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Identification and enzyme production of a cellulolytic *Bacillus*-strain isolated from moose (*Alces alces*) rumen

Master's Degree in Applied and Commercial Biotechnology

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DEDICATION

This Thesis work is dedicated to the Lord of lords and the King of kings, to the Almighty God whom I serve. Thank you for your grace and mercy.

Table of contents

Abbreviations

(CBM)- Carbohydrate-binding module

(SLH)- S-layer homology

LPXTG- (Leu-Pro-any-Thr-Gly)

(NREL)- Colorado State's Natural Resource Ecology Laboratory

(ABB) - Anaerobic Basal Broth

(ABB-) – Anaerobic Basal Broth without supplements

(ABB+cellobiose) - Anaerobic Basal Broth supplemented with 0.5% cellobiose

(ABB+xylan) - Anaerobic Basal Broth supplemented with 0.5% xylan

(BHI) – Brain Heart Infusion

(BHI-) - Brain Heart Infusion without supplements

(BHI+cellobiose) - Brain Heart Infusion supplemented with cellobiose

(BHI+xylan) - Brain Heart Infusion supplemented with xylan

(MEC)- Multi-enzyme complex

(CMC) –Carboxy Methyl Cellulose

(SDS-PAGE) – Sodium dodecyl sulfate polyacrylamide gel electrophoresis

(DTT)- Dithiothreitol

(BME)- 2-mercaptoethanol (beta-mercaptoethanol)

List of figures

List of Tables

List of Appendix

Abstract

A major obstacle to industrial-scale production of fuel from lignocellulose lies in the inefficient deconstruction of plant material, due to the recalcitrant nature of the substrate toward enzymatic breakdown and the relatively low activity of currently available hydrolytic enzymes. Improvement of the process of cellulase production and development of more efficient lignocellulose-degrading enzymes are necessary in order to reduce the cost of enzymes required in the biomass-to-bioethanol process.

Cellulases are required for cellulose degradation in nature and almost all of the biomass produced is mineralized again by enzymes which are provided by microorganisms. The crystalline material is hydrolyzed by a number of simultaneously present, interacting enzymes (endoglucanase, exoclucanase and β-glucanase), or alternatively by a multienzyme complex. Cellulosome complexes are intricate multi-enzyme machines produced by many cellulolytic microorganisms. They are characterized by having a scaffolding protein, and are typically anchored to the cell membrane through a dockerin-protein. The goal of this work involves the production, identification and initial purification of a cellulolytic and hemicellulolytic enzymes from bacterial isolates from moose (*Alces alces*) rumen. Five bacterial isolates (MRB 1-5) were comparatively analysed for effective producer of cellulase enzyme. Isolates were screened for cellulolytic activity using Carboxy Methyl Cellulose (CMC) agar plates and DNS reducing sugar assay, these techniques are time-efficient and reliable in identification of cellulolytic microorganisms. Screening also included growth curve characteristics under anaerobic and aerobic conditions. Among the five bacterial isolates, isolate MRB 3 was found to be the most effective cellulase producer both qualitatively and quantitatively. MRB 3 was identified by use of of DNA isolation and 16sRNA analysis as a strain of *Bacillus licheniformis*, tentatively named AA1.

CMC- zymogram analysis of SDS-PAGE gels demonstrated two catalytically active bands at approximately 65 kDa and 45kDa. Most of the samples purified from *B. licheniformis* AA1 cultures showed several protein bands on SDS-PAGE with the highest band at approximately 200kDa. The presumed MEC is not attached to the cell wall but is secreted into the supernatant. The CMC-ase active, high molecular protein band and lower fragments observed in this organism, further promote the hypothesis that a MEC is present in *B. licheniformis* AA1. In shaking cultures supplemented with 0.5% CMC or beechwood xylan, *B. licheniformis* AA1 was able to regulate enzyme expression based on the substrate. A stepwise release of enzyme activity by affinity washing of cellulose-bond enzyme showed that the cellulase-activity could bind to insoluble Avicel. The protein and enzyme activity was

concentrated by about two fold from culture supernatants by crossflow filtration with 95% recovery of total enzyme activity. However, significant amounts of activity passed through both 50 and 10 kDa UF membranes, indicating the presence of low-molecular cellulases. Purification of MEC from a culture supernatant was not successful. The target protein failed to bind on these otherwise standard high-yielding columns assumable not because of charge incompatibility but due to the large size of the MEC.

It is concluded that a strain of *B. licheniformis* was isolated from the rumen of the moose (*Alces alces*) and was named *B. licheniformis* AA1. It is likely that a MEC was isolated in this organism because SDS-PAGE and zymograms were repeatedly carried out with different forms of purified MEC and results showed consistency, indicating a composition that is non-random. In addition, the inability to successfully isolate the MEC through the ion exchange chromatography was presumed to be due to size exclusion. Further experiments to verify the existence and composition of a MEC consisting of cellulases and hemicellulases in this organism are suggested.

1. Introduction

1.1 Background

1.1.1 Biofuel and fossil fuel

Carbon dioxide, the major byproduct of fossil fuel combustion, is a potent greenhouse gas that remains in the atmosphere indefinitely. To reduce the carbon dioxide emissions, governments, car manufacturers and utility companies have been looking into developing an alternative energy sources. Among the leading contenders are biofuels — renewable, clean-burning fuels made from plant- and animal-based source materials like sugarcane, corn, soybeans, discarded vegetable oil or animal fat. "Biofuels have a potential to reduce our dependence on imported gasoline and diesel fuel," said William Parton (2007), researcher from Colorado State's Natural Resource Ecology Laboratory (NREL) (Parton, 2007).

For over 100years, the world has depended on fossil fuels for the production of transportation fuels. However, the oil crisis in 1973 and subsequently in 1979, which saw dramatic increases in the crude oil price, brought about the initiatives by governments for large scale research and production of alternative liquid transportation fuels (United Nations, 2008). Many countries are now into biofuel production and one of the countries that are most advanced in terms of biofuel production is Brazil where 40% of the country’s petroleum requirements are supplied by bioethanol from sugarcane (Goldemberg, 2008).

The US Department of Energy Office of the Biomass Program has set a target for biofuel production in the US to supply 60 billion gallons per year by 2030, which amounts to 30% of liquid fuel consumption for vehicles (at 2004 level) (Himmel et al., 2007). The European Union has also set a target for biofuel production by 2030 which amounts to 25% of transportation fuel requirements (Himmel et al., 2007). Bioenergy has been recognised as a renewable energy source that could have a potential impact on greenhouse gas emissions (Champagne, 2007; Goldemberg, 2008; United Nations, 2008)

1.1.2 Alternatives to fossil fuel

It is known that about 97% of current world requirements for liquid transportation fuel are derived from petroleum (Mielenz, 2001). The main biofuels with the potential to replace petroleum are biodiesel, bioethanol, biobutanol and purified biogas also known as biomethane, swamp gas, landfill gas, or digester gas—is the gaseous product of anaerobic

digestion (decomposition without oxygen) of organic matter recovered from landfill/ anaerobic digesters.

Bioethanol refers to the bioconversion of sugars from plant sources to ethanol via fermentation processes. Biodiesel is the production of fuel diesel from plant oils and usually via a chemical transesterification process although some enzymatic processes are also used (Antczak et al., 2009; Basha et al., 2009; Sharma and Singh, 2009). Biobutanol has been seen as an alternative to bioethanol and has several advantages over bioethanol (Ezeji et al., 2007; Wackett, 2008). Bioetanol has been produced by fermentation by naturally occurring solventogenic clostridia such as *Clostridium acetobutylicum* and *Clostridium beijerinckii (Montoya et al., 2000; Qureshi et al., 2006).*

1.1.3 First and second generation biofuel (and effect on food security)

A biofuel is a type of fuel whose energy is derived from biological carbon fixation (United Nations, 2008). Biofuel has been categorised into different types based on the substrates from which it is produced from. First generation biofuels are known as the production of biofuel from crops that are also used as food, e.g. sugar cane, sugar beet, maize, palm oil, oilseeds (United Nations, 2008). The technology for this process is well-established and successful on a large scale. However, the use of food crops for this purpose has become an issue of great concern as it threatens food security worldwide. In the USA, the use of corn on a large scale in bioethanol production has been reported to have a significant impact on the price of corn and thus the price of food (Gomez et al., 2008). Thus it is clear that first generation biofuel technologies can be problematic and is likely to be unsustainable for large scale production of biofuel as world population grows and food demands increase. Other critical issue is the imbalance of fossil fuel energy used for making the bioethanol and distribution energy to transport the ethanol which includes basic transportation of material and heating, and full irrigation (when there is little or no rainfall) which require about 100 cm of water per growing season (USDA., 1997a). Corn production currently is irrigated in the US (USDA., 1997a). Although not all of these require full irrigation; so therefore, it might not really be a big issue. In the US, the mean irrigation for all land growing corn grain is 8.1 cm per ha during the growing season. The total energy input to produce a liter of ethanol is 6,597 kcal. However, a liter of ethanol has an energy value of only 5,130 kcal. Thus, there is a net energy loss of 1,467 kcal of ethanol produced (Pimentel and Patzek, 2005). Land use has also been a debate for a while now; it could be a problem for both first and second generation biofuel production. At present, world agricultural land based on calories supplies more than 99.7% of all world food (calories), while aquatic ecosystems supply less than 0.3% (FAO, 2001). Already

worldwide, during the last decade per capita available cropland decreased by irrigation 12%, and fertilizers 17% (Brown, 1997). Increasing ethanol production could mean diverting valuable cropland from producing corn needed to feed people to producing corn for ethanol factories (Pimentel and Patzek, 2005). In this regard, there is an ongoing debate regarding the benefits of first generation versus second generation technologies (Cockerill and Martin, 2008; Moore, 2008).

Second generation biofuels are the bioethanol production from plant biomass and can provide a solution to many of the problems currently facing first generation biofuel technologies. Since lignocellulose contains about 75% polysaccharide sugars, it can be a valuable feedstock for production of bioethanol (Gomez et al., 2008; Lynd et al., 1991). Lignocellulose in plant biomass can be obtained from various sources, such as agricultural waste, wood, grass and even dedicated crops such as seaweed or switch grass (Champagne, 2007; Duff and Murray, 1996; Gomez et al., 2008; Jasinskas et al., 2008; United Nations, 2008). These are not food crops and thus will not threaten food security while at the same time dedicated crops such as seaweed can be cultivated without fertilizer. Switch grass can also be cultivated on marginal soils with limited fertilizer. The net biomass production worldwide is estimated to be 60 x 10^{12} tons per year in terrestrial and 53 x 10^{12} tons per year in marine ecosystems (Schwarz, 2001a). Recent studies have shown that "biomass is the only domestic, sustainable and renewable primary energy resource that can provide liquid transportation fuels" (Bayer et al., 2007).

1.2 Lignocellulosic biomass and cellulose

The chemical structure of lignocellulosic biomass might not be fully understood yet; Lignocellulose refers to plant dry matter (biomass), and is therefore called lignocellulosic biomass. It is the most abundantly available raw material on the Earth for the production of bio-fuels, mainly bioethanol. It is composed of carbohydrate polymers (cellulose, hemicellulose), and an aromatic polymer (lignin) (Beg et al., 2001). The chemical structure of lignocellulose has to be clarified in order to overcome the obstacles in degradation of lignocellulosic biomass (Raven et al., 1999). Cellulose is the main component of the plant cell wall, consisting of chains of glucose in microfibrils. Classification of hemicellulose is based on sugar moieties found in the hemicellulose fraction (Beg et al., 2001). Hemicellulose is made up of different structure which includes xylan, mannan, galactan and arabinan polymers (Beg et al., 2001). If bioethanol is to be successfully produced in large quantity, the research has to focus on the polysaccharide component of plant cell

walls and the extent to which they can be utilised for saccharification and fermentation into bioethanol. The importance of other structural components such as lignin lies in the degree to which they prevent access to enzymes and therefore degradation of plant cell wall polysaccharides (Raven et al., 1999). Figure 1.1 below is a model showing the chains of lignin interspersed with the components of the plant cell wall.

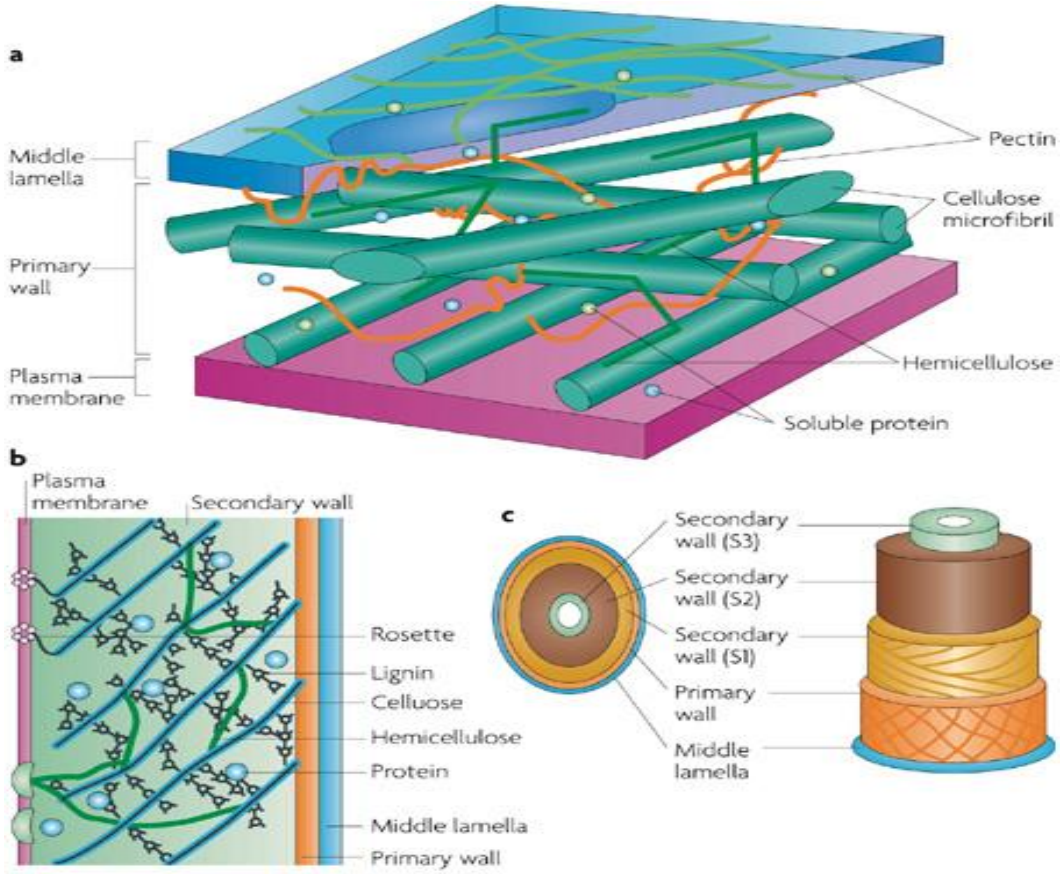


Figure 1.1 Plant plasma membrane and cell wall. A-cell wall containing cellulose microfibrils, hemicellulose, pectin and soluble proteins. B- Cellulose synthase enzymes in form of rosette complexes, which float in plasma membrane. C- showing sites where lignification occur (S1, S2, S3 layers of the cell wall). Source; (Nature Reviews Genetics)

1.2.1 Cellulose

It is said that about half of the carbonaceous compounds in terrestrial biomass are cellulose, which is the most prominent single organic compound on earth (Schwarz, 2001b). The plant cell wall surrounds the cell membrane. It is made up of multiple layers of cellulose which are arranged into primary and secondary walls. Cellulose is the most common organic compound on Earth. About 33% of all plant matter is cellulose - the cellulose content of cotton is 90% and of wood is 50% cellulose (Klemm et al., 2005).

Cellulose is a polymer, polysaccharide, made of repeating glucose molecules attached end to end. A cellulose molecule may be from several hundred to over 10,000 glucose units long. Cellulose from wood pulp has typical chain lengths between 300 and 1700 units; cotton and other plant fibres have chain lengths ranging from 800 to 10,000 units (Klemm et al., 2005).

Figure 1.2 shows the main structure of cellulose and its structural subunits. Cellulose has a simple chemical composition and consists of D-glucose residues linked by β-1,4-glycosidic bonds to form linear polymer chains (Carpita, 1996; Raven et al., 1999; Teeri, 1997).

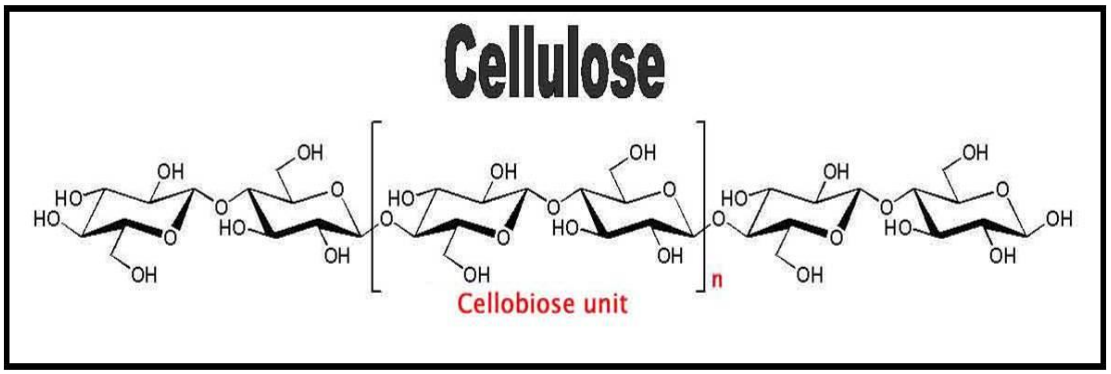


Figure 1.2. Chains of cellulose demonstrating the subunits and the potential hydrogen bonding as well as Van der Waals forces that connect adjacent chains. (SOURCE; (Raven et al., 1999).

Cellulose is similar in form to complex carbohydrates like starch and glycogen. These polysaccharides are also made from multiple subunits of glucose. The difference between cellulose and other complex carbohydrate molecules is how the glucose molecules are linked together. In addition, cellulose is a straight chain polymer, and each cellulose molecule is long and rod-like. This differs from starch, which is a coiled molecule. A result of these differences in structure is that, compared to starch and other carbohydrates, cellulose cannot be broken down into its glucose subunits by any enzymes produced by animals (Klemm et al., 2005). The structural subunit of cellulose is cellobiose, formed by two adjacent glucose residues, figure 1.2 (Schwarz, 2001a). Glucose residues form a chair conformation which form the hydroxyl groups and are forced into equatorial orientation while aliphatic hydrogen atoms are found in axial positions, causing strong hydrogen bonding to take place between adjacent cellulose chains (Himmel et al., 2007). Further, weaker, hydrophobic interactions take place between cellulose sheets (Himmel et al., 2007). Cellulose is a chemically homogeneous linear polymer of up to 10,000 D-glucose molecules, which are connected by ß-1,4-bonds. As each glucose residue is tilted by 180° towards its neighbours, the structural subunit of cellulose is cellobiose (Schwarz, 2001b). The chemical uniformity provokes spontaneous crystallization of the cellulose molecules, the tightly packed microfibrils (Schwarz, 2001b). Cellulose is a

sturdy material ideally suited to insure the structural stability of land plants where it is a main component of the primary cell wall, especially in wood (Schwarz, 2001b).

1.2.2 Cellulose-degrading enzymes

A major obstacle to industrial-scale production of fuel from lignocellulose lies in the inefficient deconstruction of plant material, owing to the recalcitrant nature of the substrate toward enzymatic breakdown and the relatively low activity of currently available hydrolytic enzymes (Hess et al., 2011). Although the success of protein engineering to improve the performance of existing lignocelluloses degrading enzymes has been limited (Wen et al., 2009), retrieving enzymes from naturally evolved biomass-degrading microbial communities offers a promising strategy for the identification of new lignocellulolytic enzymes with potentially improved activities (Rubin, (2008).).

Cellulases are required for cellulose degradation in nature. There are several enzymes displaying three main functional activities required to hydrolyse cellulose into glucose monomers and three main enzymes are responsible for this namely exo-glucanases (also termed cellobiohydrolyases, exo-1,4-β-glucanases, EC 3.2.1.91), endo-glucanases (also termed endo-1,4-β-glucanases, EC 3.2.1.4) and cellobiases (also termed β-glucosidases, EC 3.2.1.21). However, this model is old as it assumes that all exo-glucanases and endo-glucanases are identical, endo and exo activities also overlap. This is not the case and organisms that degrade cellulose effectively actually produce several exo-glucanases and endo-glucanases with different specificities that are required to act in synergy before degradation is achieved (Schwarz, 2001a).

1.2.3 Introduction to cellulose hydrolysis

Almost all of the biomass produced is mineralized again by enzymes which are provided by microorganisms. The polysaccharide hydrolysis is one of the most important enzymatic processes on earth, and cellulose synthesis and hydrolysis is a great part of the carbon cycle. Although crystalline cellulose is chemical homogeneous, no single enzyme is able to hydrolyze it, whereas soluble cellulose derivatives are easily degraded by a single endo-ß-1,4-glucanase (Schwarz, 2001b). The extensive, level surface of the insoluble crystalline microfibrils is an unusual, resilient substrate for hydrolytic (soluble) enzymes (Schwarz, 2001b). Enzyme mechanisms generally depend on single molecules fitting in their substrate pocket - with cellulose the substrate is much larger than the enzyme (Schwarz, 2001b).

The crystalline material is hydrolyzed by a number of simultaneously present, interacting enzymes, or alternatively by a multi-enzyme complex (MEC). Only by cooperation with non-catalytic specific binding modules (the carbohydrate binding proteins or modules) the enzymes are able to disrupt the crystal surface at the solid-liquid interphase, to make single cellulose fibers accessible for hydrolysis. The investigation of the hydrolysis mechanisms of cellulases opens up a new way of looking at enzymatic activity: the dualism between mechanical and structural "preparation" of the insoluble (crystalline) substrate followed by the hydrolytic activity on a released molecule (Schwarz, 2001a).

Hydrolysis of crystalline substrates requires the presence of specialised carbohydrate binding modules (CBM) for significant enzyme activity (Schwarz, 2001a). And not all organisms with cellulolytic capability will possess all three of these enzyme activities. The cellulase system is thus far more complex than originally proposed. Synergy between enzymes is important and the main forms of synergy that has been identified in cellulase systems have been between different exo-glucanases, between endo and exo-glucanases, between exo-glucanases and β-glucosidases and the synergy observed internally between the CBM and the catalytic site (Lynd et al., 1991). The active site mechanism by which glycosyl hydrolases function is a general acid catalysis mechanism in which “two amino acid residues participate in a single-displacement or double-displacement reaction resulting in inversion or retention of configuration at the anomeric carbon atom of the hydrolysed glycoside” (Davies and Henrissat, 1995; Henrissat, 1991).

1.2.4 The role of carbohydrate binding modules (CBMs)

An enzyme is known to have two binding sites involved in binding: the active, catalytic site and the CBM which are linked through a PTS (proline, threonine, serine) linker region (Schwarz, 2001a). CBMs, formally called cellulose binding domains, are amino acid sequences involved in recognition of and binding to polysaccharides. These modules are generally found as part of polysaccharide-degrading enzymes, with the exception of the scaffoldin protein in the cellulosome. The CBM is able to cause disruption of cellulose fibres without any corresponding hydrolytic activity (Boraston et al., 2004; Shoseyov et al., 2006). However, the ability of a CBM to disrupt a substrate such as crystalline cellulose has only been shown in a few cases and does not apply in general to all CBMs (Boraston et al., 2004; Din et al., 1994; Hilden and Johansen, 2004).

1.3 History of discovery of cellulosome

In the early 1980s, Raffi Lamed and Ed Bayer met at Tel Aviv University, Israel commencing their work that led to the discovery of the cellulosome concept. At the time, they weren't looking for enzymes or cellulosomes at all (Alber et al., 2010). They simply sought a 'cellulose-binding factor' or 'CBF' on the cell surface of the anaerobic thermophilic bacterium, *C. thermocellum*, which they inferred would account for the observation that the bacterium attaches strongly to the insoluble cellulose substrate prior to its degradation. They employed a then unconventional experimental approach, in which they isolated an adherence-defective mutant of the bacterium and prepared a specific polyclonal antibody for detection of the functional component. Surprisingly, they isolated a very large multi-subunit supramolecular complex, instead of a small protein. A combination of biochemical, biophysical, immunochemical and ultrastructural techniques, followed by molecular biological verification, led to the definition and proof of the cellulosome concept. The birth of the discrete, multi-enzyme cellulosome complex was thus documented (Alber et al., 2010).

1.3.1 Cellulosome complexes

Cellulosome complexes are intricate multi-enzyme machines produced by many cellulolytic microorganisms. They are designed for efficient degradation of plant cell wall polysaccharides, notably cellulose — the most abundant organic polymer on Earth (Bayer et al., 2004; Bayer et al., 2008; Doi and Kosugi, 2004; Fontes and Gilbert, 2010). The cellulosome consists of a multi-functional integrating subunit called scaffoldin, responsible for organizing the various cellulolytic subunits (e.g., the enzymes) into the complex. Within a cellulosome, multiple endoglucanases, cellobiohydrolases, xylanases and other degradative enzymes work synergistically to attack heterogeneous, insoluble cellulose substrates (Bayer et al., 2004; Bayer et al., 2008; Doi and Kosugi, 2004; Fontes and Gilbert, 2010). This is accomplished by the interaction of two complementary classes of module, located on the two separate types of interacting subunits, i.e., a cohesin module on the scaffoldin and a dockerin module on each enzymatic subunit (Bayer et al., 2004; Bayer et al., 2008; Doi and Kosugi, 2004; Fontes and Gilbert, 2010). The high-affinity cohesin-dockerin interaction defines the cellulosome structure. Attachment of the cellulosome to its substrate is mediated by a scaffoldin-borne cellulose-binding module (CBM) that comprises part of the scaffoldin subunit. Much of the understanding of its catalytic components, architecture, and mechanisms of attachment to the bacterial cell and to cellulose, has been derived from the study

of *Clostridium thermocellum* (Bayer et al., 2004; Bayer et al., 2008; Doi and Kosugi, 2004; Fontes and Gilbert, 2010).

A common characteristic of all cell wall degrading microorganisms is that they harness extensive consortia of extracellular enzymes that act in synergy to degrade the recalcitrant amorphous and crystalline substrates present in these composite structures. Cellulases and hemicellulases are elaborate enzymes, which are synthesized by anaerobes, particularly clostridia and rumen microorganisms which in most cases assemble into a large multi-enzyme complex (molecular weight > 3MDa) and are called cellulosome (Fontes and Gilbert., 2010).
Cellulosomes have many potential biotechnological applications as the conversion of cellulosic biomass into sugars by cellulosomes could result in the production of high-value products such as ethanol or organic acids from inexpensive renewable resources (Roy and Akihiko, 2004). Rapid advances in cellulosome research are providing basic information for the development of both *in vitro* and *in vivo* systems to achieve such goals (Roy and Akihiko, 2004).
Cellulosomes are protuberances produced on the cell wall of cellulolytic bacteria when growing on cellulosic materials (Lynd et al., 2002b). These protuberances are stable enzyme complexes that are firmly bound to the bacterial cell wall but flexible enough to also bind tightly to microcrystalline cellulose. Cellulosomes from different clostridia (*C. thermocellum, C. cellulolyticum*, *C. cellulovorans*, and *C. josui*) and *Ruminococcus* species in the rumen have been studied in detail (Lynd et al., 2002b). Cellulosomes are remarkably stable, large complexes that can vary from 2 to 16 MDa and even up to 100 MDa in the case of polycellulosomes (Be´guin and Lemaire, 1996; Coughlan, 1990.). The cellulosomes are extensively glycosylated (6 to 13% carbohydrate content), particularly on the scaffoldin moiety.
It was first believed that cellulosome complex only degrade cellulose but recent study has shown that it also contain an array of hemicellulosome (Morag et al., 1990), and pectinase (Tamaru and Doi, 2001), with enzyme activities which include polysaccharide lyases, carbohydrate esterases, and glycoside hydrolases. Cellulosome has a complex structure described as a ‘scaffordin’.

1.3.2 Cellulosome components

The scaffoldin subunit contains one or more cohesin modules connected to other types of functional modules. In a given scaffoldin, the latter types of modules may include a cellulose-specific carbohydrate-binding module (CBM), a dockerin, X modules of unknown function, a S-layer homology (SLH) module or a sortase anchoring motif (Gilbert, 2007). Sortase refers

to a group of prokaryotic enzymes that modify surface proteins by recognizing and cleaving a carboxyl-terminal sorting signal (Schneewind et al., 2001) (Pallen et al., 2003; Schneewind et al., 2001). The recognition signal for most substrates of sortase enzymes, consists of the motif LPXTG (Leu-Pro-any-Thr-Gly), a highly hydrophobic transmembrane sequence and a cluster of basic residues such as arginine. Cleavage occurs between the Thr and Gly, with transient attachment through the Thr residue to the active site Cys residue, followed by transpeptidation that attaches the protein covalently to the cell wall (Pallen et al., 2003; Schneewind et al., 2001). Sortases has been reported to occur in almost all Gram-positive bacteria and the Occasional Gram-negative (e.g. *Shewanella putrefaciens*) or Archea (e.g. *Methanobacterium thermoautotrophicum*), where cell wall LPXTG-mediated decoration has not been reported (Pallen et al., 2003; Schneewind et al., 2001). Figure 1.3 below shows the structure of the *C. thermocellum* cellulosome system, showing the dockerin, cohesin, scaffoldin subunits of a typical cellulosome system.

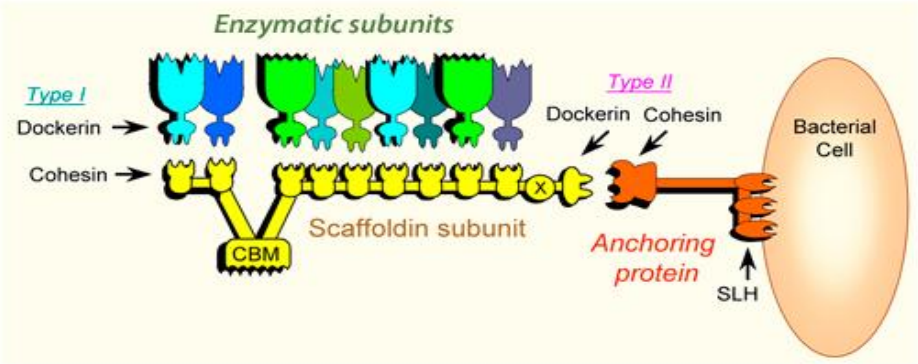


Figure 1.3. Architecture of the *C. thermocellum* cellulosome system, showing the dockerin, cohesin, scaffoldin subunits of a typical cellulosome system. (SOURCE; (Bayer et al., 1994).

Cohesin modules are the major building blocks of scaffoldins, which are responsible for organizing the cellulolytic subunits into the multi-enzyme complex (Gilbert, 2007).

Dockerin modules anchor the catalytic enzymes to the scaffoldin. The dockerin displays internal two-fold symmetry, consisting of a duplicated F-hand motif (a calcium-binding loop preceding an a helix). The dockerin can also be found in the C- terminus of scaffoldins (Gilbert, 2007). An enzyme is generally classified as cellulosomal when it has a dockerin domain which allows it to bind to the scaffoldin protein via a cohesin domain (Bayer et al., 1998a).

Catalytic subunits contain dockerin modules that serve to incorporate catalytic modules into the cellulosome complex. These catalytic modules include: glycoside hydrolases, polysaccharide lyases, and carboxyl esterases (Gilbert, 2007).

1.3.3 Cellulosome systems

It has become clear that cellulosomes are not restricted to *C. thermocellum*, but are also present in other cellulolytic bacteria (Lamed et al., 1987a). Bacterial cellulosomal systems can be categorized into two major types: simple cellulosome systems contain a single scaffoldin and complex cellulosome systems exhibit multiple types of interacting scaffoldins. The arrangement of the modules on the scaffoldin subunit and the specificity of the cohesin(s) and/or dockerin for their modular counterpart dictate the overall architecture of the cellulosome. Several different types of scaffoldins have been described, namely: the primary scaffoldins which incorporate the various dockerin-bearing subunits directly into the cellulosome complex, adaptor scaffoldins which increase the repertoire or number of components into the complex, and the anchoring scaffoldins which attach the complex to the bacterial cell surface (Alber et al., 2010).

1.3.4 Currently known cellulosome-producing anaerobic bacteria

Table 1, list of currently known cellulosome-producing anaerobic bacteria and their year of discovery.

Name of bacteria	**Year**	**Reference**
Acetivibrio cellulolyticus	1999	(Ding et al., 1999)
Bacteroides cellulosolvens	2000	(Ding et al., 2000)
Clostridium acetobutylicum	2001	(Nölling et al., 2001)
Clostridium cellobioparum (suspected, not proven)	1987	(Lamed et al., 1987b)
Clostridium cellulolyticum	1999	(Pagès et al., 1999)
Clostridium cellulovorans	1992	(Shoseyov et al., 1992)
Clostridium josui	1998	(Kakiuchi et al., 1998)
Clostridium papyrosolvens	1995	(Pohlschröder et al., 1995)
Clostridium thermocellum	1983	(Lamed et al., 1983)
Ruminococcus albus (dockerins identified, cohesins as yet undetected)	1987	(Lamed et al., 1987b)

Cellulosomes exist as extracellular complexes that are either attached to the cell wall of bacteria or free in solution, where the insoluble substrate can be broken down into soluble products and taken up by the cell. The extracellular cellulase system of an organism such as *Trichoderma reesei*, which produces free enzymes into the surrounding medium, has been well studied (Lynd et al., 2002a). *B. licheniformis* are also able to grow fast and secrete high amounts of protein into the extracellular medium (Schallmey et al., 2004). van Dyk et al., (2010b) reported that a MEC in *B. licheniformis* SVD1 is secreted into the surrounding medium rather than being cell wall anchored. The large size and heterogeneity of cellulosomes from the best-characterized organisms (i.e., *C. thermocellum*, *C. cellulolyticum*, and *C. cellulovorans*) have greatly complicated efforts to probe cellulosome structure and function. Other cellulosome systems (such as those from *Acetivibrio cellulolyticus* and *Ruminococcus flavefaciens*) appear to be even more intricate (Alber et al., 2010).

In the simple cellulosome systems, the scaffoldins contain a single CBM, one or more X2 modules and numerous (5 to 9) cohesins. These scaffoldins are primary scaffoldins, which incorporate the dockerin-bearing enzymes into the complex (Alber et al., 2010). In several cases, the simple cellulosomes have been shown to be associated with the cell surface, but the molecular mechanism responsible for this is still unclear. The X2 module may play a role in attachment to the cell wall. The genes encoding for many important cellulosome subunits are organized in "enzyme-linked gene clusters" on the chromosome (Alber et al., 2010).

Complex cellulosome systems have been described in different bacterial species (Demain et al., 2005; Gilbert, 2007)**.** In these systems, more than one scaffoldin interlocks with each other in various ways to produce a complex cellulosome architecture. At least one type of scaffoldin serves as a primary scaffoldin that incorporates the enzymes directly into the cellulosome complex. In each species, another type of scaffoldin attaches the cellulosome complex to the cell surface via a specialized module or sequence, designed for this purpose. In the complex cellulosome systems, the scaffoldin genes are organized into "multiple scaffoldin gene clusters" on the chromosome (Demain et al., 2005; Gilbert, 2007). A systematic representation of *C. thermocellum* is shown in figure 1.3 below by " (Bayer et al., 1994).

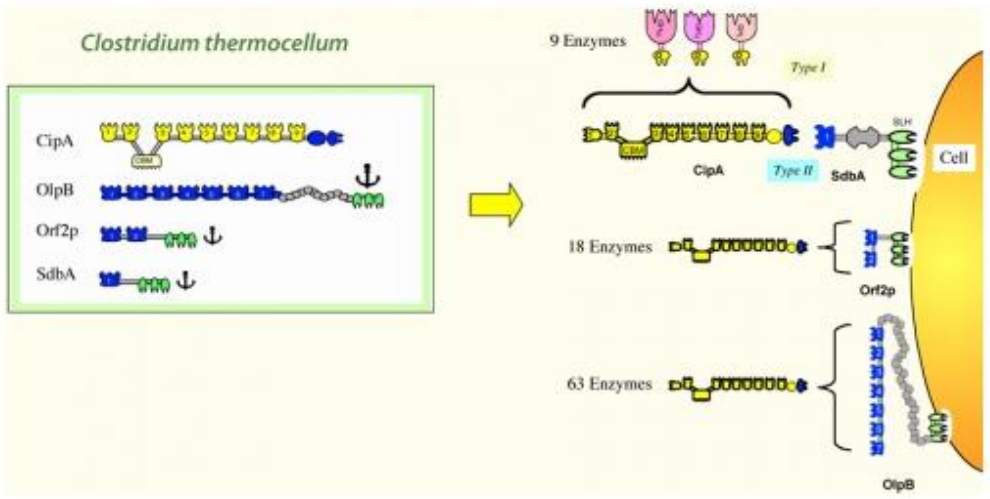


Figure 1.4, A schematic representation of C. thermocellum cellulosome components. (SOURCE; (Bayer et al., 1994).

1.3.5 Cohesin-dockerin interactions and cellulose assembly

Cohesin-dockerin interactions can be viewed as a kind of plug-and-socket mechanism in which the dockerin plugs into the cohesin socket. In general, the interaction is inter-species and intra-species (type) specific, although some cross-reactivity has been found in a few cases. The cohesin-dockerin interaction is one of the most potent protein-protein interactions known in nature, in most cases approaching the strength of high-affinity antigen-antibody interactions (Ka ~ 10^{11} M^{-1}) (Alber et al., 2010).

So far, cohesins have been phylogenetically distributed into three groups according to sequence homology; the type-I cohesin, the type-II cohesin and the recently discovered type-III cohesin. The dockerins that interact with each cohesin type are, by definition, of the same type (Alber et al., 2010). The presence of varied binding specificities between dockerins and cohesins indicate that some enzymes will bind preferentially to cohesins and will thus be more prevalent in the cellulosome, pointing to a regulated assembly. If assembly simply occurred in a random fashion (Bayer et al., 1998a) speculated that one would expect a hetereogeneous population of cellulosomes to be produced. However, in *C. thermocellum*, where binding affinities of cohesins appear to be similar, the organism produced a homogeneous batch of cellulosomes, contrary to expectations (Bayer et al., 1998a).

1.3.6 Structural characterization of cellulose components

One of the greatest efforts in the cellulosome research field is to understand the structure-function relationship in cellulosome assembly. Thus far, crystallographic structures of only

selected cohesins have been determined, all of which share the typical jelly-roll topology that forms a flattened 9-stranded beta-sandwich. In addition, crystal structures for type-I and type-II cohesin-dockerin complexes have been described. The structure of a multi-modular complex from *C. thermocellum* was also solved, composed of the type-II cohesin module of the cell surface protein SdbA bound to a trimodular C-terminal fragment of the scaffoldin subunit CipA (Alber et al., 2010). Figure 1.4 shows the structure of the *C. thermocellum* CipA scaffoldin CohI9-X-Docll trimodular fragment in complex with the SdbA CohII module (Adams et al., 2010).

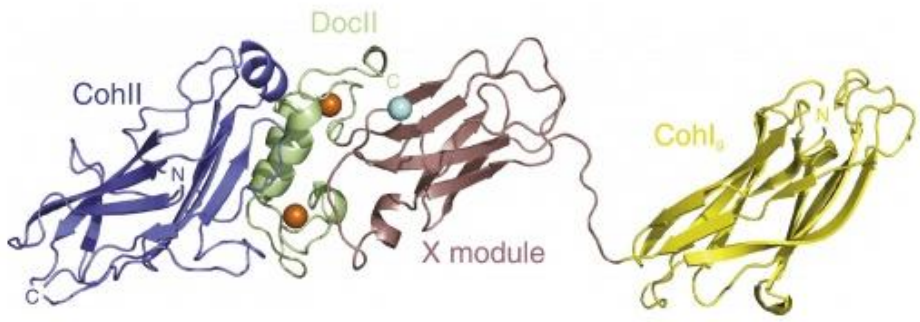


Figure 1.5. Structure of the C. thermocellum CipA scaffoldin CohI9-X-Docll trimodular fragment in complex with the SdbA CohII module. (SOURCE; (Adams et al., 2010).

1.3.7 Cellulosome and free extracellular enzymes

Free extracellular enzymes are very effective but the discovery of cellulosomal complexes have shown to have great scientific and commercial interest after it was demonstrated that the cellulosome from *C. thermocellum* had 50 times the specific activity on cotton compared to free cellulases from *Trichoderma reesei (Johnson et al., 1982).* It appeared as though the presence of enzymes within the cellulosome complex provided an advantage to the organism. Schwarz proposed four reasons why the cellulosome complex conferred an advantage to an organism in degradation of cellulose (Schwarz, 2001a), Firstly, synergy can take place because enzymes are present in the correct ratio. The synergy between enzymes in the cellulosome has been suggested as one of the main reasons why the cellulosome is so efficient at degradation of complex substrates. A synergistic association is said to exist when the combined activity of two or more enzymes together is greater than the theoretical sum of the individual activities of the enzymes on the same substrate (Hoshino et al., 1997; Teeri, 1997). Secondly, "Non-productive adsorption (the adsorption to the lignin part)" is avoided as enzyme components are spaced in an optimal fashion. Thirdly, competitive binding is avoided

as the non-enzymatic component contains a strong binding domain and not the individual enzymes. Fourthly, the presence of different enzymes ensures that complex substrates can be degraded (Schwarz, 2001a).

The combination of enzymes as well as the role of the non-catalytic scaffolding protein with specialised carbohydrate binding modules ensures that the cellulosome operates in a different manner which makes it more efficient and highly effective, particularly when acting on complex or crystalline substrates. (Boisset et al., 1999) demonstrated that the cellulosome degraded crystalline cellulose in a completely different manner than free enzymes of the same type. Having enzymes within a complex gives a further advantage to microorganisms, specifically in anaerobic environments. When microorganisms grow on large substrates such as polysaccharides, they are forced to secrete extracellular enzymes into the medium as they are unable to take large substrates up in the cell. Where cells are present at low concentrations, such enzymes could diffuse away from the cell, resulting in product formation that is inaccessible to the cell, thus failing to support growth. By having the extracellular enzymes in a complex at the cell interface, such as the cellulosome, cells can overcome this problem (Shoham et al., 1999).

1.3.8 Identification criteria of MEC

Multi-enzyme complex have been identified in different microorganisms, but how truly can a MEC be identified? For us to be able to identify or classify a multi-enzyme complex as a cellulosome/xylanosome, it is useful to look at the evidence that has been used to prove that a cellulosome/xylanosome exists in an organism. In the past, 'structures' have been used to identify the presence of cellulosome in an organism. The main structural features of the cellulosome are the scaffolding protein containing cohesin domains, and the presence of dockerin domains on cellulosomal enzymes which are the conserved molecules. Bioinformatics analysis can be used to identify the presence of the dockerin and cohesin domains and the presence of scaffoldin protein where the genomic or genetic information for the organism is available (Bayer et al., 1999). This may not be 100% accurate because cohesin domains and a dockerin domain were detected in *Archaeoglobus fulgidus* even though the presence of a cellulosome in this organism has not been established (Bayer et al., 1999), but this is still the best known method as at now. Another method that can be used is the presence of high molecular weight protein as demonstrated through size exclusion chromatography, reaction with antibodies for CipA from *C. thermocellum*, and the presence of cell protuberances using electron microscopy (Bayer et al., 1998a; Schwarz, 2001a). At

present, there is no known structural basis for the identification of xylanosome (van Dyk et al., 2010a).

SDS-PAGE and zymograms have been used effectively to identify enzymes present within the MEC but these processes have some limitations as many enzymes will not retain activity after the complete denaturation from heat and denaturing agents used in traditional SDS-PAGE (Morag et al., 1990). Morag et al., (1990) reported that enzymes such as exoglucanase and β-xylosidase in the *C. thermocellum* cellulosome did not exhibit activity after boiling. Another method that was used successfully by (Zverlov et al., 2005) to identify components of the *C. thermocellum* cellulosome was two-dimensional electrophoresis followed by MALDI-TOF/TOF. Identification was aided by the availability of the genome sequence for *C. thermocellum*.

The advantage of analysing the composition of a purified complex, as opposed to a gene sequence, is that it gives information about the actual enzymes that the organism has harnessed for the degradation of a specific substrate, thus can provide valuable insight into the enzymes present and their ratios for the synergistic degradation of complex substrates (van Dyk et al., 2010a). Identification of enzymes from a genome or gene sequence only provides the theoretical possibility that they may occur within the MEC, but does not indicate under which circumstances they will be present within the MEC (van Dyk et al., 2010a).

1.4 Bacillus licheniformis as enzyme producer

The genus *Bacillus* consists of a large number of diverse, rod-shaped Gram positive (or positive only in early stages of growth) bacteria which are capable of producing endospores that are resistant to adverse environmental conditions such as heat and desiccation (Claus and Berkeley, 1986). Typically, the cells are motile by peritrichous flagella and are aerobic. The genus consists of a diverse group of organisms as evidenced by the wide range of DNA base ratios of approximately 32 to 69 mol% G + C (Claus and Berkeley, 1986) which is far wider than usually considered reasonable for a genus (Norris et al., 1981). They have been used in the production of enzymes, recombinant proteins, antibiotics, insecticides and amino acids (Arbige et al., 1993). They are attractive species for use in industry as they are generally non-pathogenic, except species such as *B. anthracis*. They are also able to grow fast and secrete high amounts of protein into the extracellular medium (Schallmey et al., 2004).

Bacillus licheniformis is a saprophytic bacterium that is widespread in nature and thought to contribute substantially to nutrient cycling due to the diversity of enzymes produced by

members of the species. It has been used in the fermentation industry for production of proteases, amylases, antibiotics, and specialty chemicals for over a decade with no known reports of adverse effects to human health or the environment, it has been classified as a GRAS (generally regarded as safe) organism by the US Food and Drug Administration (Schallmey et al., 2004).

B. licheniformis is a ubiquitous bacterium thought to be of importance in the environment as a contributor to nutrient cycling due to the production and secretion of protease and amylase enzymes (Claus and Berkeley, 1986). Although the actual numbers in existence in the environment for this species have not been determined, in general, bacilli occur at population levels of 10^6 to 10^7 per gram of soil (Alexander, 1977). They are generally simple to cultivate and relatively easy to manipulate genetically (Arbige et al., 1993). Many strains of bacilli also produce enzymes that are tolerant of alkaline pHs and high temperatures, thus making them very useful in applications such as detergents (Schallmey et al., 2004). Schallmey estimated that commercial enzymes from *Bacillus* spp. make up about 50% of the enzyme market (Schallmey et al., 2004). They are therefore seen as preferred hosts for many commercial protein products. They are generally simple to cultivate and easy to manipulate genetically (Arbige et al., 1993). *B. licheniformis* require a very simple medium for growth and production of extracellular endoglucanase and xylanase activity (van Dyk et al., 2010b). *Bacilli* are able to grow under anaerobic conditions although growth under aerobic conditions is superior. *B. licheniformis* is able to grow weakly under anaerobic conditions and it belong to Group II bacilli which are able to grow in the absence of oxygen, particularly if nitrate is present (Priest, 1993).

B. licheniformis are also able to grow fast and secrete high amounts of protein into the extracellular medium (Schallmey et al., 2004). Numerous reports exist in literature for the isolation of endo-glucanases from various strains of *B. licheniformis (Liu et al., 2004).* The cellulolytic system of some strain of *Bacillus* has been reported to bind to Avicel. Activity on a crystalline cellulose substrate such as Avicel has generally been considered as a characteristic feature of cellulosomes, although (Bayer et al., 2004), have pointed out that the discrete nature of the cellulosome lies in its molecular structure rather than simply its cellulolytic activity (Bayer et al., 2004). In *C. acetobutylicum*, for instance, biochemical and genetic evidence exists for the presence of a cellulosome, yet no cellulolytic activity was detected in this organism (Schallmey et al., 2004).

B. licheniformis SVD1 has been reported to have the ability to utilise arabinose and galactose to some extent, arabinose and galactose are generally sugars that occur as substituents on other polysaccharides such as mannan or xylan (van Dyk et al., 2010b). The cellulolytic system of *B. licheniformis* displayed a variety of enzyme activities on a range of substrates and included endoglucanase, xylanase, mannanase and pectinase activity (van Dyk et al., 2010b).

B. licheniformis has been used in the fermentation industry for decades in the production of proteases, amylases, antibiotics, or specialty chemicals. Commercially thermostable α-amylase has been made from *B. licheniformis,* used for the liquefaction of wheat flour (Das Neves et al., 2006) and corn meal (Mojovic et al., 2006). The hydrolysates were then saccharified and fermented to ethanol in a further processing step using *Saccharomyces cerevisiae (Das Neves et al., 2006; Mojovic et al., 2006).*

1.5 **AIMS AND TOOLS**

The synthesis of organic carbon is the main biological process and primary source of energy in life. In plant, cell wall polysaccharide, primarily cellulose and hemicelluloses, are the major source of carbon and energy. The challenges for the breakdown of structural carbohydrates have been that only a restricted number of microorganisms have acquired the capacity to deconstruct these structural carbohydrates (Fontes and Gilbert., 2010). The chemical and physical complexity of plant cell walls restricts their accessibility to enzyme attack (Brett. and Waldren, 1996). One of the main bottleneck in the use of plant biomass for biofuel remains the saccharification step (Fontes and Gilbert., 2010). Himmel and co-workers (2007) identified the slow enzyme kinetics in the hydrolysis of cellulose to sugars, low yields of free sugars from other polysaccharides and removal of interspersed lignin as three main areas requiring improvement. If these obstacles could be overcome through research, biomass is able to provide the solution for provision of an alternative source of liquid transportation fuel. A further aspect is the utilisation of all sugars, both hexose and pentose, in fermentation since the main organism utilised for ethanol production, *Saccharomyces cerevisiae* (bakers yeast), is only able to utilise glucose for fermentation (Himmel et al., 2007). While the main component of plant biomass is cellulose, consisting of glucose monomers, a large component is hemicellulose with a variety of pentose sugars such as xylose and arabinose, and hexose sugars such as mannose and galactose. The ability to utilize all these sugars for fermentation would greatly enhance the productivity and efficiency of the conversion process.

Conversion of lignocellulosic biomass to fermentable sugars represent a viable alternative for production of renewable fuels such as ethanol, nonetheless, hydrolysis of structural polysaccharides is still the rate limiting step in the conversion of lignocelluloses into fuel which will require the development of more efficient enzyme system that act in synergy. The cellulosome is a highly organised cellulolytic multi-enzyme complex that has been discovered in several anaerobic bacteria such as in clostridia but little is known about their occurrence in aerobic bacteria genera as bacillus. Some MECs have been identified in aerobic bacteria such as *Bacillus circulans* and *Paenibacillus curdlanolyticus*, but the nature of these MECs is not clear (Bayer et al., 1998a). The cellulosome appears to facilitate an enhanced synergy and efficiency of its enzymes, as compared to free enzymes, for the degradation of recalcitrant substrates such as cellulose and plant cell walls. The hydrolysis of crystalline substrates like cellulose is an unsolved biochemical problem, and the action of multi-enzyme complexes is an interesting research subject.

This project work concentrates on using enzyme assay and protein methods to isolate, culture (fermentation), extract and analyse various cellulolytic/hemicellulolytic activities (cellulases/hemicellulase) of bacterial isolates from rumen of moose (*Alces alces*) to produce possible multi-enzyme complex termed cellulosome. Colony PCR amplification of 16s rDNA and Sanger-sequencing method and Bioinformatics tools was used for the identification of one of the bacterial isolates. DNS assay was used for determination of enzyme activities. SDS-PAGE and zymograms was used to identify the type of enzymes present in the isolate.

1.5.1 Research objectives

The objectives of this study were;

- To find efficient enzyme systems from ruminant animals foraging on cellulose, i.e. the moose.
- To screen and identify bacterial isolates from the rumen of the moose (*Alces alces*) for the presence of cellulolytic and hemi-cellulolytic activity
- To describe and characterise the nature of cellulase and hemicellulase present in the organism which could be
  -Cell wall bound/ free extracellular enzymes, or
  -Complex/ Non-complex enzymes
- To determine if the enzyme producing organisms can be effectively cultured in a fermentor
- To investigate an initial purification method of enzyme activity.

2. Materials and Methods

2.1 Samples collection

Bacterial Isolates, total five (MRB 1-5) isolated from moose (*Alces alces*) rumen were provided by University of Bergen and TransHerba AS for cellulase screening. Samples were stored on blood agar plates in 4^{o}C, and others frozen at -80^{o}C for long time storage until use. Samples from blood agar plates were inoculated in Brain Heart Infusion (BHI) and Anearobic Basic Broth (ABB) for 48hrs to induce growth and were later inoculated on CMC plates for cellulase screening.

2.1.1 Chemicals and Biochemicals

Brain Heart Infusion (BHI) (53286), Cellobiose, (22150), carboxy methyl cellulose (CMC), (C4888), Congo Red (C6767), T. *Reesei* (C2730, molecular weight ranging from appromately 50 kDa to 60 kDa) and Birchwood xylan (X4252) were the products of Sigma-Aldrish, Munich, Germany. Anearobic Basal Broth (ABB), (OXOID LTD CM0957, HAMPSHIRE, ENGLAND). Avicel, (E. Merck D-6100, Darnstadt, F.R Germany). D-glucose assay kits, (BOEHRINGER MANNHEIM/ R-BIOPHARM Enzymatic BioAnalysi/ Food Analysis, Cat. 10716251035)

Protein ladder; (Protein ladder, Precision Plus Protein^{TM} unstained standard, 1ml, Cat. 1610363, USA)

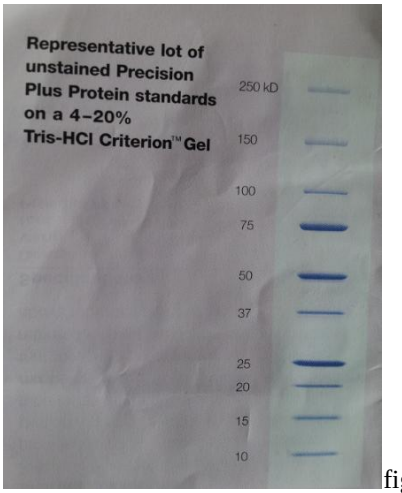


figure 2.0, BIO-RAD Precision Plus Protein^{TM} standards unstained.

All other chemicals are of analytical standard.

2.1.2 Methods flow-chat

The method flow-chart shown in figure 2.1 below shows the experimental work, which includes; screening of isolates, identification, enzyme assay and purification techniques.

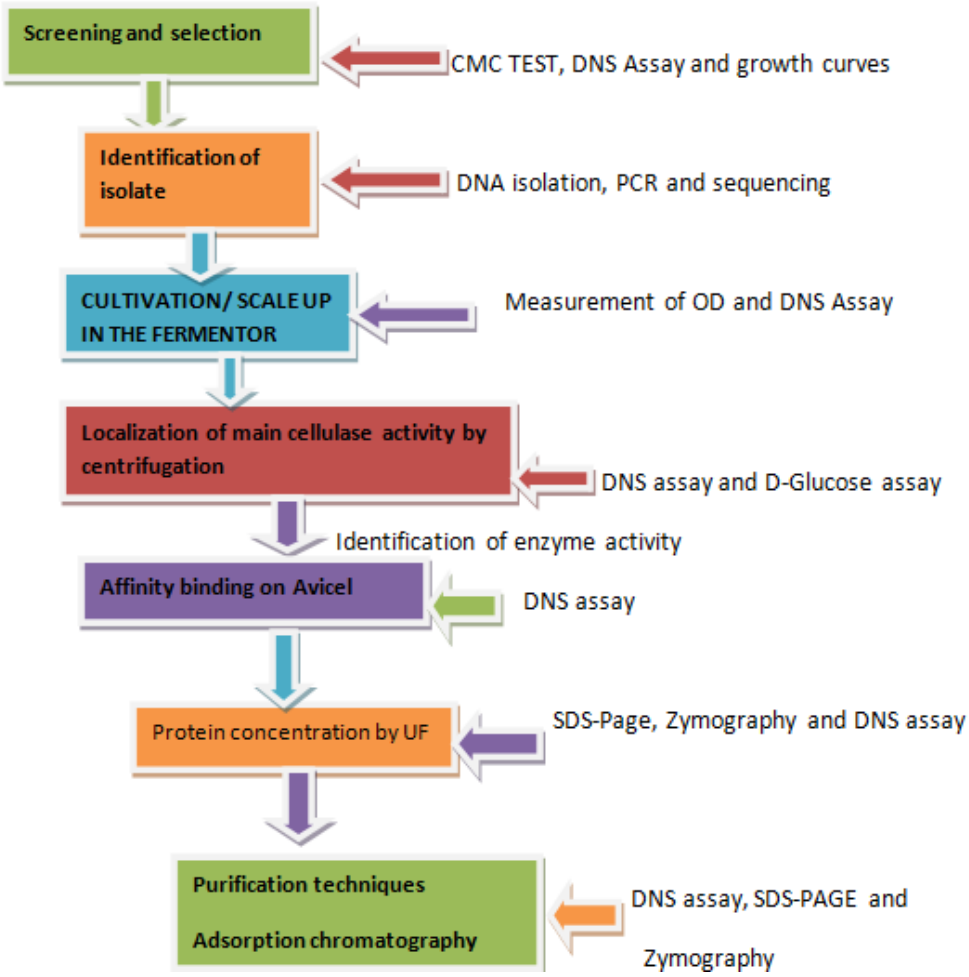


Figure 2.1, Project work flow showing main methodology and analytical steps

2.2 General procedure

2.2.1 Grams staining

Gram staining is a differential staining method which helps to group bacteria according to their Gram character (Gram positive or Gram negative). The Gram reaction depends on the growth phase of the organism, young and growing bacteria gives most consistent reaction (Bergey et al., 1994).

Procedure; Bacterial culture broth was placed on a glass slide with a syringe and heat fixed, crystal violet (primary stain) was added and allowed to stay for 1 min. The slide was washed with water, Gram's iodine (mordant) was added for 1 min. The oidine was drained off and

alcohol was added (decolorizer) for 15 seconds. The slide was washed with water and Safranin (secondary stain) was added for 1 min. The glass slide was washed with water, dried and observed under microscope.

Standard observations

- Purple cells - Gram positive or
- Pink cells – Gram negative

2.3 Experimental procedures

2.3.1 Screening procedures

Cellulase screening of samples was performed according to a modified method of kasana et al., (2008). Two microlitres of culture grown in BHI and ABB for 48hrs was spot plated (inoculation method of plates were of scatter spots and spaced regular or irredular spots) on CMC agar plates (0.2% $NaNO_3$, 0.1% K_2HPO_4, 0.005% KCl, 0.05% MgSO4, 0.2% Carboxymethylcellulose (CMC) Sodium Salt, 0.02% Peptone, 1.7% Agar) earobically and in a 3.5l Oxoid anaerobic jars (Bio-Medical scientific services, UK) to determine the most suitable growth condition for the isolates. Plates incubated at 37^{o}C for 72hrs were flooded with Gram's iodine (2.0g KI and 1.0g Iodine in 300ml distilled water) for 7mins and plates were observed for zone of clearance around the colony. Selected colonies were stored on blood agar for further analysis.

2.3.2 Sample description

Selected colonies were stored on blood agar and cultured aerobically in BHI and ABB supplemented with 0.5% cellobiose for further analysis.

Isolation of enzyme complex was performed by the modified method of (Morag et al., 1992). After the bacteria was grown in a medium of ABB supplemented with 0.5% cellobiose under anaerobic condition at 37^{o}C for 24hrs, the culture was harvested by centrifugation at 4000 Xg for 20mins at 4^{o}C. The supernatent was used as crude enzyme.

2.3.3 DNS assay for screening of isolates

Enzyme assay for cellulase and hemicellulase

The total cellulase system consists of three enzymatic activities: endoglucanases, exoglucanases, and b-d-glucosidases. One of the methods used to measure cellulase activities

is called DNS assay. Reducing sugars have the property to reduce many reagents and one of such reagents is 3,5-dinitrosalicylic acid (DNS). 3,5-DNS in alkaline solution is reduced to 3 amino 5 nitro salicylic acid, which absorbs light strongly at 540 nm (Miller, 1959).

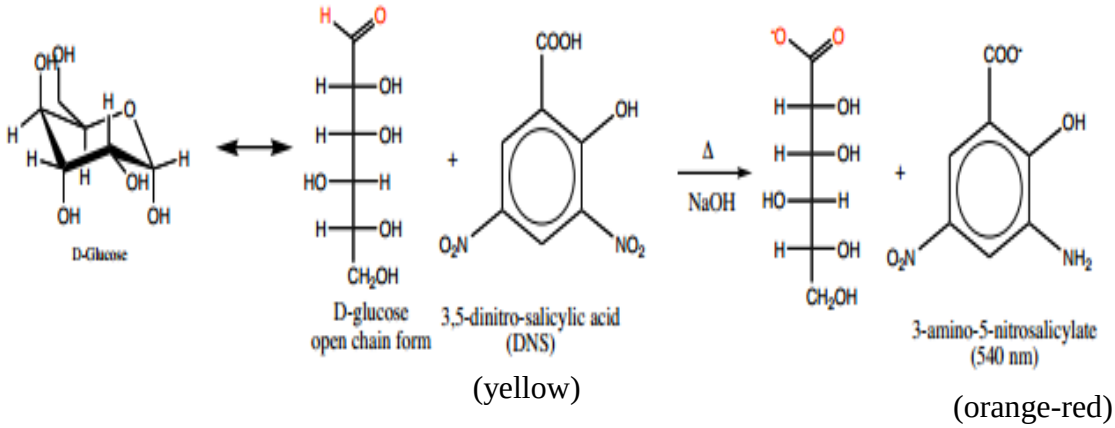


Figure 2.3, showing the how 3,5-DNS in alkaline solution is reduced to 3 amino 5 nitro salicylic acid by the reducing sugar (D-Glucose) and how the colour is observed at 540nm.

The general cellulase activity was assayed through out this study using dinitrosalicylic (DNS) assay according to the modified method of (Morais S. et al., 2011). The enzyme activity was determined by the release of reducing sugar of glucose or xylose depending on substrates. Protein samples of 500µl was mixed with 500µl of CMC or xylan in 500µl citrate buffer, pH 4.5 for CMC-ase or pH 6.5 for xylanase and incubated at 50^{o}C for 30 mins. One unit of enzyme activity is defined as the amount of enzyme that produced 1µmol of reducing sugars in 1 min under assayed condition.

The concentration of glucose is measured by detecting the reducing end of the sugar. Amount of absorbance directly relates to amount of reducing sugar. One unit of enzyme activity is defined as the amount of enzyme that produced 1µmol of reducing sugars in 1min under assayed condition (Miller, 1959). Results are presented with raw value from Absorbance 540 which represents the amount of reducing sugars under this assayed condition.

2.3.4 Identification of isolate MRB 3

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

Colony PCR; Sample collection

An aliquote (200 µL) of cultivated isolate MRB 3 was added into a micro-centrifuge tube and 800 µL H_2O was added and centrifuged at full speed for 2mins. The supernatant was discarded, and the bacterial pellet was transferred with sterile pipette tip into the PCR mix.

Genomic DNA was prepared according to the modified method of (Ausubel et al., 2002). PCR on genomic DNA of MRB 3 was performed using the following universer primers: Mangala F-1 and 16S U1510 R. The PCR reaction was performed in a Sprint thermal cycler using the following cyclical parameters: 95 ºC for 15min, 95 ºC for 30 sec, 55 ºC for 30 sec, 72 ºC for 1min 20sec (cycle repeated 30 times), end at 10 ºC for indifinite time. The presence of a PCR product and its molecular weight was confirmed using 1% agarose gel electrophoresis at 100V for 1hr, with 5 µl 1kb quantification ladder (BioLabs N3232) and 10 µl of PCR product applied to wells diluted with 6X loading buffer. Subsequently, quantification of resolved bands was analysed using Kodak Image Station 4000 MM.

Exo I treatment

The qualitative PRC product was treated with Exo I for sequencing as follows: 1µl of a 5x sequencing buffer 1.1, 0.5µl PRC product, 0.1µl Exo I and 3.4µl dH_2O to make a total reaction volume of 5µl.This was incubated at 37°C for 60 mins, 80°C for 15mins and held at 10°C.

Big Dye exterminator

This was done to purify the sequencing product. The setup was as follows: 10µl of product from sequencing PCR, 45µl SAM (from kit) and 10µl Xterminator. This mix was placed on 1500rpm shaking for 30mins and then 25rmp centrifugation for 2 mins.

Sequencing PCR

The sequencing reaction was set up (to a total volume per sample of 10µl) as follows: 5µl Exo I- treated PCR product, 0.5µl of 5x sequencing buffer 1.1,1µl Big Dye 1.1, 0.32µl of 10pmol/µl sequencing primer and 3.18µl dH_2O.

The PCR was run as follows: 96°C for 1 min followed by 25 cycles of 95°C for 15s, 50°C for 5s and 60°C for 4 mins and then held at 10°C.

The PCR product was sequenced using the same primers and Genetic analyser (Applied Biosystems), incorporating the Big Dye Terminator Cycle Sequencing kit (Applied Biosystems). Electropherograms of the sequences generated were inspected with sequence scanner. Forward and reverse sequences were aligned using a ClustalW alignment tool (http://www.ebi.ac.uk/Tools/clustalw2) and the combined sequence was used for a nucleotide BLAST search (http://www.ncbi.nlm.nih.gov/blast).

2.3.5 Growth of enzyme-producing bacteria

The selected bacterial isolate was cultivated in 500ml shaking bottle in a 250ml medium containing ABB supplemented with 0.5% cellobiose and later scaled up in 3.5l anaerobic fermentor (Bioengineering AG, Switzerland) containing 2.5l of the same medium under anaerobic condition at 37^{o}C and pH 6.8 which was found to reduce to pH 5.2 as cells reach stationary phase. The anaerobic fermentor contains sterilizer, pH regulating system and heater. Optical density was measured at 3 hours interval using spectrophotometer at 600nm. The isolate was cultured for 24hours and harvested at stationary phase. The DNS assay, for reducing sugars was used to determine the best time of harvest.

2.3.6 Preparation of cell lysates by Sonication

Isolation of enzyme complex was performed by the modified method of (Morag et al., 1992). The pellet from centrifuged cells was collected and frozen, and was later used for sonication according to modified method of (Phitsuwan. et al., 2012). Lysis buffer (50mM Tris-HCL pH 7.5, 200mM NaCl, 5mM DTT, 1mM PMSF). Pellet was resuspended in chilled lysis buffer in ratios of cell wet weight to buffer volume of 1:1 to 1:4. Cell suspension was sonicated with 10 short burst of 10 sec followed by intervals of 30 sec for cooling and cell debris was removed by centrifugation at 4^{o}C for 20mins at 4000rpm using (Beckman) and later checked for enzyme activities.

2.3.7 Localization of main cellulase activity

To determine if the main cellulase activity was found in the supernatant or in the pellet, was carried out according to the modified method by Phitsuwan, et al., (2012). After the isolate was cultured, it was then harvested by centrifucation as described above. Pellet and supernatant was collected for further analysis. DNS assay (Xylanase and CMCase activities were determined by the release of reducing sugar of glucose and xylose from CMC and xylan substrates respectively) according to the modified method of (Morais S. et al., 2011) and D-glucose was carried out according to D-glucose assay kits manual, where the increase in NADPH is measured by means of its light absorbance at 340. Cellulase from T*richoderma reesei* in a ration 1.4 dilution was used as positive control.

2.3.8 Polyacrylamide gel electrophoresis (PAGE)

SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis, is a technique used to separate proteins according to their electrophoretic mobility based on the length of a polypeptide chain and its charge. SDS is an anionic detergent applied to protein sample to linearize proteins and to impart a negative charge to linearized proteins. In most proteins, the binding of SDS to the polypeptide chain imparts an even distribution of charge per unit mass, thereby resulting in a fractionation by approximate size during electrophoresis (Rath et al., 2009).

Proteins were separated by PAGE in the presence of sodium dedecyl sulphate (SDS) according to the method of (Laemmli, 1970). The stacking and the separating gel were 10% or 12% and 3% polyacrylamide respectively. 15µl of samples were loaded accordingly. Electrophoresis was carried out at 180V for 45mins. After electrophoresis, the proteins were stained with Commassie Brilliant Blue for 1hr and destained with H_2O until bands are vissible. Gels were viewed using Kodak Image Station 4000 MM and the molecular weight of the protein bands were determined. (Precision Plus Protein™ unstained standard ladder, 1ml,) was used as the molecular weight standard. Cellulase from T. *reesei* (1.4 dilution) was used as positive control.

2.3.9 Comparing bacteria growth and enzyme activities on different substrates and pH

Different culture media were prepared from Anaerobic Basal Broth (ABB), Brain Heart Infusion (BHI) and Basic Bacillus Broth (BBB), using 0.5% cellobiose and xylan substrates as supplements for this study. Bacterial isolate (50ml) was cultured in a 500ml medium of (ABB, BBB and BHI supplemented) and incubated at 37^{o}C for 90hrs with a shaking at 200 rpm under aerobic condition. The three culture media were compared for growth for 24hrs of time and growth curve was determined by measuring optical density.

2.3.10 Binding of cellulolytic system to Avicel

Isolation of enzyme activities was performed by the modified method of Morag et al., (1992). After the bacteria were grown on ABB supplemented with cellobiose, at 37^{o}C for 24hours, the culture was harvested by centrifugation at 4100rmp for 30mins at 4^{o}C, the supernatant was

used as crude enzymes. Then, 5% Avicel was added to the 50ml crude enzyme and the mixture was stirred for 30mins at 4^{o}C. The supernatant was discarded and the pellet of enzyme-insoluble cellulose were washed in step wise with 5ml of 1M NaCl, 50mM Sodium Acetate buffer (pH 5), 50mM $NaHCO_2$ (pH 7) and 50mM Tris-HCl (pH 7.5) respectively and the resulting wash solution was analysed for released enzyme activity using DNS assay.

2.3.11 Protein Concentration

Sample concentration was done using Amicon Ultra Spinn Catridges (Centricon Amicon Ultra, Millipore, USA) with a 4ml volume and molecular weight cut-off of 50kDa, Centrifuged at 7500 xg at 25^{o}C for 10mins, and the retentate collected for enzyme activity. The concentration was performed according to the manufacturer's manual. Ultra filtration in a cross-flow was used to concentrate larger volume samples of 100ml using Millipore pellicon ultrafiltration cassettes (50cm^2 Pellicon Biomax. USA) with molecular cut-off at both 10kDa and 50kDa according to manufacturer' manual.

Concentration factor CF was used to calculate the final concentration of the protein (or enzyme activity) in the retentate relative to the start concentration.

$$CF = \left(\frac{\text{concentration in retenate}}{\text{concentartion in start solution}}\right) = \text{CF increase in A540} = C_{conc}/C_{start} \sim A_{conc}/A_{start}$$

In the case of total retention of protein (or enzyme), that is R ~ 1, then CF is approximately equal to VCF: $CF = VCF^R$

Retention R, was used to indicate to what extent is the protein retained by the membrane. R has a value between 0 and 1.
An efficient concentration step is achieved when R is 0,9-1,0.

$$R = 1 - \left(\frac{\text{proteinconcentration in permeate}}{\text{proteinconcentration in start solution}}\right) = 1 - \left(\frac{C_p}{C_b}\right)$$

Percentage of recovery was used to calculate the recovery percentage.

% Recovery ~ Total*Activity = Retentate*Activity + Permeate*Activity, (Strætkvern, 2013).

2.3.12 Protein capture on ion exchanger column

Ion exchange chromatography (usually referred to as ion chromatography) uses an ion exchange mechanism to separate analytes based on their respective charges. It is usually performed in columns but can also be useful in planar mode. Ion exchange chromatography uses a charged stationary phase to separate charged compounds including anions, cations, amino acids, peptides, and proteins. In conventional methods the stationary phase is an ion

exchange resin that carries charged functional groups that interact with oppositely charged groups of the compound to retain (Still et al., 1978). An ion-exchange resin is an insoluble matrix (or support structure) normally in the form of small (1–2 mm diameter) beads, usually white or yellowish, fabricated from an organic polymer substrate. The material has a highly developed structure of pores on the surface of the sites with easily trapped and released ions. The trapping of ions takes place only with simultaneous releasing of other ions; thus the process is called ion-exchange. There are multiple types of ion-exchange resin which are fabricated to selectively prefer one or several different types of ions. However, most are made of sulphonated cross-linked polystyrene molecules (IUPAC, 1993).

Protein capture was done using ion exchanger column (GE Healthcare Bio-science AB. Sweden). The concentrated ultrafiltration (10kDa) sample was loaded onto 1ml ion exchange column and later scaled up on 30ml Sepharose S fast flow. Both cation exchange and anion exchange methods were tested as the pI of the enzyme motions was not known. For cation exchange, Capto S (GE Healthcare Bio-science AB. Sweden) was used and Capto Q (GE Healthcare Bio-science AB. Sweden) for anion exchange. Anion exchange was carried out at pH 7, pH 8 and pH 9, while cation exchange was carried out at pH 5. For anion exchange, 50mM Tris-HCl buffer was used for equilibration while 50 mM sodium acetate buffer was used for equilibration for cation exchange. Sample volumes of 5 ml were loaded onto the column and elution of protein was carried out with a stepwise gradient of increased NaCl concentrations from 0 mM NaCl to 1 M NaCl. An equilibration system containing 0.1% Triton x100 in 20mM TrisHCl at pH 7.5 was also attempted for anion exchange (Capto Q GE Healthcare Bio-science AB. Sweden). All liquids were applied to the scouting column assay using a syringe except for Sepharose S fast flow. Fractions were collected and the absorbance of each fraction was measured at 280 nm.

Fractions from ion exchange chromatography were examined for enzyme activities. Xylanase and CMCase activities were determined by DNS assay, a measure of the reducing sugar of glucose and xylose from CMC and xylan substrates respectively. Results were presented with raw value of Absorbance 540 which represents the amount of reducing sugars under this assayed condition.

2.3.13 SDS PAGE AND ZYMOGRAPHY

SDS PAGE was performed on 10% and 12% separation gel according to (Laemmli, 1970) under non-reducing conditions. Various samples (concentrated samples using ultrafiltration method, Avicel binding experiment and fractions from ion exchange chromatography) were

treated accordingly. Samples were heated in 90^{o}C for 45seconds and cooled on ice and 15µl of samples were loaded accordingly. Electrophoresis was carried out at 180V for 45mins. After electrophoresis, the proteins were stained with Commassie Brilliant Blue for 1hr and destained with H_2O until bands are vissible. Gel were viewed using Kodak Image Station 4000 MM and the molecular weight of the protein bands were determined. (Precision Plus Protein^{TM} unstained standard ladder, 1ml) was used as the molecular weight standard.

Zymography is an electrophoretic technique, based on SDS-PAGE, that includes a substrate co-polymerized with the polyacrylamide gel, for the detection of enzyme activity (Lantz and Ciborowski, 1994). Samples were prepared in the standard SDS-PAGE treatment buffer, but without a reducing agent in order for the enzyme to retain its native state (and therefore its proteolytic activity).

Parallel SDS-PAGE gels containing 0.1% substrate (CMC or birchwood xylan) was used to detect enzyme activities. Zymography was performed according to (van Dyk et al., 2010b). After electrophoresis, 2.5% (v/v) Triton X-100 in 50mM sodium acetate buffer (pH 5.5) or 50mM Tris-HCl buffer (pH 7.5) were used to renature gels (Xylanase or CMCase respectively). Gels were then incubated in same buffers above at 37^{o}C for 12-48hrs and were subsequently stained with 0.3% Congo Red for 15mins and then destained with 1MNaCl until bands were noticed. Gels were then counterstained with 5% acetic acid. Zymogram gels were viewed using Kodak Image Station 4000 MM.

3. Results

3.1 Overview of screening procedures

Various methods were used in this study for the screening of bacterial isolates from moose rumen for the selection of the candidates for cellulase production which includes;
CMC screening of Isolate MRB 1-5 was performed to identify the bacteria with possible cellulase activity (Figure 3.1). Positive plates show clear zones on Gram's iodine on CMC agar plates. Enzyme activity (endoglucanase), growth pattern, yield and how easy it is to cultivate the isolates were considered in the selection process for this project work, (figure 3.2). DNA assay was used, which is determined by the release of reducing sugar of glucose or xylose depending on substrates. Results are presented with raw values from absorbance 540 which represents the amount of reducing sugars under this assayed condition.

3.2 Growth, Screening and Selection.

After incubation, the zone clearing was observed. Bacterial isolates MRB 2, 3, 4, 5 showed zone clearing on CMC plates (figure 3.1).

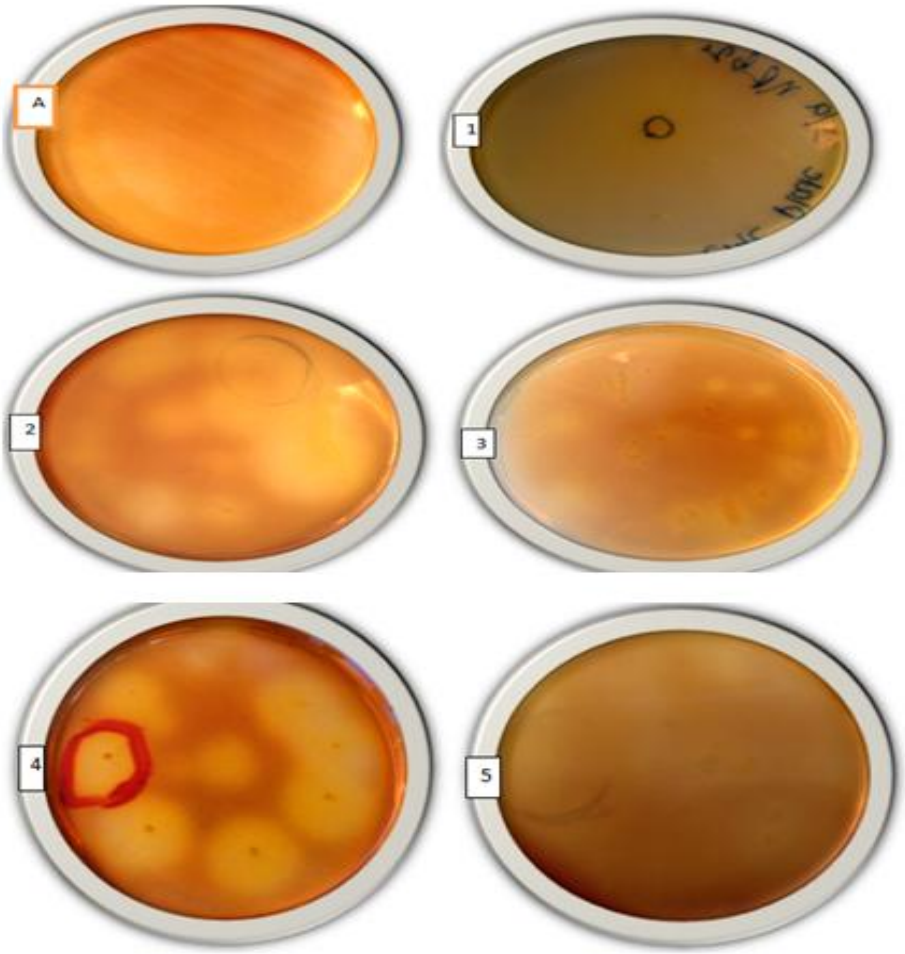


Figure 3.1. Effect of Gram's iodine on cellulolytic zone in CMC agar plates after 48 hours of incubation. Isolates were inoculated from blood agar master samples, and inoculation method of plates were of scatter spots and spaced regular spots. (A) uninoculated plate, (1) MRB 1, (2) MRB 2, (3) MRB 3, (4) MRB 4, (5) MRB 5.

Figure 3.1 A, is the uninoculated plate which represents the negative control, showed no clearing zone on CMC plates. Figure 3.1.1, represents isolate MRB 1, isolate was spot plated but irregularly on CMC agar plates and isolate showed no clearing zone on CMC plates. Figure 3.1.2, represents isolate MRB 2 which was spot plated but in irregular spacing pattern, showed strong clear zones on CMC agar plates with high intensity. Figure 3.1.3, represents isolate MRB 3 which was spot plated in irregular spacing pattern, showed strong clear zone on CMC agar plates. Figure 3.1. 4 represents isolate MRB 4 which was spot plated in regular spaced pattern on the CMC agar plate, isolate showed strong clear zone on CMC agar plates with high intensity. Figure 3.1. 5 represents MRB 5 which was spot plated irregularly on CMC agar plates, isolate showed clear but weak zones on CMC agar plates. Zones appeared clearer than what is obtainable in images. MRB 2, 3, 4, 5 showed strong clear zones on Gram's iodine in CMC agar plates. No clear zone was observed in isolate MRB 1.

3.3 Morphology and growth characteristics of isolates

Bacterial Isolates MRB 1-5, were analysed and evaluated to identify the most promising canditates for cellulase and hemicellulase production. Figure 3.2 showed the growth curves for isolates MRB 1-5. Growth rate and yield were observed during the cultivation process as a selection tool for the most promising isolates (figure 3.2).

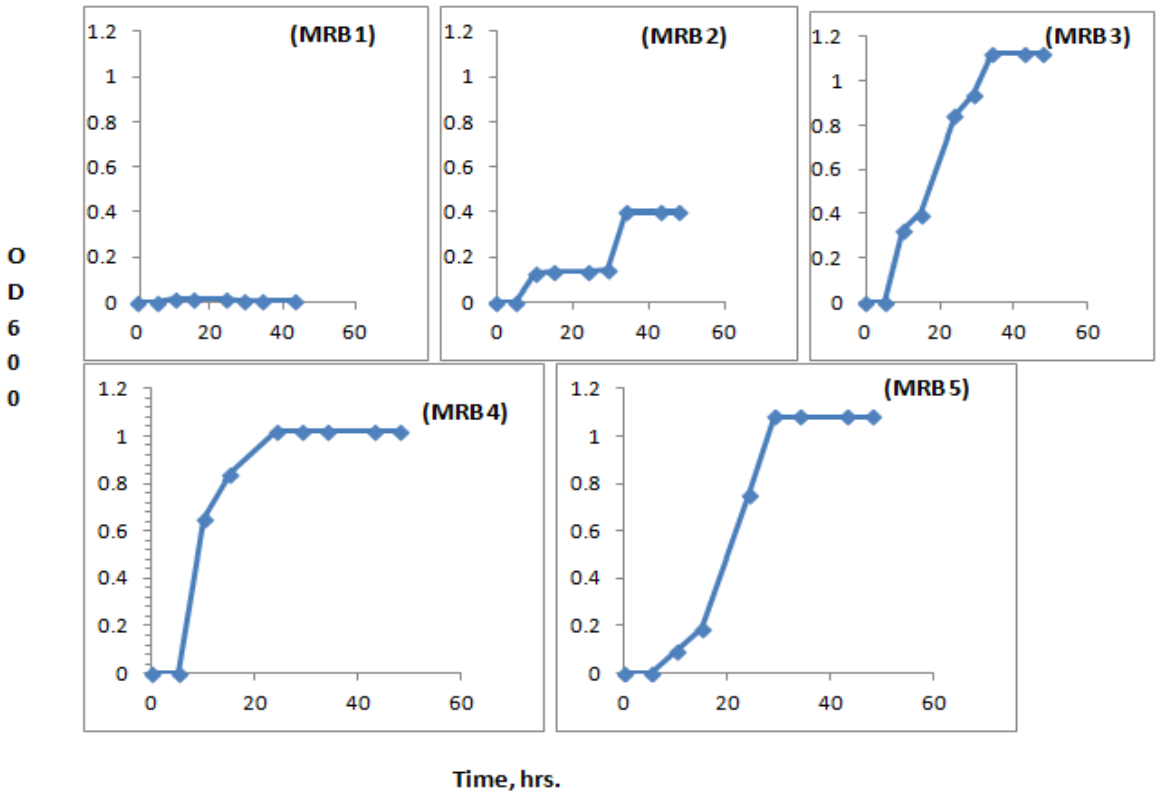


Figure 3.2, Growth curves of bacterial isolates MRB 1, MRB 2, MRB 3, MRB 4 and MRB 5 cultivated in 400ml BHI medium at 37^{o}C earobically. All growth was carried out under the same growth condition.

Isolate MRB 1: MRB 1 appeared to be rod-shaped Gram negative bacteria, and grows best under anaerobic condition; isolate gives no clear zone on CMC agar plates but enzyme activity of culture broth determined by DNS assay appeared to be positive, which is contradicting but might be due to technique reasons or that this isolate did not react with Gram's iodine. Growth rate was slow and yield was low compared to other isolates. Strictly anaerobic, which makes it difficult to cultivate in other condition or environment and appear to have more than 6 hours lag phase.

Isolate MRB 2: MRB 2 appeared to be cocci-shaped and Gram positive, it grows under both aerobic and anaerobic condition/environment, and gives strong clear zones on CMC agar plates. Growth rate was high but with low growth yield compared to the other isolates. Easy

to cultivate with more than 6 hours lag phase. Enzyme activity of culture broth determined by DNS assay appeared to be positive.

Isolate MRB 3: MRB 3 appeared to be rod-shaped, Gram positive, it grows under both aerobic and anaerobic condition, gives clear strong CMC-zone. Growth rate was high and yield is also high. It is easy to cultivate with a short lag phase. Enzyme activities determined by DNS assay appeared to be positive and high in relation to growth yield.

Isolate MRB 4: MRB 4 appeared to be curly chains and Gram negative, it also appeared to be facultative bacteria able to grow well under aerobic and anaerobic condition. It gives strong clear zone on CMC agar plates. Growth rate and yield were both high. It is easy to cultivate with less than 6 hours lag phase. Enzyme activity determined by DNS Assay was positive but low compared to growth rate and yield.

Isolate MRB 5: MRB 5 is a facultative bacteria able to grow under earobic or anaerobic condition. It showed clear but week zone on CMC agar plates and gave strong positive result on enzyme activity (DNS Assay). It appeared to be Gram positive. Growth rate and product yield was also high.

In summary, Growth factor, yield and enzyme activity were used for the screening of the bacterial isolates MRB 1-5 to identify the most promising isolate for this project work with high cellulase activity. Enzyme activity was carried out using DNS method and Isolate MRB 3 was chosen based on growth pattern, yield, observed clean zones on CMC screening, (figure 3.1) and good indication of enzyme activities on DNS assay.

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

3.4.1 Qualitative PCR.

PCR, agarose gel and sequencing were performed to identify the bacterial isolate MRB 3. Confirmation of a PCR product was done by running the qualitative PCR product on a 1% agarose gel. Results showed a product size of approximately 1.2kb, (figure 3.3).

1 2

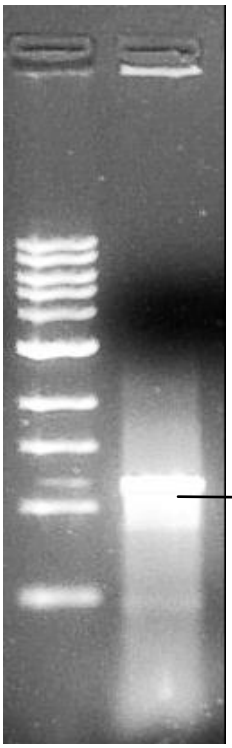


Figure 3.3. Agarose gel electrophoresis result for the PCR product showing a very strong band of PCR product. Lane 1- DNA ladder, lane 2-PCR product. 1kb quantification ladder (BioLabs N3232) was used.

3.4.2 Sequencing and identification.

The PCR product was sequenced and the sequence was obtained and analysed using Sequence Scanner. The full sequence of 720 base pairs was obtained, (figure 3.4).

GCTGGCTCCAAAAGGTTACCTCACCGACTTCGGGTGTTACAAACTCTCGTGCGTGTGACGGGCGGTGTGTACAA
GGCCCGGGAACGTATTCACCGCGGCATGCTGATCCGCGATTACTAGCGATTCCAGCTTCACGCAGTCGAGTTGC
AGACTGCGATCCGAACTGAGAACAGATTTGTGGGATTGGCTTAGCCTCGCGGCTTCGCTGCCCTTTGTTCTGCC
CATTGTAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGACGTCATCCCCACCTTCCTCCGGTTTG
TCACCGGCAGTCACCTTAGAGTGCCCAACTGAATGCTGGCAACTAAGATCAAGGGTTGCGCTCGTTGCGGGAC
TTAACCCAACATCTCACGACACGAGCTGACGACAACCATGCACCACCTGTCACTCTGCCCCCGAAGGGAAGCC
CTATCTCTAGGGTTGTCAGAGGATGTCAAGACCTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATGCT
CCACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTCAGTCTTGCGACCGTACTCCCCAGGCGGAGTGCTT
AATGCGTTTGCTGCAGCACTAAAAGGGCGGAAACCCTCTAACACTTAGCACTCATCGTTTACGGCGTGGACTA
CCAGGGTATCTAAATCCTGTTCGCTCCCCACGCTTTCGCGGCCTCAGCGTCAGTTACAGACCAGAGAGTCGCCT
TTCGCCACTGGTGTTCCTCCACATCTCTACGCATTTCACCGGC.

Figure 3.4. Obtained sequenece of 720bp from the sequencing of PCR product which was used for the identification search.

After performing a nucleotide BLAST search, the organism had the highest sequence homology and score with a number of strains of *Bacillus licheniformis.* Table 3.0 showed that the sequence of isolate MRB 3 comforms with that of strains of *B. licheniformis*. The bacterial isolate MRB 3 was named *B. licheniformis* AA1 for this study.

Table 3.0, Results of Blast search of the obtained necleotides in BLAST

Homology (Description)	Max Identity	Query cover	Accession	E value
B. licheniformis strain SCC B37 16S ribosomal RNA gene, partial sequence	100	100	KC609000.1	0.0
B. licheniformis strain SCC B37 16S ribosomal RNA gene, partial sequence	100	100	KC609000.1	0.0
B. licheniformis gene for rRNA, partial sequence	100	100	AB196353.1	1e-118
B. licheniformis gene for 16S rRNA, partial sequence	100	100	AB196350.1	1e-118
B. licheniformis strain CICC 10092 16S ribosomal RNA gene, partial sequence	100	100	AY842876.1	4e-118
B. licheniformis strain CICC 10097 16S ribosomal RNA gene, partial sequence	100	100	AY859479.1	4e-118
Uncultured soil bacterium clone 12-48 16S ribosomal RNA gene, partial sequence	100	100	JN417553.1	5e-117
Uncultured bacterium clone GZ84 16S ribosomal RNA gene, partial sequence	100	100	JX133468.1	5e-117
Bacillus aerius strain MHRS1 16S ribosomal RNA gene, partial sequence	99	100	KC443115.1	2e-116
Firmicutes bacterium K23 16S ribosomal RNA gene, partial sequence	99	100	KC887942.1	2e-116

3.5 Growth of enzyme-producing bacteria

Isolate MRB 3 was cultivated in a 500ml shaking bottle in a 250ml medium containing ABB supplemented with 0.5% cellobiose and later scaled up in 2.5l anaerobic fermentor in the same medium under anaerobic condition at 37^{o}C for 24 hours, at pH 6.8 which eventually reduced to pH 5.2 as the culture reach maturity. The isolate was cultured for 24hours and harvested. Figure 3.5 below shows the growth curve obtained from the measurement of optical density and assayed for enzyme activity.

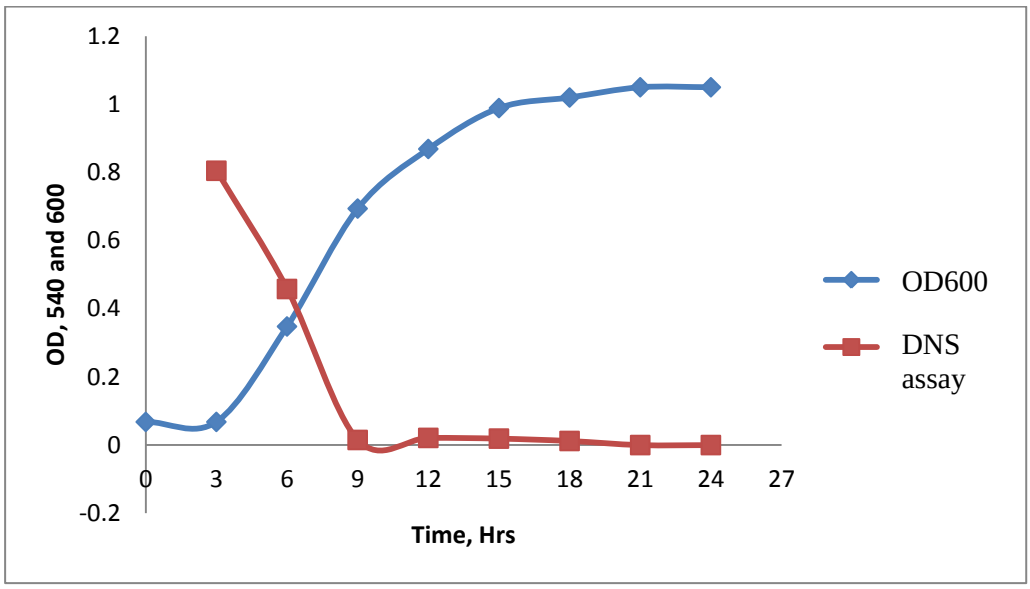


Figure 3.5, Growth curve and enzyme activity of cultivated bacterial isolate MRB 3 in a 2.5l anaerobic fermentor containing ABB medium supplemented with 0.5% cellobiose, under anaerobic condition at 37^{o}C for 24 hours. DNS assay, measurement of reducing sugar is presented as raw value from Absorbance 540 which represents the amount of reducing sugars under this assayed condition.

Determining the best time to harvest the culture was based on, the DNS assay to describe enzyme activity in relation to growth yield. Figure 3.5 showed that the enzyme activity decreases with time between 0hrs and 12hrs while growth increases which showed that the subtrates in the culture medium was been used up. The observed enzyme activity started to increase from 12hrs upwards which should account for the enzyme activity because the substrates is expected to have been used up at this stage. it also showed that samples could be harvested as early as from 18hours and could also be left to stay for 24hours or more as it was for this experiment.

3.6 Localization of main cellulase activity

3.6.1 DNS Assay and D-glucose

The harvested culture was analysed to determine the kind of protein found in the isolate; which could be bonded or unbonded protein.

After the bacteria was grown in 0.5% cellobiose under anaerobic condition at 37^{o}C for 24hrs, the culture was harvested by centrifugation at 4000rmp for 20mins at 4^{o}C. The supernatant was used as crude enzyme and pellet were stored for further analysis. Sonication was done, pellet and supernatant were stored for further experiment.

Culture broth, supernatent (after centrifugation), pellet (after centrifugation), pellet and supernatent (sonication of pellet and subsequent centrifugation) were all analysed for enzyme activity using DNS assay and D-glucose assay, (figure 3.6). Cellulase from *Trichoderma reesei* (1;4 dilution) was used as positive control. DNS assay showed that supernatant after centrifugation has the highest enzyme activity of all the samples, followed by the culture broth and pelleted cells. Only minor amounts of enzyme activity was released after sonication of pellet and subsequent centrifugation. Enzyme activity in the pellet was very low as observed in the DNS assay. D-glucose assay which show the release of monomeric glucose, showed very low activity in the supernatant (after centrifugation) and absent in all the remaining samples, which may suggest that there may not be a need for sonication in this experiment. Supernatant (after centrifugation) being positive to DNS assay and with very low D-glucose activity as an overlapping result, showed that the enzyme activity is in the supernatent (after centrifugation). Significant endoglucanase and very low exoglucanase activity was observed in the supernatant (after centrifugation). Very low endoglucanase activity was observed in the pellet (after centrifugation) and no exoglucanase activity was observed.

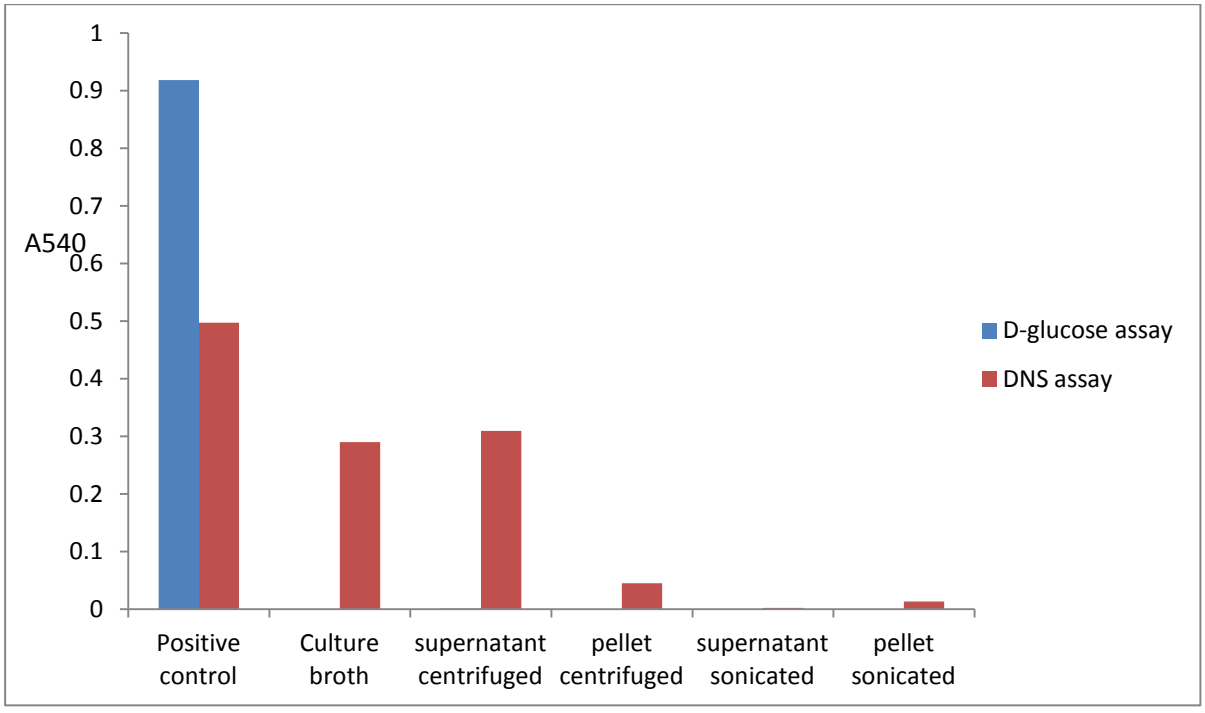


Figure 3.6, Separation of enzyme activity after centrifugation and sonication of cell culture. DNS assay, measurement of reducing sugar presented as raw value of absorbance 540nm and D-glucose was performed on supernatant (after centrifugation), culture broth, pellet (after centrifugation), supernatant (after sonication and subsequent centrifugation) and pellet (after sonication and subsequent centrifugation). Cellulase from *Trichoderma reesei* (1.4 dilution) was used as positive control.

Figure 3.6 shows the result for enzyme activities of the samples. The result suggests that the enzyme activities obtained from DNS assay and D-glucose for supernatant (after centrifugation) over-lap, which could showed that the main enzyme activities could be found in the supernatant because the activities does not over lap in other samples (pellet, and sonicated samples).

3.7 **Polyacrylamide gel electrophoresis (PAGE)**

To analyse proteins profile of various supernatants and pellets, samples were analysed in non-reducing SDS-PAGE (fig 3.7).

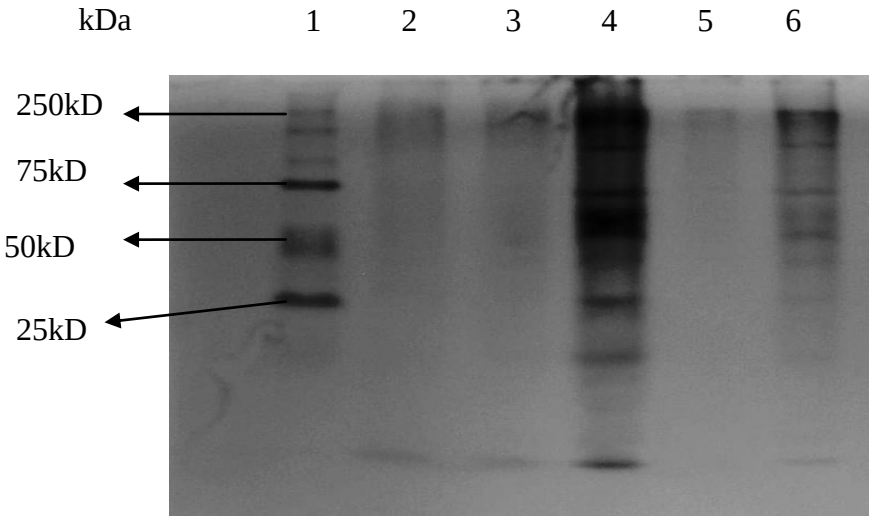


Figure 3.7, SDS-PAGE (10% gel, stained with Coomassie Brilliant Blue) analysis of isolate MRB 3 samples under non-reducing condition, lane 1, molecular mass standard, lane 2, culture broth, lane 3, supernatant (after centrifugation), lane 4, pellet (after centrifugation), lane 5, supernatant (pellet sonicated and subsequent centrifugation), lane 6, pellet (sonicated pellet and subsequent centrifigation).

Lane 4 showed distinct heavy stained bands with the highest band at approximately 200kDa, and subsequetly at around 75kDa and some low bands at 25kDa and 10kDa. Non separated bands were also observed at the bottom of the lane. Lane 6 showed visible strong bands at around 200kDa, 75kDa and 50kDa. Weak bands were also observed at around 40kDa and 30kDa. Lane 2, 3, 5 showed no visible band which may be due to the staining agent used. Maybe a more sensitive staining like silver staining would have reviewed the presence of protein bands in these lanes. No signal was observed in the supernatant samples and culture broth. Lack of bands in supernatant may suggest that the high molecular mass enzyme did not enter the gel as the proteins were run under non-denaturing condition before loading on gel

but another SDS PAGE was run with denatured proteins so that the proteins can be separated into parts.

3.8 Comparing bacteria growth and enzyme activities on different substrates and pH

After identification, *B. licheniformis* AA1 was grown on various culture media (ABB, BHI and BBB) to compare growth characteristics, observe pH change and to observe how substrates can influence or induce enzyme activities. Previous work by (Lynd et al., 2002a) has also suggested that cellobiose can be an inducer of endoglucanase activity.

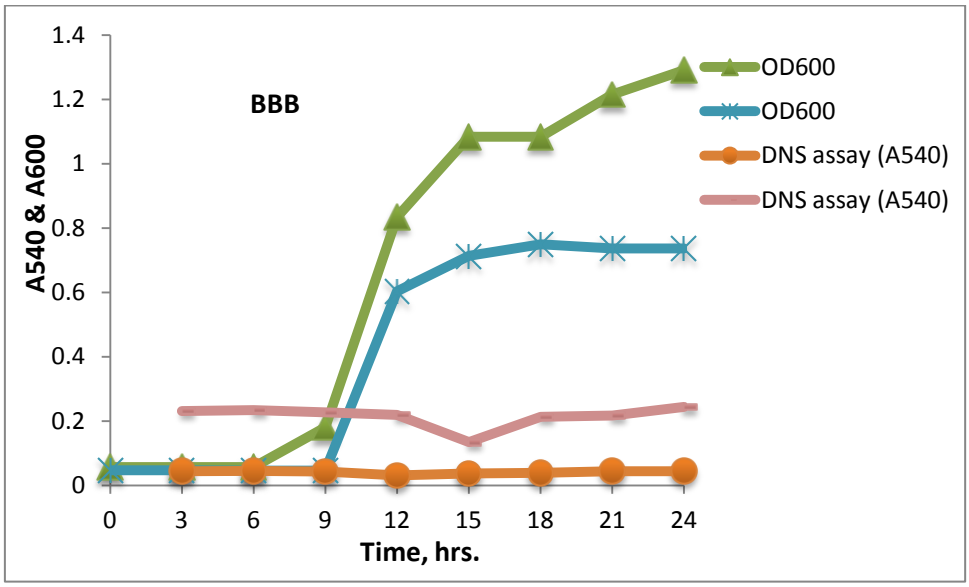


Figure 3.8; Growth curve and enzyme activity (DNS) of *B. licheniformis* AA1 grown on different substrates. Isolate was cultivated under aerobic condition at 37°C in a 500ml shaking bottle. -- Growth curve observed in BBB medium supplemented with 0.5% cellobiose (BBB+). –DNS assay for BBB supplemented with 0.5% cellobiose (BBB+). -- Growth curve observed in BBB medium without supplement (BBB-) –DNS assay for BBB medium without supplement (BBB-).

Comparing growth rate, yield and enzyme activity in BBB medium (supplemented with 0.5% cellobiose and un-supplemented), figure 3.8 showed that cellobiose when added to the growth medium can induce endoglucanase activity by an increase in the enzyme activity as also demonstrated by (Lynd et al., 2002a). Growth appeared to be differentiated with BBB+ (BBB supplemented with cellobiose) having a better growth rate and yield, while BBB- (BBB without supplement) have a low growth rate and yield (fig 3.8). Growth yield and enzyme activity were induced by the presence of cellobiose as the carbon source in the medium by an increase in endoglucanase or xylanase activity. This could be said to be true because low

enzyme activity was observed in the control medium (BBB-) which does not contain cellobiose as supplements. There was low growth and low enzyme activity in Basic Bacillus Medium without supplement (BBB-).

How *B. licheniformis* AA1 responded to different sources of carbon in the medium in terms of growth, yield and resulting enzyme activities (stimulation of enzyme activities) was investigated (fig. 3.9a and b).

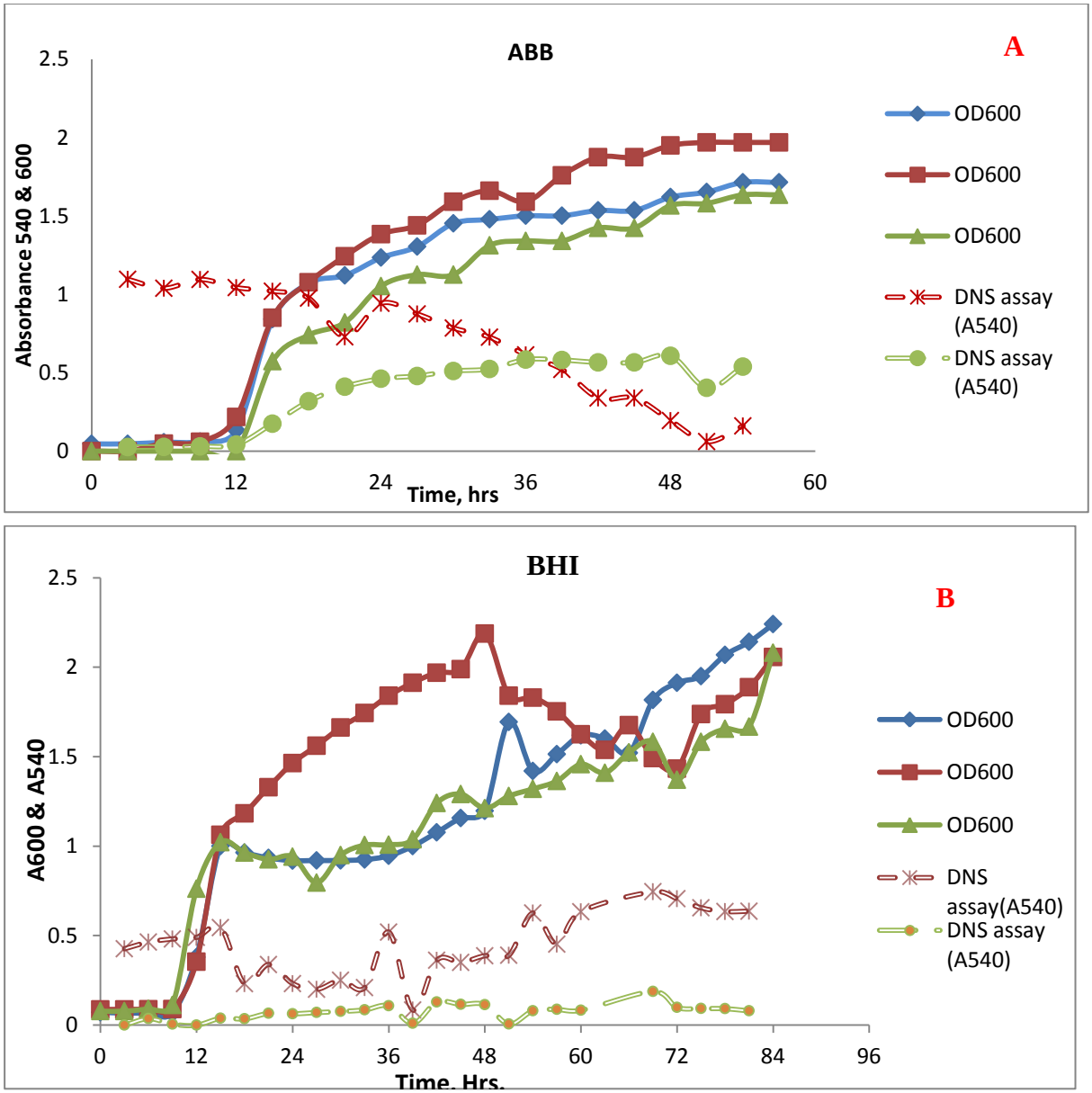


Figure 3.9a and b, Growth curve and enzyme activities (DNS) of *B. licheniformis* AA1 grown on ABB and BHI supplemented with 0.5% Xylan and 0.5% cellobiose. Activity curves are normalised against the control culture (ABB- and BHI-). DNS Assay for 63hrs and 66hrs Sample are excluded due to technical issues. - Growth curve observed in the control culture ABB or BHI medium without supplements. -Growth curve observed in ABB or BHI medium supplemented with 0.5% cellobiose. -Growth curve observed in ABB or BHI medium supplemented with 0.5% xylan. - Enzyme activity (DNS) observed in ABB or BHI medium supplemented with 0.5% cellobiose. -Enzyme activity (DNS) observed in ABB or BHI medium supplemented with 0.5% xylan. A—represents ABB medium and B—represents BHI medium.

Figure 3.9a, showed how substrate can influence the growth of a bacteria in a medium (ABB or BHI, supplemented with 0.5% cellobiose or 0.5% xylan) and how enzyme activities can be induced by an inducer. Cellobiose has been reported to be an inducer of endoglucanase activity (Lynd et al., 2002a). Figure 3.9a showed that cellobiose appear to induce englucanase activity during the log phase of the cultivation and at a late stage during the cultivation, while birchwood xylan also appear to induce xylanase activity parallel to growth during the log phase (activity was induced parallel to growth) in *B. licheniformis* AA1

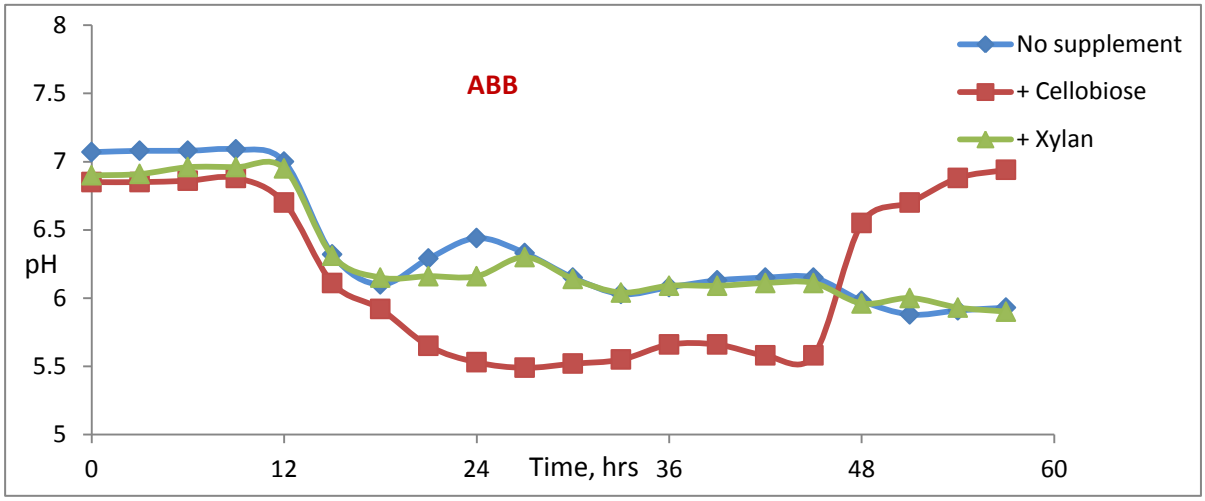
Figure 3.9b shows how different subtrates in a medium (BHI, supplemented with 0.5% cellobiose or 0.5% xylan) can influence *B.* l*icheniformis* AA1. How *B. licheniformis* AA1 responded to different sources of carbon in the medium in terms of growth, yield and resulting enzyme activities (stimulation of enzyme activities).

Figure 3.9A and B, showed that cellobiose and xylan serves as endoglucanase and xylanase inducer respectively. During the log phase, *B. licheniformis* appear to have more growth yield in the medium containing cellobiose than the medium containing xylan and in the medium containing no supplement. This could be that, *B. licheniformis* AA1 can use the cellobiose supplemented in BHI medium for growth and yield better than the xylan. Growth curve and yield was similar on ABB medium. Growth rate was similar in ABB medium for both supplemented medium and the control but the resulting enzyme activity was greater in both supplemented media (ABB+cellobiose and ABB+Xylan) than in the control (ABB-). In BHI medium, growth rate was found to be similar and parallel in BHI+Xylan and control (BHI-). Utilization of cellobiose could be said to be more pronounced in the BHI than in ABB medium in terms of growth rate and yield. While the growth yield corresponded to enzyme activity observed in ABB supplemented with xylan medium, reverse could be said in the BHI supplemented with xylan medium. It was also observed in figure 3.9b that, growth yield corresponded to high enzyme activity observed in BHI medium supplemented with cellobiose, reverse could be said in ABB medium supplemented with cellobiose. In figure 3.9b, It was also observed that, late in the log phase, at about 50hrs, growth was seen to decline in BHI+ Cellobiose and BHI+Xylan media but the corresponding enzyme activity increases in both, this could be because the source of carbon in the medium has decreased but it resulting enzyme activity did not decrease. Increase in enzyme activity during the log phase of the cultivation could be said to be significant in both ABB supplements and BHI supplements because at this stage of the cultivation, the cellobiose and xylan is expected to have been used up and the resulting enzyme activity is therefore seen to be significant. Little or no enzyme activity was observed in the control samples (ABB- and BHI-) which suggest that the supplements (Xylan and Cellobiose) could have acted as inducers of the enzyme

activities. In both experiment, it was observed that, all cultures ended up with the same yield, it could be that *B. licheniformis* grows well in both medium, (BHI and ABB) but enzyme activity is only induced in the presence of either cellobiose or xylan, because little or no activity was observed in the control (BHI- and ABB-) during the cultivation. It was observed that cellobiose and xylan induced enzyme activities in both ABB and BHI, although the effect appears to be more pronounced in ABB than in BHI.

Figure 3.9A and B, shows the pH of the culture measured at 3hrs interval during the cultivation of *B. licheniformis* AA1 in ABB and BHI media. This was carried out to observe any effects of pH change on growth behaviour in different media.

A



B

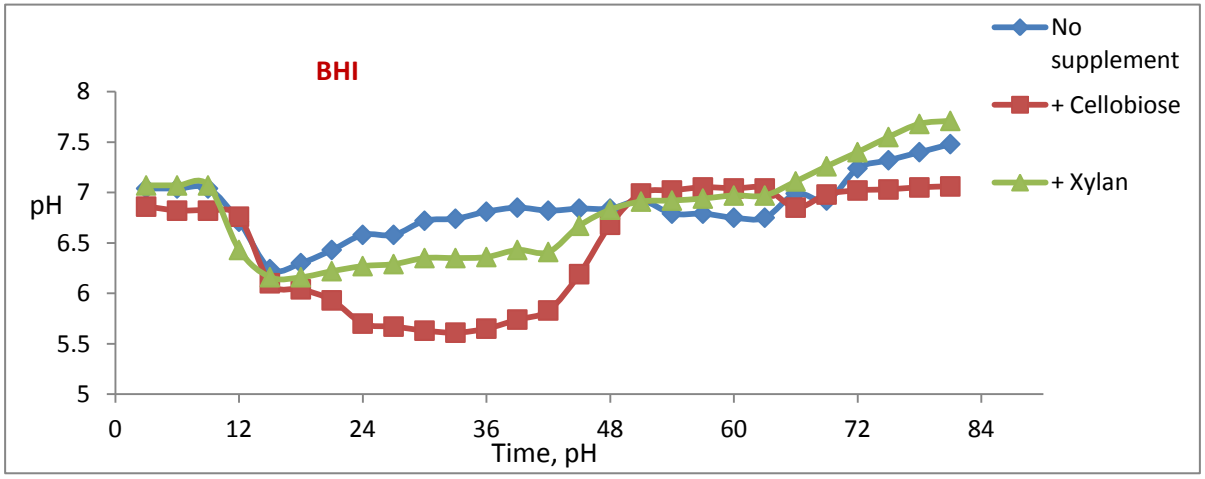


Figure 3.10, Fluctuation of pH during the cultivation of *B. licheniformis* AA1 on A- ABB or B- BHI medium supplemented with 0.5% cellobiose or 0.5% xylan as substrates. The control is without any supplement. A- represents pH from ABB medium and B- represents pH from BHI medium. The cultivation was carried out in a 500ml medium at 37^{o}C under a shaking, earobic condition. -pH from BHI or ABB medium without any supplement (BHI- and ABB-). -- -pH from BHI or ABB medium supplemented with cellobiose. -pH from pH from BHI or ABB medium supplemented with Xylan.

The pH measurement during cultivation in ABB medium, showed that pH varies from as low as pH 5.6 and as high as pH 7.2, (fig 3.10A). During the cultivation, pH drop was observed during the exponential growth phase in all the culture media (ABB-, ABB+cellobiose and ABB+xylan) and constantly reduced in ABB- and ABB+xylan until stationary phase. The pH drop is greatest during the exponential growth on ABB supplemented with cellobiose (ABB+cellobiose). The pH was found to increase late in the cultivation (stationary phase) for ABB+cellobiose medium. It was observed that pH decrease continuously as growth yield increases in all the culture media (ABB-, ABB+cellobiose and ABB+xylan) except for ABB+cellobiose which later increases late in the cultivation (stationary phase).

The pH measurement during the cultivation in BHI medium, showed that pH varies from as low as pH 5.6 and as high as pH 7.7, (fig 3.10B). During the cultivation, pH drop was observed during the exponential growth phase in all the culture media (BHI-, BHI+cellobiose, BHI+xylan). The pH drop is greatest during the exponential growth on BHI supplemented with cellobiose (BHI+cellobiose). The pH was also found to increase late in the cultivation for all the culture media.

3.9 **Binding of cellulolytic system to Avicel**

In order to isolate cellulase specifically, the protein was isolated by adsorption-desorption washing on Avicel. The enzyme activity which bond to Avicel was washed using different buffers. Figure 3.11, shows to what extent each buffer caused release of the enzyme activity from the insoluble Avicel. DNS Assay was used to determine the level of enzyme activities/protein released and later analysed on protein gel.

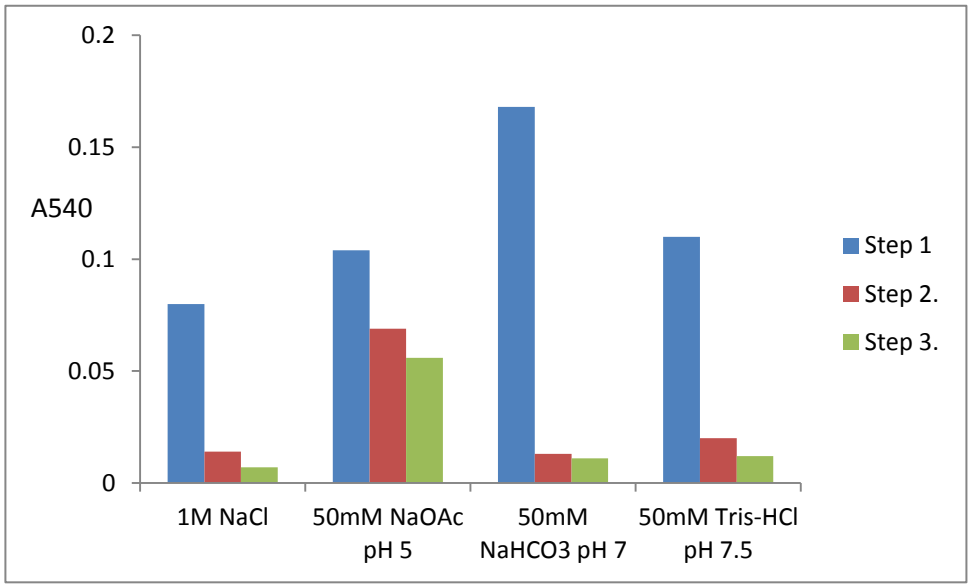


Figure 3.11, Effect of buffers on desorption cellulase from Avicel, showing level of activity in three consecutive washes of Avicel using same washing volume. 5% Avicel was added to the 50ml crude enzyme and the mixture was stirred for 30mins at 4^{o}C. The supernatant was discarded and the pellets of enzyme-insoluble cellulose were washed in three consecutive times with 5ml volume of buffers each.

Figure 3.11 shows the resulting enzyme activity from affinity Avicel washing. The result showed a significant release of enzyme activity. Sodium Bicarbonate (50mM) appear to have the hightest release of enzyme activity, with the first wash step having the highest value, followed by the second washing step and finally the third. Tris-HCl (50mM) has the second highest release of enzyme activity, with the first washing step showing the highest value, followed by the second washing step and then the third. Sodium Acetate (50mM) has the third highest released enzyme activity. 1M NaCl have the least released enzyme activity of all the buffers. A step wise reduction in the amount of enzyme activity released, showed that the enzyme activity is released in a step wise fashion. All buffers showed a step wise release of enzyme activity. Sodium Bicarbonate appeared to have the highest strength because it released the highest enzyme activity from the insoluble Avicel, while 1M NaCl has the least. The result showed that the cellulolytic system form B. *Licheniformis* AA1 could bind to Avicel.

3.10 Concentrating enzyme fraction in culture supernatant

3.10.1 Concentration in Spinn cartridge (Amicon);

After harvesting of bacterial culture by centrifugation, the culture supernatant was concentrated in spinn cartridge (Amicon) with a cut-off of 50kDa and the resulting solution was analysed for enzyme activity (DNS) (figure 3.12).

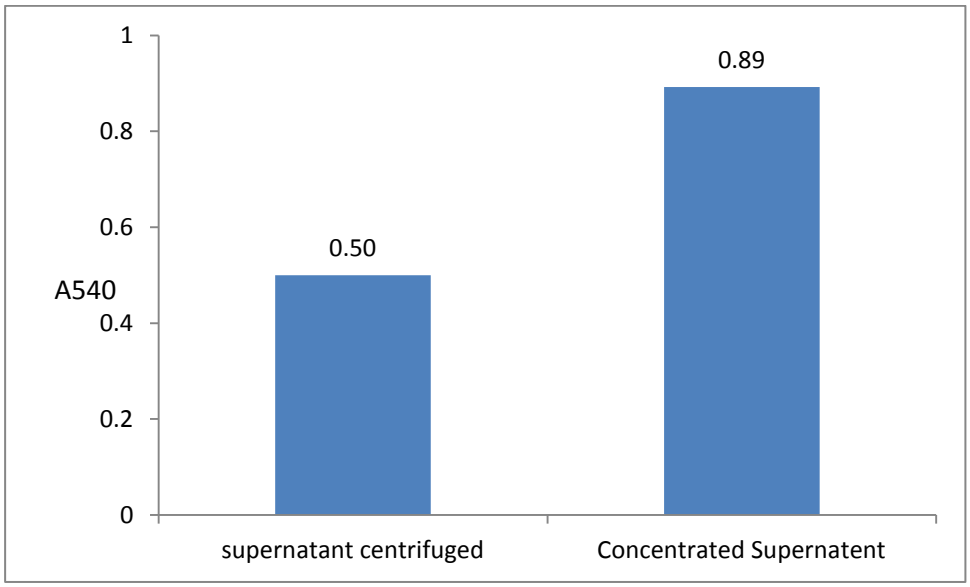


Figure 3.12 Effect of concentration of culture supernatant in 50kDa spinn cartridge (Centricon Amicon ultra, USA), supernatent (centrifuged from harvested culture).

The concentrated sample showed a less than twofold increase in enzyme activity (figure 3.12). The sample was concentrated and the resulting enzyme activity (xylanase activity) increased. The DNS assay showed a 1.8 times concentration factor.

3.10.2 Ultrafiltration methods

Ultrafiltration in a cross-flow device was used to concentrate larger volumes of samples with a nominal molecular weight cut-off of 10 kDa and 50kDa. The concentration was performed to concentrate both the protein and enzyme activity (figure 3.13), however, only the resulting enzyme activity was checked. DNS assay was used to determine the enzyme activity. Cross-flow ultra filtration membrane cassettes resulted in the recovery of 95 percent activity from the culture filtrate. Figure 3.13 (A and B) shows the effect of cross flow ultra filtration (10kDa and 50kDa) on concentrating enzyme activity, (DNS assay).

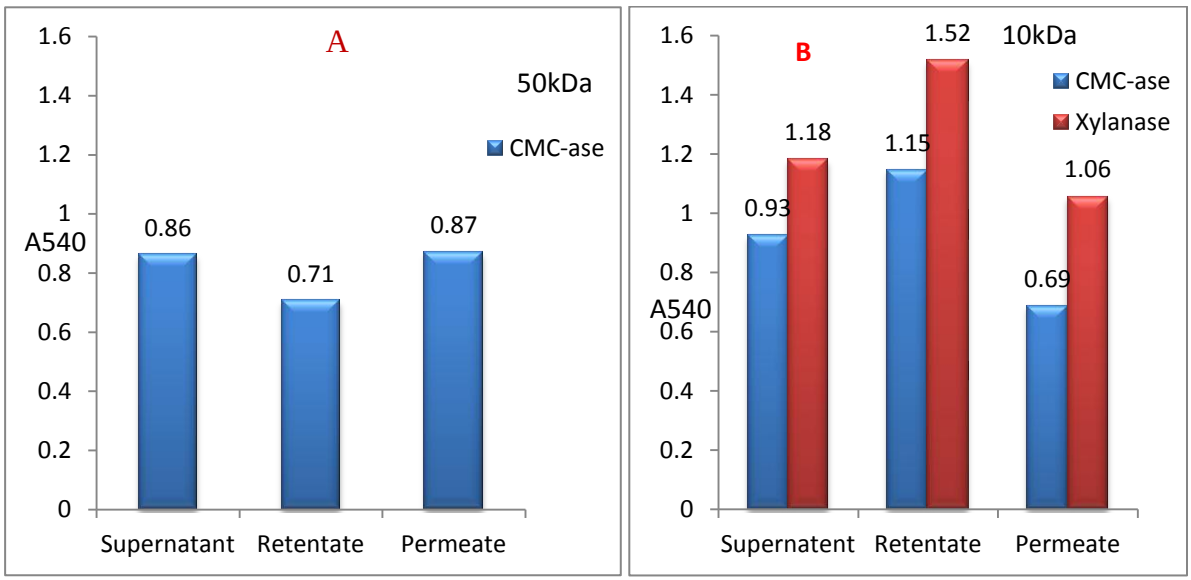


Figure 3.13; Effect of cross-flow ultrafiltration (A- 50kDa and B- 10kDa) on concentration enzyme activity. 100ml sample volume was loaded, 15ml was collected as the retentate and 85ml passed through as permeate for 10kDa while 10ml was collected as the retentate and 90ml passed through as permeate for 50kDa.

Using ultrafiltration with a cut-off of 50kDa, the concentration factor was found to be 0.82 times for the retentate sample. The percentage of recovery was found to be 99% for CMC-ase and the retention value was zero (0). It was observed that most of the CMC-ase activity passed into the permeate fraction, thus the enzyme activity was not efficiently retained.

To achieve a better concentration result, the cut-off was reduced to 10kDa. Using ultrafiltration with a cut-off of 10kDa, the concentration factor was found to be approximately 1.3 times for the retentate sample. The percentage of recovery was found to be 45% for CMC-ase and 95% for xylanase. The retention value was found to be 0.33 and 0.13 for CMC-ase and xylanase respectively, which showed that a concentration step is achieved. Using a lower cut-off had a significant effect as illustrated by a low retention value (0.01).

Table 3.2, Effect of cross flow ultra filtration (A- 50kDa and B- 10kDa) on concentration enzyme activity.

	50kDa	10kDa
Volume start (ml)	100	100
Volume retentate (ml)	10	15
Volume permeate (ml)	90	85
CF CMC- ase	0.82	1.24
Cf xylanase	Not applicable	1.3
Recovery CMC- ase	99	45
Recovery xylanase	Not applicable	95

Table 3.3, Retention R, value showing to what extent the proteins are retained by the membrane.

	CMC-ase	Xylanase
R_{10}	0.33	0.13
R_{50}	0.01	Not applicable

Reducing the cut-off of the cross-flow ultra filtration from 50kDa to 10kDa was not enough to retain the protein which might be due to technical reasons. The protein and enzyme activity was concentrated by about two fold from culture supernatants by crossflow filtration with 95% recovery of total enzyme activity. However, significant amounts of activity passed through both 50 and 10 kDa UF membranes, indicating the presence of low-molecular cellulases.

3.11 Electrophoresis and Zymography

SDS PAGE and zymography were run to determine the size of the proteins and enzyme activities respectively. Zymography was done using beechwood xylan and CMC as substrates included into the gel according to the modified method by (van Dyk et al., 2010b) figure 3.14 A and B.

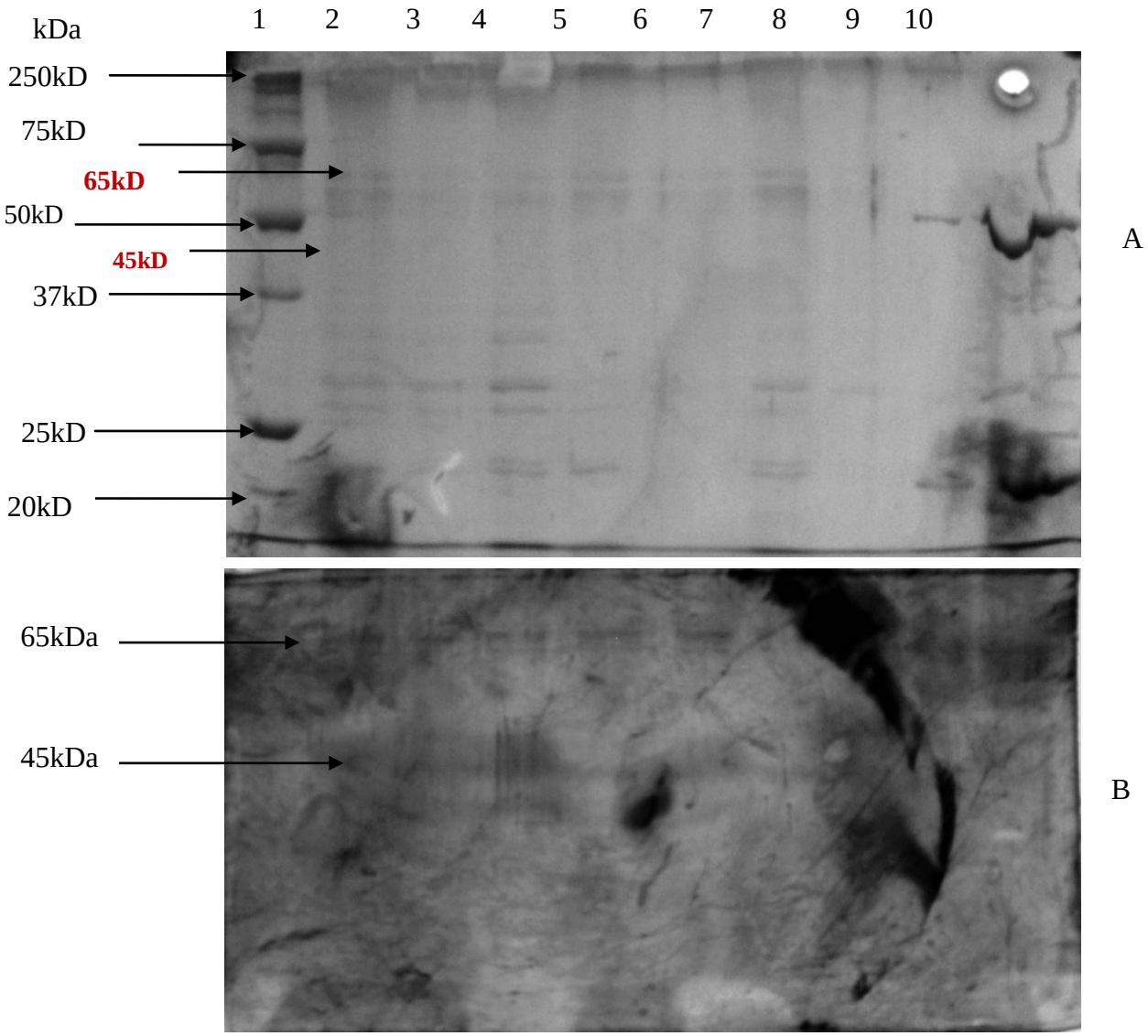


Figure 3.14, SDS-PAGE (12%) (A) and Zymography (B) analysis of fractions from UF concentration and Avicel binding. lane 1 Protein ladder, lane 2 Retentate, lane 3 Permeate, lane 4 supernatant, lane 5 $NaHCO_3$ washing, lane 6 1M NaCl washing, lane 7 Sodium Acetate washing, lane 8 empty, lane 9 and 10 positive control (Cellulase from *T. Reesei*, *in* 1;4 dilution).

Figure 3.14a showed SDS-PAGE analysis. Retentate in lane 2 showed distinct heavy stained bands with the highest band at approximately 200kDa, subsequetly at around 65kDa and another band at about 45kDa. Permeate in lane 3 showed weak fractions of bands with the highest observed at approximately 200kDa, and subsequently at about 65kDa and at about 45kDa. The supernatant in lane 4 gave weak bands at approximately 200kDa, 65kDa and at about 45kDa. Strong bands were observed at approximately 35kDa and 25kDa. Sodium bicarbonate ($NaHCO_3$) wash in lane 5 showed strong bands at approximately 55kDa, and 45kDa and weak bands were observed at about 20kDa. Very weak bands were observed at approximately 55kDa and 45kDa in lane 6 which contained 1M NaCl washing sample. Acetate buffer washing sample in lane 7 gave strong bands at approximately 55kDa and 45kDa. Further bands were observed at approximately 25kDa and 10kDa.

The CMC zymogram patterns observed in fractions from UF concentration, (figure 3.14b) was the same for all the samples, which displayed two bands with activity at approximately 65kDa and at 45kDa. Samples from Avicel binding experiment displayed two bands with activity at approximately 65kDa and at 45kDa.

3.12 **Purification Techniques.**

3.12.1 ION EXCHANGE CHROMATOGRAPHY.

In trying to find the appropriate method of purification for the samples, since pI of the sample is not known, different strategies were deployed to scout for the suitable conditions for capturing the enzyme activity concentrated by UF. Cation exchanger at pH 5 was used for the elution, large peak was observed at the start of elution with acetate buffer. Figure 3.15 below showed the protein measurement at absorbance 280 nm. Other peaks were observed during step elution with 0.25M NaCl, 0.5M NaCl and 1M NaCl (fig 3.15).

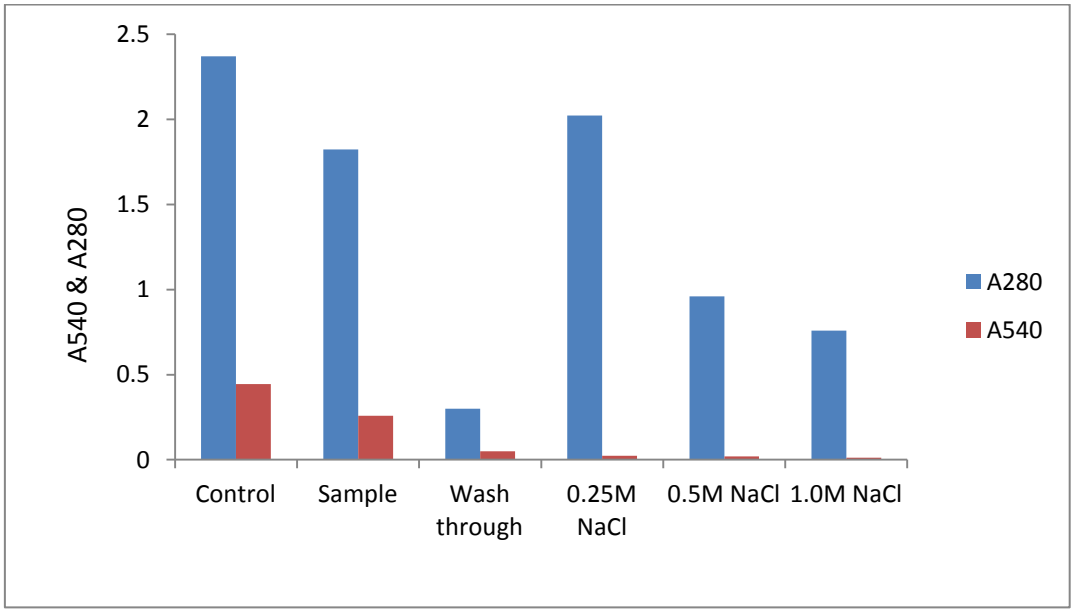


Figure 3.15, Concentrated culture supernatant applied to cation exchanger column (A- Capto S 1ml) using different concentration of NaCl to elute sample (Retentate on ABB supplemented with cellobiose substrates). 5ml volume sample was applied through a syringe.

Figure 3.15 shows that high peak was observed during elusion with acetate buffer and discrete peaks were observed during the step elution with different concentration of NaCl. Very low

xylanase and endoglucanase activities were observed in elution with different concentration of NaCl, which suggests that there was no activity binding.

Using 30ml column S-sepharose ff, a large peak was observed at the start of elution with 50 mM sodium acetate buffer. Other peaks were observed during step wise elution with NaCl (fig 3.15b).

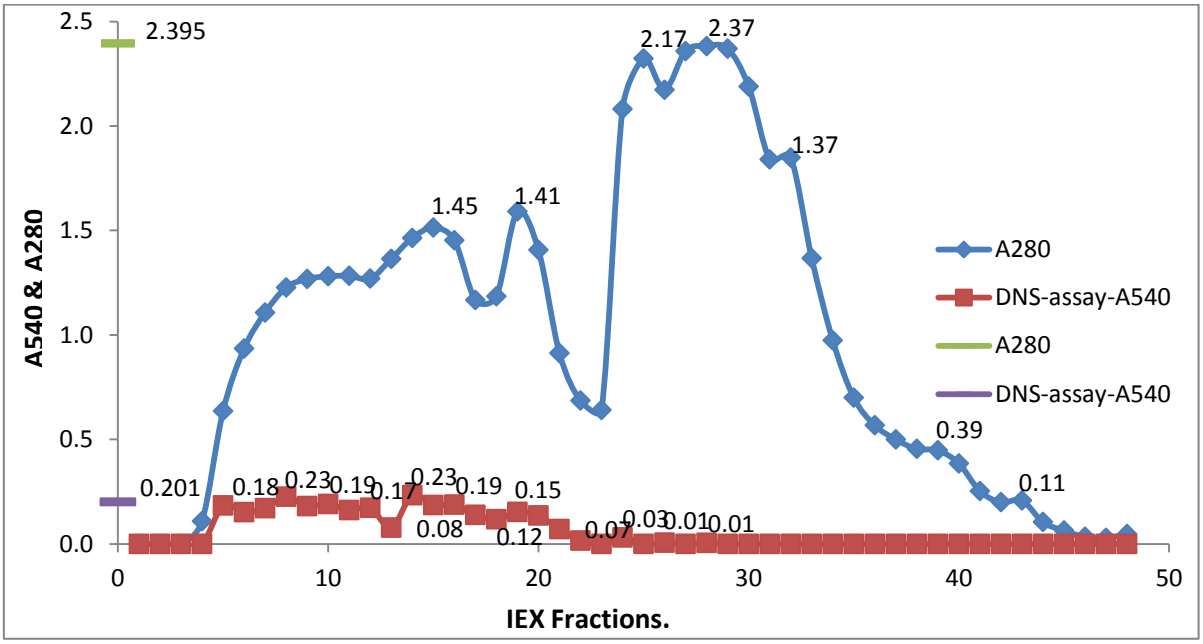


Figure 3.15b, chromatogram from S-Sepharose FF of concentrated supernatant from (ABB supplemented with xylan substrates), using different concentration of NaCl to elute sample. Collection fractions of 2 ml.

Significant protein was observed during the sample loading. Discrete peak was observed during the washing step at fraction number 18 to 20 and enzyme activity passed without binding during the loading step. Large protein peak was observed in response to charge and salt concentration, however no enzyme activity was desorbed indicating that the column did not bind the target proteins (fig 3.15b). Measures were taken either by dilution or dialysis to maintain low ionic strength (< 5mS/cm) of the sample for binding to occur.

Alternatively, an anion exchanger at pH 7, 8 and 9 were used for the elusion in an atempt to purify the protein. Addition of 0.1% Triton x100 to the buffer at pH 7.5 was also used in an attempt to dissociate the proteins from a complex into smaller entities.

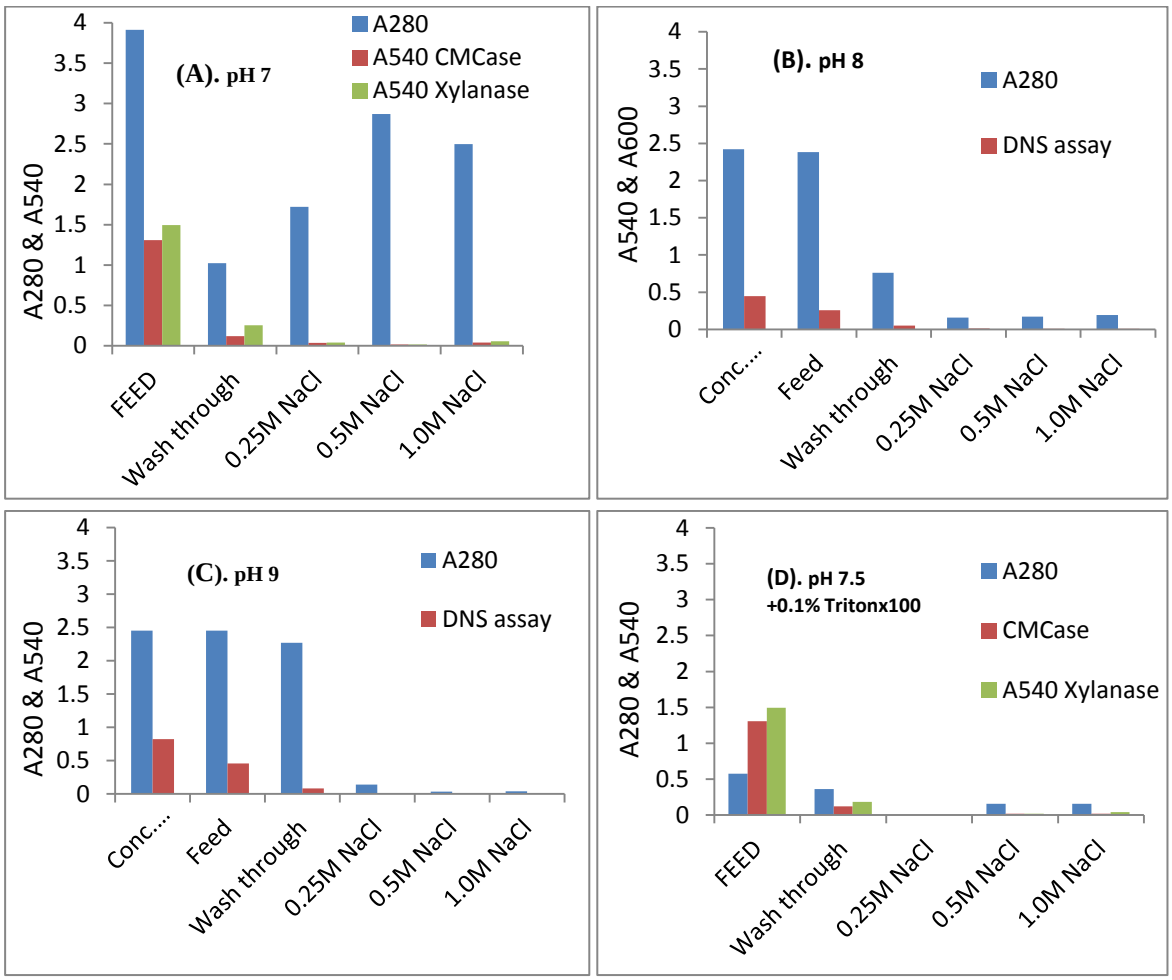


Figure 3.16 A, B, C and D, Concentrated culture supernatant applied to an anion exchanger column (Capto Q 1ml) using different loading pH and concentrations of NaCl to elute sample (Retentate on ABB supplemented with cellobiose substrates), A- pH 7. B- pH 8. C- pH 9. D- 20mM TrisHCl containing 0.1% Triton x100 at pH 7.5.

Running the anion exchange at pH 7, large peak was observed at the start of elution with 50Mm Tris-HCl. Other peaks were observed during step wise elution with NaCl. No significant enzyme activity was desorbed. Very low xylanase and endoglucanase activity were observed in elution with different concentration of NaCl. Figure 3.16a showed the protein measurement at absorbance 280 nm and the resulting enzyme activity. This suggests that there was no activity binding which may be due to steric hindrance.

Running the anion exchange at pH 8, large peak was observed at the start of elution with 50Mm Tris-HCl. Other peaks were observed during the step wise elution with NaCl but the resulting enzyme activity was very low (fig 3.16b).

Running the anion exchange at pH 9, large peak was observed at the start of elution with 50Mm Tris-HCl. Other peaks were observed during the step wise elution with NaCl but the resulting enzyme activity was also very low (fig 3.16c).

Addition of 0.1% Triton x100 to the buffer in an attempt to dissociate the proteins showed a different result as 0.1% Triton x100 gave a very different A280 pattern than untreated. Although traces of eluted activity in 0.5M and 1.0 M steps were observed, the enzyme activity was very low. large peak was observed at the start of elution with 50Mm Tris-HCl. Other peaks were observed during step wise elution with NaCl. Very low xylanase and endoglucanase activity were observed in elution with different concentration of NaCl but not significant in relation to the peak observed, which suggests that there was no activity binding. Figure 3.16d showed the protein measurement at absorbance 280 nm and resulting enzyme activity.

For all the pH tested, there was a very low xylanase and endoglucanase activity observed which suggests that, there was no activity binding which may be due to steric hindrance.

Elute from ion exchange chromatography were loaded on 12% gel (figure 3.17). Commassie staining of the gel showed only weak bands in the molecular weight range of approximately 75kDa for all elution fractions. Zymography was not attepmted for this gel analysis.

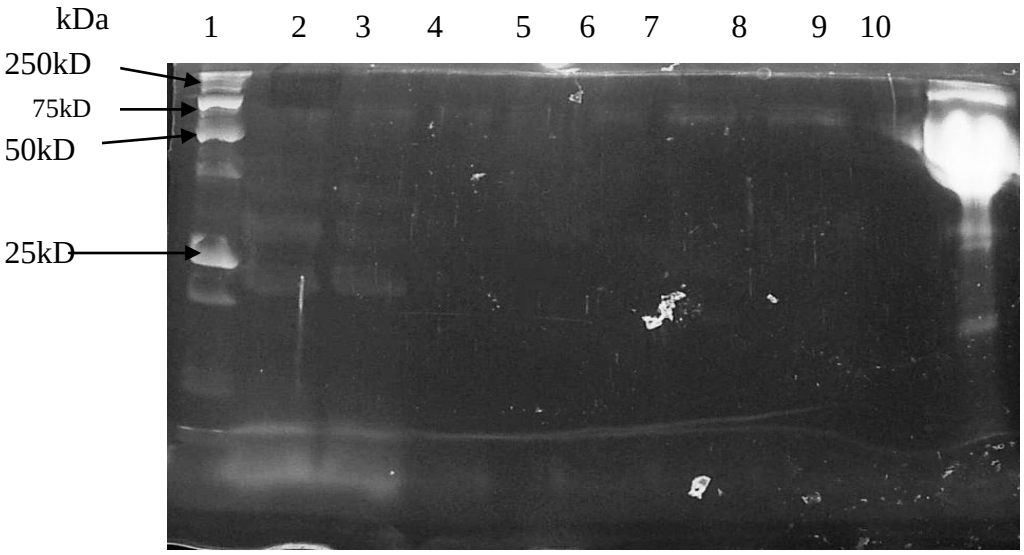


Figure 3.17. SDS PAGE (10%) analysis for fractions from cation exchange. Lane 1 ladder, lane 2 Concentrated supernatant sample, lane 3 concentrated feed sample, lane 4 wash through, lane 5 0.25M NaCl, lane 6 0.5M NaCl, lane 7, 1M NaCl, lane 8 1M NaCl, Lane 10, positive control (Cellulase from T. *reesei*). Image is shown as negative to enhance contrast.

Strong bands were observed in the supernatant and concentrated feed sample, showing different fractions with the highest band at approximately 200kDa, while non specific bands were observed in all the eluted fractions at approximately 75kDa.

4. Discussion

This study involved the production, identification and initial purification of a cellulolytic and hemicellulolytic enzyme in bacteria isolated from moose rumen.

4.1 Growth, Screening and Selection

Bacterial isolates (MRB 1-5) were provided from University of Bergen and TransHerba As and were inoculated on CMC agar plates. Isolates MRB 1 and 5 showed no visible colony after 24hrs which may suggest that CMC agar plates does not contain the minimum required nutrients for these isolates, isolates MRB 2, 3, and 4 showed visible bacterial colonies which were viewed under microscope to compare its shape to the original master bacterial isolates. The result showed that we have the same bacteria growing in the plates as observed through the microscope. The inability to sufficiently grow some of the isolates on CMC agar plates may be because the bacterial isolates were strict anaerobes but were grown aerobically. The support for this point could be seen when the bacterial isolates MRB 1-5 were cultivated in liquid medium, they appeared to grow and settle close to the bottom of the cultivation bottle where there likely is minimum oxygen available. All isolates showed visible bacteria colonies after 48hrs when grown in an anaerobic jar.

Isolates MRB 2, 3, 4 and 5 showed clear and strong CMC zones, whereas isolate MRB 1 did not show any clear CMC zone for unknown reasons even though it showed good indication of endoglucanase (CMC-ase) activity in DNS assay. Isolate MRB 1, 2, 3, 4 and 5 all showed positive results in DNS assay. This result could be said to be valuable because they show consistency with respect to zone clearing except for MRB 1.

Isolate MRB 3, 4, and 5 showed the best growth performance according to growth curve derived from the measurement of optical density at OD600. Bacterial Isolate MRB 3 was selected as the most promising isolate because of its growth rate, yield, observed clear zones on CMC screening and good indication of enzyme activity on DNS assay. MRB 4 had the highest growth yield, was easy to cultivate and also showed strong CMC zone. MRB 5 also showed high yield and it gave clear zone on CMC plates. MRB 1 did not give any clear zone on CMC agar plates, although it was positive on DNS assay. Calculating the specific enzyme activity (reducing sugar/optical density) indicated that MRB 2 appeared to have the highest specific production of enzyme. It also demonstrated clear zones on CMC plates, but gave a lower cell yield compared to MRB 3 and MRB 4. Its growth rate appeared to be slow with

low yield as observed on the growth curve which might be due to the aerobic condition under which it was grown. The validity of using RS/OD calculation for quantifying enzyme activities might be questionable since we are not dealing with purified enzymes or measured protein concentration. Such calculation can be valid if the enzymes are purified and the concentration is also known.

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

The identification of *B. licheniformis* AA1 took place through 16s rDNA sequencing. This identification was possible because many of the genomes of bacilli have been sequenced due to their commercial importance. The genome sequences of *B. subtilis* 168, *B. cereus* ATCC 14579, *B. anthracis* A202, *B. thuringiensis* subsp. *israelensis (Schallmey et al., 2004)* and *B. licheniformis* ATCC 14580/DSM 13 are completed and available (Rey et al., 2004; Veith et al., 2004). *B. licheniformis* AA1 falls within Group II of bacilli which are facultative anaerobes but may have limited growth under anaerobic conditions (Priest, 1993).
The bacterial isolate MRB 3 was identified as *B licheniformis and* named *B. licheniformis* AA1 for this study. The genome sequence for the following strain of *B. licheniformis* has been completed, namely DSM 13 /ATCC 14580 (Rey et al., 2004; Veith et al., 2004). A whole range of cellulolytic and hemicellulolytic enzymes have been identified and isolated in various strains of *B. licheniformis*. Further genetic analysis was not attempted in this study.

B. licheniformis AA1 was tested on three different growth media and appeared to require a very simple medium for growth and production of endoglucanase and xylanase activity. The organism was identified as Gram positive and rod-shaped. Cultivating *B. licheniformis* AA1 in aerobic and anaerobic condition shows that the organism is a facultative anaerobe, but growth under anaerobic conditions showed reduced growth as observed during the fermentation in the 2.5l anaerobic fermentor. For this study, it was decided to continue culturing under aerobic conditions for this reason as it has also been reported earlier in literature by Priest (1993) that *B. licheniformis* was only able to grow weakly under anaerobic conditions and that it belongs to Group II bacilli which are able to grow in the absence of oxygen, particularly if nitrate is present as electron acceptor (Priest, 1993).

4.3 **Localization of enzyme activities**

Cellulolytic microorganisms that produce enzymes generally do so in two ways, either secreted as free extracellular enzymes or in the form of a cell wall bound MEC. The extracellular cellulase system of *Trichoderma reesei*, which produces free enzymes, has been well studied (Lynd et al., 2002a). Examples of microorganism having complexed, cell wall bound enzymes are *Clostridium cellulolytium* and *Ruminococcus albus*. Yet another system is represented by strains of *Bacillus licheniformis* secreting apparently free enzymes but organised into large multi-enzymes complexes (van Dyk et al., 2010a). In order to examine the enzyme activities for *B. licheniformis* AA1, one need to determine if the bacteria secretes enzyme into the extracellular medium or not. Is the enzyme secreted into the medium?
Harvesting of bacteria by centrifugation provided different samples such as (supernatant and pellet) from the bacteria. By centrifugation of the culture broth, it was showed that the enzyme is secreted into the medium. The supernatant contained the highest enzyme activity of the samples, followed by whole culture broth and then pelleted cells. Only minor amounts of enzyme activity was released after sonication of pellet and subsequent centrifugation. Analysis of D-glucose assay showed a very low activity in the supernatant which could suggest that β-glucanase splitting cellobiose into monomeric glucose is absent or low in *B. licheniformis AA1*. The significant CMC-ase activity of *B. licheniformis* AA1 found in the supernatant supports the hypothesis by (Schallmey et al., 2004) that *B. licheniformis* is able to grow fast and secrete high amounts of protein into the extracellular medium.

The SDS-PAGE analysis of the supernatant and pellet samples showed bands at varying intensity for the pelleted cell but a lack of bands for the supernatant. The proteins were analysed under non-denaturing system which could have been the reason why any high molecular protein complex in the supernatant did not enter the gel. Similar results were found in the works of Jiang et al (2004), in which the MEC appeared at the top of gel or could not enter the gel, possibly due to its high molecular mass. The lack of discrete bands in the supernatant may show that a MEC is secreted from *B. licheniformis* AA1 but it cannot yet be concluded. MEC can be demonstrated through size exclusion chromatography shown by Bayer et al (1998) and Schwarz (2001) while establishing the presence of a high molecular weight protein in *C. thermocellum*.

When proteins were denatured at 95^{o}C for 45 seconds before electrophoresis, different fragments of a presumptive MEC were seen on the gel. The gel showed many fractions of

band which maybe an indication that protein complex is present in *B. licheniformis* AA1. This was also reported in literature by Phitsuwan et al (2012) when they isolated MEC from *Tepidimicrobium xylanilyticum* BT14.

4.4 Substrate regulation of growth and enzyme production

Although Han et al., (2003) stated that *B. licheniformis* could be cultured on a medium without additional carbohydrates or sugars; they argued that carbon source can influence enzyme activities. It is important to understand how bacteria regulate expression of the various hydrolytic enzymes in order to produce optimal enzyme mixtures for the degradation of different plant materials. Due to the complexity of plant materials, the combination of enzymes and the ratios required in order to degrade it is not completely understood and thus remains a fundamental problem in biotechnology applications.

Following identification, *B. licheniformis* AA1 was cultivated on Anaerobic Basal Broth (ABB), Brain Heart Infusion (BHI) and Bacillus Basic Broth (BBB) supplemented with 0.5% cellobiose or 0.5% xylan as well as without supplement to compare growth curve, observe pH change and to observe how substrates can influence or induce enzyme activities.

The experiments, showed that cellobiose can induce endoglucanase activities as demonstrated also by Lynd et al., (2002), and beechwood xylan could also act as a mechanism to increase xylanase activities as also reported in literature by van Dyk et al., (2010). The growth curve of *B. licheniformis* AA1 demonstrated faster growth in a medium containing cellobiose or xylan than in a medium without. From the results, it could be concluded that growth rate and enzyme activity is influenced or induced by either the presence of cellobiose or xylan as the carbon source in the medium. Beechwood xylan can induce xylanase and endoglucanase as shown by increased activity in DNS-xylan assay. The same experiments showed that cellobiose and xylan serves as endoglucanase and xylanase inducer respectively. Cellobiose and xylan induce enzyme activities in both ABB and BHI medium, although it appears to be more pronounced in ABB than it is in BHI and BBB. Cultivating *B. licheniformis* AA1 on different medium helped to study the various responses of this organism in different medium. ABB supplemented with 0.5% cellobiose/xylan medium is comparable to the BBB medium in respect to yield, growth rate and length of lag period, although, BBB gives 3 hours shorter lag phase but higher than that of BHI. Growth yield and enzyme activities seem to be more pronounced in ABB supplemented with 0.5% xylan which support the hypothesis by van Dyk et al., (2010) that xylan induces xylanases.

Inducers are generally small molecules that are able to enter the cell and Sephorose (β-1,2-glucobiose) has been identified as an inducer of cellulases in *Trichoderma reesei* although it is not clear whether this compound was a natural inducer of cellulases (Lynd et al., 2002a). There are many inducers and some have been hypothesised to be cellobiose, δ-cellobiose-1,5-lactone and xylobiose (Lynd et al., 2002a). It could be argued that, by constitutively expressing low levels of enzymes, such enzymes are, in the presence of substrate, able to cleave the substrate to produce small sugars that again are able to act as an inducer to elevate the levels of expression of enzymes (Lynd et al., 2002a). Depending on the location of genes on a genome, an inducer may activate higher levels of expression of several enzymes at the same time if such enzymes are situated within an operon. Thus an inducer such as cellobiose could often result in upregulated expression of both cellulases and hemicellulases (Lynd et al., 2002a). It was observed that xylan in this culture medium was able to induce both endoglucanase and xylanase activity and same can be said of cellobiose containing medium. Han et al., (2003) also reported this hypothesis that cellobiose act as an inducer of cellulolytic and some hemicellulolytic enzymes. This was also found to be the case in this study as the presence of cellobiose and xylan in the culture medium induced cellulases and hemicellulases simultaneously. From this data, it can be argued or said that *B. licheniformis* AA1 was able to regulate enzyme expression based on the substrate it was cultured on. These kind of responses are called cross-specificity as defined by (Wong et al., 1988) which occurs where an enzyme has activity on two distinct substrates and (Wong et al., 1988) also reported that a cellulase from *Trichoderma viride* displayed activity on both carboxymethylcellulose and xylan.
The pH measurement during the cultivations showed that *B. licheniformis* AA1 grows in the pH ranging from as low as pH 5.5 and as high as pH 7.5 in both ABB and BHI medium.

4.5 Can the cellulolytic system of *B. licheniformis* AA1 bind to Avicel?

During this study, it was observed that the endoglucenase/CMC-ase activity was able to bind to insoluble Avicel; The enzyme proteins were isolated by adsorption on Avicel which was subsequently desorbed using different buffers. The experiment showed how each buffer was able to release the enzyme activity from the insoluble Avicel and putative enzyme bands were observed on SDS-PAGE. Sodium Bicarbonate (pH 7) had the highest release of enzyme activity, Tris-HCl (pH 7.5) the second highest and Sodium Acetate (pH 5) ranked third on released enzyme activity. 1M NaCl showed the least released enzyme activity of all solutions tested. Proteins were bound to Avicel with varying strength, possible with different mechanisms of interaction. The result showed that the cellulase form *B. licheniformis* AA1

could bind to Avicel, although the resulting enzyme activities were low which might suggest that the sample needs to be concentrated more before the enzyme activity can be really significant. But at this level of testing and with the traces of enzyme activity displayed, the result is significant both by DNS assay and visible bands obtained on SDS-PAGE.

The ability to bind to insoluble substrates is considered important feature of MEC due to the fact that degradation of insoluble substrates is inextricably linked to the enzyme/complex's ability to bind and thus remain in close proximity to the substrate while it is hydrolysed. Furthermore, binding to crystalline cellulose is a feature of the cellulosome as the scaffolding protein of the cellulosome contains a CBM3a domain which is able to bind crystalline substrates (Boraston et al., 2004). It is not clear yet if MEC with predominantly xylanase activities have CBM3a domain or not, even though many have been isolated with predominantly xylanase activity. These have been termed xylanosomes rather than cellulosomes. It is a problem, that no structural basis for the composition of xylanosomes has been identified (van Dyk et al., 2010a). Although van Dyk et al., (2010) reported that MEC could be present in *B. licheniformis,* and attempted to characterise it, it is yet to be established whether the MEC is truly a cellulosome or hemicellulosome. Furthermore, a useful purification strategy for this MEC is not completely understood and thus remains a fundamental problem. The study and purification of a MEC in this organism is considered novel for this study.

4.6 Concentration and fractionation on UF-membranes

Enzyme concentration was first done using a centrifugation UF-device (4ml) with cut-off of 50 kDa and later with cross-flow ultrafiltration method with a larger volume (100ml) using cut-off of 50 kDa and 10 kDa. The cross-flow method diminished the retentate enzyme activity concentration by 0.8 times for 50kDa, but increased the concentration by 1.3 times on the 10 kDa membrane. However, reducing the cut-off of the ultrafiltration membrane from 50kDa to 10kDa was not enough to retain the protein which might be due to technical reasons. If a high molecular weight MEC is present in *B. licheniformis* AA1, then both 50kDa and 10kDa membranes should retain the protein. This observation does not completely rule out the presence of MEC in this organism; it could be that the protein complex assembly dissociates when exposed to mechanical and hydrodynamic shear forces as found in the crossflow techniques. Even so, the enzyme activity was concentrated by about two-fold from culture supernatants by crossflow filtration with 95% recovery of total enzyme activity in both retentate and permeate fractions. However, significant amounts of activity passed through

both 50 and 10 kDa UF membranes, indicating the presence of low-molecular cellulases. Isolation of an intact MEC from a culture supernatant was not successful.

From the DNS assays obtained from UF-fractions and Avicel binding experiment, it appears that xylanase activities are more abundant in *B. licheniformis* AA1 compared to endoglucanase activities. This result support the findings reported by van Dyk et al., (2010) of another strain of this organism. Van Dyk et al (2010) reported that it is clear that *B licheniformis* SVD1 is an organism with predominant hemicellulolytic activity because only moderate levels of cellulolytic activity was observed in *B licheniformis* SVD1. Even when the organism was cultured on cellulose Avicel and cellobiose, which are known to induce cellulases, only limited cellulolytic activity was observed according to van Dyk et al., (2010). Hemicellulose is more varied in structure and composition than cellulose and includes xylan, mannan, galactan and arabinan polymers (Beg et al., 2001). Many microorganisms produce several xylanases that appear to act in synergy to degrade substrates (Beg et al., 2001; Wong et al., 1988). The presence of different xylanases within an organism suggests different functions that allow the enzymes to improve degradation of the substrate through synergy.

4.7 Is MEC present in *B. licheniformis* AA1?

The protein system from *B. licheniformis* AA1 was found to have many distinct bands, with the highest band seen at approximately 200kDa. Most of the samples purified from *B. licheniformis* AA1 showed several protein bands on SDS-PAGE. The high molecular band and fragments observed in this organism on SDS-PAGE further promote the hypothesis that MEC is present in *B. licheniformis* AA1. The largest band in SDS-PAGE patterns from cellulosomal studies was often found to represent the scaffoldin protein. The smallest scaffoldin found to date was 90 kDa in *Ruminococcus flavefaciens* (Rincon et al., 2003). Generally, the scaffoldin is a large protein and the main scaffoldin in *Clostridium thermocellum* has a molecular weigth of 196 kDa (Bayer et al., 1998a).

CMCases or endoglucanases activity in the cellulase system of *B. licheniformis* AA1 was found to have active bands at approximately 65kDa and 45kDa. CMCases or endoglucanases were present in the Retentate, Permeate, supernatant and samples from Avicel binding experiment as a very prominent band in zymography at approximately 65kDa with additional active bands at 45kDa. This result also correlates with what has been reported in the literature; van Dyk et al. (2010) reported that CMCases or endoglucanases activity in *B. licheniformis* was found to have active bands at 21 kDa and 45 kDa. Xylanases activities could not be determined on the zymography gel due to unknown reasons and images could not be taken or

shown even though xylanase activities has been reported by (van Dyk et al., 2010b) in *B. licheniformis* SVD1.

Although we cannot yet say that MEC is present in *B. licheniformis* AA1, but there are evidence which support this hypothesis. It can be said that MEC was isolated in this organism because zymograms were repeatedly carried out with different forms of isolated MEC and results showed consistency (all samples isolated from *B. licheniformis AA1* showed fractions of active CMC-ase bands on zymography), indicating a composition that is non-random. Thus it appears that the MEC is not simply a random aggregation of proteins but a functional complex.

Rey et al., (2004) reported on the completed genome sequence of *B. licheniformis* ATCC 14580 and indicated that this strain had two putative endoglucanases belonging to glycoside hydrolase families GH9 and GH5, a probable cellulose-1,4-β-cellobiosidase of family GH48 and two genes for β-glucosidases. They concluded, therefore, that this strain had all the required enzymes for utilisation of cellulose and its conversion into cellobiose and glucose. This group furthermore confirmed that they found this particular strain capable of growing on CMC as a sole carbon source (Rey et al., 2004). van Dyk et al., (2010) also reported three low active activities on CMC for *B. licheniformis* SVD1.

Veith et al., (2004) also reported that *B. licheniformis* DSM 13 also contains three endoglucanases, namely a cellulose 1,4-β-cellobiosidase and some glucosidases, two α-glucosidases and one β-glucosidase (Veith et al., 2004). Numerous other reports exist in literature for the isolation of endo-glucanases from various strains of *B. licheniformis.*

Liu et al., (2004) even reports on a *B. licheniformis* strain GXN 151 that was able to bind to Avicel efficiently just like it was observed in this study also but this directly contradicts the results reported by (van Dyk et al., 2010a) that *B. licheniformis* SVS1 does not bind to Avicel and thus it appears as though there are large differences between various strains of this organism. Such differences were also reported by van Dyk et al., (2010).

4.8 **Purification by adsorption chromatography**

Most studies from literature have focused mainly on characteristics of cellulosome in determining the techniques that could be used for the purification of MECs. This is because one of the distinct structural features of the cellulosome is used in its purification, the general model of a cellulosome is that it contains a non catalytic scaffoldin protein to which all the

catalytic subunits bind. It is this characteristic that has been used in the purification strategies by utilising the binding of the cellulosome to insoluble (microcrystalline) cellulose to isolate it from contaminating proteins (van Dyk et al., 2010a). However, little or none is known about the characteristic of xylanosomes which makes its purification novel. From the results of the present purification strategy, it was apparent that most of the protein in the MEC did not bind to either the cation or anion exchange resin at any of the pH values tested. The purification method in this study started with the membrane concentration of the culture supernatant, followed by an attempt to purify using ion exchange chromatography. The pI of the MEC in this case was not known, therefore various pHs were tested with different resins. It is not clear why the MEC was unable to bind to the anion or cation exchange resin. The general principle of ion exchange is that proteins would be negatively charged at a pH above its pI which would allow it to bind to the anion exchange resin. At a pH below the pI, proteins would be positively charged, which would allow it to bind to cation exchange resin.

Although pH 7, 7.5, 8 and 9 were tested, the MEC did not bind to the anion exchange resin. This could indicate that the MEC was positively charged at those pHs, and that the pI of the MEC was very high, at pH 9 or above. However, this would imply that the MEC would also be positively charged at pH 5, with the result that it should have displayed a strong interaction with the cation exchange resin, but this did not take place. The most likely explanation for the MEC behaviour during ion exchange chromatography is the protein size exclusion limits of the chromatography resin. The dynamic binding capacity (DBC) for various protein sizes on the Sepharose Fast Flow resins showed that proteins 13-70kDa bound at 50-120 mg/ml gel, while protein 670 kDa bound only at 3 mg/ml (Amersham Pharmacia BiotechAB). This supports the hypothesis that the assumed large size of the MEC and not the charge was the reason why the protein failed to bind. The observation is confirmed by similar observations of van Dyk et al., (2010), and which could also be seen as an indirect supportive evidence of a MEC in *B. licheniformis* AA1.

As the MEC is expected to be more than 2,000 kDa in size, it is possible that the MEC was not able to sufficiently interact with the resin as it could not enter the pores. Typically, the majority of protein adsorption takes place inside porous resins. As a result, capture on the ion exchange resin failed and the MEC was eluted with the loading flow through and the initial application of wash buffer. The larger the protein, the greater the chance of steric hindrance and accessibility to the charged ligands – positive or negative. Sepharose Fast Flow and Capto media both have a pore exclusion limit of 4x 10^6 Dalton which implies that a 2MDa complex would face problems entering the resin pores, only having access to superficial surface binding (Amersham Pharmacia BiotechAB). Addition of 0.1% Triton to

the sample buffer in an attempt to dissociate the proteins gave a very different A280 pattern than untreated sample. Although traces of eluted activity in 0.5M and 1.0M steps were observed, the enzyme activity was not significant. For this study, the target protein failed to bind on these otherwise standard high-yielding columns because of the large size of the presumed MEC.

4.9 SUGGESTION FOR FUTURE WORK

Since there is no prior information regarding this isolate, but there is a lot of information in the literature on *B. licheniformis*, future work could include the investigation of other enzymes that may be present within the cellulolytic system of this organism such as glucuronidase, galactosidase, arabinofuranosidase, pectinases etc. Cloning of genes for various enzymes could also be undertaken to identify and characterise various enzymes.

Further work could be trying to identify the components of the MEC in *B. licheniformis* AA1 and determine the structural basis for its formation, whether or not it depends on the presence of a scaffoldin protein.

Further work could also include use of the purification method suitable for the multi-enzyme complex present in *B. licheniformis* AA1, namely gel filtration (size exclusion). Another approach, employed with cellulosomes, is exploiting Avicel-chromatography as means of isolation and purification.

Work should also involve a proper investigation into all the remaining four bacterial isolates and a possible co-culture of these isolates.

5. Conclusion

In conclusion, a strain of *B. licheniformis* was isolated and identified from the rumen of moose (*Alces alces*) and was named *B. licheniformis* AA1. Examination of the pure cultures showed that *B. licheniformis* was found to be Gram positive, rod-shaped cells. The identification of the organism was performed through PCR and sequencing of the 16S rDNA region. It was also discovered that *B. licheniformis* AA1 was able to grow under both anaerobic and aerobic conditions although growth under aerobic conditions was superior. *B. licheniformis* is found to be very important industrially and has been reported to contain many cellulolytic and hemicellulolytic enzymes which were reported by van Dyk et al., (2010). van Dyk., (2010) has also reported the presence of a cellulolytic and hemicellulolytic MEC in *B. licheniformis* SVD1 which also appears to be the case in this study. From the high enzyme activity observed in the supernatant through DNS assay, this suggests that the enzyme is secreted into the medium which support the hypothesis by (Schallmey et al., 2004) that *B. licheniformis* are able to grow fast and secrete high amounts of protein into the extracellular medium. From the result obtained from the cultivation of *B. licheniformis* AA1 on different substrates to investigate its response in regards to enzyme activities when cultured on various substrates, it can be said that *B. licheniformis* AA1 was able to regulate enzyme expression based on the substrate it was cultured on. *B. licheniformis* AA1 expressed mainly hemicellulolytic enzymes, with xylanase activity being predominant, as observed in all cultures, but endoglucanase activity was also significantly expressed. The organism was able to upregulate both hemicellulolytic and endoglucanase activities under certain substrate conditions.

It could also be concluded from the Avicel binding experiment that the cellulolytic system of *B. licheniformis* AA1 was able to bind to Avicel similar to what was reported in literature, that another strain has also been reported to have a cellulolytic system that binds to Avicel by Schallmey et al., (2004) but contrary to report in literature regarding *B. licheniformis* SVD1 by van Dyk et al., (2010),

It was found that *B. licheniformis AA1* grows in a pH ranging from 5.5 to 7.5 in ABB medium and BHI medium. The pH dropped during the most active growth phase, before readjusting back to more neutral pH, this may be due to acidic metabolic intermediate and end products of fermentation.

From the results obtained from SDS-PAGE and zymography experiments, we can conclude that *B. licheniformis* AA1 contain multi-enzyme complex but further work will be required to

ascertain if it is cellulosome/xylanosome containing a scaffold or is a random enzyme aggregation. *B. licheniformis* AA1 displayed high xylanase activity and appeared to form a large MEC showing distinct bands on SDS-PAGE with the highest band at approximately 200kDa. Based on zymogram analysis there were two endoglucanases of approximately 65kDa and 45kDa but the image for xylanase activities could not be shown for this study due to technical reasons.

Purification of MEC from a culture of *B. licheniformis* AA1 was not successful. Various purification methods were used to isolate the MEC or part of it. Using an anion exchange and cation exchanger, the behaviour of the MEC in failing to bind to the anion exchange and cation exchanger is suspected to be steric hindrance due to size of the multi-enzyme complex present.

Size dependent purification methods should be attempted to purify the multi-enzyme complex from *B. licheniformis* AA1. In conclusion, it appears as though purification of MEC from *B. licheniformis* AA1 using anion exchanger or cation exchanger might not be obtainable.

Although we cannot yet say that MEC is present in *B. licheniformis* AA1, there are evidence in this present study supporting this hypothesis. It can be said that MEC was isolated in this organism because zymograms and SDS-PAGE were repeatedly carried out with different forms of purified MEC and results showed consistency, indicating a composition that is non-random. In addition, the inability to successfully isolate the MEC through the anion exchanger or cation exchanger which was presumed to be due to size, further promotes the presence of MEC in this organism. The previous identification of MEC in *B. licheniformis SVD1* by van Dyk et al., (2010b) also supports the hypothesis that MEC might be present in *B. licheniformis AA1*. The ability of the cellulolytic system of *B. licheniformis* AA1 to bind to crystalline cellulose (Avicel) indicates that it might possess a CBM3a domain similar to scaffoldin proteins (although individual enzymes may possess such a domain). Cellulosomes generally have this activity as a main feature (Sabathe et al., 2002). Thus it appears that the MEC is not simply a random aggregation of proteins but a functional complex.

6. References

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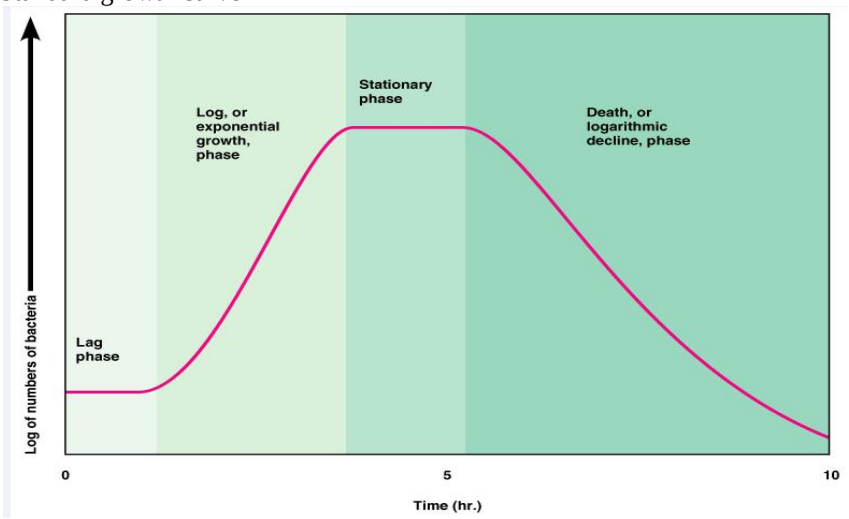
7. Appendix

Appendix I

Standard growth curve



Final OD of bacterial isolate MRB 1-5.

ISOLATES	FINAL OD/ ABB+	OD/ABB-	OD/BHI+	OD/BHI-
1	0.037	1.400	0.011	0.793
2	0.400	1.414	0.654	0.640
3	1.122	0.136	1.012	0.513
4	1.019	0.590	1.012	0.740
5	1.081	0.041	1.000	0.690

Optical density and pH of bacterial culture in ABB medium

TIME	ABB-	ABB+CELLO	ABB+ XYLAN
0	0.047 7.07	0.043. 6.85	0.009. 6.90
3	0.047 7.08	0.043. 6.85	0.009. 6.91
6	0.057 7.08	0.048 6.86	0.009. 6.96
9	0.061 7.09	0.060 6.88	0.010. 6.96
12	0.134 7.00	0.218 6.70	0.010. 6.95
15	0.842 6.32	0.851 6.11	0.574 6.31
18	1.073 6.10	1.077 5.92	0.741 6.15
21	1.121 6.29	1.243 5.65	0.821 6.16
24	1.235 6.44	1.384 5.53	1.053 6.16
27	1.305 6.33	1.440 5.49	1.126 6.30
30	1.453	1.591 5.52	1.126 6.14
33	1.479 6.03	1.661 5.55	1.313 6.04
36	1.502 6.08	1.591 5.66	1.341 6.09
39	1.502 6.13	1.759 5.66	1.341 6.09
42	1.536 6.15	1.875 5.58	1.424 6.11
45	1.536 6.15	1.875 5.58	1.424 6.11
48	1.621 5.98	1.950 6.55	1.567 5.96
51	1.653 5.88	1.969 6.70	1.581 6.00
54	1.715 5.91	1.969 6.88	1.635 5.93
57	1.715 5.93	1.969 6.94	1.635 5.90

APPENDIX II

- 16S rDNA SEQUENCE

GCTGGCTCCAAAAGGTTACCTCACCGACTTCGGGTGTTACAAACTCTCGTGCGTGTGACGGGCGGTGTGTACAAGGCCCGGG
AACGTATTCACCGCGGCATGCTGATCCGCGATTACTAGCGATTCCAGCTTCACGCAGTCGAGTTGCAGACTGCGATCCGAACT
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CAGGGTATCTAAATCCTGTTCGCTCCCCACGCTTTCGCGGCCTCAGCGTCAGTTACAGACCAGAGAGTCGCCTTTCGCCACTG
GTGTTCCTCCACATCTCTACGCATTTCACCGGC.

Returned hits from Blastn; Bacillus licheniformis 16S ribosomal RNA gene,

Sequence ID: gb|JX897676.1|Length: 1427Number of Matches: 1

Score	Expect	Identities	Gaps	Strand
1400 bits(758)	0.0	773/779(99%)	6/779(0%)	Plus/Minus

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Sbjct  1420  GCTGGCTCCAAAAGGTTACCTCACCGACTTCGGGTGTTACAAACTCTCGTG-GTGTGACG  1362

Query  61    GGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCGGCATGCTGATCCGCGATTACTAG  120
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Sbjct  1361  GGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCGGCATGCTGATCCGCGATTACTAG  1302

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Sbjct  1241  GGATTGGCTTAGCCTCGCGGCTTCGCTGCCCTTTGTTCTGCCCATTGTAGCACGTGTGTA  1182

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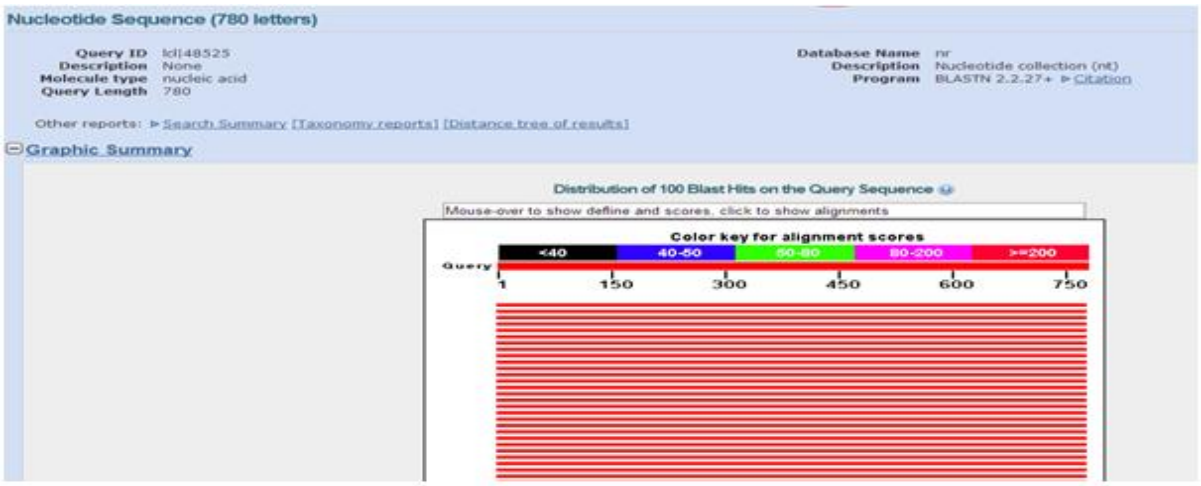


Description	Max score	Total score	Query cover	E value	Max ident	Accession
Bacillus licheniformis 16S ribosomal RNA gene, partial sequence	1400	1400	99%	0.0	99%	JX897676.1
Bacillus aerius strain VITM4I 16S ribosomal RNA gene, partial sequence	1400	1400	99%	0.0	99%	JX981918.1
Bacillus licheniformis strain LZBL-1-1 16S ribosomal RNA gene, partial sequence	1400	1400	99%	0.0	99%	JX997918.1
Bacillus licheniformis strain LZBL-6-2 16S ribosomal RNA gene, partial sequence	1400	1400	99%	0.0	99%	JX997917.1
Bacillus licheniformis partial 16S rRNA gene, strain IB-P141-2	1400	1400	99%	0.0	99%	HE995760.1
Bacillus licheniformis partial 16S rRNA gene, strain IB-P141-1	1400	1400	99%	0.0	99%	HE995759.1
Bacillus licheniformis partial 16S rRNA gene, strain IB-A4-1	1400	1400	99%	0.0	99%	HE995758.1
Bacillus licheniformis strain SM10 16S ribosomal RNA gene, partial sequence	1400	1400	99%	0.0	99%	JX897926.1
Bacillus licheniformis partial 16S rRNA gene, strain VPS50.2	1400	1400	99%	0.0	99%	HE993550.1

Description	Max score	Total score	Query cover	E value	Max ident	Accession
Bacillus licheniformis 16S ribosomal RNA gene, partial sequence	592	592	100%	7e-166	100%	JX897676.1
Bacillus aerius strain VITM4I 16S ribosomal RNA gene, partial sequence	592	592	100%	7e-166	100%	JX981918.1
Bacillus sonorensis strain VITM3I 16S ribosomal RNA gene, partial sequence	592	592	100%	7e-166	100%	JX981917.1
Bacillus sp. VITG3I 16S ribosomal RNA gene, partial sequence	592	592	100%	7e-166	100%	JX981915.1
Bacillus licheniformis strain CPH6 16S ribosomal RNA gene, partial sequence	592	592	100%	7e-166	100%	JX912559.1
Bacillus licheniformis strain CPF6 16S ribosomal RNA gene, partial sequence	592	592	100%	7e-166	100%	JX912558.1
Bacillus licheniformis strain ONF2 16S ribosomal RNA gene, partial sequence	592	592	100%	7e-166	100%	JX912557.1
Bacillus licheniformis strain LZBL-1-1 16S ribosomal RNA gene, partial sequence	592	592	100%	7e-166	100%	JX997918.1
Bacillus licheniformis strain LZBL-6-2 16S ribosomal RNA gene, partial sequence	592	592	100%	7e-166	100%	JX997917.1
Bacillus sp. N11-8 16S ribosomal RNA gene, partial sequence	592	592	100%	7e-166	100%	JX974351.1
Bacillus licheniformis strain FZWP-1 16S ribosomal RNA gene, partial sequence	592	592	100%	7e-166	100%	JX914490.1

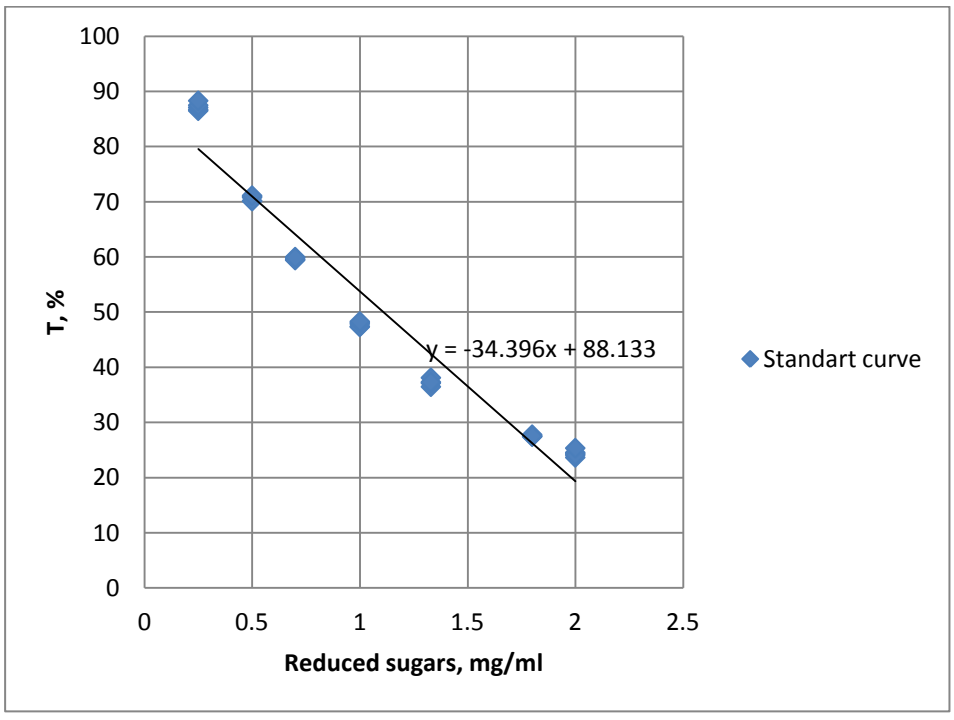
Appendix III

Results for D-glucose assay viewed on spectrophotometer.

SAMPLES.	A1	A2	dA	dAd-gl	Ccuvet, g/l	Ccorrected glucosae, g/l	F= 1	
RBG	0.103	0.115	0.012				K=	0.98419
BLANK	0.103	0.703	0.6	0.588	0.508032			
Positive cntrl	0.118	1.21	1.092	1.08	0.93312	0.918367		
whole cells	0.131	0.138	0.007	-0.005	-0.00432	0		
supernatant centrifuged	0.121	0.134	0.013	0.001	0.000864	0.00085		
pellet (centrifuged)	0.129	0.138	0.009	-0.003	-0.00259	0		
supernatent (sonicated).	0.124	0.135	0.011	-0.001	-0.00086	0		
pellet (sonicated)	0.141	0.15	0.009	-0.003	-0.00259	0		

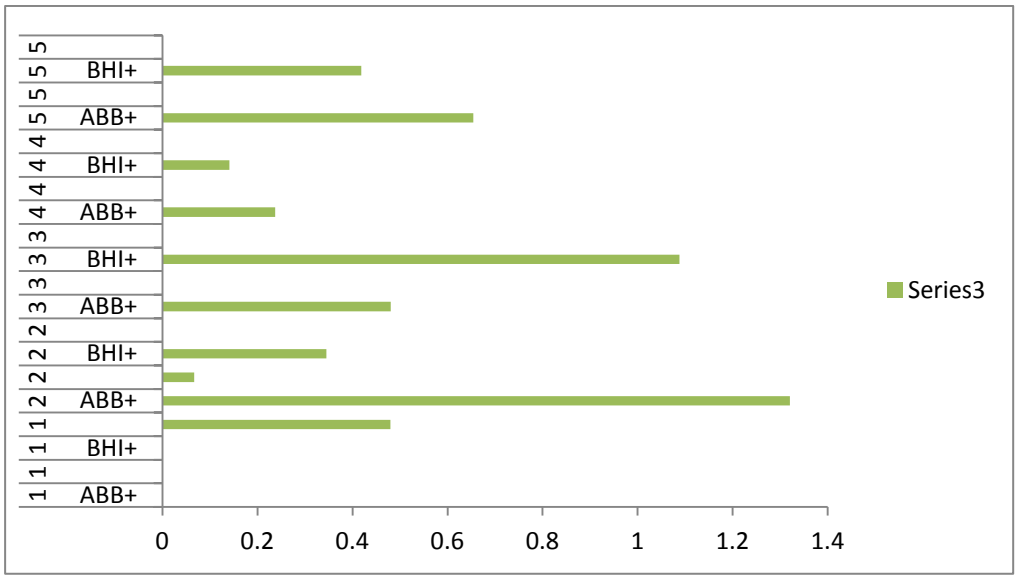
Appendix IV

Standard curve for reducing sugars



Appendix V

DNS assay for bacterial isolate MRB 1-5



Appendix VI

Oxoid Anaerobic Jar