sellami-Kamoun *et al.,* 2008). However, EDTA would chelate any divalent cations and thus it is not established which co-factor is required by xylanases within the MEC. Further work would have to be carried out to confirm the requirement of a co-factor for xylanase activity. From the data in this study it is not possible to make any conclusions with regards to the requirement of calcium for the structural integrity of the MEC.

It is generally accepted that the products of degradation often cause inhibition of an enzyme when present at high levels (Madigan *et al.*, 2003). This has been a common feature in the case of cellulases that are inhibited by cellobiose specifically, but also by glucose (Sun & Cheng, 2002). However, very few reports in literature were found to suggest that xylanases were inhibited in the presence of high levels of xylose and xylobiose. Inhibition of a xylanase by xylose was reported for two species of *Cellulomonas*, *C. fimi* and *C. uda* (Khanna & Gauri, 1993; Rapp & Wagner, 1986). However, many reports indicate a lack of inhibition by xylose. Only one report was found regarding the competitive inhibition of a

xylanase by xylobiose in *Humicola lanuginosa* (Kitpreechavanich *et al.*, 1984). It is far more common for β-xylosidase, which cleaves xylobiose into xylose, to be inhibited by xylose and xylobiose (Andrade *et al.*, 2004; Bernier *et al.*, 1987; La Grange *et al.*, 2001; Pinphanichakarn *et al.*, 2004; Xu *et al.*, 1991).

In this study, it was found that xylose and xylobiose appeared to activate the xylanases present in the MEC even after taking the controls into account. In the presence of these compounds, xylanase activity was found to be enhanced by 30-50%. No reports were found in the literature indicating a similar phenomenon. The manner in which these compounds are able to cause an activation of xylanase activity is not clear and this would have to be further investigated.

Ethanol displayed an inhibitory effect on complexed xylanase activity. However, the ethanol tolerance of xylanases was very high as 58% activity was still present at 50 g/l. The productivity of ethanologenic bacterial strains was reported by Dien *et al.* (2003) to range between 23-63 g/l. At the levels of ethanol measured in this study, high enzyme activity would still be obtained with xylanases from *B. licheniformis* SVD1 in biotechnological applications for the fermentation of bioethanol. It is reported that cellulases have been shown to be inhibited by ethanol, which, at a level of 30 g/l, caused a 25% inhibition of enzyme activity (Taherzadeh & Karimi, 2007). Wu and Lee (1997) further reported inhibition of cellulases of 9%, 36% and 64% at ethanol concentrations of 9, 35 and 60 g/l, respectively. This is very similar to the result obtained in this study where 22% inhibition of xylanases was found at 30 g/l and 42% at 50 g/l.

A K_m value for xylanase activity was calculated as 2.84 mg/ml while the maximal velocity (V_{max}) was calculated as 0.146 U (μmol/ml/min). This can be compared to K_m values for other xylanases in bacilli where a wide range of values were found from 0.32-17 mg/ml (Beg *et al.*, 2001; Sunna & Antranikian, 1997). The kinetic parameters determined for complexed xylanase activity in this study is therefore comparable with data found in literature. V_{max} values in literature are reported in units per mg protein. However, in this study, the whole complex was used rather than a purified xylanase which distorts the specific activity per mg of protein and therefore these values are not comparable with values reported in literature.

6.6 Conclusions

The physical, chemical and kinetic characterisation of complexed xylanase activity was conducted in this chapter. It was found that complexed xylanase activity had a pH optimum between pH 6.0 and 7.0, but that activity was stable over a wide range of pH from 4.0-10.0. The optimum temperature was found to be 55°C but more than 50% activity was still present at 40°C and 65°C. Temperature stability at 55°C was measured and after 2 h of incubation, activity had decreased by 54%. The parameters for pH and temperature were found to be consistent with most xylanases reported in literature.

Complexed xylanase activity was further found to be slightly inhibited by $CaCl_2$ and inhibited to a greater extent by EDTA. The inhibition by EDTA appears to indicate that a metal co-factor, which is chelated by the EDTA, is required for xylanase activity.

Complexed xylanase activity was further shown to be activated in the presence of xylose and xylobiose, both compounds which would be products of enzymatic degradation. It has been reported that these compounds, in a few cases, have been found to inhibit xylanase activity, but no reports could be found that these compounds would lead to the enhancement of xylanase activity. It is not clear what the mechanism of such activation could be and this will have to be further investigated.

Ethanol was found to inhibit complexed xylanase activity, which increased with increased concentrations of ethanol, although 58% activity was still present at concentrations of 50 g/l. Similar reports have been published on the inhibition of cellulases by ethanol, but no studies were found on the effect of ethanol on xylanases.

The kinetic parameters for complexed xylanase activity were measured and the K_m value was calculated as 2.84 mg/ml while the maximal velocity (V_{max}) was calculated as 0.146 U (µmol/min/ml). The K_m value was found to be consistent with reported values for xylanases in the literature.

Understanding the physical, chemical and kinetic characteristics of complexed xylanase activity will assist in creating the conditions for optimal performance and productivity. Should *B. licheniformis* SVD1 or the MEC itself be utilised in future for biotechnological applications, this study will greatly aid in the optimisation of conditions. For bioethanol production specifically, the high degree of pH stability, lack of product inhibition and ethanol tolerance would be beneficial characteristics in such a process.

In the following chapter, we explore further evidence to determine whether the MEC found in *B. licheniformis* SVD1 could be a putative cellulosome.

CHAPTER 7 – IS THE MULTI-ENZYME COMPLEX FOUND IN *BACILLUS LICHENIFORMIS* SVD1 A PUTATIVE CELLULOSOME? – A CRITICAL ASSESSMENT OF AVAILABLE EVIDENCE

7.1 Introduction

Over the past 26 years, the cellulosome has become the paradigm for cellulolytic multi-enzyme complexes. The interest in this complex has been in its superior efficiency in the degradation of crystalline cellulose as compared to free enzymes (Johnson *et al.*, 1982). Since the first report of its identification in *C. thermocellum* in 1983 (Lamed *et al.*, 1983), cellulosomes have been discovered in numerous other microorganisms. The cellulosome is described as a discrete and functional complex with a specific molecular structure and not simply a random aggregation of components (Bayer *et al.*, 2004). The basis of the molecular structure of the cellulosome is the large, non-catalytic scaffoldin protein containing cohesin domains to which catalytic modules, containing dockerin domains, bind. The interaction and specificity of binding between the cohesin and dockerin modules dictate the structure of the cellulosome (Bayer *et al.*, 2004). Although cellulosomes in different organisms have been shown to have many unique structural features, the basis of this cohesin-dockerin interaction has remained unchanged. Thus the key method for identification of cellulosomes in an organism has been bioinformatic analysis of gene sequences to identify proteins with dockerin sequences, or a protein containing cohesin modules that could serve a function as a scaffoldin protein. However, the presence of cohesin domains alone is not sufficient for predicting a cellulosome. For instance, cohesin domains were identified in the genome of an archaeal species, *Archaeoglobus fulgidus*, although this organism does not possess a cellulosome or even identifiable coding sequences for glycosyl hydrolases (Bayer *et al.*, 1999). While this method is most effective for identifying potential cellulosomes, gene sequences are not always available and generation of a whole genome sequence, being very costly, is not always feasible. Thus other features have been used to identify the presence of a possible cellulosome in an organism.

Schwarz (2001) lists several features used to identify cellulosome production in organisms. One of these is biochemical evidence of a complex through the use of size exclusion

chromatography. The molecular weight of cellulosomes is varied and has been reported as ranging from 650 kDa to 2.5 MDa (Doi *et al.*, 2003). However, large protein complexes with cellulolytic or hemi-cellulolytic activity have been found that were not cellulosomes and therefore size is merely one factor to be considered (Jiang *et al.*, 2006).

A further feature is reaction *with C. thermocellum* CipA scaffoldin protein antibodies should these be available (Schwarz, 2001). Thirdly, the presence of cell protuberances when using electron microscopy is often viewed as indicative of cellulosome production (Scwharz, 2001). An example of these cell protuberances as found in *C. thermocellum* is illustrated in Figure 7.1. Figure 7.1A displays the cell surface, when the organism was cultured on cellobiose, with the cellulosome visible as a resting structure on the cell surface. When the same cells were brought into contact with cellulose, these resting structures formed protracted structures to interact with the cellulose while remaining attached to the cell (Figure 7.1B). The cell protuberances therefore appear to be only present and visible under certain conditions.

Investigating the presence of cell protuberances as possible evidence for cellulosome production assumes by implication that the MEC is associated with the cell for at least a part of the growth period of the organism. However, even in cellulosomal organisms, this is not always the case. In some organisms, such as *C. papyrosolvens*, cells lacked cellulosome clusters on their surface and only low enzyme activity was associated with the cells (Cavedon *et al.*, 1990). In *C. cellulovorans*, the cellulosome was reported not to form when the organism was cultured on cellobiose, although the components were secreted into the medium and assembly took place in the presence of cellulose (Matano *et al.*, 1994). Thus the absence of cell surface structures does not mean that an MEC cannot be a cellulosome. At the same time, where cell surface structures do exist, this does not constitute sufficient evidence for a cellulosome. Lamed *et al.* (1987) found that all cellulolytic bacteria had cell surface structures and, while some of these organisms were later shown to produce cellulosomes, this was not the case for all the organisms examined.

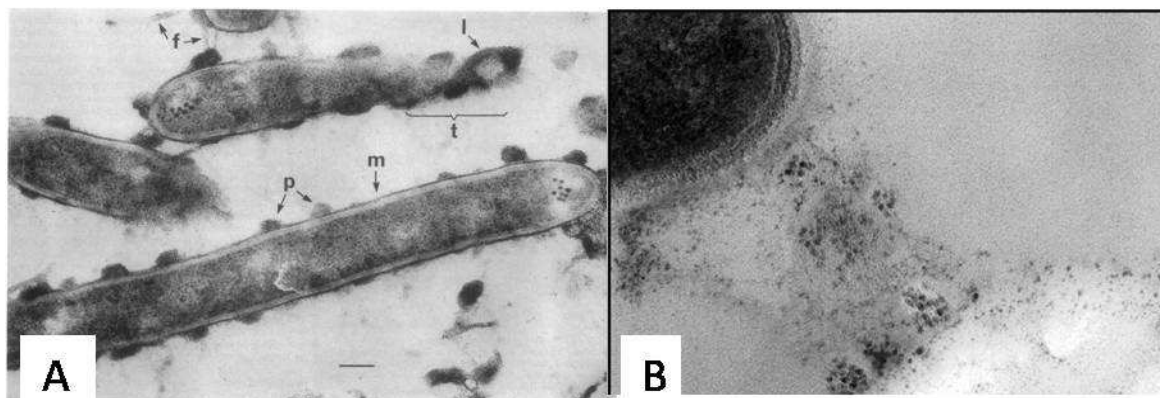


Figure 7.1. Transmission electron micrograph of cells from *C. thermocellum* displaying the cellulosome on the cell surface. In image A, cells were grown on cellobiose while in image B, cells are interacting with cellulose and form protracted structures between the cells and the cellulose. Cells were stained with cationized ferritin. Three types of features are noted: the monolayer (m) of cationized ferritin particles around the entire cell surface, the fibrous structures (f) which sometimes connect two adjacent cells, and the nodulous protuberances (p) which appear in large numbers over the entire cell surface. (Figures taken from Bayer & Lamed, 1986).

Cationized ferritin was used to stain bacterial cell surfaces for the production of the images in Figure 7.1. It is a cationic stain that is able to react with bacterial cell surfaces which are often negatively charged due to the presence of anionic polysaccharides in the glycocalyx (Kenyon *et al.*, 2005). Without this stain, it would not be possible to view the cellulosome on the cell surface. However, it should be considered that the entire glycocalyx will react with this stain and this can be seen in Figure 7.1A where the glycocalyx is visible as a thin layer around the entire cell.

Activity on a crystalline cellulose substrate such as Avicel has generally been considered as a characteristic feature of cellulosomes, although Bayer *et al.* (2004), have pointed out that the discrete nature of the cellulosome lies in its molecular structure rather than simply its cellulolytic activity. In *C. acetobutylicum*, for instance, biochemical and genetic evidence exists for the presence of a cellulosome, yet Sabathe *et al.*, (2002) could detect no cellulolytic activity in this organism.

With the exception of *R. flavefaciens*, all scaffoldin proteins of cellulosomes have been found to contain a CBM able to bind crystalline cellulose (Doi *et al.*, 2003) (see also section 1.2.8). This allows the entire complex to bind to the substrate to facilitate its efficient degradation. This feature has also been used in some cases to study MECs to identify cellulosomal characteristics (Pason *et al.*, 2006).

By comparing the MEC in this study with distinct characteristics of cellulosomes, it may be possible to gain a greater understanding of the MEC and assist with the elucidation of its structural basis.

7.2 Objectives

- To investigate the presence of a cellulosome in *B. licheniformis* SVD1 through:
    - Examining evidence for the presence of cell protuberances using transmission electron microscopy
    - Examining the ability of the MEC from *B. licheniformis* SVD1 to bind to insoluble substrates
- To perform a bioinformatic analysis of the genome of *B. licheniformis* DSM 13 and ATCC 14580 to identify the presence of signature sequences such as cohesins and dockerins within these strains.

7.3 Methods and materials

7.3.1 Transmission Electron Microscopy (TEM)

Cells of *B. licheniformis* SVD1 were cultured in the medium as described in section 2.3.4 using 0.5% (w/v) cellobiose or birchwood xylan. After 48 hours of incubation, cells were separated from the medium by centrifugation of 1 ml aliquots for 1 min at 16,000 *x g*. Cells were then prepared for TEM as described in Bayer *et al.* (1985). Cells were firstly washed in 0.9% (w/v) NaCl (saline) by centrifugation. Pelleted samples of cells were resuspended in 0.7 ml of saline. Karnovsky's fixative (0.3 ml) (1.6 g $Na_2H_2PO_4$, 0.27g NaOH, 10 mL 40% (v/v) formaldehyde, 1.43 mL 70% (v/v) glutaraldehyde, 88.5 mL distilled H_2O) was added and incubated for 20 minutes at 4^oC. The fixed cells were then washed 3 times with saline and resuspended in 0.7 ml saline. Cells were then treated with cationized ferritin stain by addition to the cell suspension of 0.5 mg of cationized ferritin in saline. The cells were incubated at room temperature for 1 hour, centrifuged, washed twice with saline and fixed with Karnovsky's fixative. Cells were centrifuged and 1% (w/v) OsO_4 in phosphate buffer added and incubated for 1 hour at 4^oC. Cells were then washed twice with distilled water. Cells were dehydrated with graded solutions of ethanol - 30%, 50%, 70%, 80%, 90% (v/v)

for 10 minutes each and then placed in absolute ethanol for 2 x 10 minutes. Cells were then embedded in gradual solutions of Araldite-Taab 812 resin. Propylene oxide was added for 2 x 20 minutes, propylene oxide:resin (75:25) for 90 minutes and then propylene oxide:resin (50:50) for 90 minutes. Propylene oxide:resin (25:75) was then added for 90 minutes before samples were placed in pure resin overnight. Stained cells in resin were placed in an oven at 60°C for 36 hours to allow the resin to dry. These cells, embedded in resin, were cut with a microtome in sections of approximately 100 nm thick. Sections were mounted on copper grids (300 mesh) and viewed on a JEOL 1210 transmission electron microscope.

7.3.2 Binding studies

Insoluble xylan was prepared by the method of Kittur *et al.* (2003). Birchwood xylan (10 g) was suspended in 200 ml distilled H_2O and stirred for 2 h before it was centrifuged at 12,000 *x g* for 10 minutes. The supernatant was decanted and retained as soluble xylan. The pellet was washed twice again and centrifuged after which it was lyophilised. After lyophilisation the pellet was ground to a fine powder using a mortar and pestle.

For the binding assay, 50 mg of Avicel and insoluble birchwood xylan were placed in 1.5 ml microcentrifuge tubes. Potassium phosphate buffer (50 mM) at pH 6.5 was added to equilibrate the Avicel and xylan and the mixture was left to stand at room temperature for an hour. The phosphate buffer was removed through centrifugation. One ml of the purified MEC (0.15 mg/ml protein) to which was added 1 mg/ml of bovine serum albumin (BSA), was added to each tube and the tubes placed at 4°C on a rotational shaker for one hour. BSA was added to prevent non-specific binding to the tube (Goldstein *et al.*, 1993). Controls were set up with 1 ml potassium phosphate buffer at pH 6.5 alone, 1 ml potassium phosphate buffer (pH 6.5) with Avicel or birchwood xylan, and 1 ml purified MEC with BSA. After shaking, the supernatant was removed through centrifugation at 16,000 *x g* for 20 seconds and assayed for activity on birchwood xylan. The assay was performed according to section 2.3.2. The percentage binding was calculated as the activity that was lost after binding took place with respect to the activity of the purified MEC control. All binding assays were performed in triplicate.

7.3.3 Bioinformatic analysis

In order to determine whether the *B. licheniformis* DSM 13 /ATCC 14580 genome contained any sequences characteristic of or indicative of a cellulosome, a sequence analysis of the genome, as available on the NCBI website (http://www.ncbi.nlm.nih.gov), was performed. Protein sequences for scaffoldins, dockerins and cohesins were obtained from the NCBI website or from the Prosite website (http://ca.expasy.org).

The Swiss-Prot or Genbank accession codes for protein sequences were as follows: scaffoldin from *C. thermocellum* (CIPA_CLOTH, Q06851, CIPB_CLOTM, Q01866), *C. cellulolyticum* (AAC28899.2), *C. josui* (BAA32429.1), *Ruminococcus flavefaciens* (ScaB, CAC34385.1) and *Acetovibrio cellulolyticus* (ScaB, AAP48995.1, ScaC, AAP48996.1).

Accession codes for dockerins were as follows: *Clostridium* cellulosome repeated domain signature (Prosite, PS00448), *C. cellulolyticum* (Type I dockerin, ZP_01574324.1), *C. acetobutylicum* (Dockerin from cellulase C, AAK78540.1).

Accession codes for cohesins were as follows: *C. thermocellum* (A3DCL1_CLOTM), *C. cellulolyticum* (A0UZJ0_CLOCE), *C. acetobutylicum* (Q97KK4_CLOAB), *Ruminococcus flavefaciens* (A0AEF2_RUMFL), *A. cellulolyticus* (55669585).

A PSI-Blast search for these modules was done in Genbank against the genome sequence for *B. licheniformis* DSM 13/ATCC 14580. Multiple sequence alignments were generated by the ClustalW program (http://www.ei.ac.uk/clustalw) and secondary structure predictions were performed using the Predictprotein site (http://dodo.cpmc.columbia.edu/predictprotein).

7.4 Results

7.4.1 Electron Microscopy

The results of transmission electroscopy are displayed in Figure 7.2. Figure 7.2 A, B, C and E are images of cells that were cultured on cellobiose while images in Figure 7.2 D and E are cells that were cultured on birchwood xylan.

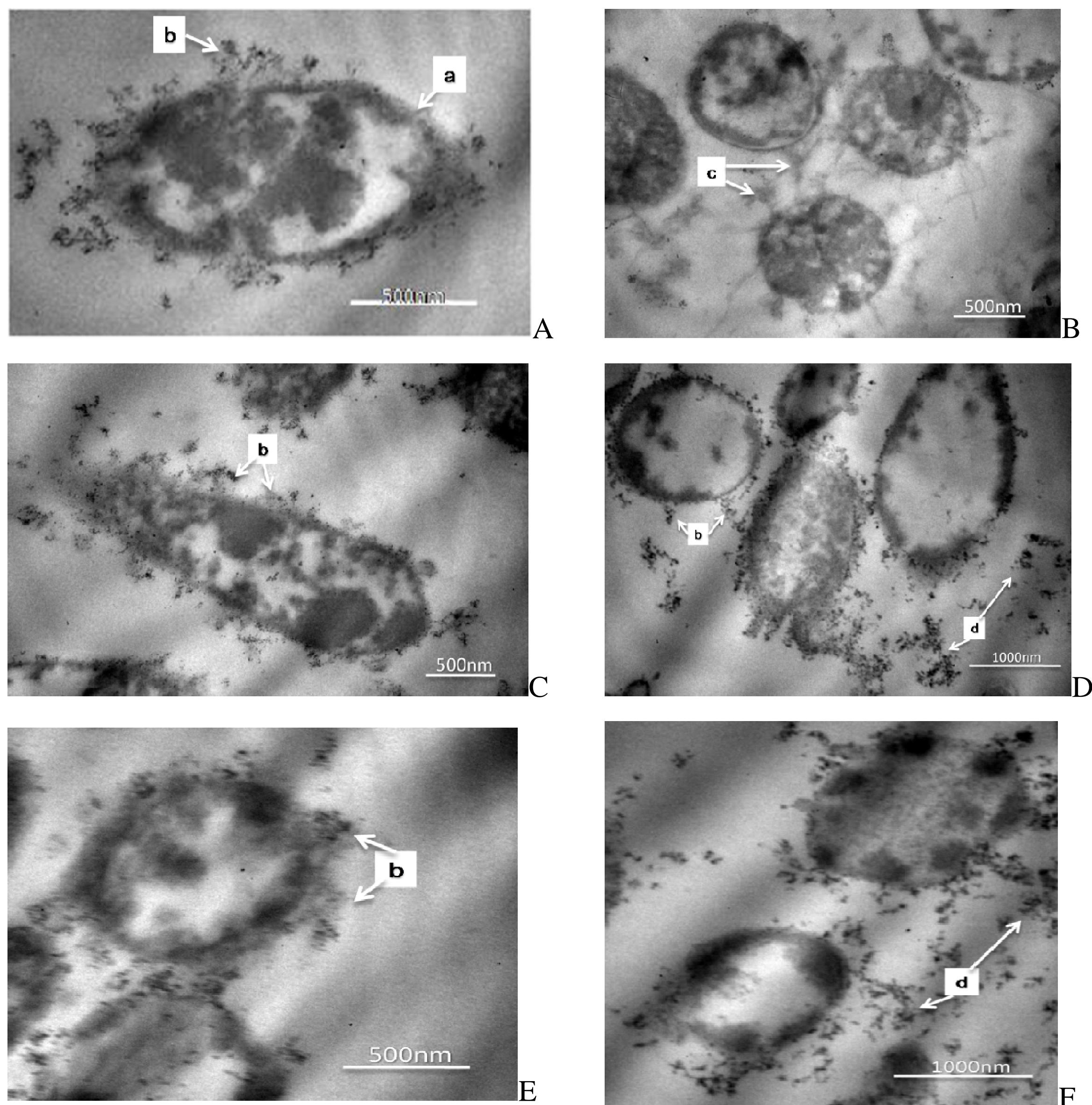


Figure 7.2. Transmission electron micrographs of cells from *B. licheniformis* SVD1 stained with cationized ferritin. Images A, B, C and E were cells cultured on cellobiose while images D and F were cells cultured on birchwood xylan.

Various features, denoted on Figure 7.2 as (a-d), are visible on the surfaces of the cells. Feature (a) represents the smooth surface of the cell while (b) appears to be some form of structure protruding from the cell surface at various positions in both the cellobiose culture and the birchwood xylan culture. These structures are, however, more prominent on the cells in the cellobiose culture. A third feature that is visible (c) is present in Figure 7.2B where the cells appear to be connected by thin, extended structures. The fourth feature (d) appears to be structures that are not connected to the cells.

7.4.2 Binding studies

The MEC was found to display binding towards insoluble xylan, but did not display binding towards Avicel. At the levels of protein used in the binding assay, 100% xylanase activity was lost from the supernatant after binding to xylan took place, but no xylanase activity was lost in the assay tubes containing Avicel.

7.4.3 Bioinformatic analysis

Although the genome sequence of *B. licheniformis* SVD1 was not available, a bioinformatic analysis of the genome of a different strain, which was available, was conducted to determine the presence of cellulosomal components within the species. The sequence analysis of the genome of *B. licheniformis* DSM 13/ATCC 14580 revealed that there was no significant homology in the amino acid sequences of the cellulosomal scaffoldin, cohesin or dockerin domains investigated, indicating that *B. licheniformis* does not contain coding sequences for proteins that could potentially form a cellulosome complex. Some sequence homology was found with scaffoldin proteins that were blasted against the *B. licheniformis* genome. Upon investigation, the sequence homology was only with respect to the CBM domain of the scaffoldin which displayed sequence homology with two endoglucanases within the *B. licheniformis* DSM 13 /ATCC 14580 genome. Although the sequence homology between the CBMs was 30-40%, it was only with respect to a small part of the scaffoldin. Sequence homology with the entire scaffoldin amounted only to 2-3%.

7.5 Discussion

When the electron micrographs from Figure 7.2 are compared with the micrographs in Figure 7.1 from *C. thermocellum*, differences can be observed. The cells of *B. licheniformis* SVD1 displayed some form of structure on the cell surfaces, but it does not appear to be as distinct as the nodulous protuberance on the cells in Figure 7.1. The most prominent of these cell surface structures can be seen in Figure 7.2E on cells from a cellobiose culture. The thin, extended structures visible in Figure 7.2B show great similarities with the micrographs in Figure 7.1A(f) where comparable structures appeared to connect adjoining cells. In the *B. licheniformis* cells cultured on birchwood xylan, extensive structures were visible that were not connected to the cell surface (Figure 7.2D and F (d)). At the same time, less of the cell surface structures were present in these cells that were cultured on birchwood xylan.

While it is apparent that some structures could be observed on the surface of *B. licheniformis* cells, it is difficult to interpret these results as evidence for cellulosome production. It cannot be expected that they would look identical to the cell protuberances observed in Figure 7.2 for *C. thermocellum*, as the complexes in each organism are different in size, structure and composition and therefore could react differently to the cationized ferritin stain. However, one would have to establish whether these structures displayed enzyme activity. Perhaps future work could include the production of antibodies against the MEC and using these to identify whether it is in fact the MEC that was present on the cell surface. One should also consider that in Figure 5.1A it was observed that cell-associated xylanase activity was present only at low levels. Furthermore, it must be considered that the MEC was purified from the extracellular supernatant and not isolated from the cell fraction. Thus if these cell surface structures were in fact the MEC, it would have to occur both in cell-associated and cell-free forms. This feature has been known to occur in the case of the cellulosome (Lynd *et al.*, 2002). However, one cannot exclude the possibility that the features observed on the cell surface of *B. licheniformis* may be as a result of exopolysaccharide formation which was observed in Chapter 2. Further research will have to be carried out to investigate this possibility.

In this study it was found that the MEC was able to bind to insoluble xylan but not to crystalline cellulose. The ability to bind insoluble substrates is considered important due to

the fact that degradation of insoluble substrates is inextricably linked to the enzyme/complex's ability to bind and thus remain in close proximity to the substrate while it is hydrolysed. Furthermore, binding to crystalline cellulose is a feature of the cellulosome as the scaffolding protein of the cellulosome contains a CBM3a domain which is able to bind crystalline substrates (Boraston *et al.*, 2004). Cellulosomes have been found to bind only very weakly to insoluble xylan (Kosugi *et al.*, 2001). Bayer *et al.* (1998), however, pointed out that: "the cellulose-binding function (is now considered) an optional, though often characteristic, feature of a cellulosome, because in some cases, the molecular basis for binding may not be strictly associated with the cellulosome components". The inability of the MEC in this study to bind crystalline cellulose indicates that it probably does not possess a CBM3a domain similar to scaffoldin proteins (although individual enzymes may possess such a domain). This MEC therefore appears to be structurally very different from the generally accepted model of the cellulosome.

Together with the absence of a CBM for binding crystalline cellulose, it was also found that the MEC in *B. licheniformis* displayed an absence of true activity on crystalline cellulose (see Chapter 5). While a few cellulosomes have been found that do not possess activity on crystalline cellulose (Avicelase activity), cellulosomes generally have this activity as a main feature (Sabathe *et al.*, 2002). While many MECs have been isolated with predominantly xylanase activity, these have been termed xylanosomes rather than cellulosomes (see Section 1.3.2). It is problematic, however, that no structural basis for the composition of xylanosomes has been identified.

The bioinformatic analysis was conducted on a different strain of *B. licheniformis*, namely DSM 13 /ATCC 14580, as this was the only genomic sequence available for *B. licheniformis*. Thus it may only have limited value considering that it was demonstrated in Chapter 5 that many differences exist between these strains and *B. licheniformis* SVD1 used in this study. Cohesins and dockerins are considered "signature components" of cellulosome architecture (Bayer *et al.*, 1998). No homology was found with these components in the genome of *B. licheniformis* DSM 13 /ATCC 14580, indicating that this strain does not produce a cellulosome. Strain DSM 13/ATCC 14580 may differ substantially from strain SVD1 and thus SVD1 may well code for a cellulosome. It would, however, only be possible to conclusively determine this if the genome sequence for strain SVD1 was obtained.

A further aspect for consideration, which has already been discussed in Chapter 5, is the likelihood that a possible scaffoldin protein exists in the MEC in *B. licheniformis* SVD1. The largest protein species observed in the MEC was 79 kDa. Although scaffoldin proteins have been found in cellulosomes in *A. cellulolyticus* and in *R. flavefaciens* as small as 89 kDa (ScaD) (Xu *et al.*, 2003; Xu *et al.*, 2004) and 90 kDa respectively (Rincon *et al.*, 2003), the scaffoldin proteins in other cellulosomes have ranged from 120 kDa in *C. josui* (Kakiuchi *et al.*, 1998) to 196 kDa in *C. thermocellum* (Bayer *et al.*, 1998). With respect to *A. cellulolyticus* and *R. flavefaciens* it should be noted that they have multiple scaffoldin proteins which are able to combine into a superstructure, whereas cellulosomes with larger molecular weight scaffoldin proteins generally only have one scaffoldin. However, it appears as though the protein species present in the MEC in *B. licheniformis* SVD1 are most likely too small to constitute a scaffoldin protein.

If it is concluded that the MEC in *B. licheniformis* SVD1 is not a cellulosome, there are two possible alternative explanations for this phenomenon. Firstly, it is possible that the MEC has a different structural basis to the cellulosome. The second alternative is that the MEC is simply a random aggregation of proteins. It was found that, although the protein species in the MEC from different purifications displayed small differences, the majority of proteins were consistently present. Furthermore, zymograms were repeatedly conducted with different batches of purified MEC and results were always consistent, indicating a composition that is non-random. Thus it appears that the MEC is not simply a random aggregation of proteins but a functional complex.

7.6 Conclusions

All available evidence was examined to determine whether the MEC in *B. licheniformis* SVD1 was a putative cellulosome. TEM demonstrated that cell surface structures were present in *B. licheniformis* SVD1 cultured on cellobiose and birchwood xylan. Furthermore, it was established in previous chapters that a large complex of 2,000 kDa was isolated based on size exclusion chromatography.

However, the MEC had no activity on crystalline cellulose (“true” Avicelase activity) and was unable to bind to crystalline cellulose. Thus it did not appear to possess a CBM capable

of binding to cellulose. Furthermore, the largest protein species present in the MEC appeared too small to encode for a scaffoldin.

Thus the evidence examined appears to indicate that *B. licheniformis* SVD1 does not code for a cellulosomal complex. However, it is argued that there may be a different structural basis for the formation of the complex as it does not appear to be simply a random aggregation of components. It is conceivable that genetic diversity in microorganisms and evolutionary pressures could have resulted in formation of other types of MECs with a different structural basis to the cellulosome. Further investigation would have to take place to identify the components of the MEC in *B. licheniformis* SVD1 and determine the structural basis for its formation.

CHAPTER 8 – GENERAL CONCLUSIONS AND FUTURE WORK

This study involved the purification and characterisation of a cellulolytic and hemi-cellulolytic MEC in *B. licheniformis* SVD1. The characterisation of the MEC was done against the background of the total cellulolytic and hemi-cellulolytic system found within this organism.

The ability of microorganisms to degrade complex lignocellulose substrates has great importance and significance for second generation biofuel technologies, the basis of which is the conversion of these substrates into fermentable sugars. As lignocellulose is structurally and chemically complex, a whole range of enzymes is required for its saccharification into monomer sugars. These enzymes include cellulases (exo-glucanases, endo-glucanases and β-glucosidases), xylanases (endo-xylanases, β-xylosidases, α-L-arabinofuranosidases, endo-1,5-α-L-arabinases, acetyl xylan esterases, ferulic acid esterases, α-glucuronidases) , mannanases (β-mannanases, β-mannosidases, α-galactosidases, acetyl-mannan esterases, β-glucosidases) and pectinases (pectin methyl esterases, polygalacturonases, pectate lyases, pectin lyases).

Microorganisms that produce these enzymes generally do so in two ways, either as free extracellular enzymes or in the form of a MEC. The extracellular cellulase system of an organism such as *Trichoderma reesei*, which produces free enzymes, has been well studied (Lynd *et al.*, 2002). However, greater interest has been shown over the last two decades in the cellulase system of organisms such as *C. thermocellum*, which produces a MEC termed a cellulosome. The interest in the cellulosome stems from the superior efficiency it appears to display in degradation of recalcitrant substrates. However, other types of multi-enzyme complexes have also been isolated from aerobic organisms such as *Bacillus circulans*, *Bacillus megaterium* and *Paenibacillus curdlanolyticus* (Beukes & Pletschke, 2006, Kim & Kim, 1993, Pason *et al.*, 2006). It has not been established whether these complexes are cellulosomes or if they have a different structural basis. There is great interest in MECs from organisms such as bacilli as these bacteria are easy to cultivate, generally non-pathogenic and easy to manipulate genetically (Arbige *et al.*, 1993; Schallmey *et al.*, 2004). *B. licheniformis*, specifically, is an important industrial bacterium and is reported to contain many cellulolytic

and hemicellulolytic enzymes, although, prior to this study, there were no reports in the literature of the presence of a cellulolytic or hemicellulolytic MEC in this organism.

The crude fraction as well as the MEC in this study showed activity on a variety of substrates, indicating the presence of numerous enzymes in *B. licheniformis* SVD1 which could make it suitable for degradation of lignocellulose substrates. When the organism was cultured on birchwood xylan, the data from enzyme assays, combined with zymogram analysis, indicated that there were three endoglucanases, seven xylanases, three mannanases and two pectinases in the crude fraction, while the MEC had two endoglucanases, seven xylanases, two mannanases and one pectinase. The pectinases in the crude fraction could be identified as a pectin methyl esterase and a lyase, but the methyl esterase was absent in the MEC. The activity on cellulose, both crystalline (Avicel) and amorphous (CMC), was however quite low. Based on its enzyme activity profile, *B. licheniformis* SVD1 appears to be more suitable for the degradation of hemi-cellulose substrates.

Several of these enzymes have previously been identified in various strains of *B. licheniformi*s (See Table 1.2). However, it is apparent that many differences exist between different strains of *B. licheniformis*. Rey *et al.* (2004) reported on the completed genome sequence of *B. licheniformis* ATCC 14580 and indicated that this strain had two putative endoglucanases belonging to glycoside hydrolase families GH9 and GH5, a probable cellulose-1,4-β-cellobiosidase of family GH48 and two genes for β-glucosidases. While the zymogram data from this study on strain SVD1 indicated the presence of three enzymes active on CMC, activity on this substrate in enzyme assays was found to be low. Growth on CMC was also found to be poor (data not shown).

With respect to cellulases, Veith *et al.* (2004) reported on the genome sequence of *B. licheniformis* DSM 13. This strain, similarly, also contains three endo-glucanases, a cellulose 1,4-β-cellobiosidase and some glucosidases, two α-glucosidases and one β-glucosidase (Veith *et al.*, 2004). Numerous other reports exist in literature for the isolation of endo-glucanases from various strains of *B. licheniformis* (see Table 2 in section 1.4 for a summary). Liu *et al.* (2004) even reported that a *B. licheniformis* strain GXN 151 was able to degrade Avicel efficiently. This directly contradicts the results found in this study and thus it appears as though large differences exist between various strains of this organism. In fact, when

conducting TLC with the crude fraction from *B. licheniformis* SVD1 and using Avicel as a substrate, no cellobiose or glucose was found to be released, indicating that true Avicelase activity was not present.

Only one xylanase was reported to be present in the genome of *B. licheniformis* DSM 13 or ATCC 14580 although many genes appear to be present in these genomes for the degradation of arabinan substrates (arabinan endo-1,5-L-arabinase, arabinan endo-1,5- α -L-arabinosidase) and for cleavage of arabinose from substrates (α-L-arabinofuranosidase) (Rey *et al.*, 2004; Veith *et al.*, 2004). There are many other reports in literature of endo-xylanases isolated from various strains of *B. licheniformis* (see Table 1.2 of section 1.4). When compared to the results in this study, there appears to be up to seven xylanases present in *B. licheniformis* SVD1 while the presence of α-L-arabinofuranosidase was predicted from the activity on oatspelt xylan. No reports in literature for *B. licheniformis* seem to indicate the presence of such a large number of xylanases in any other strain.

Our data indicates the presence of three different mannanases in *B. licheniformis* SVD1. However, reports in literature suggest only one mannosidase in the genome of *B. licheniformis* DSM 13 or ATCC 14580 although Rey *et al.* (2004) reported the presence of a β-mannanase (Rey *et al.*, 2004; Veith *et al.*, 2004) (See Section 1.2.6). Other reports in literature indicate the presence of β-mannanases in *B. licheniformis* although these reports do not indicate whether more than one mannanase could be present (see Table 1.2).

B. licheniformis SVD1 appeared to possess a pectin methyl esterase as well as a pectin or pectate lyase from zymogram data. Veith *et al.* (2004) reported on the presence of four pectate lyase genes in *B. licheniformis* DSM 13, as well as a gene encoding for a pectin methyl esterase. Other reports in literature all indicate the presence of pectate lyase in other strains of *B. licheniformis* but no reports of a pectin lyase are available (see Table 1.2 of section 1.4).

By comparing the composition of the MEC in *B. licheniformis* SVD1 with the enzyme composition of various cellulosomes, it is apparent that the predominant enzymes that have been identified in cellulosomes are generally cellulases (exo- and endo-glucanases). The cellulosome in *C. cellulovorans* has 8 cellulases (Doi *et al.*, 2003), the cellulosome in *C.*

cellulolyticum has 10 cellulases (Desvaux, 2005) and the cellulosome in *C. thermocellum* has 14 cellulases (Schwarz, 2001). Xylanases only occur in some cellulosomes, with the cellulosome in *C. thermocellum* possessing 5 xylanases (Schwarz, 2001) and the cellulosome in *C. cellulovorans* 1 xylanase (Doi *et al.*, 2003). In many cellulosome no xylanases have been identified. Mannnases appear to occur more frequently in cellulosomes with 1 mannanase having been identified in the cellulosome of *C. cellulovorans*, *C. acetobutylicum* and *C. cellulolyticum* (Doi *et al.*, 2003), as well as in *C. thermocellum* (Schwarz, 2001). Pectinases have not been readily identified in cellulosomes and a pectinase is only reported for the cellulosome of *C. cellulovorans* (a pectate lyase – Doi *et al.*, 2003) and *C. cellulolyticum* (a rhamnogalacturonase – Desvaux, 2005).

It is apparent that *B. licheniformis* SVD1, with high activity on hemi-cellulolytic substrates, probably occupies a niche in the environment for the utilisation of hemi-cellulose substrates. This may appear to make it unsuitable for utilisation in biotechnological applications for biofuel production which generally uses glucose from cellulose for fermentation into bioethanol as *Saccharomyces cerevisiae* is only able to utilise glucose (Himmel *et al.*, 2007). However, research is ongoing to use all the sugars from degradation of lignocellulose for conversion into ethanol which would make the process more efficient. One method is the genetic modification of *S. cerevisiae* to be able to utilise pentose sugars such as xylose, mannose and galactose for fermentation (Becker & Boles, 2003; Ho *et al.*, 1999; Jeffries, 2006; Katahira *et al.*, 2004; Kuyper *et al.*, 2005). An alternative approach is the utilisation of Gram-negative bacteria such as *Escherichia coli*, *Klebsiella oxytoca* and *Zymomonas mobilis* (Dien *et al.*, 2003; Fortman *et al.*, 2008). *E. coli* and *K. oxytoca* are able to use a variety of sugars and research has focused on engineering these organisms to produce ethanol. *Z. mobilis* is able to produce ethanol, but only from glucose or fructose and research has focused on engineering this organism to utilise arabinose and xylose as well (see reviews in Dien *et al.*, 2003; Fortman *et al.*, 2008). Thus it is clear that organisms with mainly hemi-cellulolytic activity may also have a role to play in biotechnological applications. In addition, several advantages exist for using bacilli in biotechnological applications as they are generally simple to cultivate and relatively easy to manipulate genetically (Arbige *et al.*, 1993). *Bacillus subtilis*, for example, was successfully used for the expression and production of mini-cellulosomes (Arai *et al.*, 2007; Cho *et al.*, 2004; Murashima *et al.*, 2002). In the same

manner it could be possible to use *B. licheniformis* SVD1 to express efficient cellulases to complement its existing enzyme system.

The cellulosome has become the paradigm of cellulolytic and hemi-cellulolytic MECs and it is inevitable that, upon isolation of a MEC from an organism, that such MEC must be compared to a cellulosome and an investigation conducted as to whether this MEC could be a putative cellulosome. The production of a large complex with cellulolytic and hemi-cellulolytic activity is the first piece of evidence to be considered. The MEC purified in this study from *B. licheniformis* SVD1 was approximately 2,000 kDa in size. The molecular weight of MECs based on size exclusion chromatography has been reported as ranging from 650 kDa to 2,500 kDa in cellulosomes (Doi *et al.*, 2003) and 400 kDa and 1450 kDa in the xylanolytic MEC in *P. curdlanolyticus* (Pason *et al.*, 2006). By comparison, the MEC in *B. licheniformis* SVD1 is therefore quite large and is similar in size to the cellulosome in *C. thermocellum*.

With respect to its apparent enzyme composition it appears as though the MEC from *B. licheniformis* SVD1 differs from cellulosomes. This MEC has predominantly xylanase activity and the presence of 7 xylanase active bands was shown through zymogram data. This MEC furthermore does not appear to possess true activity on crystalline cellulose which has been considered a feature of cellulosomes, although not a defining characteristic (Bayer *et al.*, 2004). It was furthermore demonstrated that the MEC in *B. licheniformis* SVD1 was unable to bind to crystalline cellulose and therefore does not appear to possess a CBM3a domain which is found on the scaffoldin protein in cellulosomes. Although binding to crystalline cellulose has been characteristic of cellulosome, this evidence cannot be viewed in isolation as “the molecular basis for binding may not be strictly associated with the cellulosome components” (Bayer *et al.*, 1998). Preliminary TEM analysis established the presence of cell surface structures on cells of *B. licheniformis* SVD1 with some similarities to cell protuberances in *C. thermocellum.* Such cell protuberances have been considered indicative of cellulosome production (Schwarz, 2001). However, unless these cell surface structures can be identified as the MEC through, for example, utilisation of antibodies against the MEC, no final conclusions can be made as to the nature of these cell surface structures.

The best method by which the presence of a cellulosome can be established in an organism is through the identification of cohesin and dockerin domains (Bayer *et al.*, 2004). The genome sequence of *B. licheniformis* SVD1 was not available and therefore the genome sequence of strain DSM 13/ ATCC 14580 was utilised for bioinformatic analysis to determine the presence of cohesin or dockerin sequences in this organism. No homology was found for these sequences, indicating that strain DSM 13 and ATCC 14580 do not encode for a cellulosome. This does not exclude the possibility that strain SVD1 could encode for a cellulosome, but this can only be investigated once a genome sequence for this strain is available. With the evidence available at present, the MEC in this organism appears to be a functional complex, but should perhaps rather be termed a xylanosome than a cellulosome.

B. licheniformis SVD1 was unable to utilise xylose sugars, but was able to utilise glucose and mannose; and, to a lesser extent, cellobiose, arabinose and galactose. The inability of *B. licheniformis* SVD1 to utilise xylose although it produced high levels of xylanases, and most likely a β-xylosidase based on TLC results, was a peculiar result which may be caused by a mutation in this strain. This is hypothesised based on reports in literature that *B. licheniformis* has a *xyl* regulon encoding for xylose utilisation (Scheler *et al.*, 1991) and the genome sequences for *B. licheniformis* strains DSM 13 and ATCC 14580 both contain a gene for xylose isomerise, the key enzyme required for xylose utilisation (Rey *et al.*, 2004; Veith *et al.*, 2004). This characteristic may have some practical application in biotechnological processes such as bioethanol production. Utilisation of sugars by the organism itself would reduce the amount of sugars available for bioethanol production and by choosing an organism with these characteristics, higher levels of sugars could be available for fermentation into bioethanol.

This study was furthermore able to show that *B. licheniformis* SVD1 was able to regulate expression of various enzymes when the organism was cultured on different growth substrates. While the predominant activity remained hemi-cellulolytic, activity on crystalline and amorphous cellulose could be increased on certain growth substrates. The ability of an organism to regulate its enzyme profile based on the growth substrate is an important feature as the composition of substrates can be complex and varied. By adjusting the enzyme composition, it is postulated that an organism may be able to regulate the ratio of enzymes in order to achieve optimal synergy for the efficient degradation of substrates. The work done

in this study in this regard is only a preliminary investigation and further work could be carried out using a variety of substrates.

Understanding the physical, chemical and kinetic characteristics of complexed xylanase activity will assist in creating the conditions for optimal performance and productivity. Should *B. licheniformis* SVD1 or the MEC itself be utilised in future for biotechnological applications, these parameters would greatly aid in optimisation of conditions. For bioethanol production, specifically, this study has demonstrated that complexed xylanase activity displays great pH stability, lack of product inhibition and ethanol tolerance which are all beneficial characteristics in such a process.

The knowledge gained through this work could form the basis of further study of the cellulolytic and hemi-cellulolytic system of *B. licheniformis* SVD1, with specific reference to the presence of an MEC within this organism. Some of the proposed future work could include the sequencing of the genome or shotgun cloning of specific genes coding for various enzymes could be undertaken. Together with the biochemical data from enzyme assays and zymograms, this will allow identification of enzymes present within this organism and will allow characterisation of these individual enzymes without the possible influence of other enzymes present in the system or MEC. If the coding sequences of individual enzymes are known, their presence within the MEC could be confirmed using antibodies against such enzymes. An analysis of coding sequences could furthermore provide information of the possible structural basis for the formation of the MEC.

Only some of the possible cellulolytic and hemi-cellulolytic enzymes produced by *B. licheniformis* SVD1 were measured. As such a system is likely very complex, future work could include the investigation of other enzymes that may be present within the cellulolytic system of this organism such as glucuronidases, galactosidases and arabinofuranosidases. Sequencing of the genome or cloning of genes for various enzymes would assist in the accurate identification of various enzymes present in this strain. Utilisation of antibodies to detect and identify protein species from electrophoresed gels could provide a more accurate determination of the relative expression levels of proteins and will be able to detect proteins at very low levels.

In order to more accurately determine whether constitutive expression of enzymes is taking place, *B. licheniformis* SVD1 could be cultured in the absence of a carbohydrate substrate. It would also be useful to culture the organism on a monomeric sugar such as glucose and xylose to determine the effect of this on the expression of enzymes and whether catabolite repression takes place under certain circumstances.

The availability of gene sequences will also allow more accurate determination of enzyme levels by monitoring mRNA levels using a technique such as qRT-PCR. At the time when this study was performed, such information was not available and therefore biochemical methods were the only possible means to look at enzyme expression and induction.

Future work could also include the production of antibodies to the MEC itself which could be used for labelling purposes to determine whether the cell surface protrusions can be attributed to the MEC.

Under certain conditions, colonies of *B. licheniformis* had a mucoid appearance and appeared to form an exopolysaccharide layer around cells. Preliminary observations indicated the presence of cellulolytic and hemicellulolytic activity within this layer. Future work could therefore also involve an investigation of the exopolysaccharide layer and the conditions under which it was formed, as well as the role it plays in the degradation of substrates.

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APPENDICES

Appendix 1: **16S rDNA sequence**

Appendix 2: **Blast Results**

Appendix 3: **Reagents list**

Appendix 4a: **Protein standard curves**

Appendix 4b: **Xylose standard curve**

Appendix 5: **Discontinuous denaturing gel electrophoresis (SDS-PAGE)**

Appendix 6a: **Copy of webpage http://www2.muw.edu/~lbrandon/Micro/endospore.doc with endospore stain protocol, date of access 31/08/2009**

Appendix 6b: **Copy of webpage http://www.engineeringnews.co.za/article/maize-exclusion-039defies-logic039-rethink-possible-2008-01-18, date of access 31/08/2009**

APPENDIX 1 - 16S rDNA SEQUENCE

16S rDNA sequence for *B. licheniformis* SVD1 as lodged with Genbank as Accession number EU 770587.

agcggacagatgggagcttgctccctgatgtyagcggcggacgggtgagtaacacgtgggtaacctgcctgtaagactgggataact
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aatcccacaaatctgttctcagttcggatcgcagtctgcaactcgactgcgtgaagctggaatcgctagtaatcgcggatcagcatgcc
gcggtgaatacgttcccgggccttgtacacaccgcccgtcacaccacgagagtttgtaacacccgaagtcggtgaggtaaccttttgg
agccagccgccgaaggtgggacagatgattg

APPENDIX 2 – BLAST RESULTS

Table A.1 Results of BLAST search on the website http://www.ncbi.nlm.nih.gov/blast for the sequencing results from the 16S rDNA (See section 2.3.3)

Accession no.	Description	Max score	Total score	Query coverage	E value	Max identity
AB363734.1	Bacillus licheniformis gene for 16S rRNA, partial sequence, strain: NBRC 12202	2623	2623	100%	0.0	99%
AB354236.1	Bacillus licheniformis gene for 16S rRNA, partial sequence, strain: NBRC 12107	2623	2623	100%	0.0	99%
EF471917.1	Bacillus sp. J24 16S ribosomal RNA gene, partial sequence	2623	2623	100%	0.0	99%
EF423608.1	Bacillus licheniformis strain BCRC 12826 16S ribosomal RNA gene, partial sequence	2623	2623	100%	0.0	99%
EF059752.1	Bacillus licheniformis 16S ribosomal RNA gene, partial sequence	2623	2623	100%	0.0	99%
DQ993676.1	Bacillus licheniformis strain BCRC 15413 16S ribosomal RNA gene, partial sequence	2623	2623	100%	0.0	99%
AY842871.1	Bacillus licheniformis strain CICC10181 16S ribosomal RNA gene, partial sequence	2623	2623	100%	0.0	99%
DQ351932.1	Bacillus licheniformis strain K19 16S ribosomal RNA gene, partial sequence	2623	2623	100%	0.0	99%
DQ082997.1	Bacillus licheniformis strain CICC 10104 16S ribosomal RNA gene, partial sequence	2623	2623	100%	0.0	99%
AF372616.1	Bacillus licheniformis strain Mo1 16S ribosomal RNA gene, partial sequence	2623	2623	100%	0.0	99%
DQ228696.1	Bacillus licheniformis strain ACO1 16S ribosomal RNA gene, partial sequence	2623	2623	100%	0.0	99%

APPENDIX 3 - REAGENTS LIST

Table A.2. Reagents and chemicals and the supplier used

Name of reagent	Supplier and catalog number
1,4-β-D-Xylobiose	Megazyme
1,4-β-D-Xylotriose	Megazyme
2-mercaptoethanol	Fluka (Cat. No. 63700)
3,5-Dinitrosalicylic acid	Sigma (Cat No. D0550)
Acetone	Merck (8.22251.2500)
Acrylamide	Sigma (Cat No. A8887)
Agarose	Sigma (A9539)
Ammonium persulphate	Sigma Aldrich (Cat no A3678)
Avicel PH-101	Fluka (11365)
Bacteriological agar	Biolab (Cat. No. BX1)
Birchwood xylan	Fluka (Cat. No. 95588)
Bovine serum albumin (BSA)	Sigma (A7906)
Bradford reagent	Sigma (Cat. No. B6916)
Bromophenol blue	Sigma (Cat. No. B8026)
Carboxymethyl cellulose	Calbiochem (Cat. No. 217277)
Congo Red	Sigma (Cat no. C6767)
Coomassie Brilliant Blue R250	Merck (Cat. No. 1.12553)
D-(+)-Cellobiose	Sigma-Aldrich (Cat no. C7252)
D-(+)-Xylose	Sigma (X-3877)
Dextran Blue from *Leuconostoc* ssp.	Fluka (Cat no. 31393)
Di-potassium hydrogen phoshate	Merck (1.05104.1000)
Ethanol	Merck (Cat. No. 8.18700)
Glacial acetic acid	Merck (Cat No. 1.00063)
Glycerol	Saarchem (Cat. No.2676520)
Glycine	Merck (Cat. No. 1.04169)
HPTLC plates (Silica Gel 60 F254)	Merck, Darmstadt

KAPA HiFi PCR kit	KAPA Biosystems
L-(+)-arabinose	Sigma (Cat no. A-3256)
Locust bean gum	Fluka (Cat. No. 62631)
Magnesium sulphate (anhydrous)	Saarchem (412 39 20 EM)
Mannose	Sigma (Cat. No. M2069)
Methanol	Merck (Cat. No. 8.22283)
N,N-methylenebisacrylamide	Sigma (Cat. No.M7279)
Nutrient agar	Biolab, Merck (C1)
Nutrient Broth	Biolab, Merck (C24)
Oatspelt xylan	Fluka (95590)
Orcinol monohydrate	Sigma-Aldrich (Cat no. O1875)
PageBlue™ Protein Staining Solution	Fermentas (Cat no. R0571)
PageSilver™ Silver staining kit	Fermentas (Cat no. K0681)
PBGC filters (10kDa cut-off)	Millipore
Pectin (Apple)	Sigma (P8471)
Peptone	Fluka (70169)
PeqGold protein marker II	peqLab (Cat. No. 27-2010)
Phenol	Sigma (Cat.No. P3653)
Polyethylene glycol (PEG) 20,000	Merck (Cat. No. 8.18897)
Polygalacturonic acid (PGA)	Sigma (P3850)
Ponceau S	Fluka (81460)
Potassium hydrogen phosphate	Merck (1.04877.1000)
Primers	Inqaba Biotech
Sepharose CL-4B	Sigma (CL-4B-200)
Sodium azide	Merck (Cat. No. 8.22335)
Sodium chloride	Saarchem (Cat. No. 5822320)
Sodium deoxycholate monohydrate	Aldrich (Cat no. 238392)
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)

Sulfuric acid	AECI
Toyopearl DEAE-650M	Tosoh Corporation
Trichloroacetic acid	Merck (1.00807.0250)
Tris (hydroxymethyl) aminomethane	Merck (Cat. No. 1.08382)
Triton X-100	Merck (1.08603.1000)
Yeast extract	Biolab (Cat. No. BX6)

APPENDIX 4a – Protein standard curves

Protein standard curves were generated using a modified Bradford protein assay (Bradford, 1976). Bovine serum albumin (BSA) was used as a standard with commercial Bradford's reagent (Sigma). Various concentrations of BSA were prepared ranging from 0.00625 to 1.1 mg/ml. Three different standard curves were prepared using different volumes of sample, namely 5 µl (Figure A.1), 10 µl (Figure A.2) and 25 µl (Figure A.3). These volumes were used with Bradford's reagent in the following ratios: 5:250, 10:250 and 25:230 µl. Each standard curve could accurately measure the protein concentration in a sample over a different linear range with the large sample volume of 25 µl allowing sensitivity and accuracy as low as 0.00625 mg/ml of protein. Readings were taken according to the application with 25 µl sample volumes generally used for measuring protein in fractions from chromatography applications. Where readings were found to be outside the linear range, further measurements were done using smaller samples to accurately determine protein concentration.

The protein sample was mixed with the Bradford's reagent and allowed to stand at room temperature for 5 min before readings were taken. Absorbance readings were taken at 595 nm on a Powerwave X microplate reader from Bio-Tek Instruments using KC Junior software. Samples were gently shaken on the microplate reader for 30 seconds prior to absorbance readings being taken. Standard curves were generated in Microsoft Excel®.

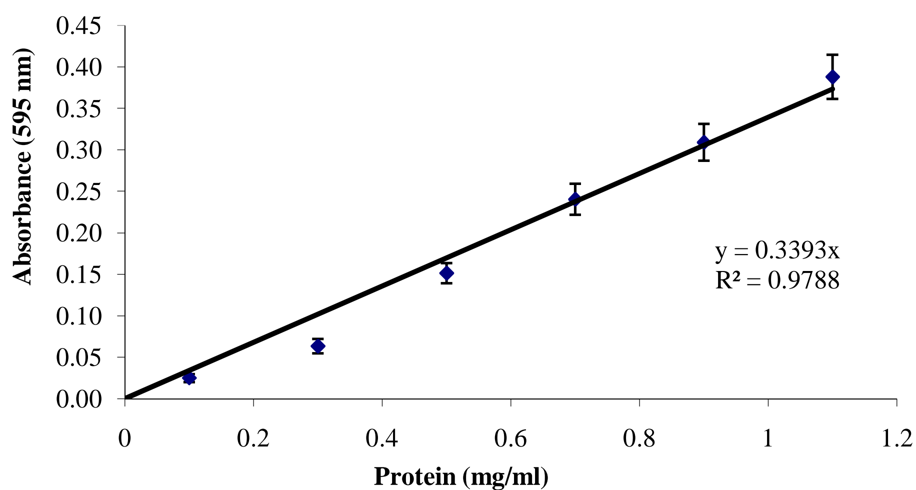

Figure A.1. Protein standard curve using 5 µl of sample and 250 µl of Bradford's reagent. Values are shown as the average ± SD (n=3).

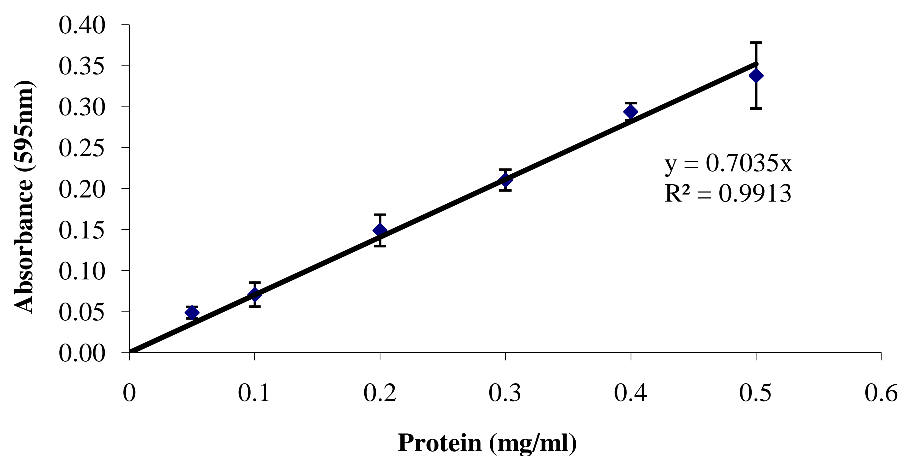

Figure A.2. Protein standard curve using 10 µl of sample and 250 µl of Bradford's reagent. Values are shown as the average ± SD (n=3).

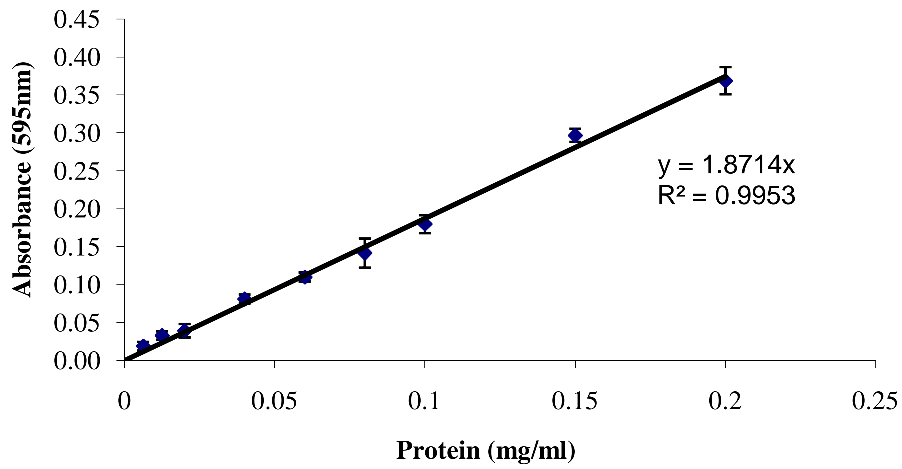

Figure A.3. Standard curve using 25 μl of sample and 230 μl of Bradford's reagent. Values are shown as the average ± SD (n=3).

APPENDIX 4b – XYLOSE STANDARD CURVE

Enzyme activity was measured by the reducing sugars formed in a modified dinitrosalicylic acid (DNS) method (Miller, 1959) using xylose as the standard.

The composition of DNS reagent was as follows:

2 g sodium hydroxide
2 g 3,5 dinitrosalicylic acid (DNS)
40 g potassium sodium tartrate (Rochelle salts)
0.4 g phenol
0.1 g sodium metabisulfite
200 ml distilled water
The sodium hydroxide was dissolved in 100 ml of distilled water before the DNS was added. Once the DNS was dissolved, the other compounds were added and the reagent made up to 200 ml.

A xylose standard curve was generated with concentrations of xylose between 0 and 6.0 µmol/ml (Figure A.4). A volume of each concentration of the xylose standard (150 µl) was added to 300 µl of DNS reagent. The mixture was then heated at 100°C for 5 min, cooled on ice for 5 min and readings taken at 540 nm on a Powerwave X microplate reader from Bio-Tek Instruments using KC Junior software. The standard curve was generated in Microsoft Excel®.

It is apparent from the standard curve that the assay does not display a linear response at low xylose concentrations. However, the standard curve was drawn through zero in order to prevent false positive readings at very low concentrations.

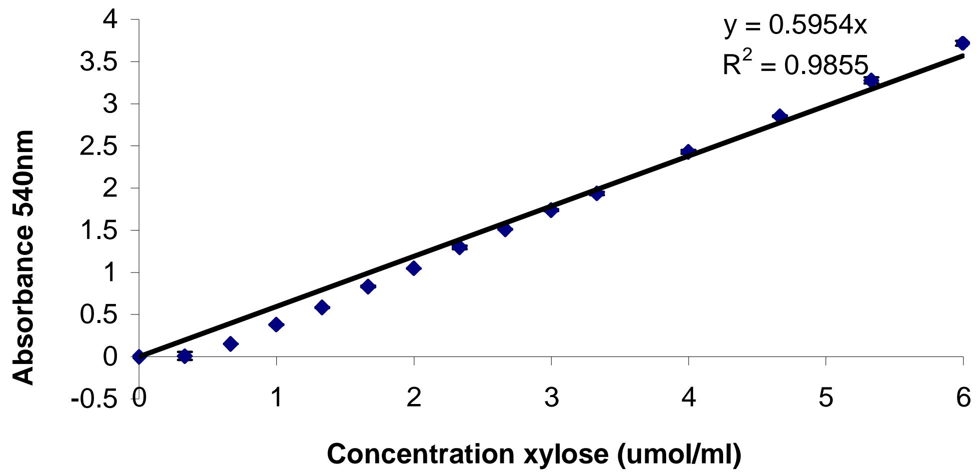

Figure A.4. Xylose standard curve. Values are shown as the average ± SD (n=3).

APPENDIX 5 – DISCONTINUOUS DENATURING GEL ELECTROPHORESIS (SDS-PAGE)

Analysis of proteins were done through sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using the method as developed by Laemmli (1970) and modified according to the BioRad Mini-Protean® 3 Cell instruction manual. All solutions for SDS-PAGE was made up as follows:

1. *10 x SDS-PAGE running buffer*

   30.3 g Tris (Hydroxymethyl) Aminomethane

   144.0 g Glycine

   10.0 g Sodium dodecyl sulphate (SDS)

   These compounds were dissolved in 1 L distilled water and stored at room temperature until required. When required for electrophoresis, the running buffer was diluted 10 times with distilled water to obtain the required volume.

2. *10% SDS stock solution*

   10 g SDS was dissolved in 100 ml distilled water.

3. *10% Ammonium persulphate (APS) solution*

   0.1 g APS was dissolved in 1 ml distilled water

   This solution was prepared freshly prior to preparing a gel.

4. *30% acrylamide stock solution*

   29.8 g acrylamide

   0.2 g bis-acrylamide

   These compounds were dissolved in distilled water and then made up to 100 ml volume. The bottle was wrapped in aluminium foil to protect it against light and stored at 4°C.

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

   6 g Tris (Hydroxymethyl) Aminomethane

   This was dissolved in distilled water and the pH adjusted with hydrochloric acid (HCl) to a pH of 6.8 before the solution was made up to 100 ml. This buffer was stored at 4^oC.

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

   18.15 g Tris (Hydroxymethyl) Aminomethane

   This was dissolved in distilled water and the pH adjusted with hydrochloric acid (HCl) to a pH of 8.8 before the solution was made up to 100 ml. This buffer was stored at 4^oC.

7. *SDS sample buffer (5x)*

   2.5 ml distilled water

   1 ml 0.5 M Tris-HCl buffer (pH 6.8)

   3 ml glycerol

   2 ml 10% SDS stock solution (w/v)

   1 ml 1% bromophenol blue (w/v)

   The sample buffer was stored at room temperature. Prior to preparing samples for electrophoresis, 5 µl β-mercaptoethanol was added to 95 µl of SDS sample buffer. SDS sample buffer (3 µl) was added to 15 µl of protein samples before boiling and electrophoresis.

8. *Coomassie Brilliant Blue protein staining solution*

   The staining solution was prepared by dissolving 0.075% (w/v) Coomassie Brilliant Blue R250 in a solution of 40% methanol and 0.7% glacial acetic acid. Staining of polyacrylamide gels was done for a minimum of 1 h or overnight on a platform shaker.

9. *Coomassie destain solution*

   Destain solution was prepared by mixing 45% methanol, 45% distilled water and 10% glacial acetic acid. Gels were placed in destain solution on a platform shaker until protein bands appeared against a clear background. Gels were then placed in distilled water.

10. Gel drying solution

7% glycerol

10% ethanol

The solution was made up to 1 l with distilled water and stored at room temperature. After destaining, gels were placed in drying solution for a minimum of 30 min before gels were dried.

11. Preparation of SDS-PAGE gels

Resolving gel

The 10% SDS-PAGE resolving gels were prepared in a small beaker by adding the following solutions in sequence:

4.04 ml distilled water

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

3.3 ml 30% acrylamide stock solution

0.1 ml 10% SDS stock solution

0.1 ml 10% APS solution

0.05 ml TEMED

The solution was lightly mixed by swirling the beaker before pouring the gel. Gels were then covered with about 50-100 µl of isopropanol and allowed to set.

For zymograms, 0.5 ml of a 2% (w/v) stock solution of birchwood xylan, CMC or locust bean gum, or 1.5 ml of a 2% (w/v) pectin solution, was added to the resolving gel solution and the distilled water reduced by the same volume. In the case of birchwood xylan, CMC and pectin, the solution was added to the resolving gel solution prior to the APS solution. However, in the case of locust bean gum, the substrate was first dissolved in the correct volume of boiled distilled water. As the locust bean gum is highly viscous, this ensured a homogeneous solution of the substrate in the gel.

The 4% stacking gels were prepared in a small beaker by adding the following solutions in sequence:

6.1 ml distilled water

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

1.3 ml 30% acrylamide stock solution

0.1 ml 10% SDS stock solution

0.1 ml 10% APS solution

0.05 ml TEMED

The isopropanol was removed from the resolving gel with some filter paper. The stacking gel solution was poured on top of the resolving gel before plastic combs were inserted to form the wells.