Isolation of xylanolytic multi-enzyme complexes from *Bacillus subtilis* SJ01

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By

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ABSTRACT

Cellulose and hemicellulose account for a large portion of the world's plant biomass. In nature, these polysaccharides are intertwined forming complex materials that require multiple enzymes to degrade them. Multi-enzyme complexes (MECs) consist of a number of enzymes working in close proximity and synergistically to degrade complex substrates with higher efficiency than individual enzymes. The cellulosome is a cellulolytic MEC produced by anaerobic bacteria that has been studied extensively since its discovery in 1983. The aim of this study was to purify a cellulolytic and/or hemicellulolytic MEC from an aerobic bacterium of the *Bacillus* genus. Several bacterial isolates were identified using morphological characteristics and 16S rDNA sequencing, and screened for their ability to degrade cellulose and xylan using a MEC. The isolate that produced a high molecular weight protein fraction with the greatest ability to degrade Avicel®, carboxymethyl cellulose (CMC) and birchwood xylan was identified as *Bacillus subtilis* SJ01. An optimised growth medium, consisting of vitamins, trace elements, birchwood xylan (as the carbon source), and yeast and ammonium sulphate (as the nitrogen sources), increased the production of CMCase and xylanase enzymes from this bacterium. The removal of a competing bacterial strain from the culture and the inhibition of proteases also increased enzyme activities. A growth curve of *B. subtilis* SJ01 indicated that xylanase production was highest in early stationary growth phase and thus 84 hours was chosen as the best cell harvesting time. To purify the MECs produced by *B. subtilis* SJ01 size-exclusion chromatography on a Sephacryl S-400 column was used. It was concluded that (for the purposes of this study) the best method of concentrating the culture supernatant prior to loading onto Sephacryl S-400 was the use of ultrafiltration with a 50 kDa cut-off membrane. Two MECs, named C1 and C2 of 371 and 267 kDa, respectively, were purified from the culture supernatant of *B. subtilis* SJ01. Electrophoretic analysis revealed that these MECs consisted of 16 and 18 subunits, respectively, 4 of which degraded birchwood xylan and 5 of which degraded oat spelt xylan. The MECs degraded xylan substrates (C1: 0.24 U/mg, C2: 0.14 U/mg birchwood xylan) with higher efficiency than cellulose substrates (C1: 0.002 U/mg, C2: 0.01 U/mg CMC), and could therefore be considered xylanosomes. Interestingly, the MECs did not bind to insoluble birchwood xylan or Avicel® and did not contain glycosylated proteins, which are common features of cellulosomes. This study is, therefore, important in

revealing the presence of MECs that differ from the cellulosome and that may have particular application in industries requiring high xylanase activity, such as the paper and pulp industry. The abundant genetic information available on *B. subtilis* means that this organism could also be used for genetic engineering of cellulolytic/hemicellulolytic MECs.

DECLARATION

I declare that this thesis in my own, unaided work. It is being submitted for the degree of Master of Science (Biochemistry) at Rhodes University. It has not been submitted before for any degree or examination at any other university.

_____

Signed: Sarah Jones

On this _____ of _____ 20____

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LIST OF OUTPUTS

1. Anticipated Publication

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LIST OF ABBREVIATIONS

16S rDNA	16S ribosomal deoxyribonucleic acid
BLAST	Basic logic alignment search tool
BSA	Bovine serum albumin
CBD	Cellulose binding domain
CBM	Carbohydrate binding module
CMC	Carboxymethyl cellulose
DNS	3,5-Dinitrosalicylic acid
FPLC	Fast performance liquid chromatography
(hemi-)cellulolytic	Hemicellulolytic and/or cellulolytic
MEC	Multi-enzyme complex
OD	Optical density
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PEG 20 000	Polyethylene glycol 20 000
PMSF	Phenylmethanesulfonyl fluoride
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEC	Size exclusion chromatography

CHAPTER 1

Background and introduction to the present study

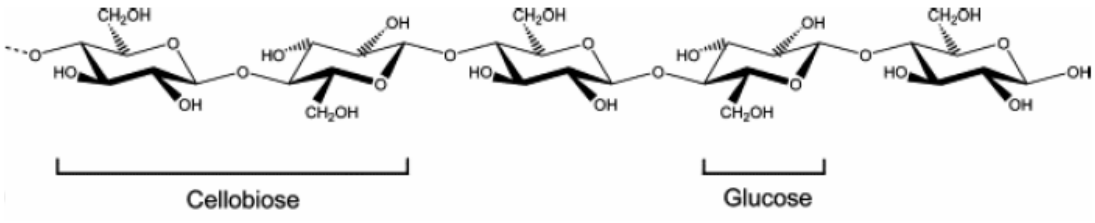
1.1 Lignocellulose

Cellulose is the most abundant material in nature and accounts for about 50% of the carbon on earth and about 40% of the world‘s biomass (Coughlan and Folan, 1979; Doi, 2008; O'Sullivan, 1997). It makes up most of the biomass in plants, where it is a structural polymer (Bayer *et al.,* 1998a; O'Sullivan, 1997), and is also found in algae, fungi and some bacteria (O'Sullivan, 1997). In plants, cellulose content ranges from 20% in grasses to 45% in wood (dry weight) and 90% in cotton fibre (Coughlan and Folan, 1979).

Research into the structure of cellulose is ongoing (Bayer *et al.,* 1998a; O'Sullivan, 1997), and has so far included extensive spectroscopy (particularly Raman and solid-state and ^{13}C-NMR), crystallography and transmission electron microscopy studies (Bayer *et al.,* 1998a; Larsson *et al.,* 1997). These studies have revealed that chains of cellulose consist of cellobiose units, which are two glucose subunits at 180° to each other, linked by β-D-1,4 glucosidic linkages (Figure 1.1a) (Beguin and Aubert, 1994; Coughlan and Folan, 1979; Gomez *et al.,* 2008; O'Sullivan, 1997). The length of these β-D-1,4 glucan chains varies depending on their source, for example 10 000 units per chain in wood cellulose and 15 000 in cotton cellulose (O'Sullivan, 1997).

Cellulose chains are packed parallel to each other in structures called microfibrils (Figure 1.1b). These are rigid and insoluble structures stabilised by intermolecular hydrogen bonds and hydrophobic interactions between the cellulose chains (Bayer *et al.,* 1998a; Beguin and Aubert, 1994; Coughlan and Folan, 1979; Gomez *et al.,* 2008). Microfibrils are composed of crystalline regions, consisting of chains packed in a highly ordered fashion, interspersed with amorphous, or less orderly, regions (Beguin and Aubert, 1994). The amount of crystalline and amorphous cellulose in a structure depends on its biological source (Beguin and Aubert, 1994; Larsson *et al.,* 1997). Microfibrils can be as small as 2 nm in the primary cell walls of plants (Bayer *et al.,* 1998a) and as wide as 20 nm in the algae *Valonia macrophysa* (Beguin and Aubert, 1994). Microfibrils can be arranged to form complex structures, for example in

secondary plant cell walls microfibrils lie next to each other forming sheets of differing orientation (Beguin and Aubert, 1994).



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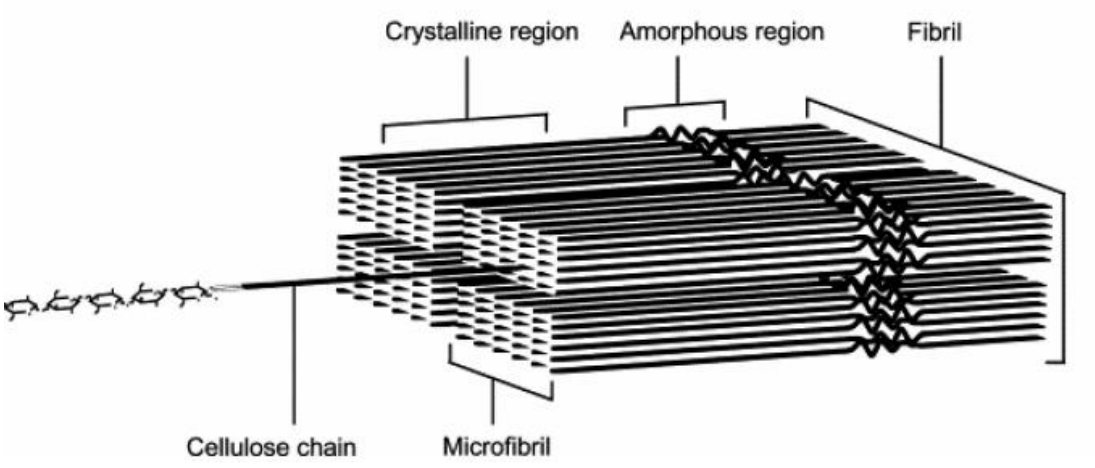


B

Figure 1.1 A: The primary structure of a chain of cellulose. **B:** A diagrammatic representation of the structure of the cellulose microfibril (Desvaux, 2005a).

Structural studies have also shown that cellulose can vary in conformation. Native cellulose is designated Cellulose I, and when treated with alkali it changes its conformation to Cellulose II, which has different intra-chain hydrogen bonding (Beguin and Aubert, 1994; Coughlan, 1992). Native cellulose (Cellulose I) comprises of varying combinations of Cellulose I_α and I_β, which differ in their intermolecular hydrogen bonding pattern (Beguin and Aubert, 1994; Coughlan, 1992).

In nature, cellulose microfibrils are intertwined with hemicellulose and lignin forming a highly complex material, called lignocellulose (Figure 1.2) (Beguin and Aubert, 1994; Coughlan and Folan, 1979; Houtman and Atalla, 1995). The inter-chain interactions of cellulose microfibrils and the complex nature of lignocellulose gives it the high tensile strength needed for plant cell walls to withstand high osmotic pressure and mechanical stress (Beguin and Aubert, 1994).

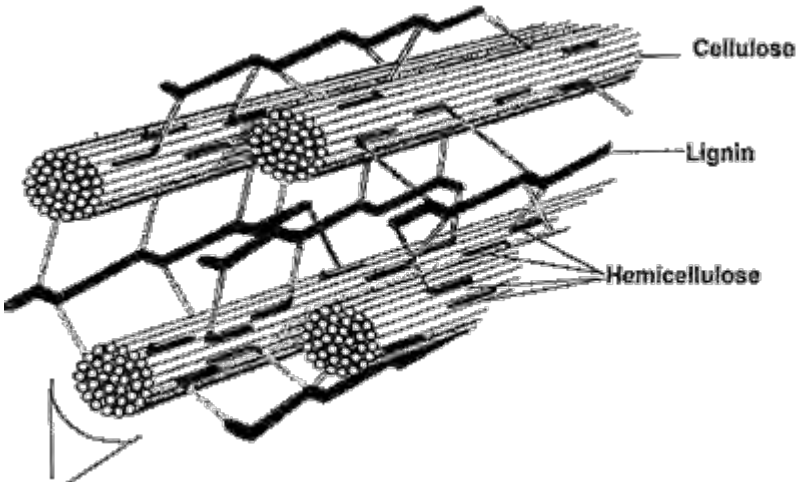


Figure 1.2 Simplified structure of lignocellulose (Felby, 2009).

Cellulose is the major component of lignocellulose, making up between 40 and 50% of lignocellulosic biomass (Beukes *et al.,* 2008; Foyle *et al.,* 2007; Gray *et al.,* 2006; Kaparaju *et al.,* 2009; Sá-Pereira *et al.,* 2002a; Tachaapaikoon *et al.,* 2006). Hemicellulose and lignin are present at approximately 25 to 35% and 15 to 25%, respectively, depending on the source (Beukes *et al.,* 2008; Foyle *et al.,* 2007; Gray *et al.,* 2006; Howard *et al.,* 2003; Sá-Pereira *et al.,* 2002a; Tachaapaikoon *et al.,* 2006). Therefore, after cellulose, hemicellulose is the next most abundant polymer. Hemicellulose is a polymer similar to cellulose, consisting of chains of sugar molecules. However, unlike cellulose, which consists only of glucose monomers, hemicellulose is heterogeneous, containing varying compositions of pentose sugars depending on the biological source (Biely, 1985; Foyle *et al.,* 2007; Gray *et al.,* 2006; Petersson *et al.,* 2007). Usually, a chain of D-xylose molecules (xylan) forms the backbone of hemicellulose with side chains containing mannose, arabinose, galactose, glucuronic acid and other sugars (Figure 1.3) (Beguin and Aubert, 1994; Gray *et al.,* 2006). Xylan is therefore the predominant component in hemicellulose. Common side chains include L-arabinofuranose linked to the *O*-3 position of D-xylose, D-glucuronic or 4-*O*-methyl-D-glucuronic acid linked to the *O*-2 position and acetyl groups at either the *O*-2 or *O*-3 position (Beguin and Aubert, 1994; Biely, 1985). Hemicellulose forms hydrogen bonds with cellulose covering the cellulose microfibrils with a coat (Figure 1.2) (Foyle *et al.,* 2007; Gomez *et al.,* 2008). The hemicelluloses link the microfibrils together and allow flexibility in the structure while still keeping the microfibrils anchored to each other (Cosgrove, 2005; Gomez *et*

al., 2008). The arabinofuranosyl side chains in hemicellulose, that have been esterified by ferulic and *p*-coumaric acid residues, form ether linkages with lignin (Beguin and Aubert, 1994; Gray *et al.,* 2006; Tabka *et al.,* 2006; Wackett, 2008).

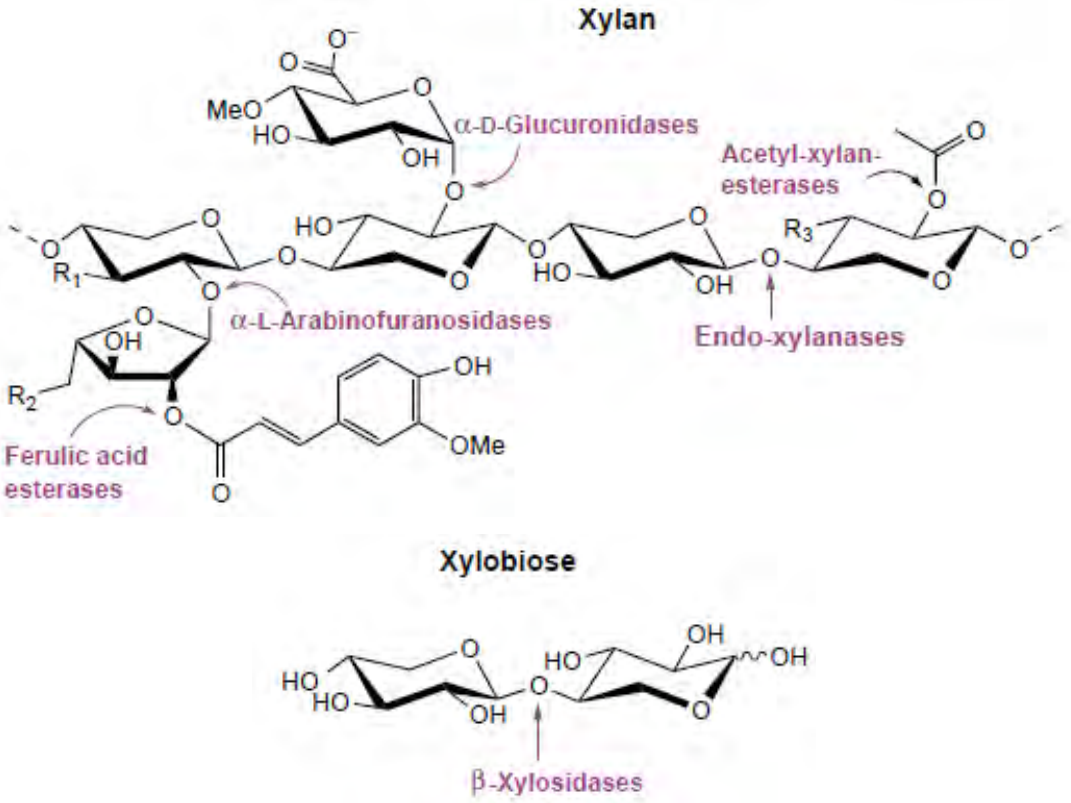


Figure 1.3 Model structure of xylan made of D-xylose units and various side chains illustrating the enzymes that degrade these structures (Biely, 1985; Shallom and Shoham, 2003).

Lignin acts as a binding material in lignocellulose, encasing the cellulose-hemicellulose complex, and making it difficult for enzymes to reach and degrade these polysaccharides (Foyle *et al.,* 2007; Gomez *et al.,* 2008). Lignin is a heterogeneous polyphenylpropane polymer (which accounts for its recalcitrant nature) and its composition depends on the source (Foyle *et al.,* 2007; Wackett, 2008). In general, lignin pre-cursors are the aromatic alcohols coniferyl, sinapyl and coumaryl (Foyle *et al.,* 2007; Houtman and Atalla, 1995; Howard *et al.,* 2003). Plant cells walls are lignified to make them waterproof and chemically durable for mechanical reinforcement and to enable the development of vascular systems for water transport in the plant (Gomez *et al.,* 2008). Treating lignocellulose with acids or ammonia depolymerises lignin, releasing cellulose and hemicellulose (Gomez *et al.,* 2008).

1.2 Lignocellulose degradation

Cellulose and hemicellulose can be degraded by chemical hydrolysis (using acids or alkalis) or enzymatic hydrolysis. Several methods of acid hydrolysis using sulphuric or hydrochloric acid at varying concentrations, temperatures and pressures have been used in industry for lignocellulose degradation (Gomez *et al.,* 2008; Madson and Tereck, 2004). Chemical hydrolysis is rapid and more complete but the use of enzymes is more efficient (i.e. fewer molecules of enzymes are required to degrade the same amount of substrate), requires milder conditions, is specific to particular bonds and does not produce toxic by-products (Coughlan and Folan, 1979; Ingesson *et al.,* 2001). At industrial or pilot scale, chemical hydrolysis causes corrosion in process tanks and fouling and polymerisation (Madson and Tereck, 2004). Enzymatic hydrolysis is therefore favoured although no system has been proven commercially viable without first removing the lignin by pre-treatment (Gomez *et al.,* 2008; Madson and Tereck, 2004).

The degradation of lignocellulose involves depolymerisation of the polysaccharide components into sugar monomers, and due to its complex structure this requires several different types of enzymes (Bayer *et al.,* 1998a). Enzymes known as cellulases are responsible for the breakdown of cellulose into glucose. This involves two steps: the conversion of cellulose into cellobiose by β-1,4-glucanase and cellobiose to glucose by β-glucosidase, as follows (Aliyu and Hepher, 2000):

$$\text{Cellulose} \xrightarrow{\beta\text{-(1,4)-glucanase}} \text{Cellobiose} \xrightarrow{\beta\text{-glucosidase}} \text{Glucose}$$

Glucanases are divided into endoglucanases and exoglucanases. Endoglucanases are responsible for the depolymerisation of amorphous cellulose (such as the commercial substrate carboxymethyl cellulose (CMC)) since they can attack β-glycosidic bonds randomly along the cellulose chain (Aliyu and Hepher, 2000). Crystalline cellulose (such as the commercial substrate Avicel®) is packed together via hydrogen bonds in such a way that water and enzymes can not reach the inner β-glycosidic bonds. Therefore, exoglucanases (or cellobiohydrolases) are required to break the outermost or terminal glycosidc bonds in crystalline cellulose (Aliyu and Hepher, 2000; Bayer *et*

al., 1998a; Teeri, 1997). Exoglucanases release cellobiose from the ends of cellulose chains, which is subsequently broken down into glucose by cellobiases (or β-glucosidases) (Aliyu and Hepher, 2000; Bayer *et al.,* 1998a; Coughlan and Folan, 1979). Recent studies into the structure and function of cellulases have shown that some cellulases can have both endo- and exoglucanase activity (Bayer *et al.,* 1998b; Howard *et al.,* 2003). Cellulases are members of the glycoside hydrolase family and are classified according to the amino acid sequences in their catalytic domains (Bayer *et al.,* 1998b; Henrissat, 1991).

Hemicellulose is more complex than cellulose since it contains a variety of sugar subunits. This means that several different enzymes are required to degrade it (Biely, 1985; Bissoon *et al.,* 2002). Hemicellulases either belong to the glycoside hydrolase family like cellulases, or they are carbohydrate esterases which catalyse the hydrolysis of ester linkages of acetate and ferulic acid side chains (Howard *et al.,* 2003). Xylan is the most common hemicellulose and xylanases hydrolyse the glycosidic bonds in the xylan backbone. Other hemicellulases include β-1,4-xylosidases (which cleave smaller xylooligomers into xylobiose); and arabinofurinosidase, glucuronidase, acetylxylan esterase and phenolic acid esterase (which all cleave particular hemicellulose side-chains) (Biely, 1985; Howard *et al.,* 2003).

Cellulases and xylanases function via an acid-base reaction mechanism, similar to that of lysozyme, involving two amino acid residues in their catalytic domains (Beguin and Aubert, 1994). The first residue is an acid catalyst that protonates the oxygen of the glycosidic bond, splitting two cellulose or hemicellulose subunits and forming an oxocarbonium intermediate (Beguin and Aubert, 1994). The second residue acts as a nucleophile, which binds with the oxocarbonium intermediate and promotes the formation of an OH^- from a water molecule which converts the intermediate into a free cellobiose or xylobiose subunit (Beguin and Aubert, 1994). Cellulases and hemicellulases also have non-catalytic domains, called carbohydrate binding modules (CBMs), which hold the enzyme‘s active site in contact with the substrate (Bayer *et al.,* 1998b; Boraston *et al.,* 2004; Gilkes *et al.,* 1991). The purpose of CBMs is to increase the concentration of the enzyme at the surface of the substrate, and thus increase the rate of hydrolysis (Boraston *et al.,* 2004). Some CBMs target specific substrates, such as cellulose or xylan, within complex plant matter (Boraston *et al.,*

2004). It has been suggested that the CBM attaches to the substrate and ‗unzips' the cellulose or hemicellulose chains by lifting a single chain away from the crystalline structure and directing it to the catalytic domain where cellobiose or xylobiose units are removed (Figure 1.4) (Coughlan, 1992). The three-dimensional structures of many cellulases and hemicellulases have been studied using X-ray diffraction analysis, NMR spectroscopy and crystallography (Bayer *et al.,* 1998b; Beguin and Aubert, 1994). These have revealed that the binding and catalytic domains are often in the form of tunnels, through which the cellulose chain can be passed, and are linked by a ‗hinge' region (Figure 1.4) (Coughlan, 1992).

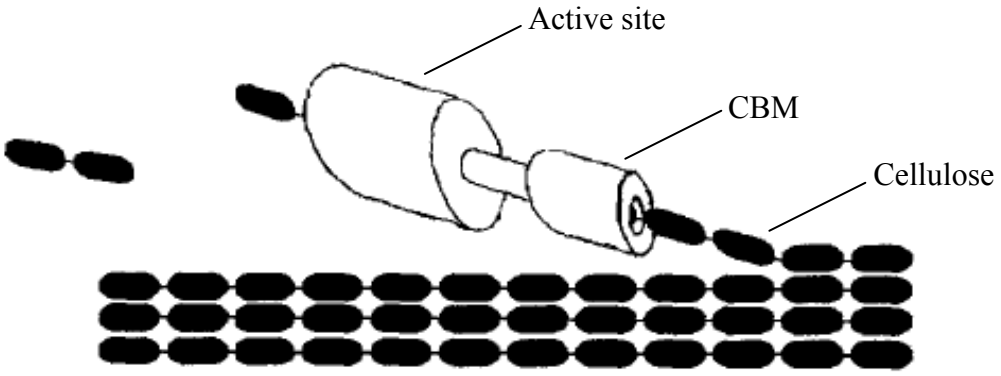


Figure 1.4 Speculative diagram of a cellulase enzyme degrading cellulose, where the carbohydrate binding module (CBM) acts as a wedge, lifting a chain of cellulose from the microfibril which then passes through the enzyme's active site (Coughlan, 1992).

In natural lignocellulosic substrates lignin covers the cellulose/hemicellulose components, shielding them from enzymes. Lignin is the most recalcitrant component of lignocellulose (Howard *et al.,* 2003). Many pre-treatments have been studied to remove some of the lignin and expose the cellulose/hemicellulose components to enzymatic attack. Examples of pre-treatments include mechanical milling; washing with higher alcohols or organic solvents; treatment with alkali, such as sodium hydroxide and ammonia; treatment with acids, such as acetic acid and hydrochloric acid; and treatment with steam so that the lignin fibres explode (Aliyu and Hepher, 2000; Madson and Tereck, 2004). Research is also being carried out into the use of microorganisms that secrete enzymes for lignin degradation (Madson and Tereck, 2004). Recently a patented method known as Lignozyme® has proven to successfully delignify wood for the paper/pulp industry (Howard *et al.,* 2003). In

nature, white rot fungi are able to breakdown lignin, allowing other fungi and bacteria access to the cellulose and hemicellulose (Beguin and Aubert, 1994).

Cellulolytic and hemicellulolytic enzymes are produced by a variety of organisms, including bacteria, fungi, protozoa, insects, mollusks and some plants (e.g. the avocado fruit) (Beguin and Aubert, 1994; Coughlan and Folan, 1979); and most organisms display several different cellulolytic and hemicellulolytic enzyme activities. Cellulolytic and hemicellulolytic microorganisms are found in all environments that contain cellulosic waste. Topsoil is the best source of aerobic (hemi-)cellulolytic microorganisms (Beguin and Aubert, 1994), and (hemi-)cellulolytic microorganisms (both aerobic and anaerobic) have also been found in rumen, manure, municipal solid waste, brewery sludge, sewage sludge, fermenting wood, hot springs, reed beds and marine sediments (Gilkes *et al.,* 1991). Examples of (hemi-)cellulolytic fungi include species of *Aspergillus*, *Penicillium*, *Schizophylum* and *Trichoderma* (Goyal *et al.,* 1991; Howard *et al.,* 2003). In particular, much research has been carried out on the cellulases, hemicellulases and ligninases of *Trichoderma reesei* and *Phanerochaete chrysosporium* (Goyal *et al.,* 1991; Howard *et al.,* 2003). Many species of hemi/celluloytic bacteria have also been isolated, some examples of which are shown in Table 1.1. Screening for bacteria that produce better, more stable enzymes with broader pH and temperature ranges is ongoing (Gilkes *et al.,* 1991; Howard *et al.,* 2003).

Table 1.1 List of cellulolytic bacteria with hemicellulase and cellulase activities isolated from a variety of sources (Doi, 2008; Howard *et al.,* 2003).

Species	Representative Enzyme Activities	Habitat
Bacillus subtilis	mannan endo-1,4-β-mannosidase; endo-β-1,4-mannanase; endo-α-1,5-arabinanase; endo-galactanase	Soil
Clostridium thermocellum	cellulase	
Streptomyces murinus	1,3-β-glucan glucohydrolase	Soil
Bacillus macerans	1,3-1,4-β-D-glucan glucanohydrolase	
Bacillus sp.	1,3-β-D-glucan glucanohydrolase	
Clostridium stercorarium	Feruloyl esterase; α L-arabinofuranosidase	
Bacillus pumilus	Endo-1,4-β-xylanase	Soil, dead plant
Thermoanaerobacter ethanolicus	β-1,4-xylosidase	
Pyrococcus furiosus	Exo-β-1,4-mannosidase	
Thermoanaerobacterium saccharolyticum	α-Glucuronidase	
Escherichia coli	α-Galactosidase	
Bacillus polymyxa	β-Glucosidase	
Fibrobacter succinogenes	Acetyl xylan esterase	Rumen
Butyrivibrio fibrisolvens	Xylan 1,4-β-xylosidase; α-N-arabinofuranosidase	Bovine rumen
Clostridium cellulolyticum	Cellulase; xylan 1,4-β-xylosidase	Decayed grass
Clostridium cellulovorans	Glucan 1,4-β-glucosidase; acetyl xylanesterase	Wood chips
Clostridium hungatei	(not specified)	Soil
Clostridium josui	Cellulase	Compost
Clostridium papyrosolvens	Glucan 1,4-β-glucosidase	Paper Mill
Ruminococcus albus	Cellulase; β-glucosidase; α-N-arabinofuranosidase	Rumen
Bacillus megaterium	(not specified)	Soil
Cellulomonas fimi	Cellulase; endo-1,4-β-xylanase; glucan 1,4-β-glucosidase; mannan endo-1,4-β-mannosidase;	Soil
Cellulomonas flavigena	Cellulase; endo-1,4-β-xylanase; β-mannosidase	Soil, leaf litter
Cellulomonas uda	Endo-1,4-β-xylanase; xylan 1,4-β-xylosidase; cellulose 1,4-β-cellobiosidase	Sugar cane field
Cellvibrio gilvus	β-glucosidase	Bovine faeces
Cellvibrio mixtus	β-mannosidase; α -glucuronidase	Soil
Pseudomonas fluorescens	L-arabinose 1-dehydrogenase; galactose 1-dehydrogenase	Soil, water
Streptomyces reticuli	Cellulase	Soil

When organisms degrade cellulose, hemicellulose or lignocellulose into sugar monomers, the sugars are subsequently absorbed by the cells by diffusion and are then subjected to further degradation (Schwarz, 2001). In aerobic environments, cellulolytic bacteria metabolise glycosyl moieties releasing carbon dioxide and water. Anaerobic digestion of glycosyl moieties leads to the production of methane, fatty acids and/or alcohols (Beguin and Aubert, 1994).

As described above, multiple enzymes are required to degrade cellulose and hemicellulose; and since cellulose, hemicellulose and lignin occur together in natural substrates, enzyme systems containing a range of enzyme activities working synergistically are required to degrade these substances (Coughlan, 1992). In nature, several different species of microorganisms can work together to degrade lignocellulose substrates (Bayer *et al.,* 1994). Synergy can be defined as the interaction between two or more components that produces a combined effect that is greater that the sum of the effects of the individual components (Beguin and Aubert, 1994; Coughlan and Folan, 1979). In other words, enzymes working together synergistically are more efficient than enzymes working individually (Beguin and Aubert, 1994; Coughlan and Folan, 1979). Bacterial (hemi-)cellulolytic enzyme systems are usually very complex, comprising of many types of enzyme activities. In some bacterial cellulase systems the components are produced as, or form aggregates. These aggregates are commonly known as multi-enzyme complexes and the most rigorously studied example is the cellulosome (Gilkes *et al.,* 1991).

1.3 Multi-enzyme complexes

In 1983 a cellulose-binding, cellulase-containing complex was discovered by Lamed and co-workers in *Clostridium thermocellum* and named the cellulosome (Lamed *et al.,* 1983). Since then, cellulosomes and similar xylan degrading complexes, named xylanosomes, have been isolated from other bacterial and some fungal species (Tables 1.2 and 1.3).

Table 1.2 Cellulosome producing microorganisms (Doi, 2008).

Species	Source	Reference
Acetovibrio cellulolyticus	Sewage sludge	(Doi, 2008)
Bacterioides cellulosolvens	Sewage sludge	(Noach *et al.,* 2005)
Butyrivibrio fibrisolvens	Bovine rumen	(Doi, 2008)
Clostridium acetobutylicum	Soil	
Clostridium cellulovorans	Wood-chip pile	(Han *et al.,* 2003; Liu and Doi, 1998; Murashima *et al.,* 2003)
Clostridium cellobioparum	Bovine rumen	(Doi, 2008)
Clostridium cellulolyticum	Decayed grass	(Desvaux, 2005a; Gal *et al.,* 1997)
Clostridium josui	Compost pile	(Doi, 2008)
Clostridium papyrosolvens	Paper mill	(Pohlschroder *et al.,* 1994)
Clostrudium thermocellum	Soil at hot springs	(Bayer *et al.,* 1983; Lamed *et al.,* 1983; Lamed *et al.,* 1985)
Ruminococcus albus	Rumen	(Doi, 2008)
Ruminococcus flavefaciens	Rumen	(Ding *et al.,* 2008; Kirby *et al.,* 1998)

Table 1.3 Xylanosome producing microorganisms.

Species	Source	Reference
Streptomyces olivaceoviridis	Soil	(Jiang *et al.,* 2004; Jiang *et al.,* 2005; Jiang *et al.,* 2006)
Butyrivibrio fibrisolvens	Rumen	(Lin and Thomson, 1991)
Chaetomium spp.	Palm Oil Mill Fibre	(Ohtsuki *et al.,* 2005)
Bacillus licheniformis	Sewage bioreactor	(van Dyk *et al.,* 2009)

The cellulosome consists of a number of cellulolytic enzymes attached to a protein scaffold, called scaffoldin, which holds the enzymes together in a protein complex (Figure 1.5) (Bayer *et al.,* 1998b). All cellulosomal enzymes have a small, 22-residue duplicated sequence known as a dockerin domain (Bayer *et al.,* 1994; Bayer *et al.,* 1998b; Doi, 2008). In free cellulases, the dockerin domain is usually replaced by a

carbohydrate binding module (CBM) (Bayer *et al.,* 1998b). Dockerins bind to cohesion domains that are present on the scaffoldin subunit. Scaffoldin also contains a CBM, also known as a cellulose binding domain (CBD), so that the entire cellulosome can attach to the substrate, as opposed to individual enzymes binding separately (Bayer *et al.,* 1994; Bayer *et al.,* 1998b; Doi, 2008). In *C. thermocellum* and other bacteria, cellulosomes are packed together in polycellulosomal organelles, called protubozymes. These can be seen bound to the outside of the cells using electron microscopy. The protubozymes mediate adhesion of the cell to the cellulose substrate, and when binding occurs the cellulosome undergoes a conformational rearrangement (Bayer *et al.,* 1994; Bayer *et al.,* 1998b).

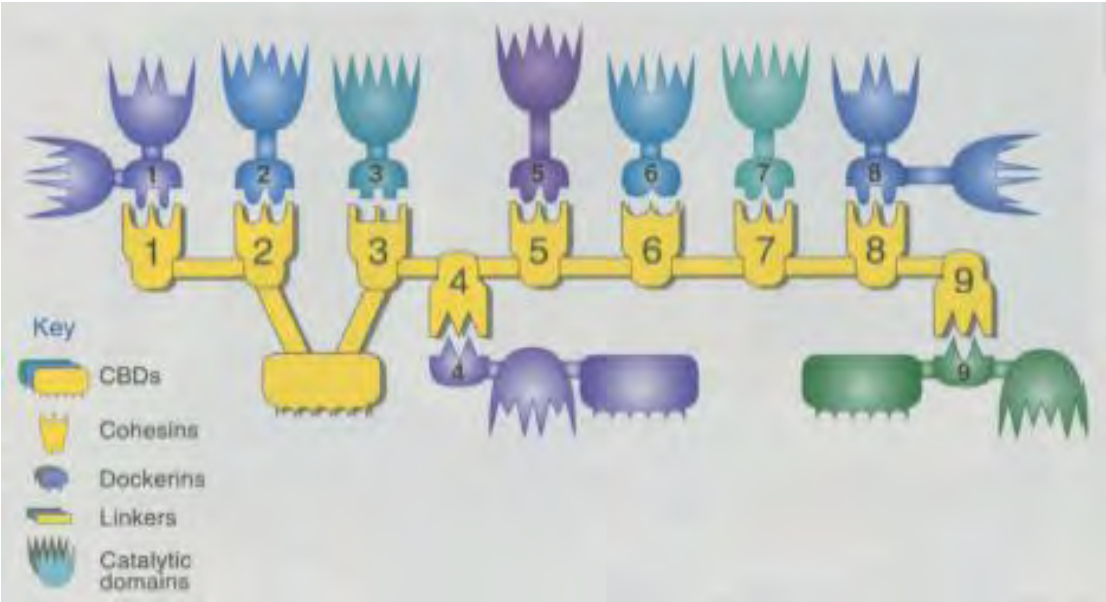


Figure 1.5 Simplified illustration of a cellulosome, where the yellow structure represents the scaffolding subunit (scaffoldin) and the catalytic (cellulolytic) subunits are in blue, green and violet (Bayer *et al.,* 1994).

The cellulosome concentrates a variety of enzymes to specific sites on a substrate which leads to improved cellulolytic hydrolysis. The organisation of the enzymes in a cellulosome increases the synergistic action of the enzymes (Bayer *et al.,* 1994; Doi, 2008; Johnson *et al.,* 1982). Different microorganisms produce cellulosomes that vary in their enzyme composition and arrangement (Bayer *et al.,* 1994; Doi *et al.,* 2003; Doi, 2008). Some cellulosomes may also have more than one CBD (Doi *et al.,* 2003) and some have more than one scaffoldin, such as in the *Acetovibrio cellulolyticus* cellulosome (Ding *et al.,* 2008).

Since the discovery of cellulosomes, extensive research has been carried out on these complexes including purification, in-depth characterisation, structural studies, DNA sequencing and expression, and cloning of the cellulosomal components (Bayer *et al.,* 1998a; Bhat *et al.,* 1997; Bhat *et al.,* 2001; Guimarães *et al.,* 2002). Along with enzyme synergy studies, research into ‗designer cellulosomes' is now being carried out to create the most efficient combination of enzymes to degrade specific substrates (Bayer *et al.,* 1994; Murashima *et al.,* 2003). Cellulosomes can be constructed by chemical incorporation of enzymes or non-catalytic components to existing cellulosomes to improve their function (Bayer *et al.,* 1994). For example, adding a β-glucosidase enzyme to a cellulosome would counteract the negative feedback effect of cellobiose on cellulose degradation (Bayer *et al.,* 1994). Also, since scaffoldin and dockerins have been expressed and isolated separately from cellulosomes, these can be used as the basis for completely new cellulosomes with the desired combination of enzymes (Bayer *et al.,* 1994; Bayer *et al.,* 2007; Caspi *et al.,* 2008; Ding *et al.,* 2008; Doi *et al.,* 2003). Recombinant DNA technology is used to fuse dockerin molecules to the desired enzymes (Bayer *et al.,* 1994; Caspi *et al.,* 2008).

Most of the cellulosome-producing organisms are anaerobic and since this growth condition is more difficult and more expensive to maintain, research is being carried out into aerobic cellulolytic organisms (van Dyk *et al.,* 2009). Currently, species of *Bacillus* are receiving the most attention since these aerobic bacteria are abundant in nature and are easy to isolate and culture (Sonenshein *et al.,* 1993). However, this research is relatively new and so few (hemi-)cellulolytic complexes have been isolated from bacilli and only three have been partially characterised. So far, (hemi-)cellulolytic multi-enzyme complexes have been isolated from *B. circulans*, *Paenibacillus curdlanolyticus*, *B. megatarium* and *B. licheniformis* (Beukes and Pletschke, 2006; Kim and Kim, 1993; Pason *et al.,* 2006a; van Dyk *et al.,* 2009; Waeonukul *et al.,* 2009). No detailed structural studies have been performed on these MECs and, although some have been called cellulosome- or xylanosome-like complexes, they may also be multi-enzyme complexes with structures different to that of the cellulosome (van Dyk *et al.,* 2009).

Kim and Kim (1993) discovered that *B. circulans* isolated from potato starch granules produced two MECs that were 669 and 443 kDa in size (Figure 1.6).

Diethylaminoethyl (DEAE) ion-exchange and size-exclusion chromatography (SEC) were used to purify the two complexes from the crude supernatant (Figure 1.6). Size-exclusion chromatography is a common method for the purification of MECs and cellulosomes because of their large size. Zymograms (also known as activity gels) of the complexes revealed that the larger complex (C1) contained at least five CMCases and two xylanases, and that C2 consisted of at least three CMCases and four xylanases. This (hemi-)cellulolytic enzyme system could hydrolyse a broad range of synthetic and natural substrates, including CMC, xylan, Avicel cellobiose, filter paper, cotton and *p*-nitrophenyl-*β*-D-cellobioside (Kim and Kim, 1993).

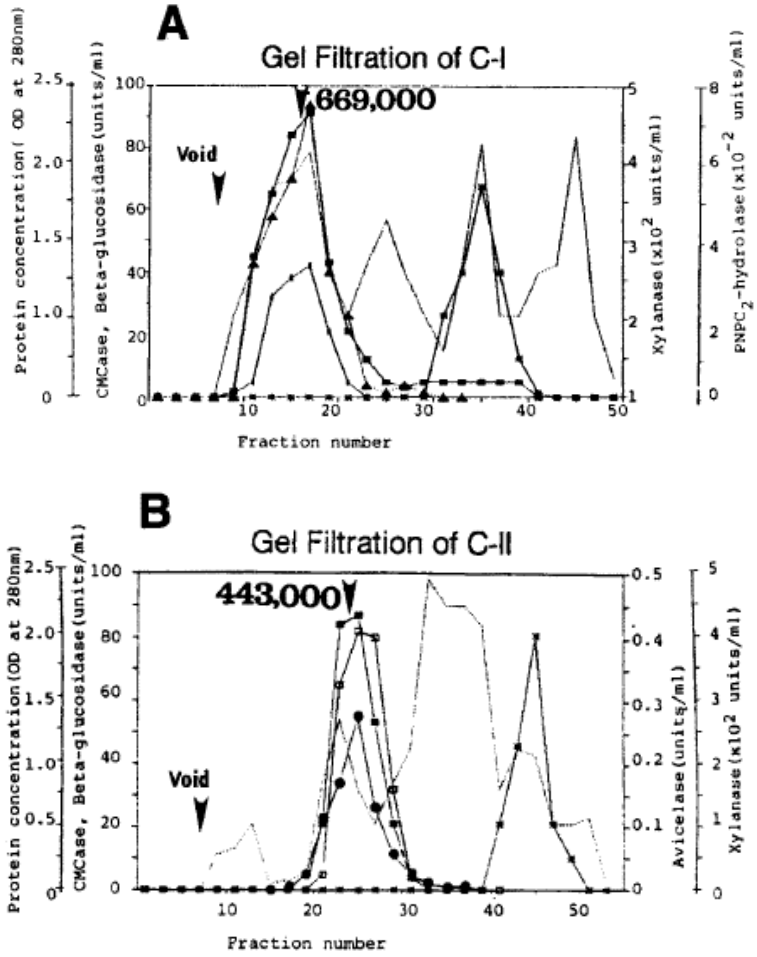
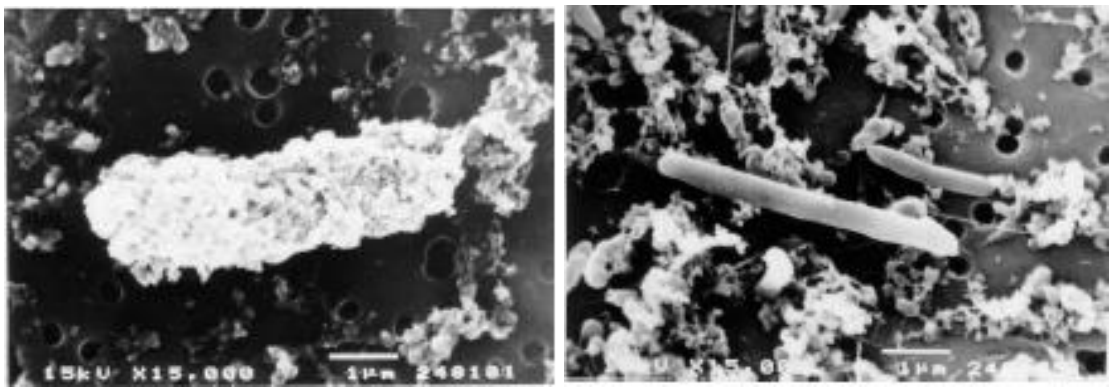


Figure 1.6 Gel filtration chromatography performed by Kim and Kim (1993) on TSK Gel Toyopearl HW-65S column of pooled fractions from DEAE Toyopearl chromatography, showing the two MECs from *B. circulans*. The elution positions of thyroglobulin (669 000 kDa), apoferritin (443 000 kDa) and the void volume are designated by arrow heads. ---Protein (absorbance at 280 nm); □Avicelase; ■CMCase; ▲ PNPC2 hydrolase; ● β-glucosidase; *xylanase (Kim and Kim, 1993).

Pason *et al.* (2006) carried out a screening of *Bacillus* species that produced cellulolytic multi-enzyme complexes. The criteria used to determine the presence of an MEC were the production of cellulases and xylanases, the presence of CBM, the adhesion of the bacterial cells to insoluble substrates and the production of a cellulolytic high molecular weight complex. Only the cells of the strain later identified as *Paenibacillus curdlanolyticus* could bind to Avicel® and xylan substrates and contained multiple CMCases and xylanases (indicated by zymograms). Purification, using affinity to Avicel® followed by SEC, revealed that *Paenibacillus curdlanolyticus* produced two MECs with molecular masses of 1450 kDa and 400 kDa, respectively. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and zymogram analysis of these complexes showed that the larger complex contained seven xylanases and five CMCases, and the smaller consisted of six xylanases and three CMCases, all of sizes between 48 and 224 kDa. Scanning electron microscopy was performed to visualise the binding of the bacterial cells to xylan (Figure 1.7) and a growth curve demonstrated that after 42 hours the cells were released from the substrate. The growth curve also indicated that CMCase and xylanase activities could only be detected in the early stationary phase of growth (between 24 and 36 hours). To further characterize the complexes from *Paenibacillus curdlanolyticus*, Waenokul *et al.* (2007) studied the effects of growing the bacteria on different carbon sources with respect to production and composition of the complexes (Pason *et al.,* 2006a).



A **B**

Figure 1.7 Scanning electron micrograph of the cell surface of *P. curdlanolyticus* B-6 harvested in late exponential growth phase and coated with xylan, indicating the adhesion of the cell to xylan (A), and with the lack of xylan showing no adhesion of the cell to xylan (B) (Pason *et al.,* 2006a).

A 2000 kDa (hemi-)cellulolytic MEC from *Bacillus licheniformis* has also been purified using SEC and partially characterised (van Dyk *et al.,* 2009). This complex contains predominantly xylanase activity and zymograms demonstrated that two xylanases of 21 and 45 kDa, and two CMCases of 25 and 30 kDa were present in the complex (Figure 1.8). A growth curve for *Bacillus licheniformis* demonstrated that, similar to *Paenibacillus curdlanolyticus*, the xylanase activity could be detected after 12 hours and increased rapidly until 60 hours. The MEC produced by this bacterium was seen to bind to insoluble xylan but not to Avicel®, indicating the presence of a CBM specific to xylan (van Dyk *et al.,* 2009).

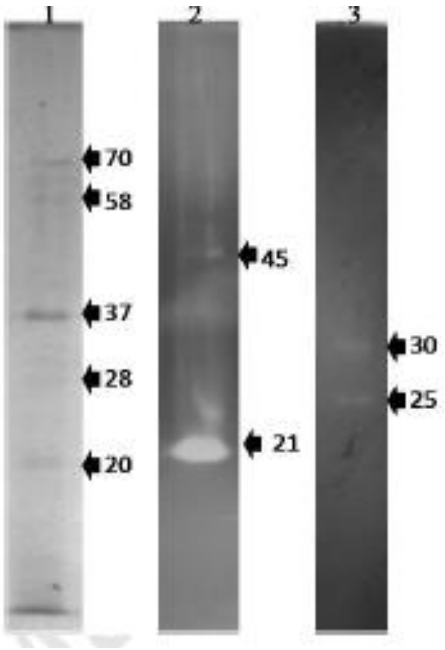


Figure 1.8 SDS-PAGE (10%) (Lane 1) and xylanase (Lane 2) and CMCase (Lane 3) zymograms of an MEC from *B. licheniformis* (van Dyk *et al.,* 2009).

1.4 *Bacillus* spp / *Bacillus subtilis*

The genus *Bacillus* consists of Gram-positive, rod-shaped, aerobic, endospore-forming bacteria (Figure 1.9). These morphological features allow members of this genus to be distinguished from other bacteria. Bacilli are very similar to clostridia except that clostridia are usually strict anaerobes, and bacilli can be strict aerobes or facultative aerobes. The ability of bacilli to grow aerobically means that they are easier to use in laboratory research and in industry.

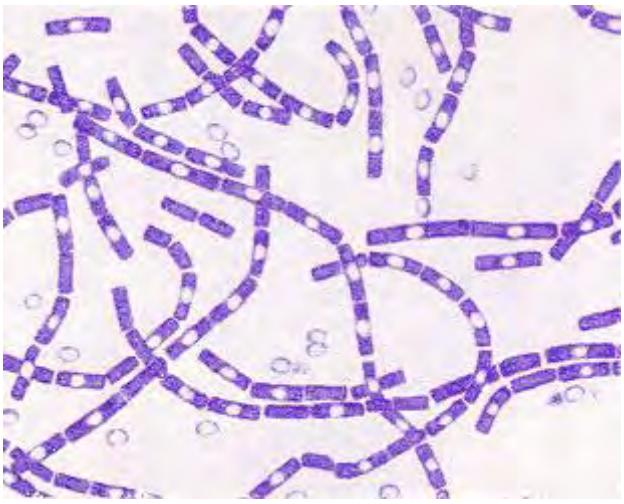


Figure 1.9 Gram stain of *Bacillus anthracis* showing Gram-positive (purple-stained), rod-shaped cells with endospores in white (Terry, 2000).

The only species of *Bacillus* that exhibits pathogenic properties is *B. anthracis*, which causes anthrax; although some of the other species are sometimes opportunistic pathogens (Harwood, 1992; Sonenshein *et al.,* 1993). Bacilli are found predominantly in soil habitats, the rumen of cattle and other animals, sewage sludge, rivers and coastal waters (Brun and Shimkets, 2000; Harwood, 1992). Many have more complex ecological niches and may be involved in intestinal life cycles, such as *B. cereus* which is found in the intestines of soil arthropods as well as in the soil after being expelled from these insects (Brun and Shimkets, 2000).

There are four genera in the family *Bacillacaea* that contain aerobic, endospore-forming bacteria, namely *Thermoactinomycetes*, *Sporoarcina*, *Sporolactobacillus*, and *Bacillus* (Sonenshein *et al.,* 1993). The genus *Bacillus* consists of more than 60 species that vary quite widely in their %GC content (Harwood, 1992; Sonenshein *et al.,* 1993). Therefore, according to Sonenshein, the *Bacillus* genus can be divided into six groups based on morphological and genetic similarities. Species of *Bacillus* are classified according to DNA homology using DNA-DNA hybridization and 16S rDNA sequencing (Sonenshein *et al.,* 1993).

Bacilli produce endospores under unfavourable growth conditions, such as high temperatures or low nutrient conditions (particularly limitations in carbon, nitrogen or phosphorous). Endospores form within the cytoplasm of the parent cell and are metabolically inactive or dormant when mature. Endospores are resistant to many

physical and chemical agents and thus survive under conditions that kill living cells (Brun and Shimkets, 2000). Endospores can survive for long periods of time, and when conditions become favourable once more they can differentiate into vegetative cells (Sonenshein *et al.*, 1993). Endospores are also important for the dispersal of *Bacillus* species, since spores, when released from the cells, are easily blown away in dust and transported via animal faeces (Sonenshein *et al.,* 1993).

Many of the uses for bacilli are linked to their ability to form spores. The resistant properties of endospores make endospore-forming bacteria important in a variety of industrial processes and endospores also offer an important molecular biological model for cell differentiation. The production of endospores is linked to the production of insect toxins and peptide antibiotics (Sonenshein *et al.,* 1993). Species of *Bacillus* are also used for the production of industrial solvents, detergents, insecticides, fine biochemicals and enzymes (Brun and Shimkets, 2000; Harwood, 1992).

Bacillus subtilis is an aerobe that can also grow in anaerobic conditions, particularly with glucose and nitrate as carbon and nitrogen sources, respectively. The endospores of *B. subtilis* are oval shaped and located centrally or subterminally. In 1936 the Marburg strain was designated the neotype strain for *B. subtilis* (Harwood, 1992; Sonenshein *et al.,* 1993) and this species is now one of the most extensively studied bacterial species, after *E. coli*. It has been used as a model system for Gram-positive bacteria in many aspects of biochemistry, genetics and physiology. *B. subtilis* was first used in a genetic transformation in 1959 by John Spizezen, and is now often used in molecular biology as a host for protein production (Harwood, 1992; Sonenshein *et al.,* 1993). Detailed genetic analyses of *B. subtilis* have been undertaken and coupled with morphological studies and metabolism and regulation studies. For example, as a model for differentiation, 75 genes involved in sporulation are known from *B. subtilis* (Brun and Shimkets, 2000). In response to fluctuating nutrient supply *B. subtilis* develops competence to take up external DNA, produces hydrolytic enzymes (proteases and carbohydrases) and forms endospores. These three events have been the main reasons for the extensive studies carried out on this bacterium (Harwood, 1992).

Apart from its importance in biochemical and genetic research, *B. subtilis* also has many industrial applications and has been used in the food, beverage and detergent industries. For example, it has been used in the production of _natto', a Japanese fermented soy product. In agriculture, *B. subtilis* can be used as a biocontrol agent against fungi that cause crop diseases, such as *Fusarium oxysporum* and *Botryodiplodia theobromae*, and to provide nutrients, such as phosphorus and sulphur, to promote plant growth (Swain and Ray, 2009). This bacterium is also extensively used in industry for the production of proteins. It is able to secrete large volumes of protein into the growth media and is non-pathogenic, which makes it a prime candidate for industrial scale protein production. The major industrial enzymes produced by *B. subtilis* are α-amylase and β-glucanase (Harwood, 1992; Swain and Ray, 2009). In addition, proteases produced in the late stationary phase of growth have been used in the detergent industry and pectinases have been used in biopreparation and bioscouring of cotton (Ahlawat *et al.,* 2009; Harwood, 1992; Wang *et al.,* 2007). Many other enzymes produced by *B. subtilis* have been reported, such as chitinase, β-galactosidase (Chiang *et al.,* 2003; Shaikh *et al.,* 2007). In particular, *B. subtilis* produces many cellulolytic enzymes such as lichenase (a type of β-glucanase), maltase, pectate lyase, endoglucanases with cellulose binding domains and several xylanases. The research conducted on these enzymes include purification and characterisation, optimisation of production, structural and active site studies, gene cloning and transformation into *E. coli* (Cantwell and McConnell, 1983; El-Helow and El-Ahawany, 1999; Harwood, 1992; Jalal *et al.,* 2009; Sá-Pereira *et al.,* 2002a; Wang *et al.,* 2009).

In the *B. subtilis* genome project, from 1989 to 1998, several laboratories from Japan, Europe and the USA collaborated to sequence the entire 4.2 Mb *B. subtilis* strain 168 genome (Devine, 1995; Harwood, 1992; Harwood and Wipat, 1996; Moszer, 1998). The *B. subtilis* genome was the first complete genome of a free-living soil bacterium to be sequenced (Wipat and Harwood, 1999). The reasons for sequencing its genome are the importance of *B. subtilis* as a model organism in research, its industrial importance and the small size of its genome (Devine, 1995). The sequencing strategy adopted for this genome project was to create ordered libraries of large, overlapping chromosomal fragments which are then individually sequenced (Harwood and Wipat, 1996). Moszer (1998) later reviewed the function of the protein coding genes in the

completed sequence in a functional analysis program, and Zuber (2001) reviewed the peptide profiles developed for 345 small polypeptide open reading frames. The genome sequence and gene analysis information, together with the knowledge already available for this organism, provide the basis for researching the behaviour and ecology of *B. subtilis* at the molecular level (Wipat and Harwood, 1999).

B. subtilis is easily isolated from the environment due to its prevalence in the soil. From the soil the bacteria are also transferred to plants, plant materials, foods, animals and aquatic environments. From eating plants and food, *B. subtilis* is transferred to animals, where it can be found in the gut and the faeces, its route back to the soil (Sonenshein *et al.,* 1993).

1.5 Applications for the enzymatic degradation of cellulose and hemicellulose

Cellulases and hemicellulases make up a large portion of the world‘s industrial enzymes due to their wide range of uses in various industries including chemicals, fuel, food, brewing and wine, animal feed, textile and laundry, pulp and paper, and agriculture. Xylanases are used in the baking industry to improve the texture, volume and shelf-life of bread and for wheat separation. The paper and pulp industry is one of the largest consumers of hemicellulases, ligninases and other lignocellulolytic enzymes. Cellulases and hemicellulases have also been used in the animal feed industry to reduce the fibre content to improve feed utilization, milk yield and body weight gain in cattle and sheep (Howard *et al.,* 2003).

Lignocellulose can be used to produce chemicals, biofuels, a cheap energy source for fermentation, improved animal feed and human nutrients (Figure 1.10) (Gravatis, 2004; Howard *et al.,* 2003; Madson and Tereck, 2004). The enzymatic hydrolysis of lignocellulose, hemicellulose and cellulose results in production of hexose and pentose sugars as well as various lignin monomers. The sugars from this hydrolysis can be used in human nutrients, or in fermentation. Glucose is a common substrate used in the fermentation processes for industrial products such as organic acids, amino acids, vitamins and several bacterial and fungal polysaccharides. Xylose, produced from saccharification of hemicellulose, is used in the production of xylitol and furfural. Xylitol is used as an artificial sweetener in food, in teeth hardening, and

as an anti-microbial agent in toothpaste and chewing gum. Furfural is used in manufacturing furfural-phenol plastics, varnishes and pesticides (Gravatis, 2004; Howard *et al.,* 2003).

The fermentation of the sugars from cellulose and hemicellulose degradation generates products such as ethanol, acetone, butanol, glycerol acetic acid, citric acid and fumaric acid (Howard *et al.*, 2003). These chemicals, along with aromatic compounds produced from the hydrolysis of lignin, can be used to make other organic chemicals which, in turn, can be used to various chemical products including polymers and resins (Howard *et al.,* 2003). Ethanol and butanol produced from degraded and fermented lignocellulose can be used as biofuels.

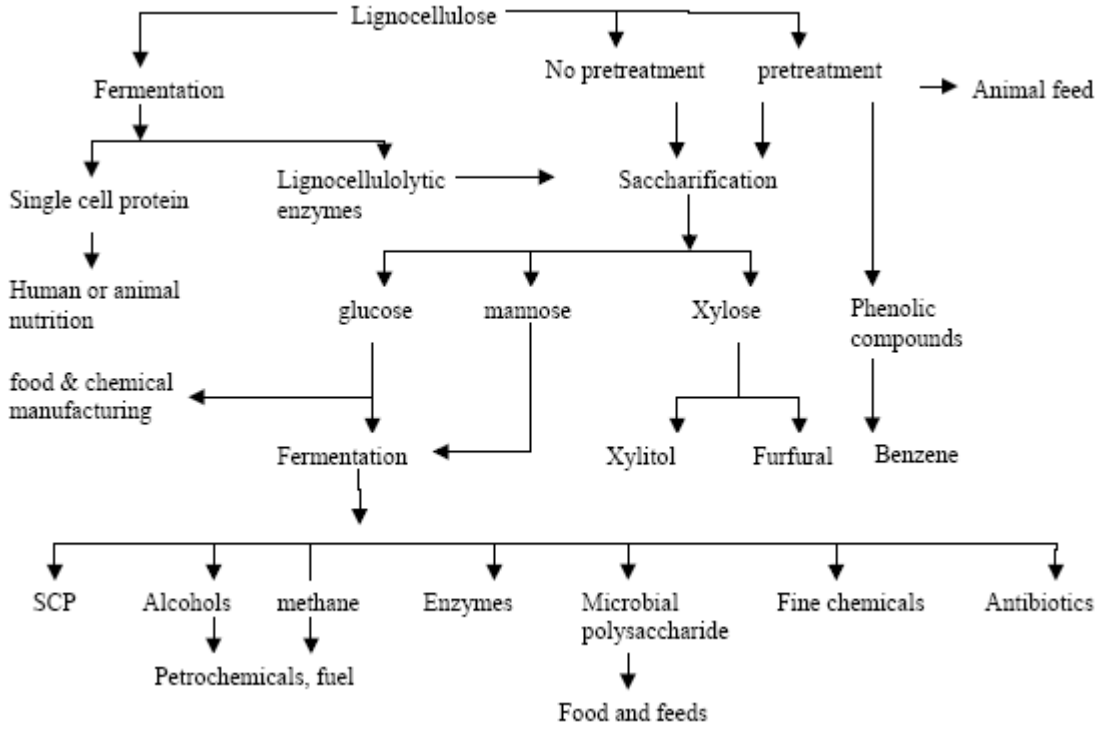


Figure 1.10 Lignocellulose bioconversion processes into valuable bioproducts (Howard *et al.,* 2003).

Bioethanol is produced in large quantities in the USA, Brazil and Europe from corn, sugar cane and wheat, respectively. These starchy crops are hydrolysed to sugars and then fermented to produce ethanol (Figure 1.11) (Linde *et al.,* 2008). Due to food security, land use limitations, and the need to meet bioethanol demands, focus is turning to lignocellulosic substances for bioethanol production (Gray *et al.,* 2006;

Gressel, 2008; Hahn-Hägerdal *et al.,* 2006). Lignocellulose (or biomass) is much harder to hydrolyse than starch but produces much higher ethanol yields (Gray *et al.,* 2006). Lignocellulose sources include crop wastes (for example corn stalks and bagasse), forest residues, municipal solid waste, non-food crops (e.g. switch grass), and the residues from bioethanol production from corn and wheat (DDGS distiller dry grain with solubles) (Bayer *et al.,* 2007; Frederick Jr. *et al.,* 2008; Gray *et al.,* 2006; Gressel, 2008; Linde *et al.,* 2008; Tabka *et al.,* 2006). Extensive research has led to the development of several pilot plants for bioethanol production from biomass and Kaparaju and co-workers have described a biorefinery for the production of bioethanol, biohydrogen and biogas (Kaparaju *et al.,* 2009; Linde *et al.,* 2008; Thomsen *et al.,* 2008). Much research is still being carried out to improve the process, including genetically engineering plants to contain less lignin (Sticklen, 2006), isolating or engineering microbes that can simultaneously hydrolyse lignocellulose and ferment sugars (SSF, simultaneous saccharification and fermentation) (Ballesteros *et al.,* 2004; Gomez *et al.,* 2008; Petersson *et al.,* 2007) and the use of multi-enzyme systems for more thorough degradation of complex substrates (Dogaris *et al.,* 2009). Research into the use of the cellulosome in commercial ethanol production is also being carried out to determine whether it would lead to a better bioconversion process than using free enzymes (Ding *et al.,* 2008).

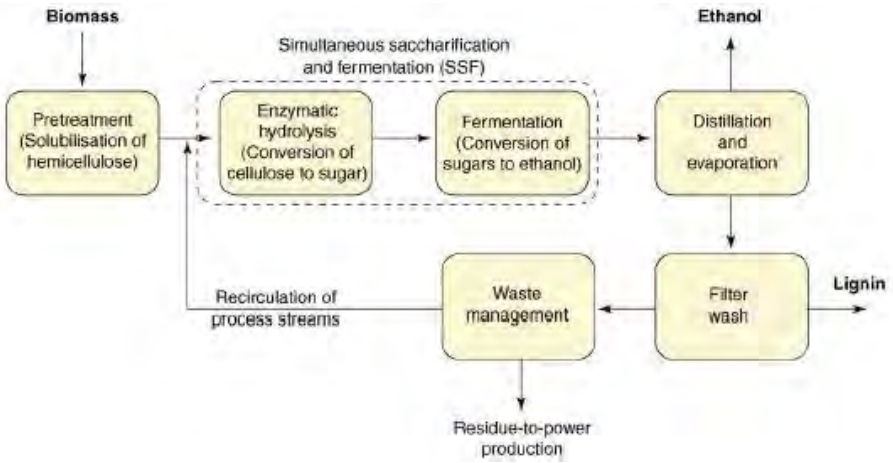


Figure 1.11 Biomass to ethanol flowchart (Hahn-Hägerdal *et al.,* 2006).

Another important application of cellulolytic degradation is waste management. Municipal solid waste (MSW), consisting primarily of newspaper, wood and cardboard, is being dumped in ever-growing landfills that contaminate groundwater

(Bayer *et al.,* 2007). Also, in agriculture, disposal of crop residues by burning, such as sugar cane residues, contributes to air pollution by introducing carbon monoxide, nitrogen dioxide and respirable particles (Beary *et al.,* 2002). The use of (hemi-)cellulolytic enzyme systems and cellulosomes is an efficient, environmentally friendly way to dispose of these and other lignocellulosic wastes (Bayer *et al.,* 2007). An added benefit of this method of lignocellulose waste disposal is the generation of valuable products such as biofuels and other chemicals.

1.6 Problem Statement

Multi-enzyme complexes such as the cellulosome degrade complex substrates with higher efficiency than individual enzymes, but the cellulosome is mainly found in anaerobic bacteria which are more difficult and costly to culture than aerobic bacteria, such as those of the *Bacillus* genus.

1.7 Hypothesis

A species of *Bacillus* isolated from a natural environment can produce a multi-enzyme complex capable of degrading cellulose and hemicellulose substrates.

1.8 Aims and Objectives

1. To screen for a species of *Bacillus* that degrades cellulose and hemicellulose using a multi-enzyme complex (MEC).
2. To optimise the production of cellulolytic and hemicellulolytic enzymes by the *Bacillus* sp.
3. To purify the (hemi-)cellulolytic MEC from the *Bacillus* sp.
4. To perform a partial characterisation of the (hemi-)cellulolytic MEC.

CHAPTER 2

Screening for a *Bacillus* strain that produces cellulolytic/hemicellulolytic MECs

2.1 Introduction

Cellulose and hemicellulose are abundant and renewable sources of carbon whose degradation has been extensively studied and applied to several industries including those discussed in Section 1.5 (O'Sullivan, 1997). Many cellulose and hemicellulose degrading MECs have been purified and studied, especially the cellulosomes from clostridia (Doi *et al.,* 2003; Doi, 2008). More recently, the purification of MECs from *Bacillus* has attracted attention, but there has not been much detailed research conducted into bacilli as MEC producers. Since bacilli are aerobic they are easier to work with in the laboratory and have the potential to produce high enzyme activities.

Bacilli occur abundantly in nature, particularly in soil and compost (Sonenshein *et al.,* 1993). Those species of the *Bacillus* genus that can degrade cellulolytic compounds can be easily separated from non-cellulolytic microorganisms by growth on Berg‘s minimal medium with cellulose as the only carbon source (Berg *et al.,* 1972; Pason *et al.,* 2006a). This is a common method for the isolation of cellulose-degrading microorganisms.

Members of the *Bacillus* genus are also easily identifiable because they are aerobic, Gram-positive, rod shaped, endospore forming bacteria with catalase activity (Sonenshein *et al.,* 1993). A commonly used method for the identification of bacteria to species level is 16S rDNA sequencing (Lee *et al.,* 2008). The 16S rDNA is a sequence common to all bacteria that has a variable region specific to a bacterial species. Flanking the variable region are conserved regions, useful for designing primers.

Pason *et al.* (2006b) carried out a selection process for (hemi-)cellulolytic MEC producing bacilli by isolating microorganisms from several sources using Berg‘s medium with 0.5% (w/v) Avicel® and testing the CMCase and xylanase activities of the culture supernatants. Similarly, in this study enzyme activity assays were

performed using Avicel®, CMC and xylan substrates to demonstrate the ability of the isolated microorganisms to degrade naturally occurring cellulosic material, which can contain a combination of crystalline cellulose (Avicel®), amorphous cellulose (CMC) and hemicellulose (which consists predominantly of xylan) (Bayer *et al.,* 1998a; Gray *et al.,* 2006). While bacilli are known to produce many extracellular cellulolytic and hemicellulolytic enzymes (Chapter 1, Table 1.1), this study focused on the screening of bacilli that produce (hemi-)cellulolytic MECs. Multi-enzyme complexes degrade compound substrates more efficiently than numerous individual enzymes because they concentrate a variety of key enzymes to specific sites on a substrate and enhance enzyme synergy (Bayer *et al.,* 1994; Doi, 2008). A useful method for the rapid detection of large protein complexes is size-exclusion chromatography using a gel matrix with a low kDa exclusion limit so that a MEC would elute in the void volume.

The aim of this chapter was to isolate a number of cellulose and hemicellulose degrading microorganisms and screen for those that were members of the *Bacillus* genus, were able to degrade Avicel®, CMC and xylan, and produced a (hemi-)cellulolytic MEC. The identified candidate organism that produced an MEC with the highest cellulolytic and hemicellulolytic enzyme activities was selected for all subsequent experiments in this study.

2.2 Materials and Methods

Four steps were used in the screening for a *Bacillus* strain with a cellulose/hemicellulose-degrading MEC:

1. A selective culture medium was used in which only organisms that could utilize cellulose as the sole carbon source could grow.
2. The organisms that grew in the selective medium were screened according to morphological characteristics that are typical of the *Bacillus* genus.
3. The cultures that were identified as bacilli were tested for the presence of a MEC.
4. A MEC-producing *Bacillus* strain was then selected based on its cellulolytic and hemicellulolytic activities.

2.2.1 Isolation of cellulolytic bacteria

Samples were collected from three compost heaps in Grahamstown, South Africa (a sawdust based compost, a grassy-garden-waste compost and compost consisting mostly of vegetable waste) as well as from soil in Grahamstown. The samples were named C1, C2, C3 and S1, respectively and were stored in 1X phosphate buffer saline (PBS). From each sample 5 ml was inoculated into 50 ml of Berg's mineral medium (Berg *et al.,* 1972) with 0.5% (w/v) Avicel® as the only carbon source, and incubated at 37°C on a rotary shaker at 200 rpm. After 7 days, a sample from each of the four cultures was heated at 80°C for 15 minutes. This heat treatment kills all microbes that do not form spores, leaving bacilli and other spore forming microbes which can withstand high temperatures for long periods of time. The heat-treated samples were then grown on nutrient agar at 37°C, and colonies of different morphologies were seen for each sample. Each colony type from each sample was picked and grown in enrichment broth containing 0.5% (w/v) Avicel®, 0.5% (w/v) peptone, 0.1% (w/v) yeast, 0.5% (w/v) NaCl, 0.1% (w/v) K_2HPO_4, 0.02% (w/v) $MgSO_4$, 0.01% (w/v) $CaCl_2$, 0.1% (w/v) tryptone soy broth and 0.1% (w/v) cellobiose at 37°C and 200 rpm.

To obtain additional bacterial strains, a second soil sample (named S2) was collected from St Francis Bay, South Africa and suspended in 1 X PBS. This time, a 1 ml portion was heat treated at 80°C for 15 minutes and then inoculated into 100 ml Berg's mineral media supplemented with 0.5% (w/v) Avicel®. After 7 days incubation at 37°C on a rotary shaker at 200 rpm, the culture was plated onto enrichment agar plates (which contained the same nutrients as the enrichment broth described above, in addition to 1.6% (w/v) bacteriological agar). Four different colony types were observed (a large white, a small orange, and white with a dark centre and a yellow colony) and each of these was picked and grown in enrichment broth in the same way as the strains isolated and cultured previously.

Each bacterial strain was named according to the compost or soil sample it was isolated from and the colour of its colonies on enrichment or nutrient agar plates. For example, C1W was the strain from the sawdust compost that had white colonies, C1O had orange colonies and S2Y was isolated from the soil from St Francis Bay that had yellow colonies.

2.2.2 Bacillus identification

Bacillus species can be indentified by the following characteristic features:

1. Aerobic
2. Rod-shaped cells
3. Gram positive
4. Endospore-forming
5. Catalase positive

As described in Section 2.2.1, all the isolated strains were cultured aerobically by shaking at 200 rpm. A light microscope was used to view the cells of each culture, and Gram stains and endospore stains were used in identifying the cultures as members of the *Bacillus* genus. For both stains a colony was picked from a nutrient agar plate, placed in a drop of water on a slide and fixed by passing the slide through a flame. The Gram stain involved staining with crystal violet and then iodine for 30 seconds each, and counterstaining with safrinin for 1 minute. This resulted in Gram positive cells appearing purple and Gram negative cells appearing pink. For the endospore stain Malachite Green was left for 5 minutes over boiling water and safrinin was used to counterstain (Bartholomew and Mittwer, 1950). With this stain cells appeared pink while endospores appeared green. The catalase test was performed by adding a drop of hydrogen peroxide to a drop of each enrichment broth culture on a glass slide (Lasec) (Krych *et al.,* 1980). The rapid formation of bubbles indicated the production of catalase by the cells.

2.2.3 Protein and enzyme assays

Strains cultured in enrichment medium were centrifuged at 12 000*g* for 15 minutes and the supernatants concentrated using polyethylene glycol (PEG) 20 000. The protein content was determined using the method of Bradford, where 5 µl of enzyme sample was added to 250 µl of Bradford reagent (Sigma) and the protein concentration calculated in mg/ml using a bovine serum albumin (BSA; Sigma) standard curve (See Appendix 1, Figure A1) (Bradford, 1976). This assay was only accurate for protein concentrations above 0.2 mg/ml, and so for lower protein concentrations 25 µl of protein sample was added to 230 µl Bradford reagent (See Appendix 1, Figure A2 for standard curve).

The cellulolytic and hemicellulolytic enzyme activities of each concentrated supernatant were determined using the dinitrosalicyclic acid (DNS) method (Miller, 1959), which measures reducing sugars. The DNS reagent was prepared according to (Wood and Bhat, 1988) where 2 g dinitrosalicyclic acid (Sigma), 0.4 g phenol, 0.1 g sodium metabisulphite (Sigma-Aldrich®) and 40 g potassium sodium tartrate were dissolved in 100 ml of 2% (w/v) NaOH and diluted to 200 ml with distilled water. The samples were first incubated at 50°C in a solution containing 250 µl 20 mM phosphate buffer, pH 7.0, 50 µl of 2% (w/v) substrate and 100 µl sample. The substrates used were Avicel® (Fluka), CMC (Calbiochem) and birchwood xylan (Fluka). An enzyme control contained 300 µl buffer and 100 µl enzyme sample; a substrate control contained 350 µl buffer and 50 µl substrate; and a blank contained 400 µl buffer. After incubation, 300 µl of the assay preparation was added to 600 µl of DNS reagent and boiled (100°C) for 5 minutes in a dry bath (Labnet, inc.). The reaction was stopped by placing on ice for 5 minutes, and the absorbance read at 540 nm (Bio-Tek Instruments, inc. PowerWave). The µmoles of glucose or xylose produced per hour during incubation at 50°C was calculated using appropriate glucose and xylose standard curves (See Appendix 1, Figure A3).

Crude enrichment broth cultures were also plated (100 µl) onto enrichment agar plates containing CMC or xylan as the carbon source and incubated at 37°C. After sufficient growth the plates were covered with 0.3 % (w/v) Congo Red (Sigma) for 15 minutes. This was decanted and replaced with 1 M NaCl for 15 minutes (Teather and Wood, 1982). Congo Red stains polysaccharides greater than 7 monomers in length, and thus clear zones that formed after washing with 1 M NaCl indicated that the polysaccharide substrates (CMC or xylan) had been degraded.

2.2.4 MEC determination

In order to determine the presence of a MEC, each culture supernatant was dialysed overnight in 50 mM Tris-HCl buffer, pH 7.0, and concentrated using PEG 20 000. The concentrated samples were filtered using 0.45 µl syringe filters. About 2 ml of each was loaded onto a Superose 12 size exclusion column (separation range: 1 – 300 kDa) on a fast performance liquid chromatography (FPLC) system (ÄKTA™). The following standards were loaded on the column: Dextran Blue (2000 kDa), β-

galactosidase (116 kDa), BSA (66.4 kDa), trypsin (24 kDa), lysozyme (14.7 kDa) (Fluka) and Vitamin B12 (1.6 kDa) (all standards were from Sigma unless stated otherwise). A peak in the void volume (7.87 ml as demonstrated by Dextran Blue) indicated the presence of a protein fraction large enough to be an MEC.

2.2.5 Species identification

Based on results from the above screening steps, the MEC-producing *Bacillus* strains with the highest enzyme activities were grown in nutrient broth overnight at 37°C and 200 rpm. A 1.5 ml sample of each was removed and a chromosomal DNA purification was performed according to the instruction manual using the Promega Wizard Genomic DNA Purification Kit (Promega Corporation). The purified DNA was electrophoresed on a 1% agarose gel (Sigma) to determine the success of the genomic DNA purification. A PCR reaction using the primers 9F and 1541R (IDT, Inc.) for amplification of the 16S rDNA sequence was performed using the following program on a ‗PCRSprint' thermocycler (Hybaid, Ltd.):

95°C, 2 minutes
98°C, 30 seconds
61°C, 30 seconds
68°C, 1 minute
} 25 cycles
68°C, 5 minutes

The PCR reaction mixture consisted of 33.5 µl triple distilled water, 10 µl 5XFid. Buffer (KapaBiosystems), 1.5 µl dNTP (KapaBiosystems), 1.5 µl of each primer, 1 µl DNA polymerase (KapaBiosystems), and 1 µl DNA (added at about 50°C). A 1% agarose gel of the PCR products was used to check for a single band of about 1500 pb (the expected size of the 16S rDNA region). The PCR products were then sequenced by Inqaba Biotec and the sequences were used in a nucleotide-nucleotide BLAST search to identify the species.

2.2.6 Secondary screening

Due to low enzyme activities obtained from the initial set of strains, a secondary screening was carried out in which new strains were added to the collection and an optimised enrichment medium was used.

Glycerol stocks containing cellulose/hemicellulose-degrading bacteria isolated by undergraduate students at Rhodes University were donated to this project. The stocks were heat-treated at 80°C for 15 minutes to remove possible non-spore-forming contaminants, and cultured at 37°C and 200 rpm. These new strains were identified as *Bacillus* spp. using the same method as in Section 2.2.2.

Based on preliminary nutrient optimisation studies (data not shown), which indicated that ammonium sulphate was a better nitrogen source for enzyme production than peptone, a more suitable enrichment medium was used in all subsequent experiments. This enrichment medium contained 0.5% (w/v) yeast, 0.5% (w/v) $(NH_4)_2SO_4$, 0.2% (w/v) NaCl, 0.025% (w/v) $MgSO_4$, 0.2% (w/v) K_2HPO_4, 0.2% (w/v) KH_2PO_4 and 0.5% (w/v) carbon source (either Avicel®, CMC or xylan). Four strains were selected from the initial set based on their cellulolytic and hemicellulolytic activities (primary screening) and these, together with the newly donated strains, were cultured in the optimised medium. Crude supernatants (after centrifugation at 12 000*g* for 15 minutes) were assayed for protein and Avicelase, CMCase and xylanase activities. These results were used to select a group of candidate *Bacillus* strains.

2.2.7 MEC-associated (hemi-)cellulolytic activities

The strains selected from secondary screening were cultured in enrichment media containing 0.5% (w/v) birchwood xylan. After centrifugation of the cultures at 12 000*g* for 15 minutes, the resulting supernatants were dialysed in 50 mM Tris-HCl, pH 7.0 and concentrated using PEG 20 000. A 2 ml fraction of each sample was loaded onto a Sepharose 4B column and eluted with 50 mM Tris-HCl buffer, pH 7.0. The presence of a protein peak with a high molecular weight was considered an indication that the organism may have been able to produce a MEC, where protein sizes were calculated using a calibration curve for the Sepharose 4B column

(Appendix 1, Figure A.4). The fractions in these high molecular weight protein peaks (‗MEC peaks') were pooled, concentrated using PEG 20 000 and measured for Avicelase, CMCase and xylanase activities using the DNS method.

2.3 Results

2.3.1 Isolation and identification of MEC-producing (hemi-)cellulolytic bacilli

Bacteria were isolated from several composts and soils and screened according to their morphological characteristics and ability to degrade cellulose and hemicellulose substrates. The best candidate strains displayed characteristics of *Bacillus* spp., contained a large molecular weight protein fraction and were able to degrade cellulose and hemicellulose substrates.

All the isolated bacterial strains were viewed under the light microscope after endospore staining and Gram staining. Figure 2.1 is a typical Gram stain used to positively identify bacilli.

Figure 2.1. Photograph of a Gram stain of culture C1W grown overnight in nutrient broth. Magnification: ×1000

The cells in Figure 2.1 were stained purple, indicating that they were Gram-positive. All the strains that displayed rod-shaped, Gram-positive and spore-forming cells, similar to those in Figure 2.1, were considered possible members of the *Bacillus* genus and were selected for further investigation.

A method for visualising cellulolytic activity is the use of Congo Red, a dye that stains polysaccharides a bright red colour. Isolated *Bacillus* strains were plated onto enrichment agar, containing CMC as the only carbon source, and after 24 hours of growth the plates were stained with Congo Red. The presence of CMC (a polysaccharide) in the agar medium meant that the agar stained red. When washed with 1 M NaCl the areas where CMC had been degraded appeared as clear zones (Figure 2.2).

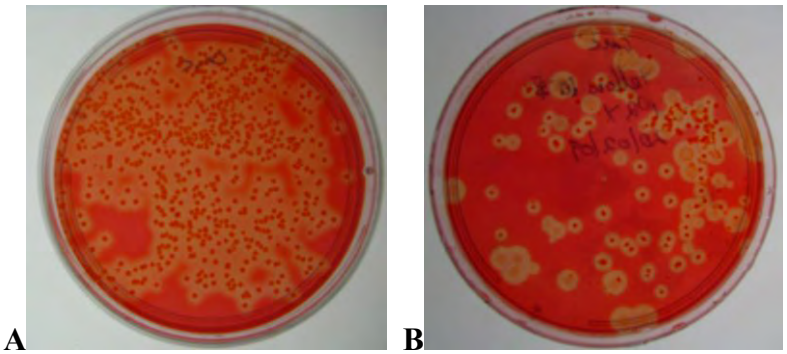


Figure 2.2. Photographs of CMC enrichment agar plates stained with Congo Red and destained with 1 M NaCl. **A:** culture C1W; **B:** 10^{-6} dilution of culture S2Y.

Figure 2.2 displays clear zones around the *Bacillus* colonies indicating that these colonies produced enzymes that degraded the CMC in the agar. All the strains that were isolated using the selective media with Avicel® as the only source of carbon produced enzymes that degraded CMC, as demonstrated in Figure 2.2.

To determine the presence of a large protein complex, extracellular fractions from each strain were loaded onto a Superose 12 size exclusion chromatography column. This column has a separation range of 1 – 300 kDa, which means that an enzyme complex would be too large to enter the resin and would be eluted in the void volume. The void volume (7.78 ml) was measured using the standard, Blue Dextran (2000 kDa). A typical Superose 12 chromatogram obtained from the detection of MECs in the selected *Bacillus* strains is displayed in Figure 2.3 below.

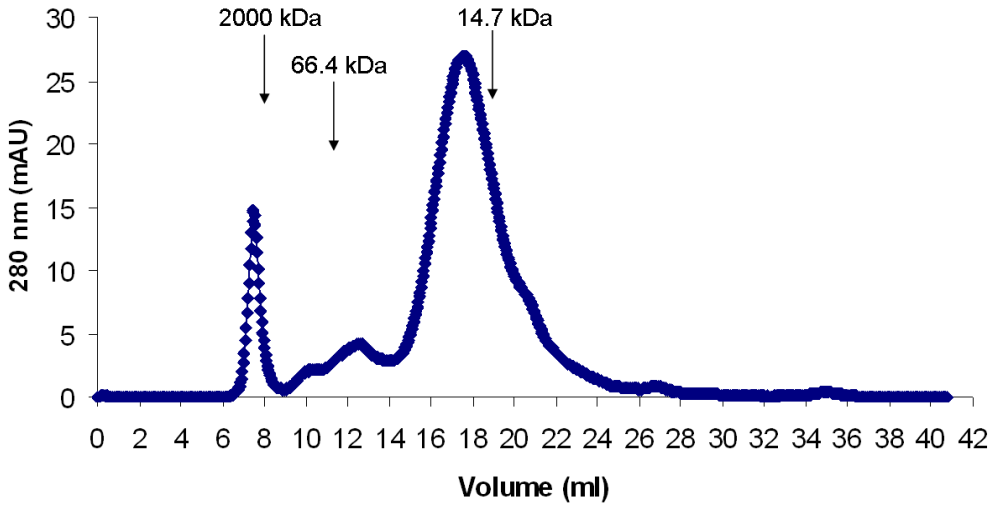


Figure 2.3 Superose 12 chromatogram of extracellular proteins from strain S2Y. Column dimensions: 1 × 30 cm; flow rate: 0.5 ml/min. ♦ A_{280} (mAU).

Figure 2.3 displays the chromatogram of strain S2Y (grown on Avicel®) that had a peak in the void volume (7.49 ml) indicating the presence of a protein fraction above 2000 kDa, which could be an MEC. It is also possible that the peaks at 10.32 ml and 12.55 ml were smaller MECs, and that the peak at 17.56 ml represented free protein, as indicated by the sizes in Figure 2.3. All the cultures that contained a peak in the void volume, similar to that in Figure 2.3, were selected for further investigation. (See Appendix 2, Figure A.10 for Superose 12 chromatograms of all cultures).

Some of the candidate strains from the above screening steps were identified to species level using 16S rDNA sequencing. Typical agarose gels of the genomic DNA isolated using a DNA purification kit, and of the 16S rDNA PCR products are shown in Figure 2.4. The 16S rDNA region from *Bacillus* spp. should be approximately 1500 bp in size. A single band of this size indicates that the PCR reaction was successful and these PCR products were sent for sequencing at Inqaba Biotec.

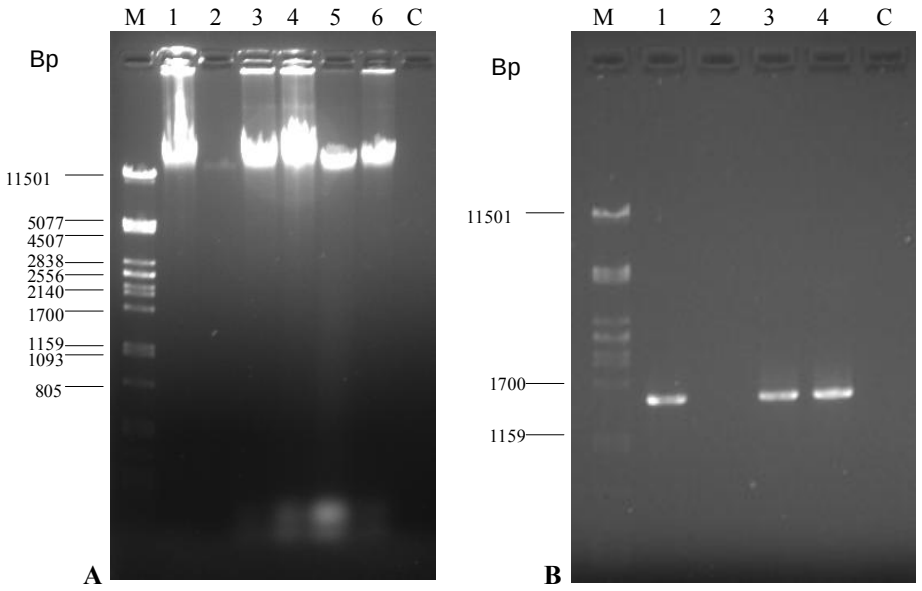


Figure 2.4 Typical agarose gels (1%) of **A:** genomic DNA purification products and **B:** PCR products from 16S rDNA amplification of bacterial strains grown overnight in nutrient broth (lanes 1-6 and 1-4). M, *Lamda Pst* molecular weight marker; C, negative control.

Figure 2.4A displays single bands above 11 501 bp which indicates the successful isolation of genomic DNA. Lanes 4 and 5 contain bands at the bottom of the gel as well. These could be fragments of DNA, or RNA that was not successfully removed. Figure 2.4B displays single bands of 1500 bp for all successful PCR reactions. Lane 2 did not contain a band which might be because the PCR primers were not compatible with this strain. Alternative primers (fD1 and rD1; or GmF5 and R907) were used for those strains that were not successfully amplified using primers 9F and 1541R, as seen in Lane 2. However, the 16S rDNA region of sample strain 19 (Table 2.1) was not successfully sequenced with any of the available primers. The 16S rDNA sequences obtained from Inqaba Biotec were analysed using nucleotide-nucleotide BLAST. The 16S rDNA sequences had between 96 and 99% identity to sequences in the database and the species were identified according to these sequence matches.

A summary of the results from all the above screening criteria and species identification are shown in Table 2.1.

Table 2.1 Relevant characteristics for screening bacterial strains isolated from various sources. Positive/present (+); negative/absent (-).

Name	Source	Cell Shape	Gram Stain	Spores	Catalase Test	MEC	Species
C1W	compost	Rods	+	+	+	+	*Bacillus cereus*
C1O	compost	Rods	+	+	+	+	
C2W	compost	Rods	+	+	+	+	*Lysinibacillus sp.* BG-B112
C2O	compost	Rods	+	+	-	+	
C3W	compost	Rods	+	+	-	+	
C3O	compost	Rods	+	+	+	+	*Bacillus thuringiensis*
S1W	soil	Rods	+	+	-	+	
S1O	soil	Rods	+	+	+	+	*Brevibacillus parabrevis*
S2W	soil	Rods	+	+	-	+	
S2O	soil	Rods	+	+	+	+	
S2Y(D)	soil	Rods	+	+	+	+	*Lysinibacillus boronitolerans*
S2Y(L)	soil	Rods	+	+	+	+	*Bacillus subtilis*
S2WD	soil	Round	+	+	-	+	
18	compost	Rods	+	+	+	+	
19	cow dung	Rods	+	+	+	+	
43	compost	Rods	+	+	+	+	*Brevibacillus borstelensis*

Table 2.1 indicates that all isolated strains were Gram-positive bacteria that produced spores and at least one MEC. Strains that were catalase negative were probably not *Bacillus* species and were disregarded in further studies.

Bacillus species that had the best ability to degrade cellulose, as shown by the enzyme activity assays (Figures 2.5, 2.6 and 2.7), were chosen for 16S rDNA sequencing. Cultures S2Y(D) and S2Y(L) were initially isolated as one culture (S2Y), but it was later discovered that S2Y consisted of a co-culture of two morphologically different strains (See Chapter 3). However, in this chapter S2Y had not yet been separated into S2Y(D) and S2Y(L).

2.3.2 Screening for candidate Bacillus *strains based on (hemi-)cellulolytic activities*

Primary screening:

The crude supernatants of strains C1W to S2WD (Table 2.1), cultured in the initial enrichment broth containing 0.5% (w/v) Avicel®, were tested for cellulolytic and hemicellulolytic activities using Avicel®, CMC and xylan substrates (Figure 2.5).

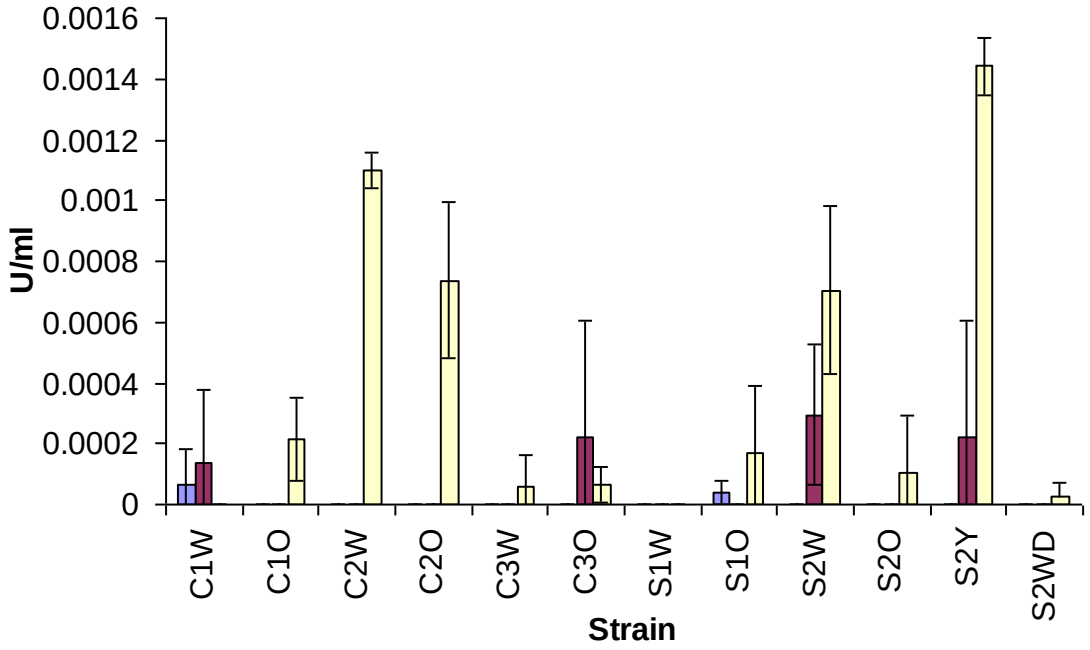


Figure 2.5 Avicelase; CMCase and xylanase activities (U/ml) of concentrated culture supernatants from strains grown in enrichment media containing 0.5% (w/v) Avicel®. U = µmol reducing sugar produced/hour. High standard deviations were due to very low activity.

Figure 2.5 demonstrates that strains C2W and S2Y had the highest xylanase activity compared to the other strains. Strains C2O and S2W also had high xylanase activities but it is possible that these were not *Bacillus* strains since they were catalase negative (Table 2.1). Strain C1W had the highest ability to degrade Avicelase which indicated the presence of exoglucanase and endoglucanase activity. Strain C30 had the next highest CMCase activity after S2W and S2Y, indicating the presence of endoglucanases. Therefore, from the information obtained in Table 2.1 and Figure 2.5, the strains C1W, C2W, C3O and S2Y were selected for further screening.

The enzyme activities in Figure 2.5 were very low and exhibited very large standard deviations. Larger U/ml values would automatically result in smaller standard deviations. The protein concentrations in the samples in Figure 2.5 were also low. The low activities and low protein might be explained by the use of Avicel® as the carbon source in the enrichment media. The strains did not grow easily on Avicel®, as indicated by low OD600 readings of the cultures (data not shown). This is probably linked to their inefficiency, and in some cases inability, to produce Avicel®-degrading enzymes. For this reason the secondary screening included growth on Avicel®, CMC and xylan to compare the enzyme activities obtained when the carbon source is different. To improve enzyme activities, a new enrichment was also used with yeast and ammonium sulphate as nitrogen sources instead of peptone.

Secondary screening:

The four selected strains (C1W, C2W, C3O and S2Y) were cultured from glycerol stocks in fresh enrichment medium with Avicel®, CMC or xylan as the carbon source. The activities in the supernatants were compared to those observed in the strains donated by Rhodes University, which had been cultured from glycerol stocks in the same media (Figure 2.6 and 2.7).

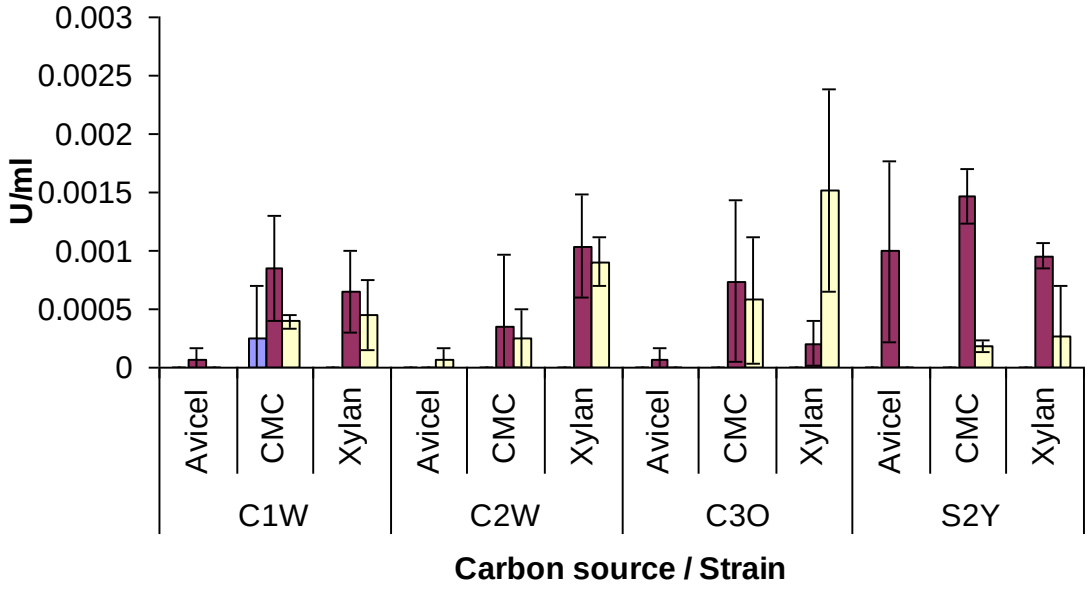


Figure 2.6 Avicelase, CMCase and xylanase activities (U/ml) of the crude supernatants of cultures grown in enrichment media containing 0.5% (w/v) Avicel®, CMC or xylan. U = µmol reducing sugar produced/hour.

Figure 2.6 demonstrates an increase in activity when the strains were cultured on CMC and xylan compared to Avicel®. However, the activities were still low, resulting in large standard deviations. Strain C1W was the only culture with Avicelase activity and when cultured on CMC it exhibited activity on all three substrates. Strain S2Y had relatively high CMCase activity when cultured on all three substrates.

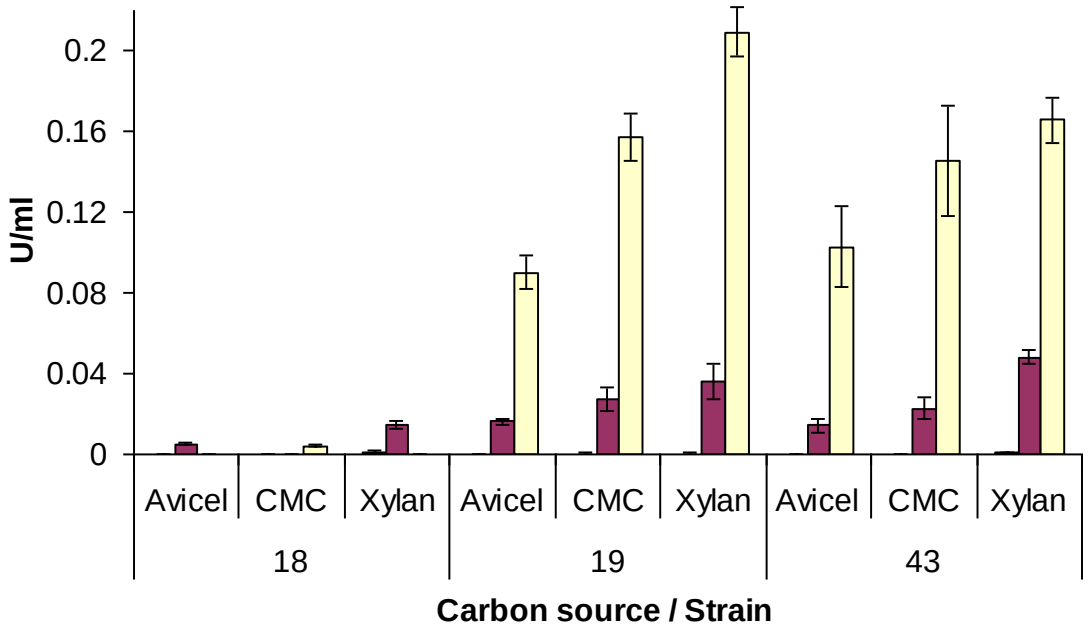


Figure 2.7 Avicelase, CMCase and xylanase activities (U/ml) of the crude supernatants of cultures donated by the Rhodes University Undergraduate Laboratories grown in enrichment media containing 0.5% (w/v) Avicel®, CMC or xylan. U = µmol reducing sugar produced/hour.

Figure 2.7 shows that strains 19 and 43 donated by Rhodes University Undergraduate Laboratories had much higher CMCase and xylanase activities compared to the strains in Figure 2.6. Once again, enzyme activity was higher when these strains were grown on xylan, followed by CMC and Avicel®. Strain 43 exhibited a small amount of Avicelase activity when grown on xylan.

From Table 2.1 and Figures 2.6 and 2.7 strains 43, 19 and S2Y were selected for further screening because they had cellulolytic and hemicellulolytic enzyme activities when grown on all three substrates. Strain C1W was also selected for further

screening because of its Avicelase activity which is important for the degradation of natural substrates.

2.3.3 MEC-associated (hemi-)cellulolytic activities

The purpose of this research is to purify a (hemi-)cellulolytic MEC from a *Bacillus* strain. To measure the cellulolytic and hemicellulolytic activities specifically associated with the MECs produced by the selected stains, the supernatants of each enrichment broth culture were first run on a Sepharose 4B size exclusion column to separate the large protein fractions from the small (Figure 2.8).

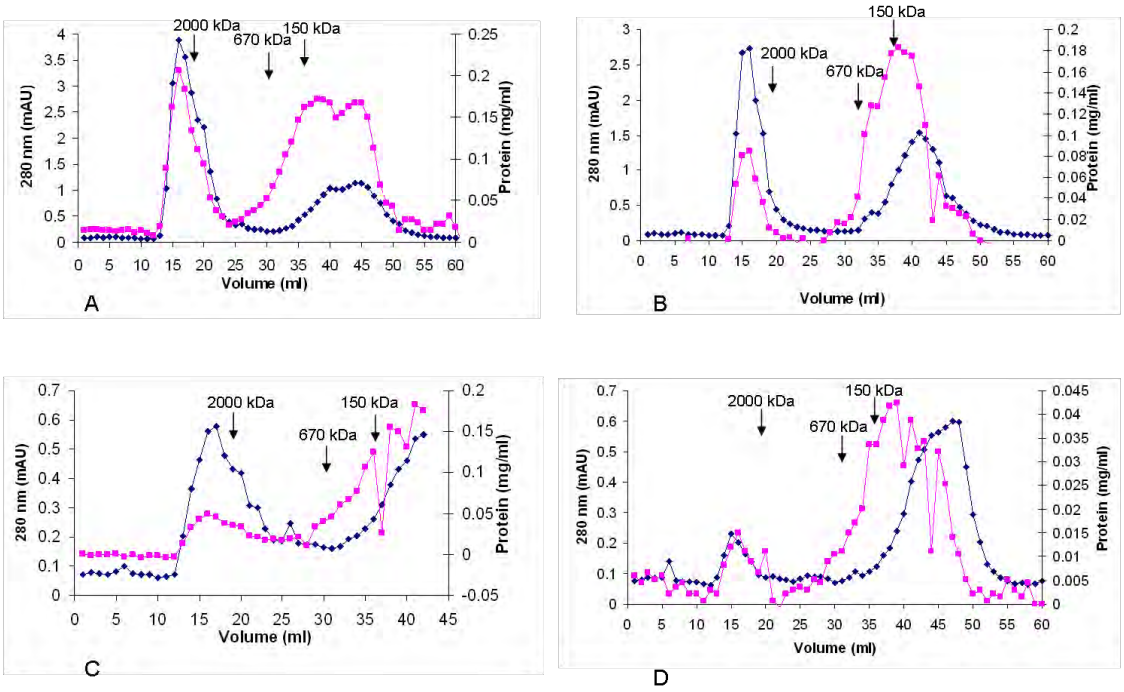


Figure 2.8 Sepharose 4B chromatograms of concentrated culture supernatants from strains **A:** C1W; **B:** S2Y; **C:** 19 and **D:** 43 grown in enrichment media containing 0.5% (w/v) birchwood xylan. Column dimensions: 28 × 1.5 cm; Flow rate: 20 ml/hour. ♦ A_{280} (mAU); ■ protein (mg/ml).

Two major peaks were seen in the Sepharose 4B chromatograms of the selected strains, one peak which represented small, free proteins (between 35 and 45 ml, Figure 2.8) and one which represented a large protein complex or aggregated protein (17 or 18 ml, Figure 2.8). According to the Sepharose 4B calibration curve (Appendix 1, Figure A5) these two peaks represented proteins of 190 kDa and smaller and approximately 2000 kDa, respectively. The fractions in each of these protein

peaks were pooled, concentrated and assayed for Avicelase, CMCase and xylanase activity (Table 2.2).

Table 2.2. Avicelase, CMCase and xylanase activities (U/mg total protein) in the 2000 kDa (MEC) and 1-190 kDa (free protein) peaks obtained from Sepharose 4B chromatography. U = µmol of reducing sugar produced/hour.

Strain	Protein Peak	Enzyme Activity (U/mg)		
		Avicelase	CMCase	Xylanase
C1W	**MEC**	**0.139**	**0.133**	**0.567**
C1W	Free protein	0.003	0.000	1.201
S2Y	**MEC**	**1.140**	**0.792**	**2.841**
S2Y	Free protein	0.000	0.012	1.116
19	**MEC**	**0.009**	**0.00**	**0.191**
19	Free protein	0.011	0.127	0.474
43	**MEC**	**0.026**	**0.004**	**0.052**
43	Free protein	0.000	0.374	0.952

Table 2.2 shows that S2Y had the highest Avicelase, CMCase and xylanase activities in its _MEC' fraction compared to the other three strains. The free protein in sample S2Y had high xylanase activity, but low CMCase and Avicelase activities. Although samples 19 and 43 had high enzyme activities in Figure 2.7, Table 2.2 shows that most of this activity was due to free proteins. Therefore, strain S2Y was selected for all subsequent experiments in this research project and, according to Table 2.1, strain S2Y (L) was renamed *Bacillus subtilis* SJ01.

2.4 Discussion and Conclusions

Isolation of cellulolytic microorganisms using a selective medium was successful, and due to the abundance of these organisms in the chosen environments (soil, compost and cow dung), it was easy to obtain a collection consisting of several different species and strains (Table 2.1). It is interesting to note that the majority of the cultures isolated displayed the morphological characteristics of bacilli (Table 2.1). This means that isolating organisms aerobically in a cultured medium that was selective for cellulose-degraders limited the number of microorganisms that were not of the genus

Bacillus. It also means that *Bacillus* was a predominant bacterial genus in environments where cellulose and hemicellulose were the major carbon sources. Those strains in Table 2.1 that were not identified as bacilli could only be distinguished by testing for catalase production. Testing for catalase activity is, therefore, a useful method to differentiate between bacilli and other similar, rod-shaped, Gram positive bacteria like clostridia which are catalase negative.

It is also interesting to note that all the bacterial strains in Table 2.1 had protein peaks in the void volume of the Superose 12 size exclusion chromatography column, indicating the possibility of MEC production. It might be concluded that all the isolated microorganisms required a MEC to degrade the available carbon sources, and that there are a large number of organisms capable of producing MECs. However, it is possible that the peaks above 2000 kDa are due to protein aggregation and are not true MECs. There are several criteria, other than size, that have been used to determine the presence of a cellulosome-type MEC, such as a cellulose binding domain that has the ability to bring the cells in contact with the substrate (Pason *et al.,* 2006b). These criteria will be addressed further in Chapter 5.

In the primary screening study it was discovered that the isolated strains grew slowly in media containing Avicel® as the only carbon source. The resulting enzyme activities of these cultures were very low (Figure 2.5), indicating that Avicel® was not a good carbon source for the production of cellulolytic and hemicellulolytic enzymes from the strains in Figure 2.5. In a similar experiment by Pason *et al.* (2006a) *Bacillus* strains cultured on Avicel® exhibited no CMCase or xylanase activities, but when grown on xylan the strains were able to degrade both substrates. In accordance with these results, Figures 2.6 and 2.7 demonstrated that using CMC and xylan as carbon sources resulted in higher production of cellulolytic and hemicellulolytic enzymes. From this observation it was concluded that an optimisation study was required to determine the growth conditions under which the best cellulolytic and hemicellulolytic enzyme activities are obtained (Chapter 3).

Due to the low enzyme activities observed in primary screening, a secondary screening was undertaken using the four strains selected from primary screening and an additional three strains that were donated to this project. The strain C1W was able

to degrade Avicel®, CMC and xylan substrates, indicating that this culture showed the greatest potential for the degradation of lignocellulose, which contains amorphous cellulose, crystalline cellulose and hemicellulose (Section 1.1). However, in Figure 2.6 the enzymes activities of C1W were very low compared to strains S2Y, 19 and 43. Strains 19 and 43 had particularly high xylanase activities, indicating their ability to degrade hemicellulose more readily than cellulose (Figure 2.7). The ability of strains S2Y, 19 and 43 to produce relatively high enzyme activity (particularly xylanase) when grown on Avicel®, CMC or xylan is important in terms of their versatility. In other words, these organisms have the potential to be used for enzyme production and enzymatic hydrolysis in various industries and in applications requiring growth on a variety of substrates, such as waste conversion.

In contrast to previous results in which the enzyme activities in the crude supernatants were measured, once the MECs of strains C1W and S2Y had been purified and concentrated these strains displayed better potential as candidates for the production of (hemi-)cellulolytic MECs than strains 19 and 43 (Table 2.2). Table 2.2 also demonstrated that the MEC fraction of S2Y had higher Avicelase, CMCase and xylanase activity than C1W. This means that the objective of the screening was achieved and strain S2Y (renamed *Bacillus subtilis* SJ01) was selected for further research. However, the crude supernatants were concentrated using PEG 20 000 prior to separation on the Sepharose 4B column, which might have caused protein aggregation resulting in a high molecular weight protein fraction. Therefore, a purification protocol that limits the possibility of protein aggregation is required (See Chapter 4).

Figure 2.8 and Table 2.2 demonstrated that the high enzyme activities in strains 19 and 43 were due to free proteins. Combinations of enzymes connected in a protein complex are thought to be more efficient at degrading complex substrates than a collection of free proteins, and so although these strains can efficiently degrade CMC and particularly xylan, they would probably be less efficient at degrading complex natural lignocellulose substrates and so will not be studied further in this research.

CHAPTER 3

Optimisation of the production of cellulolytic/hemicellulolytic enzymes from *Bacillus subtilis* SJ01

3.1 Introduction

In Chapter 2 it was apparent that changing the carbon source in the growth medium led to an increase in cellulolytic and hemicellulolytic enzyme activities produced by the *Bacillus* strains. There are many factors that can affect the rate of production of enzymes by bacteria, including available nutrients, incubation time, temperature, pH, size and age of inoculum and protease production (Battan *et al.,* 2007; Kapoor *et al.,* 2008). Several studies have been performed in which cellulolytic activity was measured while varying the factors mentioned above to find the growth conditions that are optimal for enzyme production (Battan *et al.,* 2007; Dhillon *et al.,* 2000; Kapoor *et al.,* 2008).

A bacterial culture always requires a carbon source (for the development of cell material), and a nitrogen source (for the manufacture of amino acids and nucleic acids) (Madigan *et al.*, 2003). There are various compounds that can be used to provide these nutrients. For the purposes of producing cellulolytic and hemicellulolytic enzymes, the carbon source must be a cellulosic substance that induces the production of these enzymes. As observed previously, some cellulosic substrates induced higher enzyme activities than others (Chapter 2). The same variation in enzyme production can be observed with different nitrogen sources. Common sources of nitrogen used in growth media are ammonium or nitrate compounds and yeast lysate (Battan *et al.,* 2007; Kapoor *et al.,* 2008). Peptone is also used as a nitrogen source, but interestingly, peptone induces the production of proteases which, in turn, degrade other types of enzymes that are released simultaneously (Battan *et al.,* 2007). Many *Bacillus* species, including *B. subtilis*, produce proteases (Abdulkarim *et al.,* 2005; Doi, 1991), which could lead to a decrease in cellulolytic and hemicellulolytic enzyme activities. Besides carbon, nitrogen and oxygen, many other elements, vitamins and minerals are important nutrients required for cell growth and various metabolic pathways (Dhillon *et al.,* 2000). Although these are not required in high concentrations, they may also effect

the production of particular enzymes, depending on the structure of the enzyme and the process required for its manufacture (Madigan *et al.*, 2003).

The period of time for which cells are cultured before harvesting will also affect the amount of enzymes in the harvested sample. This is because cells produce enzymes at different phases of their growth. Bacterial cultures have three phases of growth – an initial slow phase, called the lag phase, where cells are just beginning to multiply; a log or exponential phase where cells are multiplying exponentially; and finally a stationary phase in which cells stop multiplying due to a limiting factor (usually insufficient nutrients or inhibition by a product) (Harwood, 1992; Madigan *et al.*, 2003). These phases of growth can be visualised on a growth curve, by measuring the amount of cells at different times. Growth curves have also been used to study the release of enzymes into the culture supernatant by measuring enzyme activity at time intervals (Pason *et al.,* 2006a; Waeonukul and Ratanakhanokchai, 2007; Zhang *et al.,* 2004). According to Harwood (1992), towards the late exponential phase and during the stationary phase of growth, fluctuations in nutrient supply induce the production of hydrolytic enzymes in *B. subtilis,* including proteases and carbohydrases. This is in conjunction with research into *B. subtilis* and other *Bacillus* species which demonstrate that cellulases and xylanases are often released during the stationary phase of growth (Heck *et al.,* 2006; Robson and Chambliss, 1984; Sá-Pereira *et al.,* 2002b).

The aim of this chapter was to increase the cellulolytic and hemicellulolytic enzyme concentrations produced by *B. subtilis* SJ01 by eliminating competing bacteria, inhibiting proteases, optimising nutrients in the medium and optimising the incubation time.

3.2 Materials and Methods

3.2.1 Separation of co-culture

It was observed that the S2Y culture sometimes displayed light yellow colonies and sometimes much brighter, darker yellow colonies when grown on agar, indicating the possibility that S2Y may exist as a co-culture (two strains or species growing

together). One colony of each morphology, now designated S2Y(L) and S2Y(D), was picked and cultured separately in enrichment media for approximately 48 hours and then centrifuged at 12 000*g* for 15 minutes. The supernatants of each were tested for Avicelase, CMCase and xylanase activities using the DNS assay (incubating at 50°C for 18 hours), and total protein using the Bradford assay.

3.2.2 Detection of proteases

Bacteriological agar was prepared and 10% (v/v) skimmed milk added. Several plates were made from this ‗milk agar' and four small wells were bored into each plate. A 24 hour culture of *B. subtilis* SJ01, which had been grown in enrichment medium at 37°C and 200 rpm, was centrifuged at 3000*g* for 5 minutes. The crude, supernatant and pellet (100 µl of each) were inoculated into the wells of the milk plates in triplicate. A control of distilled water was used in the fourth well of each plate. The plates were incubated at 37°C for 48 hours and then examined for clear zones around the wells which would indicate the degradation of the casein in the milk.

3.2.3 Nutrient optimisation

Bacillus subtilis SJ01 was cultured in several enrichment media varying in carbon source, nitrogen source and presence of vitamins and trace elements. The enrichment media contained 2 g/l NaCl, 0.5 g/l $MgSO_4.7H_2O$, 2 g/l K_2HPO_4, 2 g/l KH_2PO_4, 5 g/l of a carbon source (Avicel®, CMC or birchwood xylan), 5 g/l of a nitrogen source (yeast, peptone, tryptone, $(NH_4)_2SO_4$ or NH_4Cl) or a combination of nitrogen sources often seen in the literature (5 g/l each: yeast + peptone, tryptone + NH_4Cl, yeast + $(NH_4)_2SO_4$, yeast + tryptone, 2 g/l each: yeast + tryptone + peptone + NH_4Cl) and 0.05 % (w/v) vitamin solution (containing 0.04 g/l biotin, 0.04 g/l folic acid, 0.2 g/l pyridoxine hydrochloride, 0.002 g/l vitamin B12 and 0.1 g/l ρ-aminobenzoic acid) or 1 % (v/v) trace element solution. The trace element solution was made up by adding 1 ml of 15 g/l nitrilotriacetic acid and 0.5 ml of a solution containing 10 g/l $MnSO_4.2H_2O$, 2 g/l $FeSO_4.7H_2O$, 2 g/l $CoCl_2.2H_2O$, 2 g/l $CaCl_2.2H_2O$, 2 g/l $ZnSO_4$, 0.2 g/l $CuSO_4.5H_2O$, 0.2 g/l $AlCl_3$, 2 g/l H_3BO_3, 0.2 g/l $Na_2MO_4.2H_2O$ and making up the volume to 50 ml with distilled water.

For the determination of the best nitrogen source 5 g/l xylan was used as the carbon source and no vitamins or trace elements were added. For the determination of the best carbon source 5 g/l yeast + 5 g/l $(NH_4)_2SO_4$ was used as the nitrogen source and no vitamins or trace elements were added. For determining the effects of vitamins and trace elements, 5 g/l xylan and 5 g/l yeast + 5 g/l $(NH_4)_2SO_4$ were used as the carbon and nitrogen sources, respectively.

For each growth medium a 20 µl volume of a *B. subtilis* SJ01 glycerol stock was inoculated into 50 ml media in triplicate and the cultures grown at 37°C on a 200 rpm shaker for 48 hours. The cell growth of each culture was determined by measuring the OD at 600 nm. Samples of each culture were centrifuged at 3000*g* for 5 minutes, the total protein was measured using the Bradford assay and the Avicelase, CMCase and xylanase activities were measured using the DNS assay for 18 hours.

3.2.4 Growth curve

The optimum enrichment media, containing 5 g/l xylan, 2 g/l NaCl, 0.5 g/l $MgSO_4.7H_2O$, 2 g/l K_2HPO_4, 2 g/l KH_2PO_4, 5 g/l yeast, 5 g/l $(NH_4)_2SO_4$, 0.05% (v/v) vitamin solution, 1% (v/v) trace element solution and 0.1% (v/v) of 0.035 g/ml phenylmethanesulfonyl fluoride (PMSF) (Fluka) dissolved in ethanol, was made up in a total of 500 ml distilled water. The vitamins and PMSF were added after autoclaving. A 3 ml sample was removed for the time = 0 hours sample and then 500 µl of *B. subtilis* SJ01 was inoculated into the media and cultured at 37°C on a 200 rpm shaker. Samples (3 ml) were then taken regularly for a total of three weeks (516 hours). The pH and OD600 of the each sample were determined and then the samples centrifuged at 3000*g* for 5 min. The total protein (Bradford assay), total reducing sugar (300 µl sample -without prior incubation - added to 600 µl DNS reagent, boiled for 5 minutes, put on ice and read at 540 nm) and Avicelase, CMCase and xylanase activities (DNS assay, incubating at 50°C for 18 hours) of the pellets and supernatants at each time interval were measured.

3.3 Results

3.3.1 Separation of the co-culture

Two colonies of different morphologies were seen in the culture S2Y when grown on enrichment agar. These were cultured up separately in liquid media and the crude supernatants tested for Avicelase, CMCase and xylanase activities (Figure 3.1).

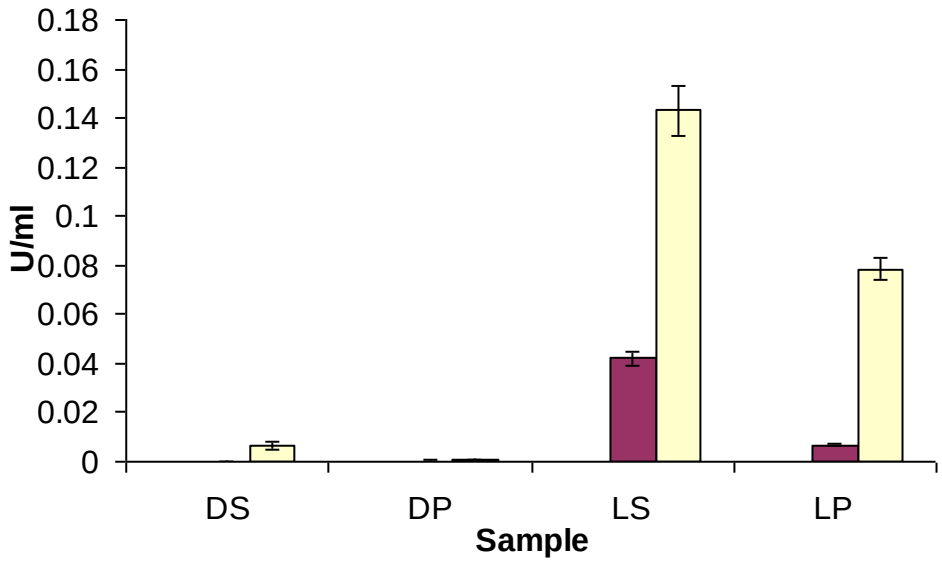


Figure 3.1 Avicelase, CMCase and xylanase activities (U/ml) in the culture supernatants (S) and pellets (P) of two strains, one with dark colonies (D) and one with light colonies (L), that previously grew together in culture S2Y. U = µmol reducing sugar produced/hour.

Figure 3.1 clearly demonstrates that the culture S2Y(D) had much lower CMCase and xylanase activities than the culture S2Y(L). According to the 16S rDNA sequences that were analysed for each of these cultures, S2Y(D) and S2Y(L) were in fact different species - *Lysinibacillus boronitolerans* and *B. subtilis*, respectively (See Chapter 2, Table 2.1). The co-occurrence of these two species is a possible reason for the variation in enzyme activities seen in S2Y during the screening process (Chapter 2). Once separated from the *L. boronitolerans* strain, *B. subtilis* SJ01 had a much higher ability to degrade Avicel®, CMC and xylan. This probably indicates that *L. boronitolerans* was competing with *B. subtilis* SJ01 and thus decreasing and destabilising the overall cellulolytic and hemicellulolytic enzyme activities displayed.

3.3.2 Detection of proteases

Another possible for reason for low enzyme activity, such as in the screening process (Chapter 2), might have been due to the release of proteases from the bacteria which degrade cellulolytic and hemicellulolytic enzymes. To test for the presence of proteases *B. subtilis* SJ01 was grown on agar containing casein from skim milk (Figure 3.2).

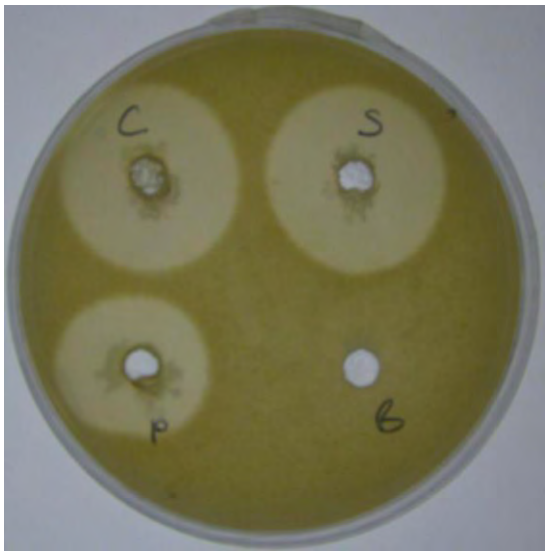


Figure 3.2 Bacteriological agar containing 10% (v/v) skim milk. Wells were inoculated with 100 µl of *B. subtilis* SJ01 grown in enrichment media; C: crude; S: supernatant; P: pellet; B: blank (distilled water).

Figure 3.2 shows that the *B. subtilis* SJ01 culture released proteases into the culture supernatant and also contained proteases in the cellular pellet. These proteases degrade the casein in milk, leaving a clear zone in the cloudy ‗milk agar'. These proteases have the ability to degrade other proteins and could potentially cause a decrease in the cellulolytic and hemicellulolytic enzyme activities. For this reason, 0.1% (v/v) PMSF (Bollag *et al.*, 1996) was added to the enrichment medium in all subsequent experiments.

3.3.3 Nutrient optimisation

To find the medium that induces the highest expression of (hemi-)cellulolytic enzymes by *B. subtilis* SJ01, varying combinations of nutrients were used in the

culture medium and Avicelase, CMCase and xylanase activities of the culture supernatants were measured (Figure 3.3).

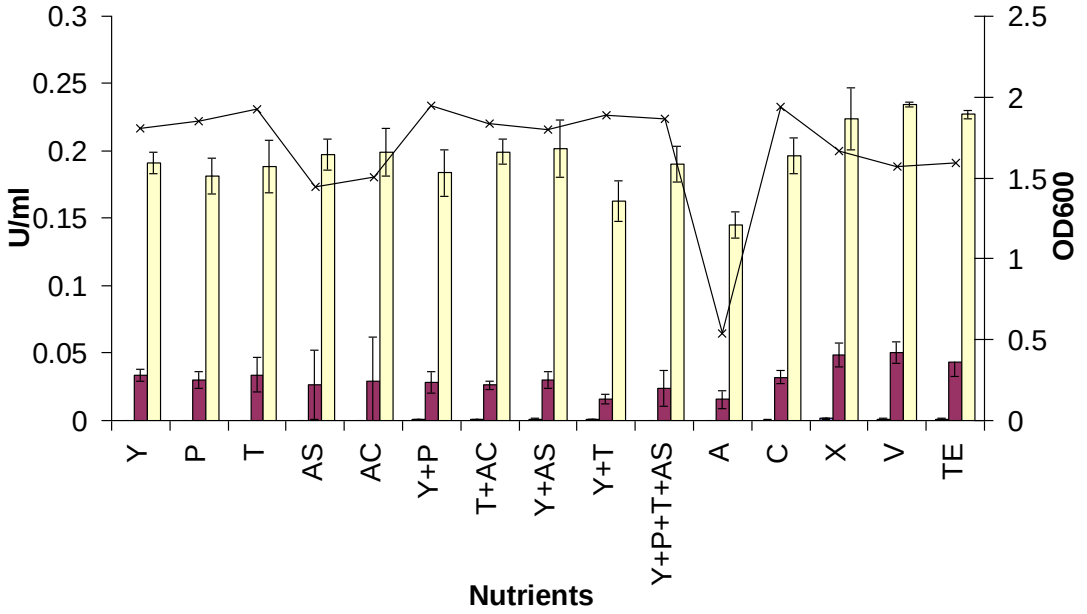


Figure 3.3 Nutrient optimisation for *B. subtilis* SJ01 where the ▮ Avicelase, ▮ CMCase and ▮ xylanase activities (U/ml), and × OD600 were measured after 48 hours growth in media containing varying nitrogen (yeast (Y), peptone (P), tryptone (T), $(NH_4)_2SO_4$ (AS), NH_4Cl (AC) or a combination) and carbon sources (Avicel (A), CMC (C) or xylan (X)) and in the presence and absence of vitamins (V) and trace elements (TE).

Figure 3.3 shows that a combination of yeast and $(NH_4)_2SO_4$ produced the highest xylanase activity (0.202 ± 0.021 U/ml). Yeast as the only nitrogen source produced the highest CMCase activity (0.034 ± 0.004 U/ml), but this was only slightly higher than the value for yeast and $(NH_4)_2SO_4$ together (0.030 ± 0.006 U/ml). All values for Avicelase were very low and barely detectable. Such low U/ml values had large standard deviations and so were not taken into account. Cell growth, measured by the OD600, in the media containing yeast and $(NH_4)_2SO_4$ was also high, and this combination of nitrogen sources was therefore used in all subsequent experiments.

When birchwood xylan was used as the carbon source *B. subtilis* SJ01 produced the highest amount of xylanase and CMCase. Although growth was slightly higher with CMC as the carbon source, a higher enzyme activity is more important for this study and thus birchwood xylan was used as the carbon source in all subsequent experiments.

The addition of both vitamins and trace elements increased the amount of xylanase produced, and the addition of vitamins also increased the CMCase activity.
These results, therefore, correlated with those of the preliminary nutrient optimisation study (Section 2.2) and the enrichment medium used in all subsequent experiments contained the same nutrients as those used previously (5 g/l xylan, 2 g/l NaCl, 0.5 g/l $MgSO_4.7H_2O$, 2 g/l K_2HPO_4, 2 g/l KH_2PO_4, 5 g/l yeast, 5 g/l $(NH_4)_2SO_4$), with the addition of 0.05% (v/v) vitamin solution, 1% (v/v) trace element solution and 0.1% (v/v) PMSF (0.035 g/ml in ethanol).

3.3.4 Growth curve

A single culture of *B. subtilis* SJ01 was grown in the optimised enrichment medium and samples taken at regular time intervals were tested for growth, (hemi-)cellulolytic enzyme activities, pH and total reducing sugars. This experiment is important to indicate the point during growth where the highest concentrations of (hemi-)cellulolytic enzymes are produced by the bacteria, and at what point the enzymes are released from the cells into the medium.

The OD600 in Figure 3.4 shows that *B. subtilis* SJ01 grew exponentially for approximately 24 hours. The OD600 then increased slowly to a peak before decreasing slightly at 84 hours (3.5 days) and then stabilising, indicating that stationary growth phase was reached.

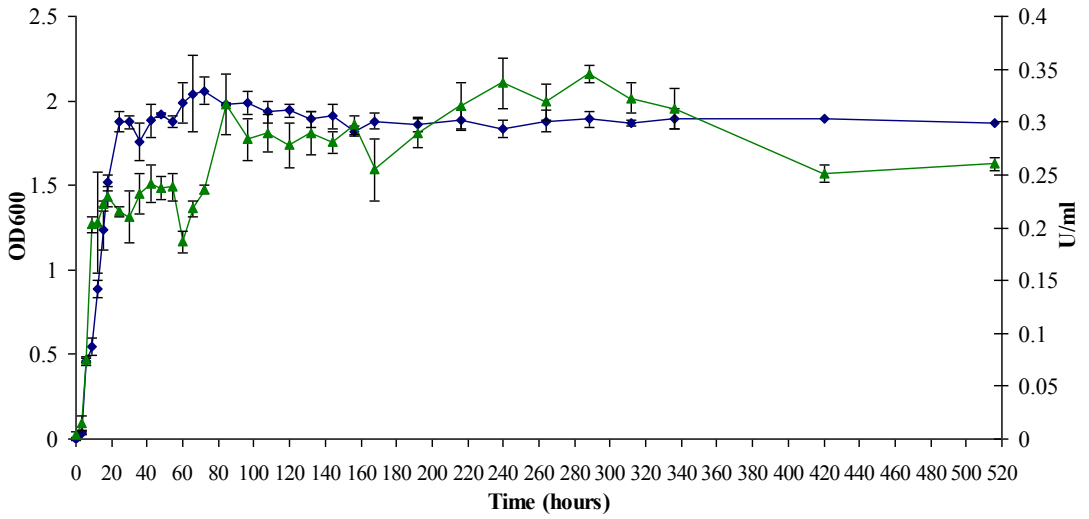


Figure 3.4 Cell growth (♦ OD600) and xylanase activity (▲U/ml) in the culture supernatant of *B. subtilis* SJ01 grown in enrichment medium for a total of 3 weeks.

Figure 3.4 demonstrates that xylanase enzymes were produced immediately by the bacteria and continued to be produced rapidly for the first 18 hours. A cyclic production of xylanase enzymes was seen, where the production increased to a peak, plateaud and then decreased slightly before starting to increase again. This was observed at least three times during the three week experiment with peaks at 18 hours, 84 hours (3.5 days) and 240 hours (10 days). Smaller cycles were observed with peaks at 42 hours and at 288 hours (12 days). The more general trend in xylanase production coincided with the growth of the bacteria, where rapid production occurred at the same time as exponential growth (0 to 24 hours) and then curved off before becoming more stable after 84 hours. Although xylanase production continued in the stationary growth phase, the rate of production was much slower. Eventually, in the third week, xylanase activity decreased probably due to depletion of the xylan substrate and because the cells began to die.

According to Figure 3.5 the pH of the culture remained relatively stable after the initial 3 to 4 days of rapid growth and enzyme production. During these 4 days the metabolic reactions demonstrated in Figure 3.4 caused the slight fluctuations in pH seen in Figure 3.5. Figure 3.5 also indicates that there was a decrease in the pH of the culture medium which coincided with a large amount of total reducing sugars released into the culture supernatant. This rapid increase in total reducing sugars occurred in the exponential growth phase and corresponded with the rapidly increasing xylanase

activity. It demonstrated that as the bacterial culture reached exponential phase, cells started to produce large amounts of xylanase enzymes required for the degradation of the only available carbon source in the media, xylan. The xylan was broken down into xylose, a reducing sugar, and so large amounts of xylose were detected in the media. These sugars were then consumed by the bacteria so that they could continue to grow and multiply, which explains the rapid decrease in total reducing sugars between 12 and 36 hours. Eventually the bacteria reached stationary growth in which they produced reducing sugars at relatively the same rate at which they consumed them, and so only small amounts were detectable in the medium. The depletion of the carbon source would also cause the decrease of reducing sugars between 12 and 36 hours.

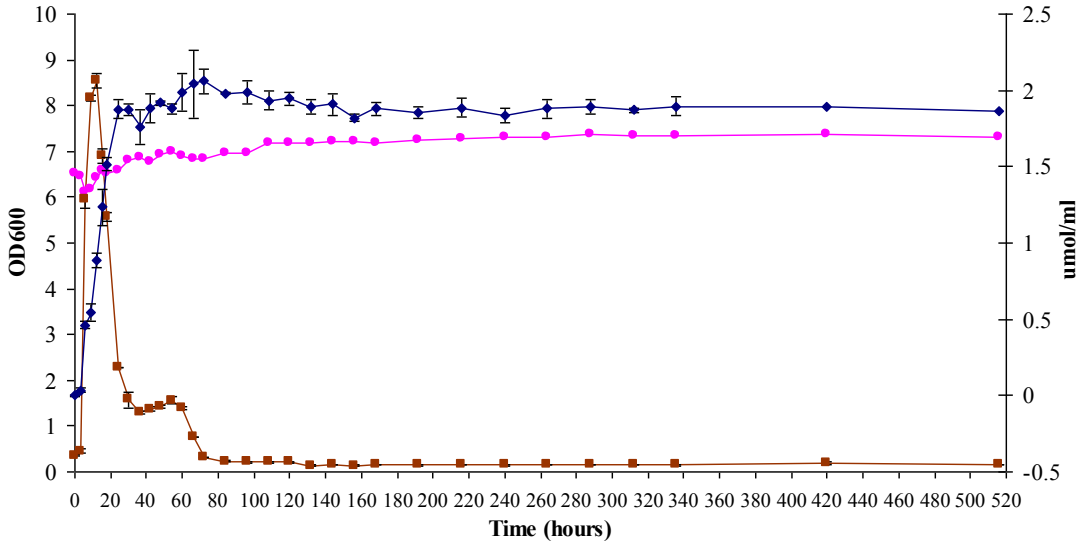


Figure 3.5 Cell growth (♦ OD600), ● pH and ■ total reducing sugar (µmol/ml) in the culture supernatant of *B. subtilis* SJ01 grown in enrichment medium for a total of 3 weeks.

Figure 3.6 demonstrates that there was a large amount of cell-associated xylanases and the production of this with time correlated strongly with the xylanase that was released into the medium. Initially, there was less cell-associated xylanase than xylanase released into the medium. At 72 hours there were larger amounts of xylanase in the pellet than in the supernatants, but this decreased again until, at 192 hours (8 days), there was again less xylanase in the cellular fraction.

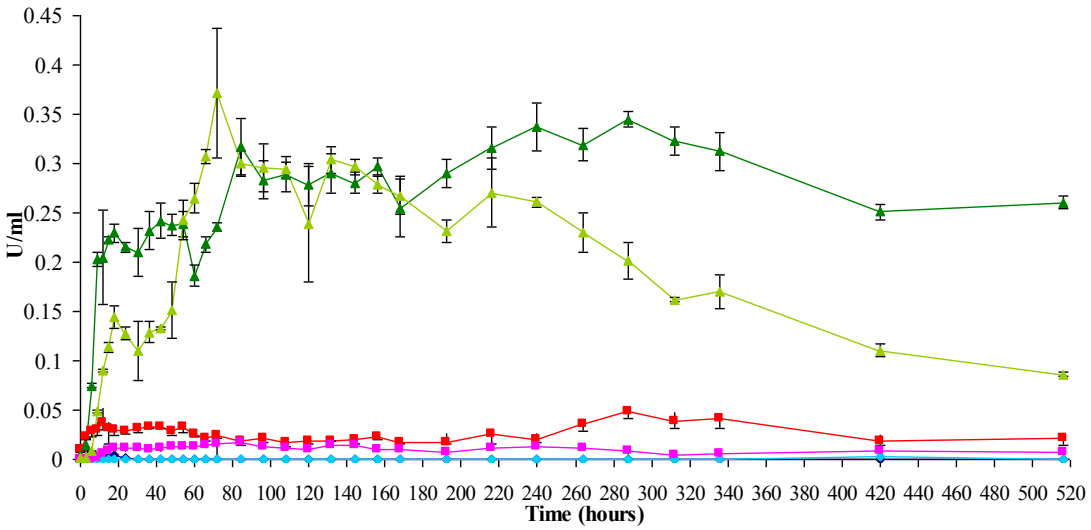


Figure 3.6 Avicelase (♦ ♦), CMCase (■ ■) and Xylanase (▲ ▲) activities (U/ml) in supernatants and pellets, respectively, of *B. subtilis* SJ01 grown in enrichment medium for a total of 3 weeks.

Figure 3.6 demonstrates that the CMCase and Avicelase activities were very low compared to xylanase and the standard deviations on these graphs were, therefore, very large, and so the trend in production of these enzymes was not discussed.

Using Figures 3.4 to 3.6 a time of 84 hours was chosen as the optimum cell harvesting time for all subsequent experiments. At this time the xylanase activity in the supernatant was 0.317 ± 0.028 U/ml, which is a 27.5% increase from the first peak at 18 hours, in other words an increase of 0.0871 U/ml in 66 hours. Although the activity at 288 hours was the highest in the growth curve, there is only an increase of 8.1% between 84 and 288 hours, which was a 0.0279 U/ml increase in 204 hours. After 84 hours the bacteria were in early stationary phase of growth, the pH was stable and the reducing sugars were being consumed at the same rate as they were produced.

3.3 Discussion and Conclusions

Microorganisms live naturally in complex ecosystems consisting of many different species (Madigan *et al.*, 2003). In a given environment where the external conditions are similar, the microorganisms living in the environment will have many of the same characteristics, for example the same ability to degrade a given nutrient. In the soils

sample collected from St. Francis Bay, South Africa there were initially two types of colonies detected. However, with further culturing and plating a third type of colony was discovered in the S2Y culture. It is possible that the two species in the S2Y culture were living symbiotically, making them difficult to separate initially; or perhaps during initial culturing the one species out-competed the other making the latter unnoticeable on the agar plates. Since *B. subtilis* SJ01 had much higher CMCase and xylanase enzyme activities than the *L. boronitolerans* strain (Figure 3.1), it is possible that *L. boronitolerans* was relying on *B. subtilis* SJ01 to degrade the available carbon source and provide nutrients in the form of sugars. To relieve *B. subtilis* SJ01 of the competition imposed by the co-cultured species, the two types of colonies were cultured separately from each other.

Bacillus species, including *B. subtilis*, produce large quantities of proteases and have been used in the commercial production of these enzymes (Doi, 1991). The production of proteases by *B. subtilis* SJ01, visualised by the degradation of casein in Figure 3.1, is detrimental to this study because the proteases may degrade the cellulolytic and hemicellulolytic enzymes that are produced simultaneously (Doi, 1991). Protease inhibitors are often used to prevent proteolysis and thus protect the enzyme of interest (Bollag *et al.*, 1996). A common protease inhibitor is phenylmethylsulfonyl fluoride (PMSF) which inhibits serine and thiol proteases (Bollag *et al.*, 1996). Ethylenediamine tetracetic aid (EDTA) is often used to inhibit metalloproteases (Bollag *et al.*, 1996), but it could not be used in this research because it might have caused the denaturation of the MEC if metal ions were involved in stabilising the complex.

Many different media have been used for the culturing of *Bacillus* species for cellulase and xylanase production (De Paolis and Lippi, 2008; Gessesse, 1998; Kapoor *et al.,* 2008; Kim and Kim, 1993; Mawadza *et al.,* 2000; Mayende, 2006). There have also been many studies to determine the optimal nutrients required for cellulase and/or xylanase production (Battan *et al.,* 2007; Dhillon *et al.,* 2000; Pham *et al.,* 1998; Sá-Pereira *et al.,* 2002a). In this chapter, a selection of the nutrients from the literature was tested to see which induced the highest (hemi-)cellulolytic enzyme activities in *B. subtilis* SJ01. The highest enzyme activity (0.224 ± 0.023 U/ml

xylanase and 0.0488 ± 0.009 U/ml CMCase, Figure 3.3) was seen with birchwood xylan as the carbon source, which indicated the ability of *B. subtilis* SJ01 to degrade and grow on this substrate. Battan *et al.* (2007) and Pham *et al.* (1998) obtained a similar result in which a higher xylanase activity (in U/ml) was obtained when birchwood xylan was used as the only carbon source than when alternative carbon sources were used. This also correlates with the large amount of literature describing the production of xylanases from many different species of *Bacillus* (Archana and Satyanarayana, 1997; Battan *et al.,* 2007; Dhillon *et al.,* 2000; Dhiman *et al.,* 2008; Kapoor *et al.,* 2008; Pham *et al.,* 1998) and from *B. subtilis* in particular (Jalal *et al.,* 2009; Sá-Pereira *et al.,* 2002a; Sá-Pereira *et al.,* 2004), indicating that xylanase enzymes are common in these bacteria.

A combination of yeast and ammonium sulphate was chosen as the optimum nitrogen source based on high xylanase activity (0.202 ± 0.021 U/ml, Figure 3.3). Various optimal nitrogen sources have been reported in previous experiments, but Battan *et al.* (2007), Kapoor *et al.* (2008) and Pham *et al.* (1998) all showed that yeast extract induced high xylanase activities. The optimal nitrogen source will be specific to the species and will depend on the amino acids required for the production of the enzymes of interest. Results obtained by Dhillon and co-workers correlate with the results in Figure 3.3 indicating that the addition of vitamins and trace elements increased the production of CMCase and xylanase enzymes (Dhillon *et al.*, 2000).

A growth curve reveals important information regarding the growth and metabolism of bacteria. The growth curve, indicated by the OD600 in Figure 3.4, shows that *B. subtilis* SJ01 had a typical growth pattern similar to that seen in all bacteria, with an exponential growth phase followed by a stationary growth phase. In *Bacillus* species it is common for the stationary phase to be reached between 24 and 48 hours (Kapoor *et al.,* 2008; Pham *et al.,* 1998; van Dyk *et al.,* 2009), which correlated with Figure 3.4 where the exponential phase ended after about 24 hours. The literature states that extracellular enzymes are released from bacilli in their stationary growth phase (Howard *et al.,* 2003; Robson and Chambliss, 1984; Sá-Pereira *et al.,* 2002a), which again correlated with the data in Figure 3.4 in with the xylanase activity was highest in the stationary phase. Therefore, to obtain the largest yield of xylanase

activity from *B. subtilis* SJ01 the cells must first reach stationary phase before they are harvested.

In the exponential growth phase of *B. subtilis* SJ01 there was a rapid increase in reducing sugars that coincided with the increase in cell density as well as a decrease in the pH (Figure 3.5). It is common to see changes in pH in a medium during bacterial growth and, in particular, sugar metabolism is linked to a decrease in pH (Paavilainen *et al.,* 1995). Paavvilainen and co-workers showed that reducing sugars were further catabolised into acids and carbon dioxide by *B. cereus (Paavilainen et al., 1995)*. This dehydrogenation of sugar molecules was required for the growth of bacteria, and eventually the pH increased again and became stable when the redox state was re-equilibrated (Paavilainen *et al.,* 1995). These findings correlated with the trends observed in Figure 3.5.

In cellulosome research a growth curve is often used to indicate the point at which the cellulosome dissociates from the cell, by comparing the cellulosomic enzyme activities in the culture supernatant and pellet over time. Bayer and co-workers stated that the cellulosome and cellulosomal enzymes are released from the pellet into the supernatant as the cell culture matured (Bayer *et al.,* 1983). In other words, for cellulosome-type complexes it would be expected to see high cell-associated cellulolytic activity during exponential growth and high cellulolytic activity in the supernatant during the stationary growth phase (Bayer *et al.,* 1983; Bayer *et al.,* 1994). Kosugi and co-workers found that when *Clostridium cellulovorans* was cultured on xylan, most of the xylanase activity was secreted into the culture supernatant throughout growth of the bacterial cells (Kosugi *et al.,* 2001). In contrast to both of these studies, Figure 3.6 demonstrates that the xylanase activity in the pellet of *B. subtilis* SJ01 remained high in early stationary phase, and decreased slightly only after 192 hours. At 84 hours, the optimal harvesting time with respect to xylanase production, there was high cell-associated and extracellular xylanase activity. However, the growth curve in Figure 3.6 did not indicate the amount of (hemi-)cellulolytic MECs in the pellet and supernatant, and rather the activity attributed to both complexed and non-complexed enzymes was measured. Therefore, the high cell-associated xylanase activity at 84 hours may be a result of MECs that had not yet been released from the cells, and perhaps always remains cell-associated;

or it may be due to free enzymes that remain cell-associated after the MEC has been released into the culture medium. It was, therefore, necessary to remove the enzymes from the pellet and purify this fraction along with the extracellular fraction to check for the presence of a (hemi-)cellulolytic MEC (Chapter 4).

The results in this chapter indicate that the removal of competing organisms, inhibition of protease action, addition of nutrients that are optimal for the production of (hemi-)cellulolytic enzymes, and harvesting of cells at a particular time, led to the increased cellulolytic and hemicellulolytic activities in *B. subtilis* SJ01.

CHAPTER 4

Purification of MECs from *Bacillus subtilis* SJ01

4.1 Introduction

The third objective of this research (Section 1.8) was to purify the multi-enzyme complex produced by *B. subtilis* SJ01 (selected in Chapter 2) after growth of this bacterium under optimal conditions for (hemi-)cellulolytic enzyme production (Chapter 3). Several techniques for the purification of cellulosomes and MECs have been used, many of which include affinity purification to cellulose and/or SEC (Lamed *et al.,* 1983; Morag *et al.,* 1992; Pason *et al.,* 2006a; Waeonukul *et al.,* 2009). Since it is not known whether *B. subtilis* SJ01 produces a cellulose-binding MEC, affinity purification could not be used. However, due to the large molecular weight of MECs, SEC is an established method of purification and was therefore used in this chapter (Cavedon *et al.,* 1990; Jiang *et al.,* 2005; van Dyk *et al.,* 2009).

A SEC column contains resin consisting of gel beads that are separated by pores. Smaller molecules will penetrate more deeply into this matrix by diffusing through the gel pores, and will take longer to run through the column; whereas larger molecules, which do not easily enter the gel pores, will run more quickly through the column (Bollag *et al.*, 1996; Simpson *et al.,* 2009). The gel matrix must be chosen according to its fractionation range which is determined by the pore size of the matrix. For the separation of MECs a gel matrix with a high molecular weight fractionation range is required. In Chapter 2, a Sepharose 4B gravity column was used, but a more modern and rapid method is the use of fast performance liquid chromatography (FPLC). A commonly used FPLC gel matrix for separation of MECs is Sephacryl S-300 (with a separation range of 10 to 1500 kDa) (Cavedon *et al.,* 1990; Jiang *et al.,* 2004; Lamed *et al.,* 1983; Pason *et al.,* 2006a). In this chapter a similar gel matrix, Sephacryl S-400 (20 – 8000 kDa separation range), was chosen because Chapter 3 indicated the presence of a protein complex of about 2000 kDa.

Unlike other chromatography techniques, SEC does not involve binding to the resin. This means that fragile protein complexes will not break apart during purification, which is possible with ion-exchange chromatography if there are enzymes of differing charge within a weakly bound complex. The disadvantage of SEC is the poor

resolution that results from this lack of retention (Simpson *et al.*, 2009). However, this will not affect the separation of MECs from non-complexed proteins.

Prior to loading onto a SEC column, crude enzyme preparations are concentrated. In Chapter 2 it was concluded that although a large protein fraction was eluted from size exclusion chromatography on Sepharose 4B, this fraction may be caused by protein aggregation during concentration with PEG 20 000 and may not be a true MEC. An alternative method of concentration is ultrafiltration (Cavedon *et al.,* 1990; Kim and Kim, 1993; Lamed *et al.,* 1985; Pohlschroder *et al.,* 1994; van Dyk *et al.,* 2009). This method involves a filter which retains proteins above a certain size and allows smaller molecules, such as water or buffer molecules, to pass though, thus concentrating the larger molecules. The flow of molecules across the filter is increased by the use of pressure. An important advantage of ultrafiltration is the variety of filters that can be used. If a filter with a higher molecular weight cut-off is used, then the method serves to not only concentrate proteins but also to specifically concentrate large molecules, such as MECs, away from smaller proteins which will also pass through the filter.

A more modern method of ultrafiltration is the use of centrifugal devices, also known as spin columns (Adams *et al.,* 2004; Han *et al.,* 2003). These devices use centrifugal force to push molecules through a filter, and in the same way as conventional ultrafiltration, the filter can be chosen depending on the size of the protein of interest. For MECs, a molecular weight cut-off of 100 kDa will ensure that non-complexed proteins will pass through the filter and MECs will remain behind. The use of centrifugal force is more rapid than the use of pressure in conventional ultrafiltration.

In Chapter 3 high cell-associated and extracellular xylanase activities were observed in *B. subtilis* SJ01. It is generally accepted that cellulolytic and hemicellulolytic enzymes and enzyme complexes are extracellular as they are required to break down substrates that are too large to enter the cell (Doi, 2008; Gilkes *et al.,* 1991; Howard *et al.,* 2003). However, studies on the cellulosome show that when the culture is in exponential growth phase this complex is attached to the outside of the cell (Bayer *et al.,* 1994). The complex can also bind to the substrate, thus bringing the cell in close contact with the substrate (Bayer *et al.,* 1994). In order to determine whether the MEC of *B. subtilus* SJ01 is cell-associated or extracellular at 84 hours of growth

(stationary phase) an MEC purification was performed on the culture supernatant and on the cellular fraction after washing the enzymes off the cells with 1 M NaCl (Cavedon *et al.,* 1990).

The aim of this chapter was to purify an MEC or MECs from *B. subtilus* SJ01 using conventional ultrafiltration, spin columns or PEG 20 000 to concentrate proteins, followed by SEC; and to investigate whether the cellular pellet contains (hemi-)cellulolytic MECs in stationary growth phase.

4.2 Materials and Methods

4.2.1 Purification using ultrafiltration

Bacillus subtilus SJ01 was cultured by inoculating 500 ml optimal enrichment medium (Chapter 3) with 500 µl of a *B. subtilus* SJ01 glycerol stock and incubating at 37°C and 200 rpm. After 84 hours of growth (Chapter 3, Growth Curve) the culture was centrifuged at 12 000*g* for 5 minutes. The supernatant was then concentrated by ultrafiltration using an Amicon 8200 ultrafiltration cell with a 50 kDa cut-off membrane (Millipore, South Africa). Proteins larger than 50 kDa remained on the filter and were resuspended in 20 mM Tris-HCl, pH 7.0. This retentate was purified on a Hi-Prep^{TM} 16/60 Sephacryl S-400 High Resolution size-exclusion column (GE Healthcare Bio-Sciences) connected to an ÄKTA™ fast protein liquid chromatography (FPLC) system with Unicorn 5.1 software, by loading 1.2 ml at a time (1% of the column volume). The standards; Dextran (5000 – 40 000 kDa), Dextran Blue (2000 kDa), thyroglobulin (670 kDa), ferritin (450 kDa), catalase (250 kDa), gamma globulin (150 kDa), and bovine serum albumin (66 kDa); were used in a calibration curve for the Sephacryl S-400 column (Appendix 1, Figure A.5). Dextran (5000 – 40 000 kDa) was used to determine the void volume. The fractions in each of the protein peaks on the chromatogram were pooled and concentrated with PEG 20 000. As with all subsequent purification procedures, the Bradford and DNS assays were performed on all purification steps and on all the concentrated peaks from the SEC column. Specific activities and yields were calculated and a purification table was constructed.

4.2.2 Xylan spectral scan

Birchwood xylan was used as the carbon source in the enrichment medium and so was present in the ultrafiltered supernatant that was loaded onto the Sephacryl S-400 column. To check if xylan absorbs at A_{280}, a spectral scan was performed. A 0.125% (w/v) solution of xylan in water was prepared and read on a PowerWave microtitre plate reader (Bio-Tek Instruments, Inc.) using a spectral scan from 200 to 600 nm. Distilled water was used as the blank.

4.2.3 Purification using spin columns

A more modern alternative to ultrafiltration is the use of spin columns to concentrate proteins and separate larger proteins from smaller ones. A fresh culture was grown up and centrifuged in the same way as before (Section 4.2.1). The supernatant was placed in 60 ml Jumbosep™ spin columns with 100 kDa filters (Pall Life Sciences). These were centrifuged at 3000*g* for 40 minutes, and in a second experiment were centrifuged at 1000*g* for 40 minutes. The resulting retentates and filtrates were further concentrated using Macrosep™ spin columns with 10 kDa filters at 5000*g* for 90 minutes, and then loaded onto a Sephacryl S-400 column. The DNS and Bradford assays were again used to determine protein and Avicelase, CMCase and xylanase activities of the purification fractions. The Folin-Lowry protein assay was also used to determine the protein concentration. In this method 1 ml of solution A (0.05 g/l $CuSO_4.5H_2O$ and 0.1 g/l $Na_3C_6H_5O_7.2H_2O$) was added to 50 ml of solution B (20 g/l Na_2CO_3 and 4 g/l NaOH) and 250 µl of this (Solution C) was added to 50 µl of sample and incubated at room temperature for 10 minutes. Folin-Ciocalteau phenol reagent (25 µl) was added to the sample and incubated at room temperature for 30 minutes. The absorbance was read at 750 nm (Lowry *et al.,* 1951) and the protein concentration in mg/ml was calculated using a BSA standard curve (Appendix 1, Figure A.6).

4.2.4 Purification using PEG 20 000

A third method of concentrating proteins before loading on the Sephacryl S-400 column was using PEG 20 000. A fresh culture was grown up and centrifuged as

before and the crude supernatant was placed in dialysis tubing and covered with PEG 20 000 for several hours. The pores in the dialysis tubing allow water molecules to pass through but all proteins, even low molecular weight proteins, are retained.

4.2.5 Purification of cell-associated (hemi-)cellulolytic enzymes

The literature states that (hemi-)cellulolytic MECs can be found in the culture supernatant or attached to the cells. To check for the presence of MECs that were attached to the cells at 84 hours of growth, a pellet wash was undertaken in a similar method to that described by Cavedon and co-workers (Cavedon *et al.,* 1990). A fresh culture was grown up for 84 hours at 37°C and 200 rpm and centrifuged at 12 000*g* for 15 minutes. The pellet was washed twice by adding 1 M NaCl in 20 mM phosphate buffer, pH 7 and centrifuging at 12 000*g* for 15 minutes. The supernatant contained proteins that had been washed off the surface of the cells and was stored for assays. The pellet was resuspended in 10 ml 20 mM Tris-HCl, pH 7, and then 1 ml of 1% (w/v) lysozyme and 50 µl of 0.5% Triton X-100 were added. The mixture was incubated at 37°C for 30 minutes and then centrifuged at 12 000*g* for 15 minutes. The supernatant, containing proteins released from inside the cells, was stored for assays. The DNS and Bradford assays were performed on the original supernatant and pellet as well as the supernatants obtained after the NaCl wash and lysozyme treatment. The latter two supernatants were also concentrated with PEG 20 000 and run on the Sephacryl S-400 column. The fractions of the protein peaks seen on the chromatograms were pooled and concentrated and the Avicelase, CMCase and xylanase activities determined using the DNS assay.

4.3 Results

4.3.1 Purification using ultrafiltration

To purify (hemi-)cellulolytic MECs from *B. subtilis* SJ01, this strain was cultured in optimised enrichment medium and the crude supernatant concentrated using ultrafiltration with a 50 kDa filter. The ultrafiltration retentate was loaded onto a Sephacryl S-400 size exclusion chromatography column and the resulting chromatogram is displayed in Figure 4.1.

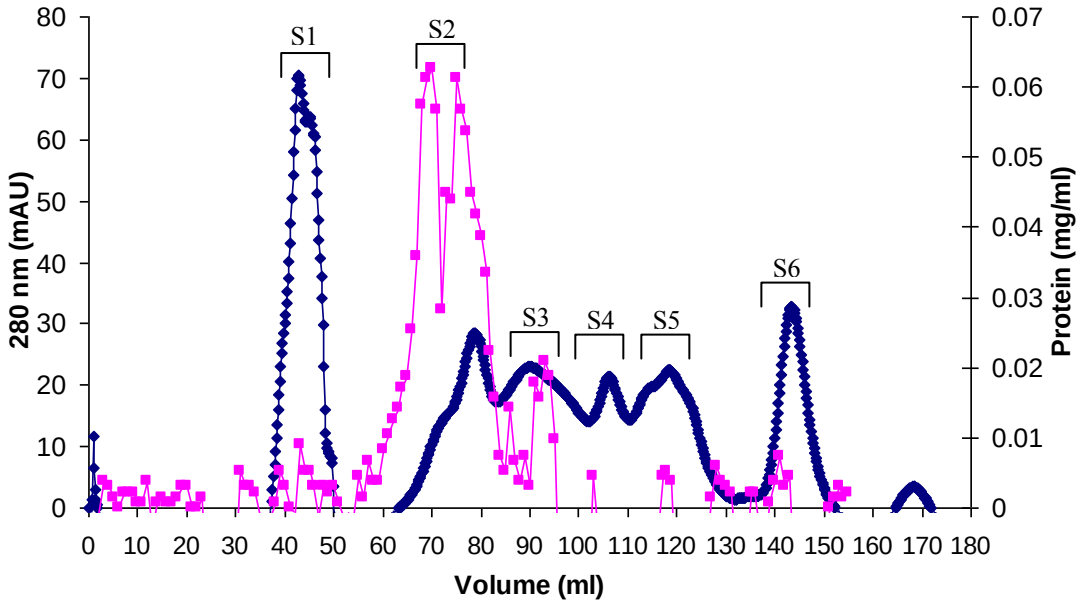


Figure 4.1 Sephacryl S-400 chromatogram of ultrafiltration retentate. Column dimensions: 60 × 2 cm; flow rate: 0.5 ml/min. ♦ A_{280} in mAU; ■ protein concentration in mg/ml. Peaks S1 (2210 kDa), S2 (372 kDa) and S3 (81 kDa) were pooled and concentrated.

The Sephacryl S-400 chromatogram in Figure 4.1 showed several A_{280} peaks, and two protein peaks that were measured using the Bradford assay (S2 and S3). Peak S2 could possibly be two peaks, but was treated as one peak in this purification procedure. The sizes of the peaks, as shown in Table 4.1, were calculated using the Sephacryl S-400 calibration curve (Appendix 1, Figure A6) and the following equations:

$$K_{av} = (V_e - V_o) / (V_t - V_o) \qquad \text{(Equation 1)}$$

where V_e is the volume at which the protein eluted, V_o is the void volume (42.17 ml), and V_t is the total volume of the column (120 ml).

$$\text{Log } M_r = -2.2323\ (K_{av}) + 3.3683 \qquad \text{(Equation 2)}$$

Table 4.1 Molecular weights (kDa) of proteins in peaks S1 to S3 from the Sephacryl S-400 chromatogram of ultrafiltration retentate.

Peak	Elution Volume (ml)	K_{av}	Log M_r	M_r (kDa)
S1	43	0.011	3.344	2210
S2	70	0.358	2.570	372
S3	93	0.653	1.910	81

Table 4.1 demonstrates that peaks S1 and S2 had molecular weights high enough for these protein fractions to be MECs. Peak S3 (81 kDa) was smaller than all recorded MECs and was more likely to be an individual protein. The peaks S4 to S6 were not included in Table 4.1 because they eluted below 100 ml (50 kDa), so were too small to be MECs and should not have passed through the 50 kDa ultrafiltration membrane.

To assess the efficiency of the 50 kDa ultrafiltration filter a sample of the ultrafiltration filtrate was run on the Sephacryl S-400 column and the resulting chromatogram is shown in Figure 4.2.

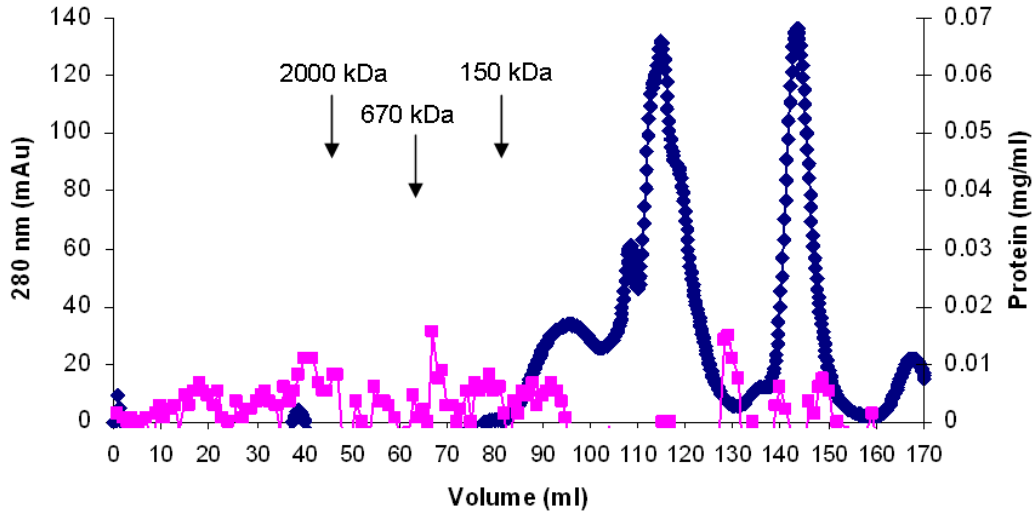


Figure 4.2 Sephacryl S-400 chromatogram of ultrafiltration filtrate. Column dimensions: 60 × 2 cm; flow rate: 0.5 ml/min. ♦ A_{280} in mAU; ■ protein concentration in mg/ml.

Figure 4.2 demonstrates that there were no protein or A_{280} peaks above 96 ml (67 kDa) and, therefore, it seemed like there were no MECs present in the ultrafiltration filtrate.

The fractions in peaks S1, S2 and S3 in Figure 4.1 were pooled and concentrated using PEG 20 000, and the protein concentration and Avicelase, CMCase and xylanase activities of each concentrated peak was measured. Using these results and results from the DNS and Bradford assays of all previous steps in the purification procedure, a purification table was completed (Table 4.2). Very low Avicelase activities were obtained and so are not shown in Table 4.2.

Table 4.2 Xylanase and CMCase purification tables for the purification of MECs from *B. subtilus* SJ01 using ultrafiltration and SEC. C, crude; CS, crude supernatant; UR, ultrafiltration retentate; UF, ultrafiltration filtrate.

Step	Volume (ml)	Protein (mg/ml)	Total Protein (mg)	Activity (U/ml)	Total Activity (U)	Specific Act. (U/mg)	Purif. Factor	Yield (%)
Xylanase								
C	500	1.43	713.50	0.244	122.15	0.17	1.00	100.0
CS	500	0.68	339.00	0.233	116.40	0.34	2.01	95.3
UR	100	2.22	221.50	0.299	29.86	0.13	0.79	24.5
UF	400	0.63	252.80	0.318	127.20	0.50	2.94	104.1
S1	42	0.05	2.08	0.001	0.03	0.02	0.10	0.0
S2	250	0.20	50.25	0.052	12.98	0.26	1.51	10.6
S3	33	0.02	0.73	0.001	0.03	0.05	0.27	0.0
CMCase								
C	500	1.43	713.50	0.025	12.36	0.02	1.00	100.0
CS	500	0.68	339.15	0.019	9.36	0.03	1.59	75.7
UR	100	2.22	221.50	0.033	3.34	0.02	0.87	27.1
UF	400	0.63	252.92	0.035	14.03	0.06	3.20	113.6
S1	42	0.05	2.09	0.000	0.01	0.00	0.19	0.1
S2	250	0.20	50.35	0.002	0.38	0.01	0.44	3.1
S3	33	0.02	0.74	0.021	0.70	0.94	54.54	5.6

According to Table 4.2 the ultrafiltration filtrate (UF) had high xylanase and CMCase activities. This indicated that there were many free, non-complexed xylanases and CMCases in the crude supernatant that were removed before loading onto the SEC column.

The ultrafiltration retentate (UR) was loaded onto the Sephacryl S-400 column and resulted in the production of peaks S1, S2 and S3. Peaks S1 and S3 had very low protein concentration and xylanase activity compared to S2. Peak S1 also had low CMCase activity and so S1 in the A_{280} chromatogram (Figure 4.1) might be due to interference at A_{280} rather than an enzyme or protein. Peak S3 had high CMCase activity, but was too small (81 kDa, see Table 4.1) to be an MEC. Therefore, Peak S3 in the Sephacryl S-400 chromatogram (Figure 4.1) was probably a non-complexed CMCase.

Peak S2 had activities of 12.98 U (where U = µmol reducing sugar produced/hour) and 0.38 U on xylan and CMC, respectively (Table 4.2), and is approximately 372 kDa in size (Table 4.1). This peak is therefore indicative of a (hemi-)cellulolytic MEC because a protein peak that is large enough to consist of multiple proteins bound together, and has activity on multiple substrates is likely to be a MEC.

According to Table 4.2, the percentage yield for the MEC (S2) seemed to be low, with a 10.6% yield for xylanase and 3.1% yield for CMCase. However, it must be kept in mind that these figures were calculated based on the crude which contained high amounts of free enzymes as well as complex-bound enzymes. Once the smaller free enzymes had been removed, the total xylanase and CMCase activities were greatly reduced. Therefore, a low yield does not necessarily indicate the loss of (hemi-)cellulolytic MEC. The same argument can be used for the fold purification since, although the total protein decreased significantly during the purification procedure, so did the CMCase and xylanase activities due to the removal of free enzymes. So, in fact, for the purposes of isolating MECs from free proteins, it is better to see a loss of activity during the purification, as indicated by the activities in fractions UR and UF in Table 4.2.

Having said this, it is noteworthy to mention that it is also possible that the xylanase and CMCase activities in peak S2 were due to entrapped enzyme contaminants, and are not MEC-associated activities. However, for the remainder of this thesis it has been assumed that this was not the case.

4.3.2 Xylan spectral scan

Peak S1 in Figure 4.1 was shown to have low protein content and low CMCase and xylanase activities and so was thought to be due to interference at A_{280}. It was suspected that this peak might be caused by the xylan substrate used in the enrichment medium (van Dyke *et al.*, 2009). To determine if xylan absorbs at A_{280} a spectral scan of a dilute solution of birchwood xylan was performed as displayed in Figure 4.3.

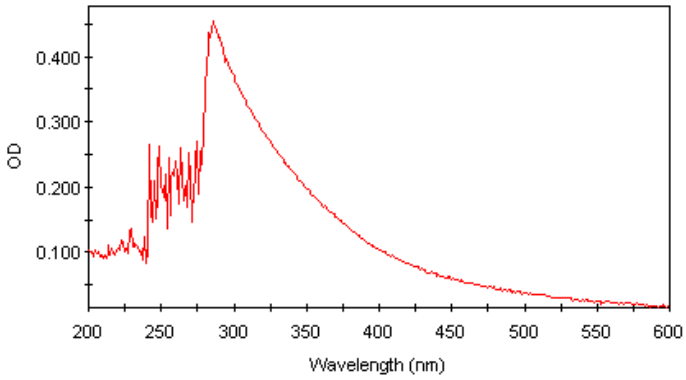


Figure 4.3 Spectral scan of a 0.125% (w/v) solution of birchwood xylan showing a peak of OD 0.455 at 286 nm.

The scan in Figure 4.3 demonstrates that xylan had an OD of 0.455 (455 mAU) at 286 nm and therefore will produce a peak in the A_{280} chromatogram during size-exclusion chromatography. Peak S1 in Figure 4.1 was, therefore, likely to be caused by birchwood xylan.

4.3.3 Purification using spin columns

Previously, spin columns have not been extensively used in the purification of MECs, where conventional ultrafiltration, ammonium sulphate precipitation or use of PEG 20 000 are more commonly used methods. However, the use of spin columns is a more rapid technique for the separation of MECs from smaller free proteins. The crude supernatant from a fresh culture of *B. subtilis* SJ01 was centrifuged in a spin column containing a 100 kDa filter. The retentate and filtrate were further concentrated and then run on the Sephacryl S-400 size-exclusion column (Figure 4.4).

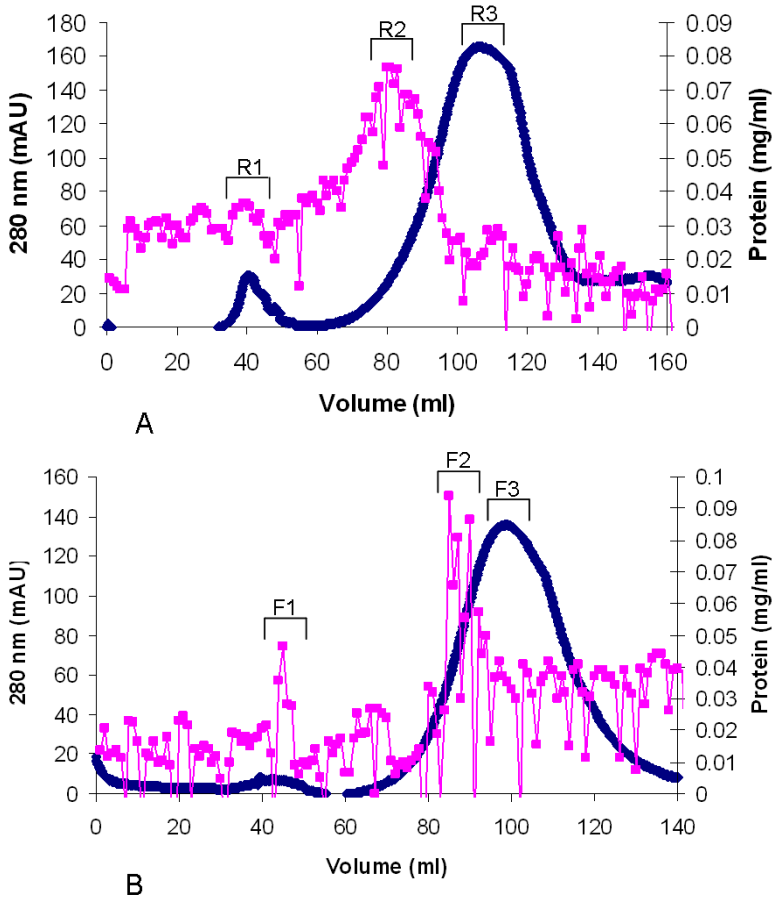


Figure 4.4 Sephacryl S-400 chromatograms of **A:** spin column retentate and **B:** spin column filtrate after centrifuging at 3000*g* for 40 minutes. Column dimensions: 60 × 2 cm; flow rate: 0.5 ml/min. ♦ A_{280} in mAU; ■ protein concentration in mg/ml. Peaks R1 (2522 kDa), R2 (219 kDa), R3 (30 kDa), F1 (1936 kDa), F2 (138 kDa) and F3 (51 kDa) were pooled and concentrated.

The peaks labeled in Figure 4.4 were pooled and concentrated and the sizes measured according to the Sephacryl S-400 calibration curve and Equations 1 and 2 (Section 4.3.1) as shown in Table 4.3.

Table 4.3 Molecular weights (kDa) of the peaks from the Sephacryl S-400 chromatograms of spin column retentate (R) and filtrate (F).

Peak	Elution volume (ml)	K_{av}	Log M_r	M_r (kDa)
R1	41	-0.015	3.402	2522
R2	78	0.460	2.341	219
R3	108	0.743	1.710	30
F1	45	0.036	3.287	1936
F2	85	0.550	2.140	138
F3	100	0.743	1.710	51

The chromatograms of the spin column retentate and spin column filtrate (Figure 4.4) were similar, both containing peaks above and below 100 kDa (Table 4.3), indicating that the spin column did not successfully separate large proteins from small proteins. The cellulolytic activity in each concentrated peak was measured as shown in Figure 4.5.

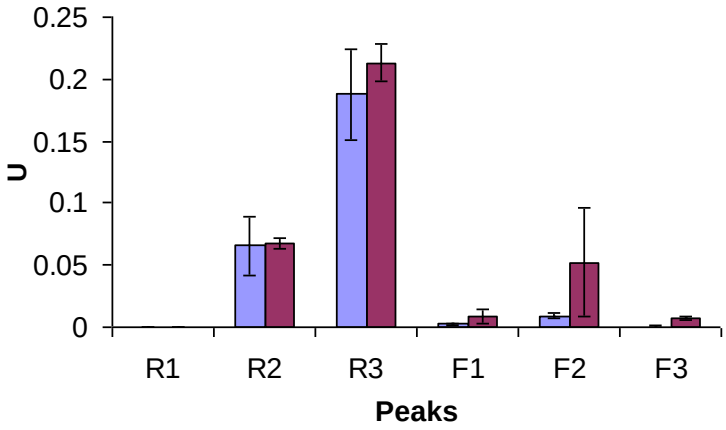


Figure 4.5 Total ▮ CMCase and ▮ xylanase activities (U) in protein peaks from Sephacryl S-400 chromatography of spin column retentate (R) and filtrate (F).

Figure 4.5 shows that there was very low or no Avicelase activity present in all of the peaks.

The low enzyme activities (Figure 4.5) and high molecular weights (Table 4.3) of peaks R1 and F1 indicate that these were due to the birchwood xylan substrate that was used in the growth medium, as described in Section 4.3.2. It is possible that some enzymes attach to this substrate resulting in the low enzyme activities seen in F1. The presence of peak F1 in Figure 4.4B was unexpected since this peak was too large (1936 kDa) to pass though the 100 kDa cut-off filter and so should not be present in the spin column filtrate.

According to Figure 4.5 and Table 4.3, peak R2 was large enough to be an MEC and displayed CMCase and xylanase activity. However, this complex was smaller (219 kDa) than the MEC in Figure 4.1 (372 kDa). Peak R3 had higher CMCase and xylanase activities than peak R2 but was only 30 kDa and so could not be a MEC. A protein of 30 kDa should have passed through the filter into the spin column filtrate, and it was also unusual that this peak had both CMCase and xylanase activities since it was too small to be an enzyme complex.

Peaks F2 (138 kDa) and F3 (51 kDa) both had low CMCase and xylanase activities (Figure 4.3). It is possible that a protein or MEC close in size to the molecular weight cut-off of the membrane (100 kDa), such as Peak F2, could pass into the spin column filtrate as demonstrated in Figure 4.4.

A purification table was completed based on the enzyme activities and protein concentration obtained for the peaks in Figure 4.4A (Table 4.4).

Table 4.4 Xylanase and CMCase purification tables for the purification of MECs from *B. subtilus* SJ01 using spin columns and SEC. C, crude; CS, crude supernatant; SCR, spin column retentate; SCF, spin column filtrate. U = µmol reducing sugar produced/hour.

Step	Volume (ml)	Protein (mg/ml)	Total Protein (mg)	Activity (U/ml)	Total Activity (U)	Specific Act. (U/mg)	Purif. Factor	Yield (%)
xylanase								
C	500	0.952	476.00	0.189	94.71	0.199	1.00	100.0
CS	500	0.338	169.00	0.197	98.65	0.584	2.93	104.2

SCR	95	0.344	32.68	0.180	17.12	0.524	2.63	18.1
SCF	160	0.408	65.28	0.270	43.22	0.662	3.33	45.6
R1	237	0.026	6.27	0.000	0.00	0.000	0.00	0.0
R2	**198**	**0.165**	**32.65**	**0.027**	**5.37**	**0.164**	**0.83**	**5.7**
R3	435	0.035	15.28	0.039	16.87	1.104	5.55	17.8
CMCase								
C	500	0.952	476.00	0.046	22.92	0.048	1.00	100.0
CS	500	0.338	169.00	0.055	27.71	0.164	3.41	120.9
SCR	95	0.344	32.68	0.053	5.07	0.155	3.22	22.1
SCF	160	0.408	65.28	0.072	11.49	0.176	3.65	50.1
R1	237	0.026	6.27	0.000	0.00	0.000	0.00	0.0
R2	**198**	**0.165**	**32.65**	**0.026**	**5.18**	**0.159**	**3.29**	**22.6**
R3	435	0.035	15.28	0.034	14.86	0.972	20.19	64.8

The xylanase yield in R2 was lower (5.67%) than when conventional ultrafiltration was used in the purification (10.6%; Table 4.2), indicating that some of the activity in this fraction was lost when spin columns were used. On the other hand the CMCase yield in Table 4.4 (22.6%) was higher than that in Table 4.2 (3.1%).

Peak R3 contained a low concentration of protein (Table 4.4) according to the Bradford assay, which did not correlate with its high enzyme activity (Figure 4.5 and Table 4.4). The Bradford reagent reacts with aromatic and positively charged amino acids only, and so it is possible that there were proteins in R3 that were undetectable using the Bradford assay.

Therefore, the spin column purification was repeated, centrifuging at 1000*g* for 40 minutes (to assess whether a lower centrifugal force improves the separation of large proteins from small ones) and measuring the protein concentration using the Folin-Lowry protein assay, which involves the interaction between copper and the amide backbone of proteins (Figure 4.6).

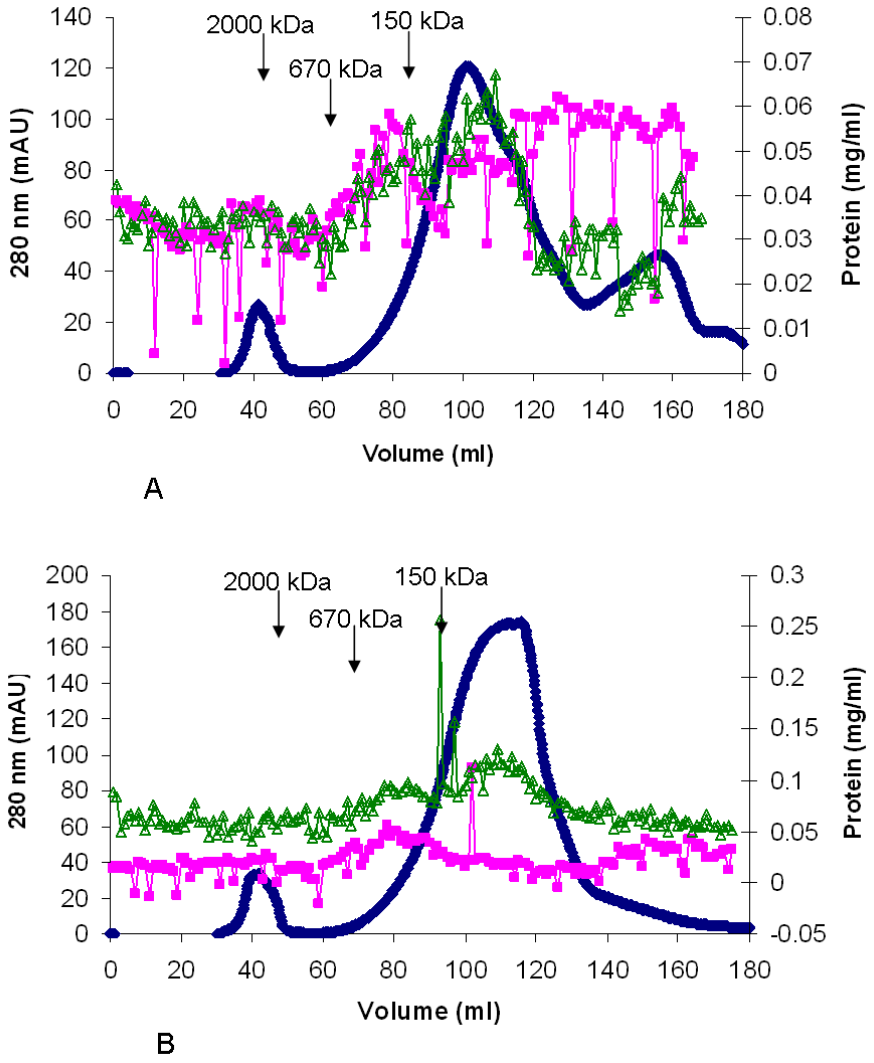


Figure 4.6 Sephacryl S-400 chromatograms of **A:** spin column retentate and **B:** spin column filtrate after centrifuging at 1000*g* for 40 minutes. Column dimensions: 60 × 2 cm; flow rate: 0.5 ml/min. ♦ A_{280} in mAU; ■ mg/ml protein measured using the Bradford assay; Δ mg/ml protein measured using Folin-Lowry assay.

Figures 4.6 and 4.4 are very similar indicating that there was no change in the separation of proteins with a decrease in the centrifugal force applied. Figure 4.6 also demonstrated that the Folin-Lowry and Bradford assays correlated closely, and so it was not necessary to perform both assays in future experiments. Fractions 90 to 110 in Figure 4.6A had a higher protein concentration (using the Bradford and Folin-Lowry assays) than the fractions of peak R3 in Figure 4.4A. The low protein concentration in S3 as shown in Figure 4.4A and Table 4.4, was therefore, not reproduced, and might be inaccurate.

4.3.4 Purification using PEG 20 000

The crude supernatant can also be concentrated in such a way that does not remove smaller proteins, such as the use of PEG 20 000 which simply dehydrates a solution. This method was performed in order to compare it with the use of ultrafiltration and spin columns and the resulting concentrate was loaded onto the Sephacryl S-400 column (Figure 4.7).

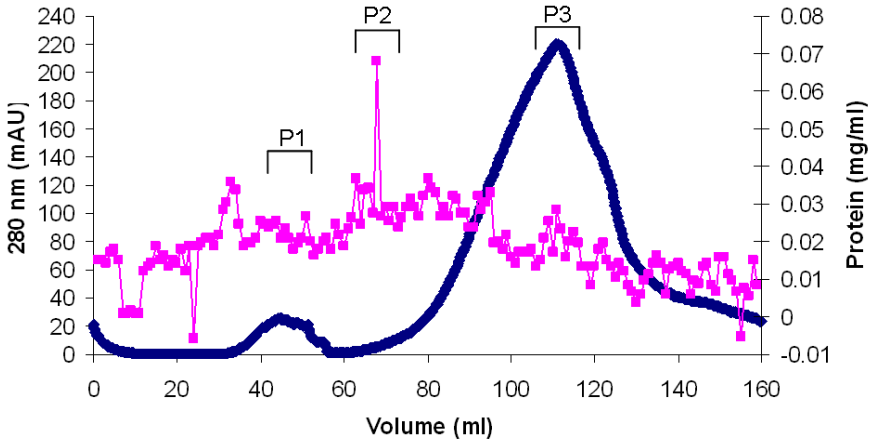


Figure 4.7 Sephacryl S-400 chromatogram of PEG-concentrated crude supernatant. Column dimensions: 60 × 2 cm; flow rate: 0.5 ml/min ♦ A_{280} in mAU; ■ mg/ml protein. Peaks P1 (1558 kDa), P2 (424 kDa) and P3 (23 kDa) were pooled and concentrated.

The peaks labeled in Figure 4.7 were pooled and concentrated and their molecular weights (Table 4.5) and Avicelase, CMCase and xylanase activities were determined in the same way as before and a purification table (Table 4.6) was constructed.

Table 4.5 Molecular weights (kDa) of the peaks from the Sephacryl S-400 chromatogram of PEG-concentrated crude supernatant.

Peak	ml	K_{av}	Log M_r	M_r
P1	48	0.075	3.201	1588
P2	68	0.332	2.627	424
P3	112	0.897	1.365	23

Table 4.6 Xylanase and CMCase purification tables for the purification of MECs from *B. subtilus* SJ01 using PEG 20 000 and SEC. C, crude; CS, crude supernatant; SCR, spin column retentate; SCF, spin column filtrate. U = µmol reducing sugar produced/hour.

Step	Volume (ml)	Protein (mg/ml)	Total Protein (mg)	Activity (U/ml)	Total Activity (U)	Specific Act. (U/mg)	Purif. Factor	Yield (%)
xylanase								
C	500	0.617	308.5	0.0914	45.700	0.148	1.00	100.0
CS	500	0.222	111.0	0.0854	42.700	0.385	2.60	93.4
CP	500	0.329	164.5	0.0757	37.836	0.230	1.55	82.8
PEG	112.5	0.758	85.3	0.1274	14.329	0.168	1.13	31.4
P1	15	0.032	0.5	0.0187	0.281	0.585	3.95	0.6
P2	**18.75**	**0.124**	**2.3**	**0.0291**	**0.546**	**0.235**	**1.58**	**1.2**
P3	11.25	0.387	4.4	0.0008	0.009	0.002	0.01	0.0
CMCase								
C	500	0.617	308.5	0.0019	0.955	0.003	1.00	100.0
CS	500	0.222	111.0	0.0085	4.228	0.038	0.26	442.7
CP	500	0.329	164.5	0.0000	0.000	0.000	0.00	0.0
PEG	112.5	0.758	85.3	0.0140	1.571	0.018	0.12	164.5
P1	15	0.032	0.5	0.0003	0.004	0.008	0.06	0.4
P2	**18.75**	**0.124**	**2.3**	**0.0059**	**0.111**	**0.048**	**0.32**	**11.6**
P3	11.25	0.387	4.4	0.0039	0.044	0.010	0.07	4.6

Due to the lack of protein and enzyme activity in Peaks S1 and R1 (Figure 4.1 and 4.4A), and the ability of birchwood xylan to absorb at A_{280} (Figure 4.3), it was concluded that these peaks were caused by the xylan substrate used in the culture medium. However, although peak P1 was similar in size to Peaks S1 and R1, it contained xylanase activity, indicating the presence of enzymes in this fraction.

Peak P2 (Figure 4.7) was large enough to be an MEC and was close in size (424 kDa, Table 4.5) to peaks S2 (372 kDa) and R2 (219 kDa) in Figures 4.1 and 4.4A. Table 4.6 demonstrates that Peak P2 also contained both CMCase and xylanase activities. The total activity (U) in this MEC fraction (P2) (Table 4.6) was considerably lower than the total activity in the MEC fractions of the samples that were purified using conventional ultrafiltration (S2, Table 4.2) and spin columns (R2, Table 4.4). The xylanase yield (1.2%) in the MEC fraction of Table 4.6 (P2) was also lower than in S2 (10.6%, Tables 4.2) and R2 (5.7%, Table 4.4), but the CMCase yield in P2 (11.6%) was higher than that in the MEC fraction purified using conventional ultrafiltration (3.1%, Table 4.2) and lower in R2 (22.6%, Table 4.4).

4.3.5 Purification of cell-associated (hemi-)cellulolytic enzymes

To assess the presence of cell-associated MECs at 84 hours of growth the crude pellet from a fresh *B. subtilis* SJ01 culture was washed with 1 M NaCl and then treated with a 1% (w/v) lysozyme solution. Xylanase and CMCase activities of each step of this pellet wash process are displayed in Figure 4.8.

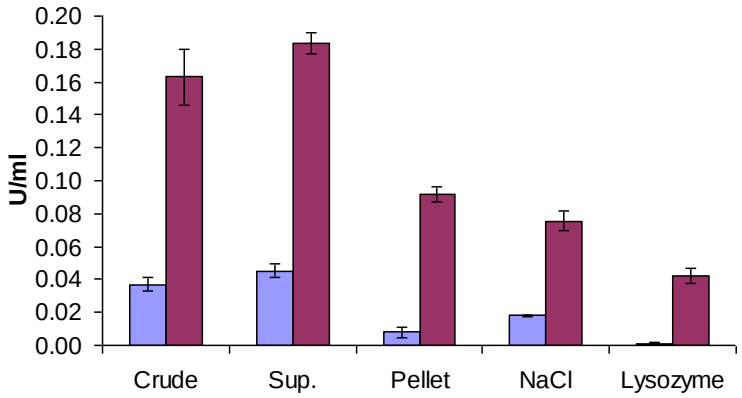
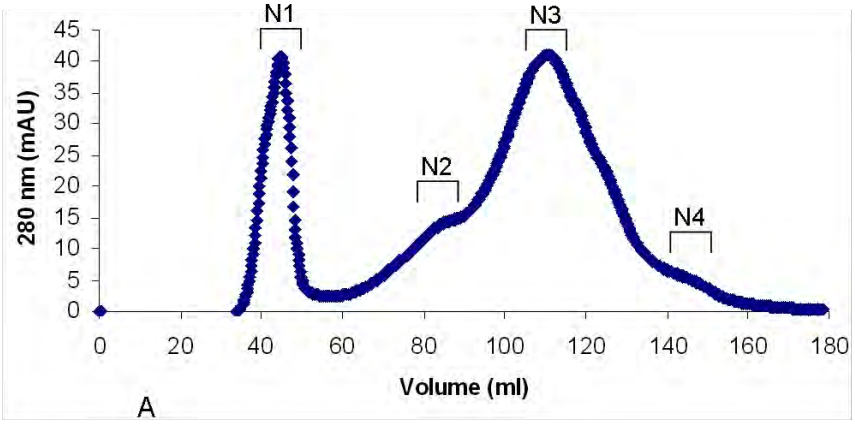


Figure 4.8 CMCase and xylanase activities (U/ml) of crude, crude supernatant (sup.), crude pellet, supernatant after pellet was washed with NaCl, and supernatant after pellet was treated with lysozyme. U = µmol reducing sugar produced/hour.

The CMCase and xylanase activities in the fractions „Pellet“ and „NaCl“ in Figure 4.9 indicate that most of the CMCase and xylanase enzymes that were attached to the crude pellet were removed after treatment with NaCl. Figure 4.9 also demonstrates that after treating with lysozyme, xylanase enzymes were released from the cells, indicating that some of the xylanases remained bound to the cell membrane after treatment with NaCl.

To determine whether the enzyme activities in the „NaCl“ and „Lysozyme“ fractions (Figure 4.9) were associated to MECs or due to non-complexed enzymes, these fractions were subjected to Sephacryl S-400 chromatography and the resulting chromatograms are shown in Figure 4.10.



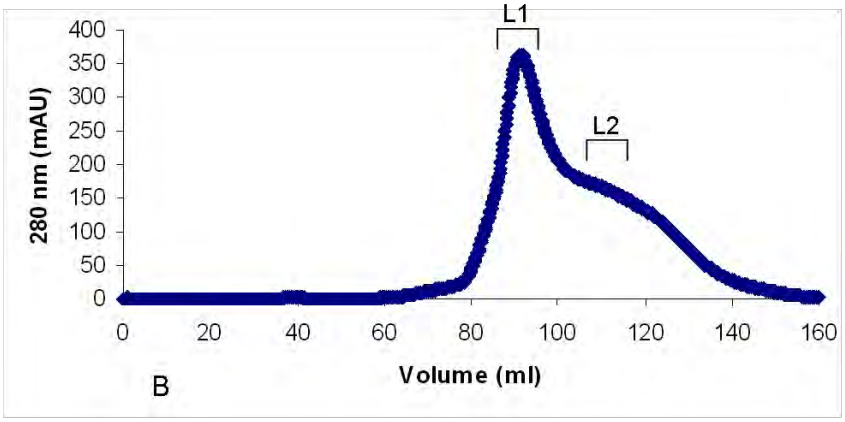


Figure 4.9 **A:** Sephacryl S-400 chromatograms of **A:** proteins after treating the crude pellet with 1 M NaCl and **B:** proteins released from cells after treatment with 1% (w/v) lysozyme. Column dimensions: 60 × 2 cm; flow rate: 0.5 ml/min. ♦ A_{280} in mAU. Peaks N1 (1936 kDa), N2 (121 kDa), N3 (25 kDa), N4 (2 kDa), L1 (87 kDa) and L2 (11 kDa) were pooled and concentrated.

The concentration of proteins in mg/ml measured using the Bradford assay was very low and so was not shown in Figure 4.9. The sizes of peaks N1 to N4, L1 and L2, as shown in Table 4.7, were determined using the Sephacryl S-400 calibration curve (Appendix 1, Figure A6) and Equations 1 and 2 (Section 4.3.1).

Table 4.7 Molecular weights (kDa) of the peaks from the Sephacryl S-400 chromatogram of proteins washed off the cell surface (N) and proteins released from the cells (L) in the crude pellet.

Peak	Elution Volume (ml)	K_{av}	Log M_r	M_r (kDa)

N1	45	0.036	3.287	1936
N2	87	0.576	2.082	121
N3	111	0.884	1.394	25
N4	147	1.347	0.362	2
L1	92	0.640	1.939	87
L2	123	1.039	1.050	11

Table 4.7 indicates that only peak N1 and possibly N2 are large enough to be MECs. The fractions making up all the above peaks were pooled and concentrated using PEG 20 000 and then assayed for Avicelase, CMCase and xylanase activities (Figure 4.10).

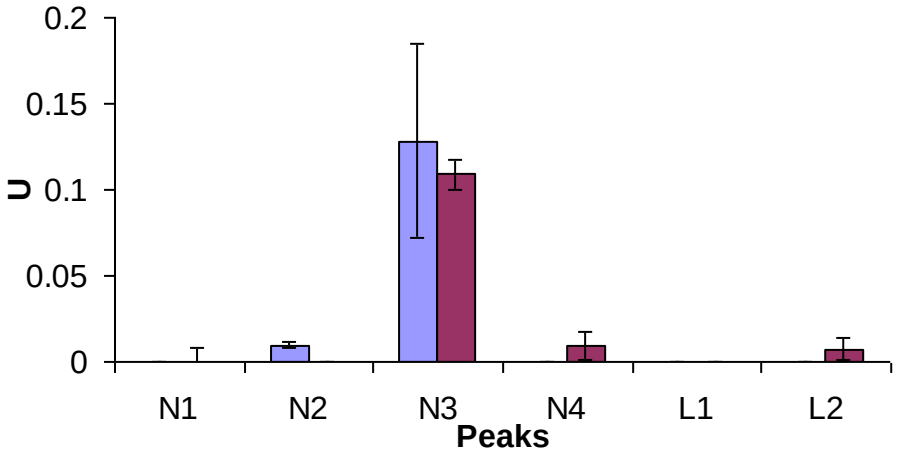


Figure 4.10 Total ▮ CMCase and ▮ xylanase activities (U) in protein peaks from Sephacryl S-400 of NaCl washed (N) and lysozyme treated (L) crude pellet. U = µmol reducing sugar produced/hour.

There was no Avicelase activity present in any of the peaks and CMCase and xylanase activities were low in all peaks except N3. Peak N3, however, is too small to be an MEC, and so it can be concluded that the xylanase activity found in the crude pellet at 84 hours of growth of the *B. subtilis* SJ01 culture (Figure 3.6) was not as a result of (hemi-)cellulolytic MECs, and was instead due a mixture of free CMCases and xylanases that are attached to the cell surface.

4.4 Discussion and Conclusions

In previous studies reported in the literature ultrafiltration has been successfully used as a concentration step in the purification of MECs and cellulosomes (Cavedon *et al.,*

1990; Lamed *et al.,* 1983; Pohlschroder *et al.,* 1994; van Dyk *et al.,* 2009). Figure 4.1 demonstrates that a 50 kDa ultrafiltration membrane was successful in retaining a 372 kDa MEC (peak S2) that displayed CMCase and xylanase activities (Table 4.2). The high CMCase and xylanase activities in the ultrafiltration filtrate (Table 4.2) and the size-exclusion chromatogram of this fraction (Figure 4.2) indicated that the ultrafiltration method also successfully removed many non-complexed enzymes. The low % yield seen in Table 4.2 confirms the loss of non-complexed CMCase and xylanase enzymes, and so in this case a low % yield indicated the success of the purification of MECs away from non-complexed enzymes. It is not possible to determine the % yield of the MEC specifically, since the yield is calculated based on the total (hemi-)cellulolytic enzyme activity and not only that which is present in an MEC.

An important discovery in this chapter was that birchwood xylan absorbs at A_{280} and so, since it was present in the culture supernatant as an insoluble substrate, it was detected on the Sephacryl S-400 chromatograms at an elution volume between 40 and 50 ml (1500 kDa – 2700 kDa). In some cases, such as in Peaks F1 and P1 in Figures 4.4 and 4.7, respectively, small amounts of xylanase activity was detected. It is possible that xylanase enzymes sometimes bind to the birchwood xylan substrate and so these enzymes co-elute with the xylan substrate during SEC.

Although spin columns are more modern and rapid ultrafiltration devices, Section 4.3.3 showed that there were problems using these devices in MEC purification. Ultrafiltration using spin columns was successful in purifying a 219 kDa MEC with CMCase and xylanase activity (Figures 4.4A and 4.5, and Tables 4.3 and 4.4). This is close to the size observed for the MEC in peak S2, from the purification using conventional ultrafiltration, and so may in fact be the same MEC. However, the filtrate from the spin columns contained a protein of 138 kDa and the xylan substrate which eluted at 41 ml (2522 kDa) (Figure 4.4B and Table 4.3), which means that the membrane was not capable of retaining all molecules above 100 kDa. The reason for this might be that the centrifugal force pushed molecules, which would not normally fit, through the pores of the filter by causing them to compress into tighter conformations. When run on the Sephacryl S-400 matrix, the pump pressure of the

FPLC was not sufficient to force the xylan into the gel pores, and so it eluted according to its native size and shape. However, it is typical for molecules close to the molecular weight cut-off of the filter, such as the 138 kDa protein, to pass into the filtrate. When the purification procedure was repeated using a lower centrifugal force (1000*g*), the same results were obtained (Figure 4.6). A 1000*g* force for 40 minutes is the minimum that can be used in order to sufficiently concentrate the crude supernatant.

A second problem observed during the purification with spin columns was the presence of a 30 kDa protein (R3, Figure 4.4A) in the retentate that displayed both CMCase and xylanase activities (Figure 4.5). The Sephacryl S-400 column was not capable of separating small proteins and so peak R3 might have contained a mixture of free xylanases and CMCases of similar size that were not properly resolved. It is possible that during ultrafiltration with spin columns these enzymes were MEC-bound and so were retained on the 100 kDa filter, but for some reason was separated from the complex prior to SEC. This scenario is not specific to spin columns, since purification using conventional ultrafiltration produced three peaks (S4, S5 and S6) in the retentate which were smaller than 50 kDa (the molecular weight cut-off of the membrane used).

The % yield for xylanase in the MEC fraction was lower when spin columns were used than when conventional ultrafiltration was used (Tables 4.2 and 4.4). The loss of activity may be due to the higher molecular weight cut-off of the spin column filter, which means that more non-complexed enzymes were removed. Loss of activity seen by a lower yield might also be as a result of some the MEC being degraded and moving into the filtrate, as illustrated by the variation in CMCase yields between the three purification methods (Tables 4.2, 4.4 and 4.6).

When the crude supernatant of *B. subtilis* SJ01 was concentrated using PEG 20 000 prior to SEC, a 424 kDa MEC displaying CMCase and xylanase activity was purified. However, the total CMCase and xylanase activities (U) of this MEC was considerably lower than that of the MECs purified using conventional ultrafiltration and spin columns (Tables 4.2, 4.4 and 4.6). This is probably because PEG 20 000 concentrated the entire crude supernatant, whereas ultrafiltration specifically concentrated

molecules above a certain size. Therefore, with ultrafiltration a larger fraction of the concentrated sample consisted of the MEC and so more MEC was loaded onto the Sephacryl S-400 column.

The xylanase activity in Peak P1 was high with respect to peaks S1 and R1 (Tables 4.2, 4.4 and 4.6). As established previously, peak P1 was due to the interference of xylan at A_{280} and the presence of xylanase enzymes in this peak indicated that, during concentration with PEG 20 000, some xylanase enzymes bound to the xylan substrate. The peak P1 is similar in size to the _MEC' fractions from the Sepharose 4B column in Chapter 2 (Figure 2.8 and Table 2.2) in which PEG 20 000 was used to concentrate the supernatant before size exclusion chromatography. It is, therefore, possible that concentrating with PEG 20 000 somehow leads to the formation of a xylanase-xylan complex.

It was concluded that the best method for concentrating the crude supernatant before SEC was conventional ultrafiltration. This is because the centrifugal force used with spin columns seems to push molecules larger than 100 kDa though the pores, and PEG 20 000 does not sufficiently concentrate the MEC, as discussed. Therefore, conventional ultrafiltration with a 50 kDa filter was used to purify sufficient amounts of MEC for partial characterisation (Chapter 5).

Bayer *et al.* (1994) and Beguin and Aubert (1994) stated that cellulosomes can be found in both the culture supernatant and attached to the cells. In a method similar to that described by Cavedon *et al.* (1990), the presence of a cell-associated (hemi-)cellulolytic MEC was determined in an 84 hour culture. A 1 M NaCl solution was used to wash the cells because the ions in this solution will displace the enzymes bound to the cell surface. Lysozyme was used to disrupt the cells and thus remove enzymes that were not washed off with NaCl, and to release enzymes that were inside the cell. Figure 4.8 demonstrated that there were xylanases and small amounts of CMCases present in the cellular pellet that were successfully washed off the cells using the above method. However, Figure 4.9 and Table 4.7 demonstrated that none of the CMCase and xylanase activities seen in Figure 4.8 were due to a MEC. The only fraction that was large enough to be an MEC was N1 (1936 kDa), but this fraction had no CMCase or xylanase activity (Figure 4.11). Therefore, in further

research, only the crude supernatant will be used for the purification of (hemi-)cellulolytic MECs.

CHAPTER 5

Partial characterisation of the MECs from *B. subtilis* SJ01

5.1 Introduction

Once purified, an enzyme or enzyme complex is studied to learn more about its characteristics and functions. The characterisation of cellulosomes and other MECs usually involves testing for features that are common to these complexes. For example, all MECs have high molecular weights, contain a number of enzymes of different sizes and activities, and can, therefore, act on multiple substrates (Kim and Kim, 1993). Cellulosomes and xylanosomes also contain a carbohydrate binding module (CBM), have a non-catalytic scaffolding protein and are cell-associated in exponential growth phase (Bayer *et al.,* 1983; Bayer *et al.,* 1994; Jiang *et al.,* 2006).

Although much work has been done on the cellulosomes of clostridia, the MECs of only three species of *Bacillus* have been partially characterised to date. The MECs purified from *Bacillus circulans* were 669 and 443 kDa, respectively (Kim and Kim, 1993), those purified from *Paenibacillus curdlanolyticus* were 1450 kDa and 400 kDa (Pason *et al.*, 2006), and a 2000 kDa MEC was purified from *Bacillus licheniformis* (van Dyk *et al.*, 2009). Techniques used to characterise these MECs included electrophoresis (native PAGE, SDS-PAGE and zymograms), scanning electron microscopy, enzyme assays, substrate binding studies, temperature and pH optimum and enzyme kinetics (Kim and Kim, 1993; Pason *et al.*, 2006; van Dyk *et al.*, 2009).

So far, we have shown that the large protein fraction isolated from *B. subtilis* SJ01 is roughly 372 kDa in size, can degrade CMC and birchwood xylan substrates and is extracellular in stationary growth phase (Chapter 4). The aim of this chapter was to determine the subunits of this complex, to determine whether any of these subunits are glycoproteins, to check for the presence of a CBM, and to test the ability of the complex to degrade various substrates.

5.2 Materials and Methods

5.2.1 SDS-PAGE

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using a 10% resolving gel and 4% stacking gel according to the method described by (Laemmli, 1970). Protein fractions were first concentrated to 20 µg using acetone precipitation, in which 1 ml of ice-cold acetone was added to 200 µl protein sample. The mixtures were vortexed and then incubated at -20°C for 10 minutes prior to centrifuging at 13 000*g* for 5 minutes. The acetone was decanted and the pellets left to dry before resuspension in 12 µl distilled water. These protein samples were mixed with 3 µl of a 5X sample buffer (containing 3.55 ml water; 1.25 ml 0.5 M Tris-HCl, pH6.8; 2.5 ml glycerol; 2.0 ml 10% (w/v) SDS; 0.2 ml 0.5% (w/v) bromophenol blue; and 5% (v/v) β-mercaptoethanol added prior to use) and boiled at 100°C for 5 minutes. The boiled samples were loaded into the wells and the gel electrophoresed at 180 V for about 45 minutes.

The SDS-PAGE gels were stained with Coomassie protein stain (0.1% (w/v) Coomassie brilliant blue G 250; 45% (v/v) methanol; and 10% glacial acetic acid) overnight, and destained with a destain solution (45% methanol; 45% distilled water; 10% glacial acetic acid) for about 3 hours.

A peqGOLD protein marker II (peqLab Biotechnolgie GmbH) was electrophoresed next to the samples. The molecular weights of the bands seen on the SDS-PAGE gels were calculated using a graph of log M_r verses R_f (Appendix 1, Figure A.7), where R_f was the ratio of the migratory distance of the protein to the migratory distance of the bromophenol blue dye.

5.2.2 Zymograms

Enzyme samples were concentrated using acetone precipitation to obtain an appropriate mass of protein dissolved in 12 µl of distilled water. To these, 3 µl of sample buffer was added and the samples boiled at 100°C for 5 minutes. For each zymogram, two SDS-PAGE gels were prepared, one containing the same ingredients as described in Section 5.2.1, and the second containing 0.1% (w/v) substrate (birchwood xylan or oat spelt xylan) in addition to the ingredients described in Section 5.2.1.

The gels were electrophoresed at 180 V for about 45 minutes, and the SDS-PAGE gel was then stained with Coomassie stain. The peqGOLD protein marker on the gel containing 0.1% (w/v) substrate was removed and stained with Coomassie stain, while the rest of the gel was placed in 2.5% Triton-X 100 prepared in 20 mM phosphate buffer, pH 7.0, for 1 hour to renature the enzymes. The gel was then incubated in 20 mM phosphate buffer, pH 7.0, at 50°C for 48 hours (for oat spelt xylan) or 24 hours (for birchwood xylan). The gel was then stained with 0.3% (w/v) Congo red for 15 minutes, destained with 1 M NaCl and counterstained with 0.5% (v/v) acetic acid. To record the results the gels were photographed using a Uviprochemi geldoc imaging system (Whitehead scientific). The molecular weights of the bands were calculated using a graph of log M_r verses R_f (Appendix 1, Figures A.8 and A.9).

5.2.3 Substrate specificity

To determine the range of cellulolytic and hemicellulolytic activity of the MECs from *B. subtilis* SJ01, 2% (w/v) solutions of several substrates (birchwood xylan, oat spelt xylan, CMC, bagasse, Avicel® and cellulose) were prepared. Volumes of 100 µl substrate and 50 µl enzyme were diluted with 250 µl of 20 mM phosphate buffer, pH 7.0 and incubated at 50°C for 60 hours. The DNS assay was used to determine the hydrolysis of cellulose and xylan substrates into glucose and xylose sugars.

5.2.4 Glycoprotein detection

A glycoprotein detection kit (Sigma, GLYCOPRO) was used to determine the presence of glycoproteins in the MECs of *B. subtilis* SJ01. A 10% SDS-PAGE containing 20 µg and 50 µg protein for each MEC sample was electrophoresed at 180 V for about 45 minutes. The gel was then fixed in 50% (v/v) methanol and washed with water before oxidizing with the oxidation component in the kit. The gel was washed again and then stained with Schiff's reagent. This reagent was replaced with a reduction solution found in the kit, prior to a final wash in distilled water. Schiff's reagent stains glycoproteins pink.

5.2.5 Substrate binding study

Insoluble birchwood xylan was prepared according to (Kittur *et al.,* 2003). Birchwood xylan (10 g) was suspended in 200 ml distilled water and stirred for 2 hours before it was centrifuged at 12 000*g* for 10 minutes. The pellet was washed, lyophilised and then ground to a fine powder using a mortar and pestle.

About 50 mg of Avicel® and insoluble birchwood xylan were added to microcentrifuge tubes (Eppendorf) containing 1 ml of 20 mM phosphate buffer, pH 7.0. The mixtures were left at room temperature for 1 hour and then centrifuged at 13 000*g* for 5 minutes to remove the buffer. Concentrated samples of each MEC (approximately 0.5 mg/ml protein) were added to the insoluble substrates to a volume of 1 ml each, with 100 µl of 10 mg/ml bovine serum albumin to prevent non-specific binding to the tube (Goldstein *et al.,* 1993). The microcentrifuge tubes were incubated on an Intelli-Mixer shaker (SkyLine, ELMI) at 4 °C at 40 rpm for 1 hour and then centrifuged at 13 000*g* for 1 minute.

Controls for the binding study were set up with 1 ml of phosphate buffer only; 50 mg insoluble substrate in 1 ml phosphate buffer; and 1 mg/ml BSA and 1 ml MEC, to

determine the protein concentration before binding to the substrate occurs. These controls were incubated and centrifuged in the same way as the experimental samples.

The Bradford and DNS assays were performed on the supernatants from the above procedure and the % binding calculated using the following equation:

$$\% \text{ binding} = \frac{(\text{concentration without substrate} - \text{concentration after binding})}{\text{concentration without substrate}}$$

5.3 Results

5.3.1 SDS-PAGE

The purification described in Section 4.2.1 was repeated several times to generate sufficient protein for a partial characterisation. In each case a similar chromatogram to that shown in Figure 4.1 was obtained, and it became evident that peak S2 may consist of two MECs, since the peak is a doublet. To determine if this was true, the two peaks that make up S2 were purified separately and then run on an SDS-PAGE gel to determine their protein composition. The two new samples from S2 were labelled C1 and C2 (Figure 5.1).

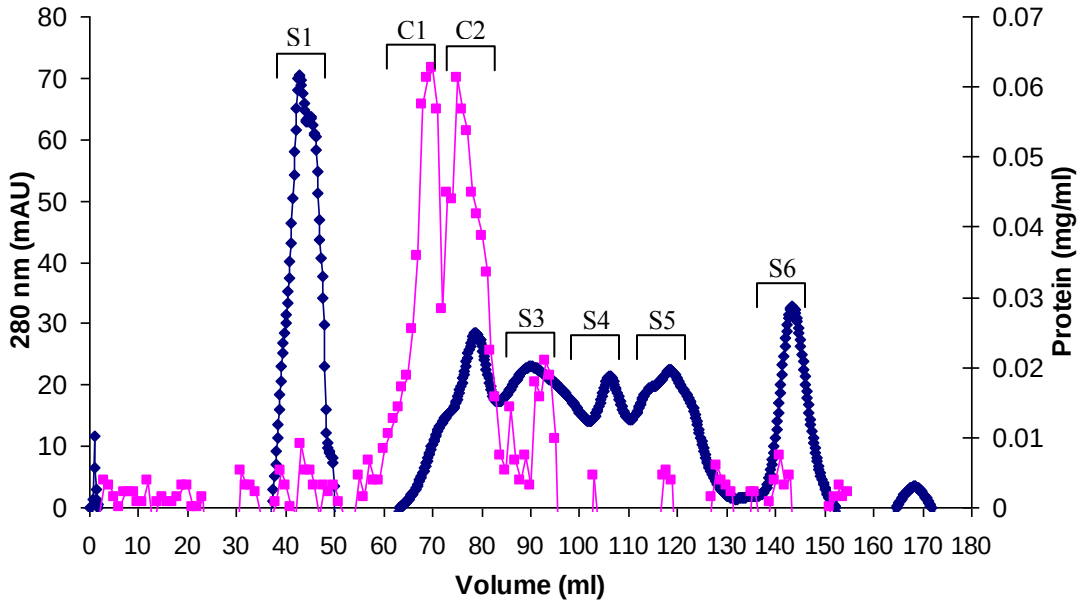


Figure 5.1 Sephacryl S-400 chromatogram of ultrafiltration retentate. Column dimensions: 60 × 2 cm; flow rate: 0.5 ml/min. ♦ A_{280} in mAU; ■ protein concentration in mg/ml. Peaks S1 (2210 kDa), C1 (371 kDa) C2 (267 kDa), S3 (81 kDa), S4 (34 kDa), S5 (14 kDa) and S6 (3 kDa) were pooled and concentrated.

According to the Sephacryl S-400 calibration curve (Appendix 1, Figure A6), the peaks C1 and C2 in Figure 5.1 were 371 and 267 kDa in size, respectively. To observe the single enzymes that make up these complexes, SDS-PAGE was performed on the ultrafiltration rententate and all the peaks labelled in Figure 5.1, as displayed in Figure 5.2.

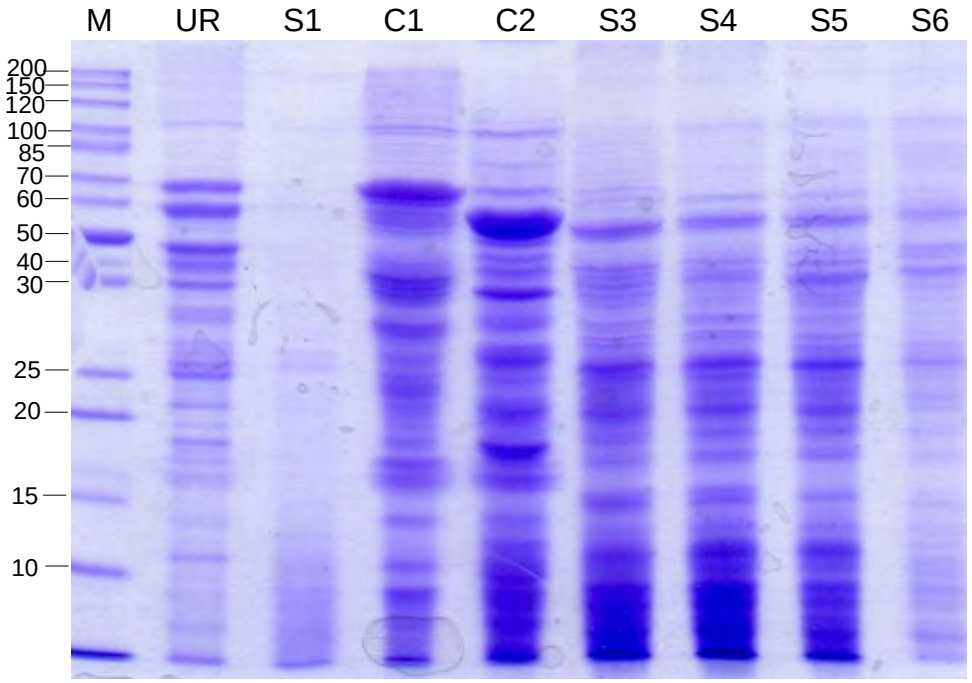


Figure 5.2 SDS-PAGE (10%) of fractions from the purification of MECs from *B. subtilis* SJ01 using ultrafiltration. M, peqGold protein marker II; UR, ultrafiltration retentate; S1, Sephacryl S-400 fraction 1; C1, multi-enzyme complex 1.

Figure 5.2 shows that S1 contained no protein, which supports the conclusion made in Chapter 4 that this fraction consisted of birchwood xylan.

The SDS-PAGE profiles of C1 and C2 in Figure 5.2 appeared to be different, indicating that they were in fact two separate multi-enzyme complexes. Figure 5.2 shows that there were many proteins common to both complexes but some of these proteins were more concentrated in one than the other.

Figure 5.2 also demonstrates that peaks S3 to S6 had the same SDS-PAGE profiles. The peaks S3 to S6 were small enough to be non-complexed enzymes (Figure 5.1) and it was expected that single bands of decreasing size would appear in each lane from S3 to S6. The presence of multiple bands in these fractions indicated the presence of a combination of small enzymes, and the SDS-PAGE profiles indicated that peaks S3 to S6 all contained the same combination of enzymes.

The sizes of the bands in Figure 5.2 were calculated using a graph of log M_r versus R_f (Appendix 1, Figure A.7). The major bands in lane C1 are 67, 40, 32, 22 and 15 kDa, respectively and the major bands in lane C2 are 54, 38, 25, 19, 16 kDa, respectively. Lanes S3 to S5 contain major bands of 61, 43, 25, 20, 16, 12 and 8 kDa, respectively.

5.3.2 Zymograms

Zymograms were performed on C1 and C2 to determine which of the protein bands in Figure 5.2 displayed enzyme activity. Avicel® and CMC substrates produced very diffuse zymograms (a gel overlay was used for Avicel®) due to the low Avicelase and CMCase activities of C1 and C2, and so only results for xylan are shown (Figure 5.3 and 5.4). Increasing concentrations of C1 and C2 were used for xylan zymograms to determine at which concentration the enzyme complexes could degrade this substrate in the gel (Figure 5.3).

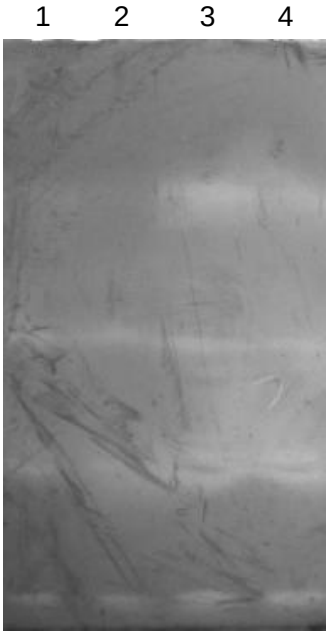


Figure 5.3 Birchwood xylan zymogram (10%) where lanes 1 to 4 contain 20 µg C1 and C2, and 50 µg C1 and C2, respectively.

Figure 5.3 shows that 20 µg of protein was too low to detect clearing zones in a birchwood xylan zymogram and that 50 µg was more appropriate. However, 100 µg of

protein was required for oat spelt xylan zymograms as no bands were seen at lower concentrations (data not shown).

Birchwood and oat spelt xylan zymograms were repeated using 50 µg birchwood xylan and 100 µg oat spelt xylan for 24 and 48 hours, respectively (Figure 5.4).

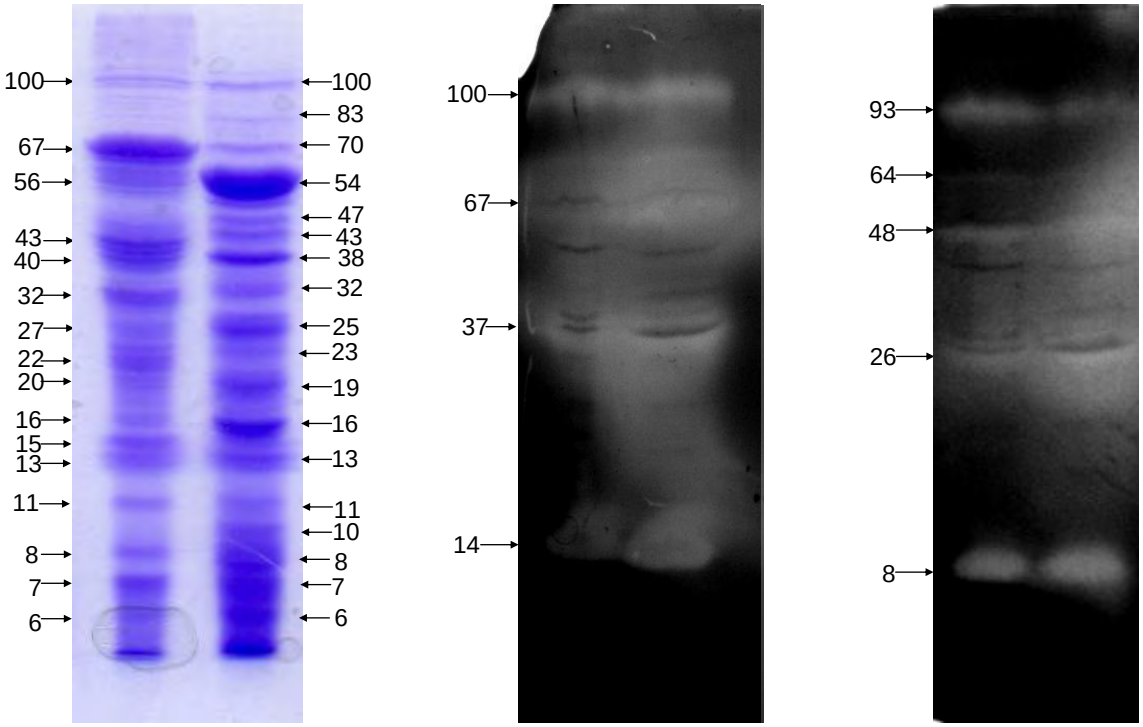


Figure 5.4 **A:** SDS-PAGE (10%) of 20 µg C1 and C2; **B:** birchwood xylan zymogram (10%) of 50 µg C1 and C2; **C:** oat spelt xylan zymogram (10%) of 100 µg C1 and C2. Sizes of the bands (kDa) are labelled with arrows.

Figure 5.4 shows that C1 and C2 contained enzymes of 100 kDa, 67 kDa, 37 kDa, and 14 kDa, respectively, capable of degrading birchwood xylan. Bands of similar sizes were visualized in these complexes when oat spelt xylan was used as a substrate with an additional band of 48 kDa.

5.3.3 Substrate specificity

To determine the range of substrates that C1 and C2 can degrade the DNS assay was performed using a variety of cellulose and hemicellulose substrates (Figure 5.5).

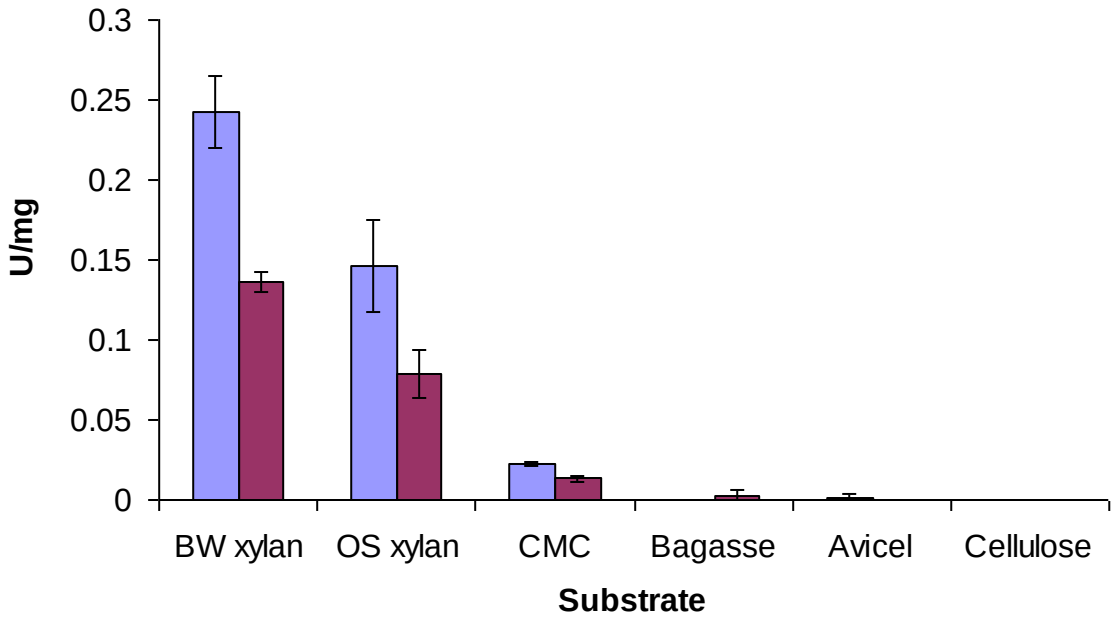


Figure 5.5 Enzyme activity (U/ml) of C1 and C2 on various substrates, determined using the DNS assay. U = µmol reducing sugar produced/hour.

Figure 5.5 demonstrates that both C1 and C2 degrade xylan substrates with much higher efficiency than cellulose substrates. The complex C2 displayed slightly lower enzyme activities than C1. However, it is interesting to note that C2 displayed a small degree of activity on sugarcane bagasse.

5.3.4 Glycoprotein detection

A glycoprotein detection kit was used to detect the presence of glycoproteins in the MECs, C1 and C2 (Figure 5.6).

Figure 5.6 Glycoprotein detection test, where a pink band indicates the presence of glycoproteins. PC is the positive control (containing peroxidase); Lanes 1 and 2 contained 20 µg C1 and C2; and Lanes 3 and 4 contained 50 µg of C1 and C2.

Lane PC in Figure 5.6 represents the positive control which contained peroxidase. The pink band in this lane indicates the presence of a glycoprotein, and since no other pink bands were seen on the gel it was concluded that no glycoproteins were detected in either C1 or C2.

5.3.5 Substrate binding study

Cellulosomes and xylanosomes have binding domains that allow for the binding of the enzymes complexes to insoluble substrates. A binding study was carried out using Avicel® and insoluble birchwood xylan to see if C1 and C2 could bind to these substrates, and these results were expressed in % binding (Table 5.1).

Table 5.1 Binding (%) of two MECs (C1 and C2) to insoluble Avicel® and birchwood xylan substrate.

	C1	C2
Avicel	0	10.92
Xylan	16.07	25.93

Table 5.1 shows that there was a small amount of binding of the MECs to insoluble substrates.

5.4 Discussion and Conclusions

Peaks C1 and C2 were poorly resolved on the Sephacryl S-400 column because they were close in size (371 and 267 kDa, respectively) (Figure 5.1). Although there were enzymes common to both complexes, their SDS-PAGE profiles were different (Figure 5.2). It was concluded that peaks C1 and C2 represented two separate MECs but, due to limited resolution on Sephacryl S-400, they co-eluted to some degree.

The limited resolution of the Sephacryl S-400 column was again seen with the smaller protein peaks, S3 to S6, which each contained the same combination of proteins (Figure 5.2). The Sephacryl S-400 column was not capable of resolving small proteins, and so peaks S3 to S6 in Figure 5.1 were mixtures of non-complexed enzymes of similar sizes, below 100 kDa.

The molecular weight of C1 (371 kDa) was similar to that of complexes isolated by Kim and Kim (1993) and Pason *et al.* (2006) from *B. circulans* and *Paenibacillus curdlanolyticus*, respectively, which were each approximately 400 kDa in size (Section 5.1). Both C1 and C2, however, were smaller than other MECs and cellulosomes described in literature (Bayer and Lamed, 1986; Desvaux, 2005b; Doi *et al.,* 1998; Pason *et al.,* 2006a; van Dyk *et al.,* 2009).

Figure 5.4 indicates that C1 and C2 exhibited the same profiles on xylan zymograms. Each complex consisted of 4 xylanases that degraded birchwood xylan and 5 that degraded oat spelt xylan. The two MECs of *P. curdlanolyticus* contained 7 and 5 xylanases, respectively, according to zymograms using 0.1% (w/v) soluble xylan (Pason *et al.,* 2006a), and the MEC from *Bacillus licheniformis* contained 2 xylanases (van Dyk *et al.,* 2009). The xylanosomes of *Streptomyces olivaceoviridis*, *Butyrivibrio fibrisolvens*

and C*haetomium* spp. contained 4, 11 and 5 xylanases, respectively (Jiang *et al.,* 2006; Lin and Thomson, 1991; Ohtsuki *et al.,* 2005; van Dyk *et al.,* 2009).

Avicel® and CMC zymograms of C1 and C2 did not produce bands (data not shown) due to the low activities on these substrates, as seen in Figure 5.5. A study was performed in which samples were boiled for 1 and 3 minutes, instead of 5 minutes, before loading onto zymograms to denature the MECs without reducing the activity of the enzymes, but the Avicel® and CMC zymograms were still unclear. The substrate specificity test in Figure 5.5 shows that C1 and C2 had predominantly xylanase activity, with small amounts of activity on CMC and bagasse. Many species of *Bacillus* produce xylanase enzymes and it is common to see xylanases as the predominant enzymes in MECs produced by bacilli (Kim and Kim, 1993; Pason *et al.,* 2006a; van Dyk *et al.,* 2009). Van Dyk and co-workers discuss the possibility of the *B. licheniformis* MEC being a xylanosome (van Dyk *et al.,* 2009). A xylanosome has a structure analogous to the cellulosome but has predominantly xylanase activity and will have a xylan binding domain (XBD) instead of a cellulose binding domain (CBD) (Jiang *et al.,* 2005). There are several characteristics that can be used to identify a cellulosome or xylanosome. Previous studies reported in the literature used bioinformatic analysis to reveal the presence of key nucleotide sequences that code for dockerin and cohesion modules; electron microscopy to reveal the presence of protuberances on the cell surface; and binding studies to indicate the presence of a CBD or XBD (Section 1.3; Schwarz, 2001; van Dyk, 2009).

The cellulosomes of *C. thermocellum* and *C. papyrosolvens* contain a non-catalytic glycoprotein called CipA which serves as a scaffolding for the enzyme subunits of the cellulosomes and mediates binding to cellulose (Bhat *et al.,* 1997; Garcia-Campayo and Béguin, 1997; Pohlschroder *et al.,* 1994). Previous research has also shown that some MECs contain catalytic subunits that are glycosylated (Desvaux, 2005b; Gal *et al.,* 1997). Figure 5.6 revealed that there were no glycoproteins in C1 and C2, which indicated the absence of the CipA scaffolding found in the cellulosome of *C. thermocellum* and *C. papyrosolvens*. Therefore, if the MECs from *B. subtilis* SJ01 have unglycosylated scaffolding proteins and catalytic domains, it would indicate that these complexes have a

structure different to many of the cellulosomes and other (hemi-)cellulolytic MECs described in literature.

Cellulosomes and xylanosomes bind to insoluble substrates via binding domains associated with the scaffoldin or the catalytic subunits (Bayer *et al.,* 1994). In previous binding studies (Jiang *et al.,* 2006) reported 50% binding of the *S. olivaceoviridis* xylanosome to insoluble xylan, (Pason *et al.,* 2006b) reported 57% binding of the *P. curdlanolyticus* (hemi-)cellulolytic MEC to insoluble xylan and Ratanakhanokchai *et al.* (1999) reported 60% binding of an endoxylanase to insoluble xylan. The small degree of binding to Avicel® and insoluble xylan by C1 and C2 (Table 5.1) indicated the absence of a substrate binding domain on the complexes themselves. However, since there was some binding, it is possible that some of the enzymes had individual binding sites and detached from the complex to bind to the substrate, leaving the bulk of the complex unbound in the supernatant (Boraston *et al.*, 2004; Ratanakhanokchai *et al.,* 1999; Sá-Pereira *et al.,* 2002a).

Therefore, although the MECs of *B. subtilis* SJ01 are of similar size and consist of similar catalytic subunits to other (hemi-)cellulolytic MECs, they do not share the same characteristics as the cellulosomes and xylanosomes isolated previously and further research is required to understand the exact nature of these MECs.

CHAPTER 6

General discussion, conclusions and future recommendations

The enzymatic degradation of cellulose and hemicellulose is an important process because these substrates are abundant sources of carbon and their degradation products have many industrial applications. One such application is the production of bioethanol or biobutanol from lignocellulosic waste material, which has gained much interest recently with the growing need to produce environmentally friendly fuels from renewable sources. Enzymatic degradation is more efficient than chemical degradation, and, in particular, complexes consisting of multiple enzymes have been shown to degrade cellulose/hemicellulose with higher efficiency that individual enzymes. The cellulosome is a multi-enzyme complex (MEC) that degrades cellulose with particularly high efficiency and has, therefore, been studied extensively. Research into the production of MECs from aerobic bacilli is now being carried out to see if these bacteria produce cellulosome- or xylanosome-type MECs. In this research a *Bacillus subtilis* SJ01 strain capable of degrading cellulose and hemicellulose was selected and two MECs produced by this bacterium were purified and partially characterised.

Many species of *Bacillus* are able to degrade cellulose and hemicellulose and Chapter 3 demonstrated that this bacterial genus was predominant in aerobic environments containing these carbon sources. Many species of *Bacillus* also produced large protein aggregates that could be MECs. The purification and partial characterisation of *Bacillus* MECs has been carried out on *B. circulans* (Kim and Kim, 1993), *Paenibacillus curdlanolyticus* (Pason *et al.*, 2006), *B. licheniformis* (van Dyk *et al.*, 2009) and now *B. subtilis* SJ01.

B. subtilis SJ01 degraded birchwood xylan more readily than Avicel® or CMC, and had improved cellulolytic and xylanolytic abilities when cultured on birchwood xylan. Many other species of *Bacillus* are also good degraders of xylan and produce higher amounts of xylanases than cellulases (Pason *et al.*, 2006a). The xylanolytic activity of *B. subtilis* SJ01 was highest in early stationary phase, at which point this activity was present in

both the culture supernatant and cellular pellet. However, *B. subtilis* SJ01 produced non-complexed xylanolytic and cellulolytic enzymes as well as its MECs, and in early stationary phase (84 hour culture) the MECs were not cell-associated. Therefore, non-complexed enzymes were responsible for the cell-associated xylanase activity. A growth curve for the MECs instead of the total xylanase activity would be a lengthy but important additional experiment to reveal if the MECs, like cellulosomes, are cell-associated in exponential phase and then released into the culture supernatant during growth of the culture (Bayer *et al.*, 1994). Electron microscopy of *B. subtilis* SJ01 cells would also indicate whether the MECs are cell-associated at different stages of growth, and if they form cell protuberances like cellulosomes (Pason *et al.*, 2006).

Two MECs of 371 and 267 kDa, respectively, were purified from *B. subtilis* SJ01 using ultrafiltration followed by size exclusion chromatography. Ultrafiltration is a preferred method for concentrating MECs because the use of a high molecular weight cut-off filter will remove most non-complexed enzymes. For better resolution of the two MECs an anion exchange chromatography step could be added to the purification procedure (Kim and Kim, 1993; van Dyk *et al.,* 2009; van Dyk, 2009). The MECs from *B. subtilis* SJ01 (C1 and C2) consisted of 16 and 18 subunits, respectively, 4 of which degraded birchwood xylan and 5 of which degraded oat spelt xylan. The MECs had low activity on Avicel®, CMC, cellulose powder and bagasse, which suggests that they may be xylanosomes. However, the MECs did not have glycosylated scaffolding proteins, as seen in the cellulosome, and did not bind to insoluble substrates, indicating the lack of substrate binding domains. The MECs from *B. subtilis* SJ01 were, therefore, different in structure to a typical cellulosome. This study is, therefore, important in the discovery of MECs that differ from those previously reported, and thus the expansion of the information on bacterial (hemi-)cellulolytic enzyme systems that already exists.

The hydrolysis of birchwood xylan by C1 and C2 was measured in μmoles of reducing sugar produced per hour per milligram of protein (U/mg). Compared to the xylanolytic MECs purified previously (Table 6.1), C1 and C2 were relatively inefficient with 0.25 U/mg and 0.14 U/mg xylanase activities, respectively. These low enzyme activities limit

the use of these MECs in research and industry. However, further studies can be performed to investigate the structure and function of these MECs, to determine their range of activity and hence increase their usefulness in addition to other xylanolytic MECs.

Table 6.1 Xylanase activities of xylanolytic MECs.

Species	Xylanase activity of MEC(s)	Reference
S. olivaceoviridis	406 µmol/min/mg	(Jiang *et al.,* 2004)
B. circulans	137 and 252 µmol/min/mg	(Kim and Kim, 1993)
P. curdlanolyticus	3.17 µmol/min/mg	(Pason *et al.,* 2006a)
B. licheniformis	0.07 µmol/min/mg	(van Dyk *et al.,* 2009; van Dyk *et al.,* 2009)
B. subtilis SJ01	0.004 and 0.002 µmol/min/mg	Chapter 5

To obtain a better understanding of the enzyme subunits making up the xylanolytic MECs of *B. subtilis* SJ01, further substrate specificity tests could be carried out on a wider variety of xylan (or hemicellulose) substrates. There are several types of enzymes that degrade hemicelluloses, including endo-β-1,4-xylanases (EC 3.2.1.8) and β-xylosidases (EC 3.2.1.37) that cleave the xylan backbone and α-L-arabinofuranosidase (EC 3.2.1.55), acetyl esterase (EC 3.1.1.6), and α-D-glucuronidase (EC 3.2.1.1) that cleave the side chains of hemicellulose (Pason *et al.,* 2006b; Ratanakhanokchai *et al.,* 1999). Endo-β-1,4-xylanases activity can be measured using birchwood or oat spelt xylan substrates. For the detection of β-xylosidases and the side chain degrading enzymes the *p*-nitrophenol assay can be used with *p*-nitrophenyl-β-D-xylopyranoside, *p*-nitrophenyl-α-L-arabinofuranoside and *p*-nitrophenylacetate as substrates (Ratanakhanokchai *et al.,* 1999; Waeonukul and Ratanakhanokchai, 2007). The xylanolytic MECs could also be assayed for their ability to degrade natural xylan substrates such as beechwood xylan, kraft pulp xylan, corncob xylan and wheat arabinoxylan (Jiang, 2004) and other natural substrates with high hemicellulose content. Analysis of the degradation products (xylooligomers) using thin layer chromatography (TLC) will also reveal the types of xylanases present in the MECs (Jiang *et al.,* 2005;

Kim and Kim, 1995). N-terminal sequencing analysis could be performed to elucidate both the catalytic and non-catalytic subunits in the MECs (Jiang *et al.,* 2005).

Bagasse is a by-product of the sugarcane industry and is a lignocellulosic material that when degraded into sugars can be used for the bioethanol industry. The MEC, C2, from *B. subtilis* SJ01 was able to degrade this substrate, although the activity was very low. The limited activity of the *B. subtilis* SJ01 MECs indicates that they have limited industrial application. As discussed in Section 1.1, in nature hemicellulose is intertwined with cellulose and lignin, and so for the degradation of natural substrates, MECs that can degrade cellulose and hemicellulose are more desirable. However, xylanases are used in the paper and pulp industry (Balakrishnan *et al.,* 2006), for the processing of fabrics (Dhiman *et al.,* 2008), in food processing, poultry feeds, degumming of plant fibres (Battan *et al.,* 2007) and clarification of juices (Dhillon *et al.,* 2000). Xylanolytic MECs can also be used in conjunction with cellulosomes for the degradation of recalcitrant lignocellulose substrates, and for the construction of designer cellulosomes. There is already a great deal of genetic information on *B. subtilis* and this bacterium is often used in genetic research which means it would be easy to genetically manipulate this species to produce MECs containing a desired combination of enzymes based on synergy studies (Bayer *et al.,* 1994; Murashima *et al.,* 2003).

In conclusion, a strain of *B. subtilis* was successfully isolated from the environment that was capable of degrading xylan via two multi-enzyme complexes. The MECs had relatively poor enzyme activity but further research, including genetic manipulation and assays on a wider variety of xylanolytic substrates, could reveal their industrial importance.

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APPENDIX 1: Standard curves

1.1 Bradford 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 reagent (Sigma). Various concentrations of BSA were prepared ranging from 0.00625 to 1.1 mg/ml. Two standard curves were generated using 5 µl (Figure A1) and 25 µl (Figure A2) sample and 250 µl and 230 µl Bradford reagent, respectively. The large sample volume of 25 µl allowed for the accurate detection of proteins at concentrations as low as 0.00625 mg/ml. The protein samples were mixed with Bradford reagent and allowed to stand at room temperature for 5 minutes before reading the absorbance at 595 nm.

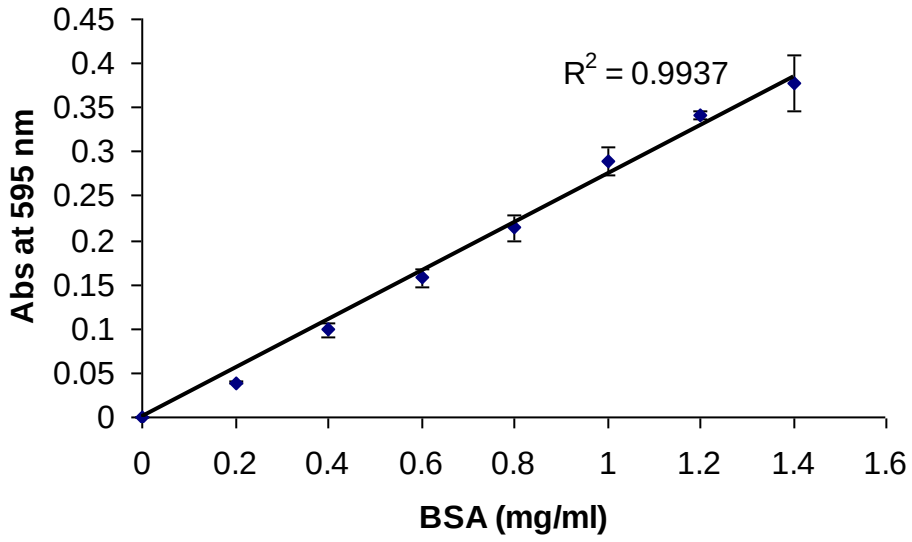


Figure A.1 Bradford standard curve for high protein concentrations.

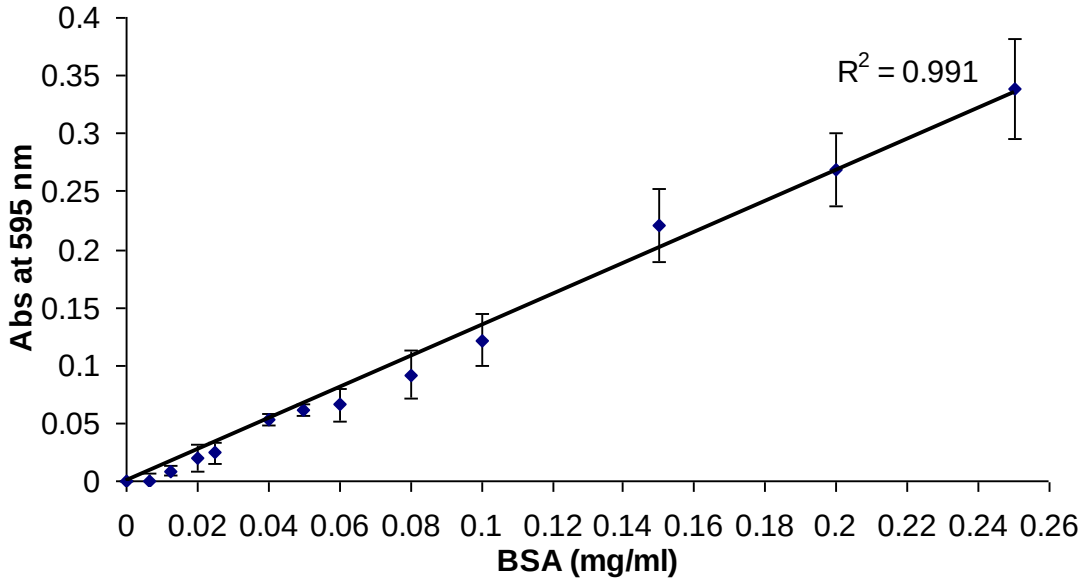


Figure A.2 Bradford standard curve for low protein concentrations.

1.2 Sugar standard curves

Enzyme activity was measured by the reducing sugars formed in a modified dinitrosalicylic acid (DNS) method (Miller, 1959) using glucose and xylose standard curves. The DNS reagent was composed of the following:

2 g sodium hydroxide
2 g 3,5-dinitrosalicylic acid (DNS)
40 g potassium sodium tartrate (Rochelle salt)
0.4 g phenol
0.1 g sodium metabisulphite
200 ml distilled water

The sodium hydroxide was dissolved in 100 ml of distilled water prior to adding the remaining compounds.

The reducing sugar standard curves were generated with concentrations of glucose and xylose between 0 and 0.6 µmol/ml (Figure A3). A 150 µl volume of each concentration of standard was added to 300 µl of DNS reagent. The mixture was heated at 100ºC for 5 minutes and cooled on ice for 5 minutes. Absorbance readings were taken at 540 nm.

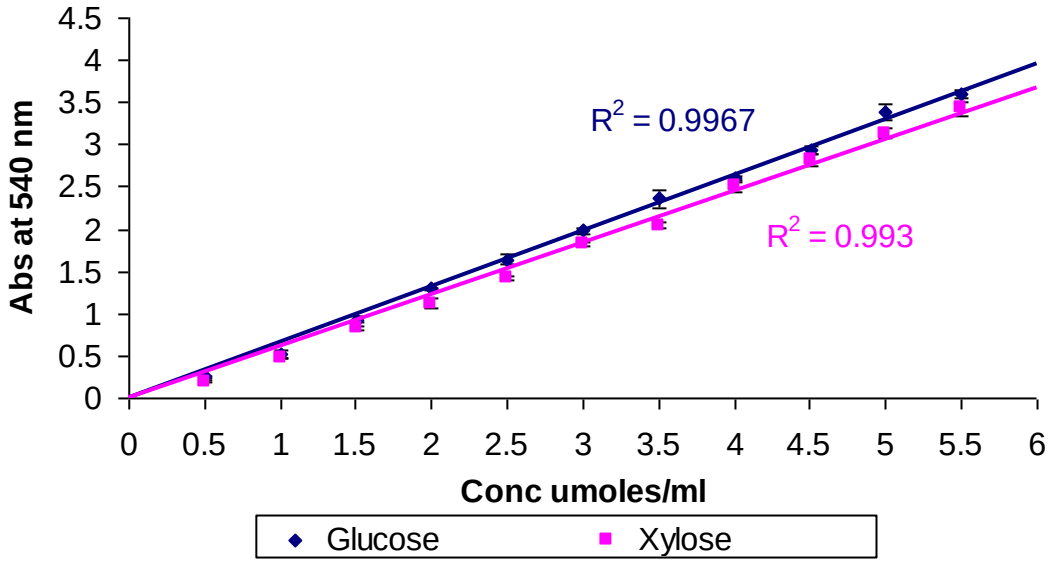


Figure A.3 DNS assay standard curves for glucose and xylose.

1.3 Sepharose 4B calibration curve

The standards Blue Dextran (2000 kDa), thyroglobulin (670 kDa), β-glucoronidase (290 kDa), γ-globulin (150 kDa) and bovine serum albumin (66 kDa) were made up to a concentration of 20 mg/ml. Each standard (1 ml) was loaded separately onto a Sepharose 4B chromatography column and eluted using 20 mM Tris-HCl, pH7.0. The elution volume of each standard was used to calculate K_{av} and calibration curve was generated (Figure A.4).

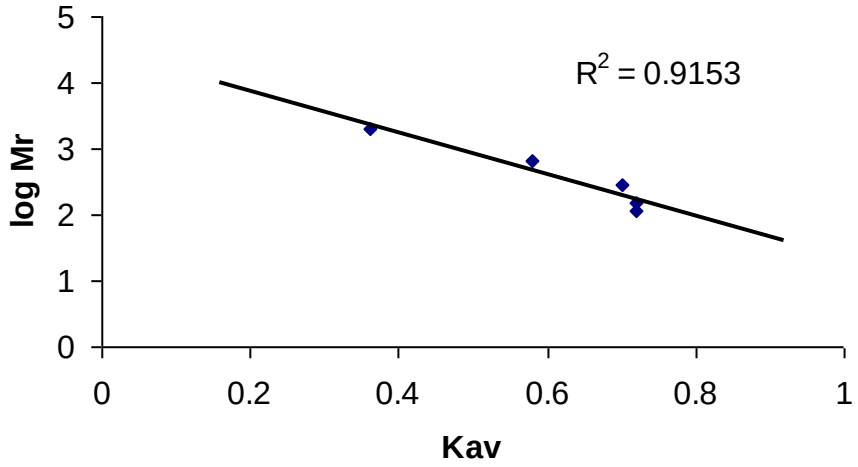


Figure A.4 Sepharose 4B calibration curve.

1.4 Sephacryl S-400 calibration curve

The standards Blue Dextran (2000 kDa), thyroglobulin (670 kDa), ferritin (450 kDa), catalase (250 kDa), γ-globulin (150 kDa) and BSA (66 kDa) were made up to a concentration of 20 mg/ml and 1 ml of each was loaded onto a Sephacryl S-400 column. The standards were eluted with a Tris-HCl, pH 7.0. The elution volume of each standard was used to calculate K_{av} and a calibration curve was generated (Figure A.5).

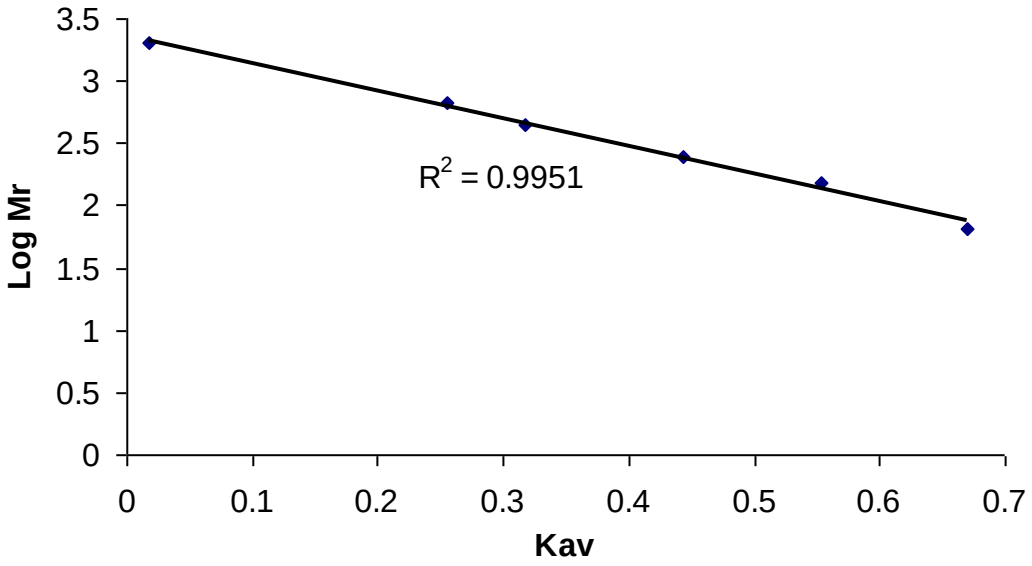


Figure A.5. Sephacryl S-400 calibration curve.

1.5 Folin-Lowry standard curve

An alternative method of protein detection was the Folin-Lowry assay. The standard curve for this assay was generated using various concentrations of BSA from 0 to 0.1 mg/ml. A 50 µl volume of sample was added to a solution containing 1 ml of solution A (0.05 g/l $CuSO_4.5H_2O$ and 0.1 g/l $Na_3C_6H_5O_7.2H_2O$) and 50 ml of solution B (20 g/l Na_2CO_3 and 4 g/l NaOH), and incubated at room temperature for 10 minutes. Folin-Ciocalteau phenol reagent (25 µl) was added and the mixture incubated for a further 30 minutes at room temperature. The absorbance was read at 750 nm to obtain the standard curve in Figure A.3.

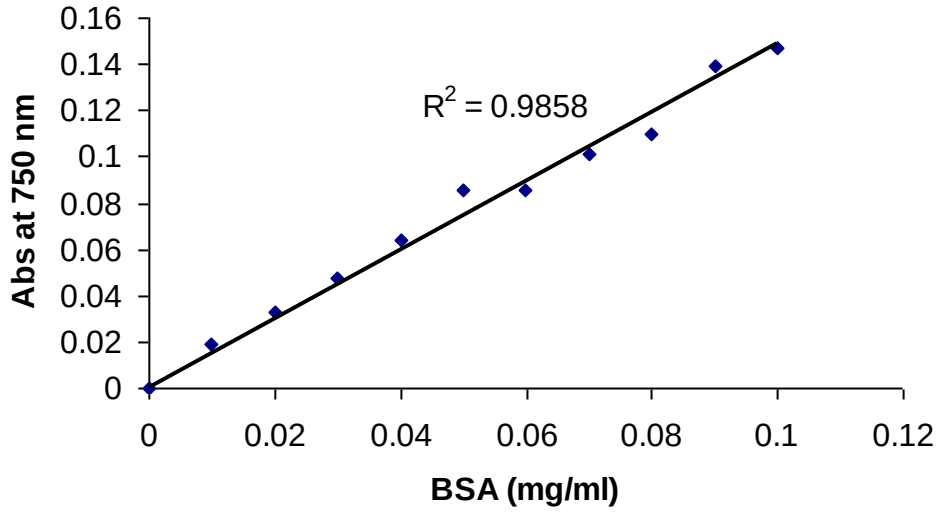


Figure A.6 Folin-Lowry standard curve.

1.6 Electrophoresis standard curves

A peqGold protein marker was electrophoresed on a 10% SDS-PAGE gel and the distance migrated by each standard was measured. The R_f values for each protein band were calculated and a standard curve of log M_r versus R_f was calculated (Figures A.7, A.8 and A.9).

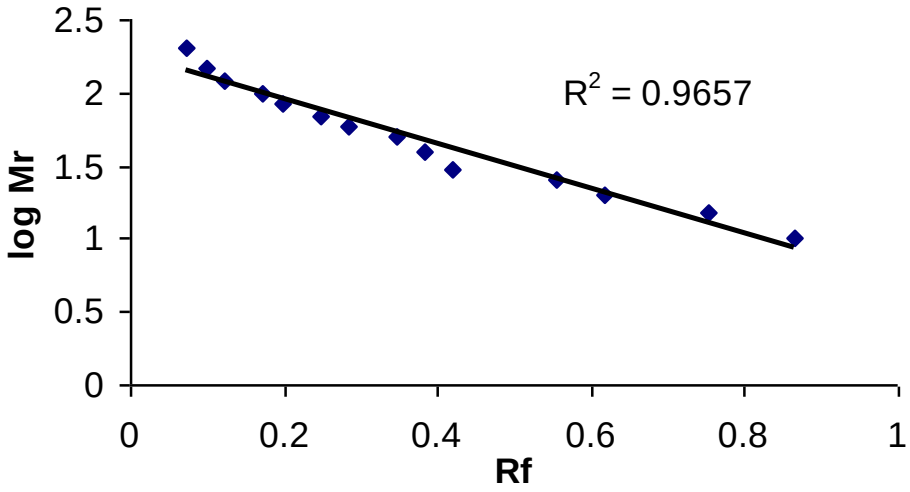


Figure A.7. Standard curve of the PeqGold protein marker run on 10% SDS-PAGE.

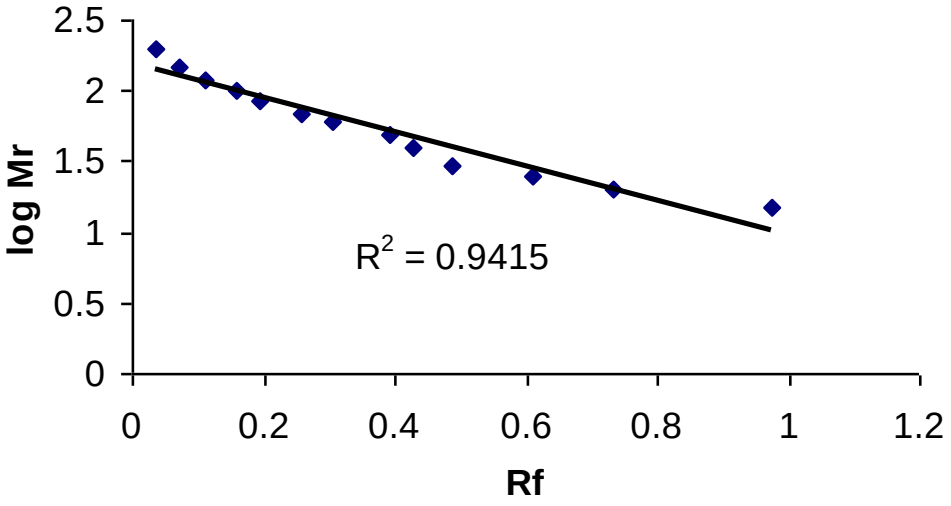


Figure A.8. Standard curve of the PeqGold protein marker run on 10% SDS-PAGE for birchwood xylan zymogram.

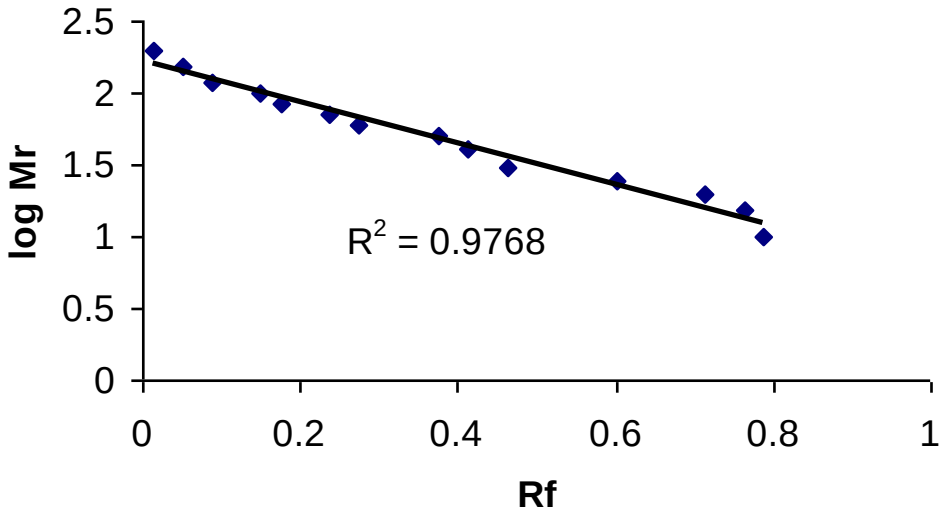
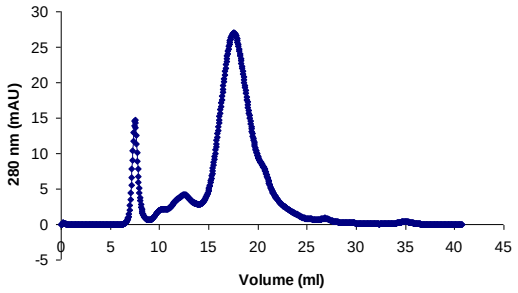


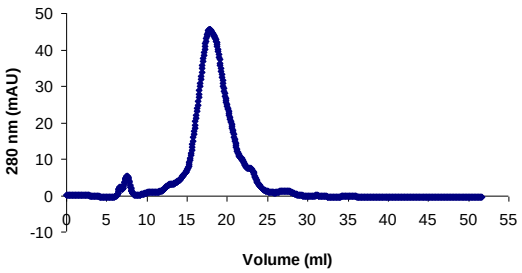
Figure A.9. Standard curve of the PeqGold protein marker run on 10% SDS-PAGE for oat spelt xylan zymogram.

APPENDIX 2: Screening for MECs using Superose 12 size exclusion chromatography

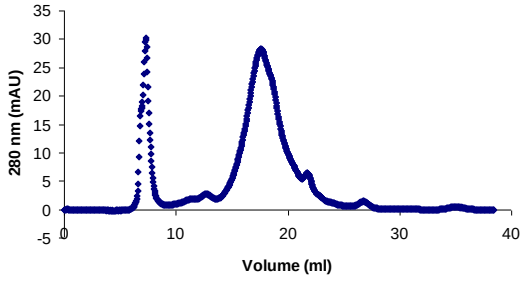
All the isolated strains in Table 2.1 were cultured in enrichment media containing 0.05 g/l (w/v) Avicel®, 0.05 g/l (w/v) peptone, 0.01 g/l (w/v) yeast, 0.05 g/l (w/v) NaCl, 0.01 g/l (w/v) K_2HPO_4, 0.02 g/l (w/v) $MgSO_4$, 0.01 g/l (w/v) $CaCl_2$, 0.01 g/l (w/v) tryptone soy broth and 0.01 g/l (w/v) cellobiose at 37°C and 200 rpm. In order to determine the presence of a MEC, each culture supernatant was dialysed overnight in 50 mM Tris-HCl buffer, pH 7.0, and concentrated using PEG 20 000. The concentrated samples were filtered using 0.45 µl syringe filters. About 2 ml of each was loaded on a Superose 12 size exclusion column (separation range: 1 – 300 kDa) on a FPLC system (Figure A.10). The following standards were loaded on the column: Dextran Blue (2000 kDa), β-galactosidase (116 kDa), BSA (66.4 kDa), trypsin (24 kDa), lysozyme (14.7 kDa) (Fluka) and Vitamin B12 (1.6 kDa) (all standards were from Sigma unless stated otherwise). A peak in the void volume (7.87 ml for Dextran Blue) indicated the possible presence of a MEC.



C1W



C1O



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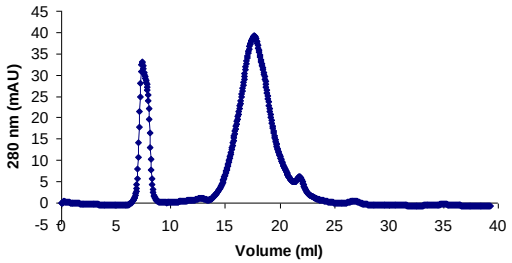

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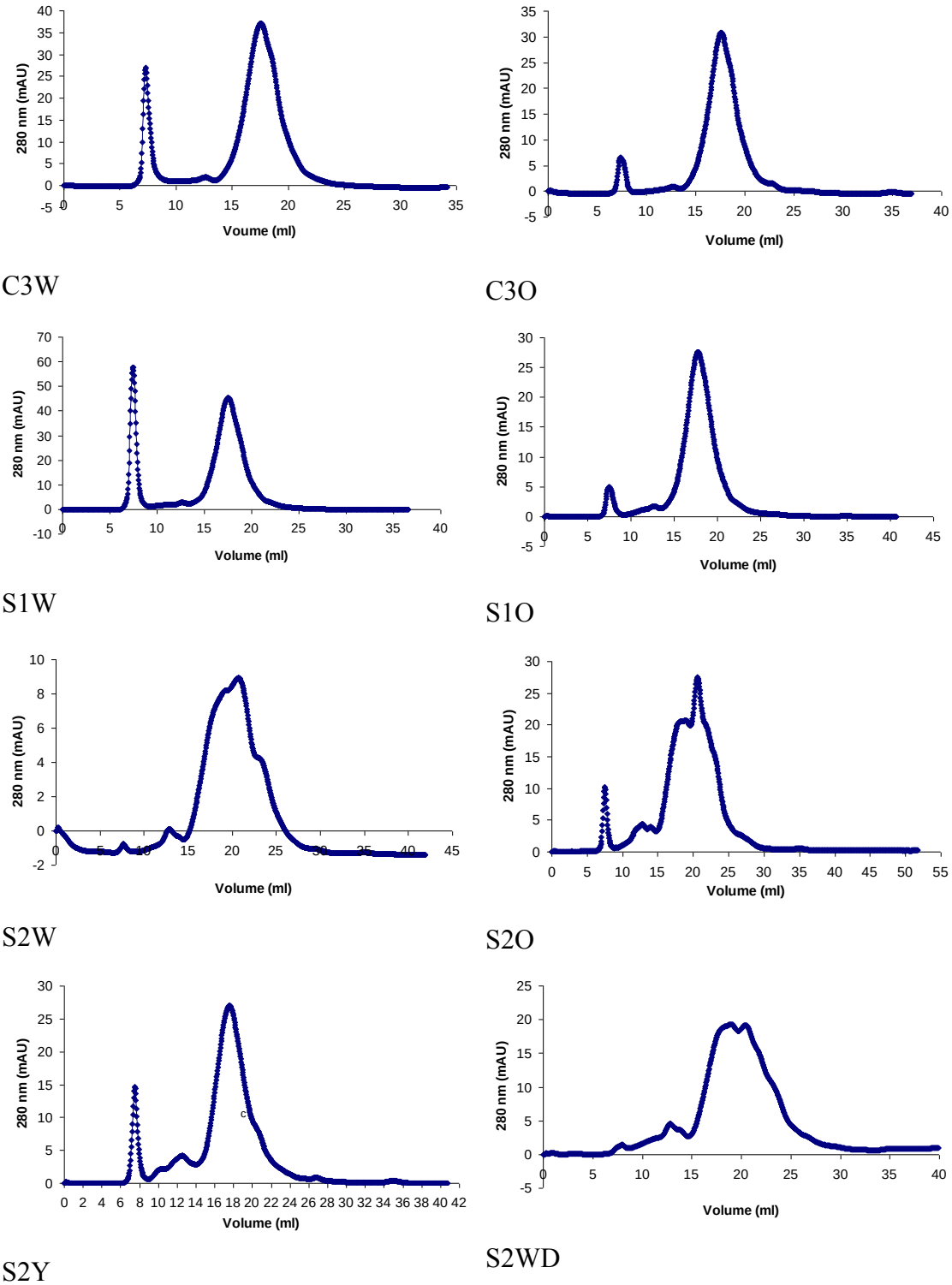

Figure A.10 Superose 12 chromatograms of extracellular proteins from several *Bacillus* strains as indicated below each graph. Column dimensions: 1 × 30 cm; flow rate: 0.5 ml/min. ♦ A_{280} (mAU). V_o = 7.87 ml.

APPENDIX 3: List of Reagents

Table A.1 Reagents and chemicals and the supplier used.

Name of reagent	Supplier and catalog number
β-galactosidase	Sigma (G5160)
β-mercaptoethanol	Fluka (63700)
Acetone	Merck (8.22251.2500)
Acrylamide	Sigma (Cat. No. A8887)
Agarose	Sigma (A9539)
Ammonium chloride	Saarchem (1122720 EM)
Ammonium persulpahte	Sigma Aldrich (Cat. No. A3678)
Ammonium sulphate	Merck (1.01217.1000)
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)
Calcium chloride	Saarchem (152 49 00 EM)
Carboxymethyl cellulose	Calbiochem (Cat. No. 217277)
Catalase	Sigma (C9322)
Cellulose	Aldrich (31.069-1)
Congo Red	Sigma (Cat. No. C6767)
Coomassie Brilliant Blue G	Merck (Cat. No. 1.12553)
Copper sulphate	Sigma (C8027)
D-(+)-Cellobiose	Sigma-Aldrich (Cat. No. C7252)
D-(+)-Xylose	Sigma (X-3877)
Dextran (5000 - 40 000 kDa)	Sigma (D5501)
Dextran Blue	Fluka (Cat. No. 31393)
3,5-Dinitrosalicylic acid (DNS)	Sigma (Cat. No. D0550)
Di-potassium hydrogen phosphate	Merck (1.05104.1000)
Ethanol	Merck (Cat. No. 8.18700)
Ferritin	Sigma (F4503)

Folin-Ciocalteau	Merck (1.09001.0500)
Gamma globulin	Sigma (49030)
Glaciel acetic acid	Merck (Cat. No. 1.00063)
Glucose	Saarchem (267 60 20 EM)
Glycerol	Saarchem (Cat. No.2676520)
Lysozyme	Sigma (L2879)
Magnesium sulphate	Saarchem (Cat. No. 412 39 20 EM)
Methanol	Merck (Cat. No. 8.22283)
Nutrient Broth	Biolab, Merck (C24)
Oat spelt xylan	Fluka (95590)
Peptone	Fluka (70169)
peqGOLD protein marker II	peqLab (Cat. No. 27-2010)
Phenylmethanesulfonyl fluoride (PMSF)	Sigma (P7626)
Polyethylene glycol (PEG) 20 000	Merck (Cat. No. 8.18897)
Potassium hydrogen phosphate	Merck (1.04877.1000)
Potassium sodium tartrate	Merck 1.08087.1000)
Sepharose CL-4B	Sigma (CL-4B-200)
Sodium carbonate	Saarchem (5822800)
Sodium Chloride	Saarchem (Cat. No. 5822320)
Sodium citrate	Sigma (W 302600)
Sodium dodecyl sulphate (SDS)	BDH biochemicals (301754)
Sodium hydroxide	Saarchem (Cat. No. 5823200)
Sodium metabisulphite	Sigma-Aldrich (Cat. No. 301754)
Thyroglobulin	Sigma (T1001)
Tris (hydroxymethyl) aminomethane	Merck (Cat. No. 1.08382)
Triton X-100	Merck (1.08603.1000)
Trypsin	Sigma (T1426)
Tryptone	Fluka (T7293)
Tryptone soy broth	Fluka (08069)
Vitamin B12	Sigma (V2876)
Yeast extract	Biolab (Cat. No. BX6)