ISOLATION OF A *CLOSTRIDIUM BEIJERINCKII* sLM01 CELLULOSOME AND THE EFFECT OF SULPHIDE ON ANAEROBIC DIGESTION

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ABSTRACT

Cellulose is the most abundant and the most resistant and stable natural organic compound on earth. Enzyme hydrolysis is difficult because of its insolubility and heterogeneity. Some (anaerobic) microorganisms have overcome this by having a multi-enzyme system called the cellulosome. The aims of the study were to isolate a mesophilic *Clostridium* sp. from a biosulphidogenic bioreactor, to purify the cellulosome from this culture, to determine the cellulase and endoglucanase activities using Avicel and carboxymethylcellulose (CMC) as substrates and the dinitrosalicyclic (DNS) method. The organism was identified using 16S rDNA sequence analysis. The sequence obtained indicated that a strain of *Clostridium beijerinckii* was isolated. The cellulosome was purified from the putative *C. beijerinckii* sLM01 host culture using affinity chromatography purification and affinity digestion purification procedures. The cellulosomal and non-cellulosomal fractions of *C. beijerinckii* sLM01 were separated successfully, but the majority of the endoglucanase activity was lost during the Sepharose 4B chromatography step. These cellulosomal and non-cellulosomal fractions were characterised with regards to their pH and temperature optima and effector sensitivity. Increased additions of sulphide activated the cellulase activity of the cellulosomal and non-cellulosomal fractions up to 700 %, while increased additions of sulphate either increased the activity slightly or inhibited it dramatically, depending on the cellulosomal and non-cellulosomal fractions. Increased additions of cellobiose, glucose and acetate inhibited the cellulase and endoglucanase activities. pH optima of 5.0 and 7.5 were observed for cellulases and 5.0 for endoglucanases of the cellulosomal fraction. The non-cellulosomal fraction exhibited a pH optimum of 7.5 for both cellulase and endoglucanase activities. Both fractions and enzymes exhibited a temperature optimum of 30 ˚C. The fundamental knowledge gained from the characterisation was applied to anaerobic digestion, where the effect of sulphide on the rate-limiting step was determined. Sulphide activated cellulase and endoglucanase activities and increased the % chemical oxygen demand (COD) removal rate. Levels of volatile fatty acids (VFAs) were higher in the bioreactor containing sulphide, substrate and *C. beijerinckii*. Sulphide therefore accelerated the rate-limiting step of anaerobic digestion.

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LIST OF ABBREVIATIONS

16S rDNA	16S ribosomal deoxyribonucleic acid
3-D	Three-dimensional
BLAST	The Basic Local Alignment Search Tool
cLM01-03	Colony 1-3, Lungisa Mayende
CBDs	Cellulose binding domains
CMC	Carboxymethylcellulose
COD	Chemical Oxygen Demand
Coh	Cohesin domains
CPD	Critical point drying
Da	Daltons
DNS	Dinitrosalicyclic acid
DTT	Dithiothreitol
MALDI-TOF	Matrix-assisted laser desorption/ionization–time of flight
MDa	Million Daltons
NCBI	National Center for Biotechnology Information
PCR	Polymerase Chain Reaction
S4B	Sepharose 4B
SEM	Scanning electron micrograph
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
sLM01	Strain 1, Lungisa Mayende
TEM	Transmission electron microscopy
VFAs	Volatile fatty acids

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CHAPTER 1

GENERAL INTRODUCTION

1.1 The structure of cellulose

Biomass encompasses a range of polysaccharides, including cellulose and hemicellulose, which serve as structural or storage compounds. Cellulose is the most abundant polysaccharide on earth and it is the major component of plant matter (Bayer *et al.*, 1998a). Cellulose is easily available and is therefore a rich and renewable resource. The total production of biomass is estimated to be 60 Gt per year of carbon in terrestrial and 53 Gt per year in marine ecosystems, where 1 Gt is 10^{12} kg (Schwarz, 2001).

The structure of cellulose is such that it is made up of linear chains of several thousands of glucose residues. These residues are linked by β-1,4 glycosidic bonds and are stabilised by internal hydrogen bonds (Uhlig and Linsmaier-Bednar, 1998; Liu *et al.*, 2006). Unlike other glucan polymers such as starch, the repeating units in cellulose are not glucose but rather cellobiose units (Desvaux, 2005) (figure 1.1 (a)). The chains are arranged in bundles that constitute 40-60 chains, which are aligned in parallel and are linked via hydrogen bonds. These crystalline aggregates are called microfibrils (figure 1.1 (b)) and are subunits of macrofibrils (Uhlig and Linsmaier-Bednar, 1998). Cellulose fibres contain different types of irregularities such as twists or voids, this facilitates in increasing their total surface area. Furthermore, native cellulose is paracrystalline in that the microfibril alternates the amorphous and crystalline regions (Desvaux, 2005).

The structure of cellulose has been investigated at either global or ultra-structural levels. At the global level the information has been largely obtained from spectroscopy and crystallography methods. At the ultra-structural level, information of the cellulose structure has been obtained due to advances in transmission electron microscopy (TEM). Using this method, high-resolution lattice images have been produced, where individual cellulose chains can be directly visualised (Bayer *et al.*, 1998a). The data has shown that

most of the amorphous cellulose corresponds to chains that are located at the microfibril surface and the crystalline components are located in the core (Bayer *et al.*, 1998a).

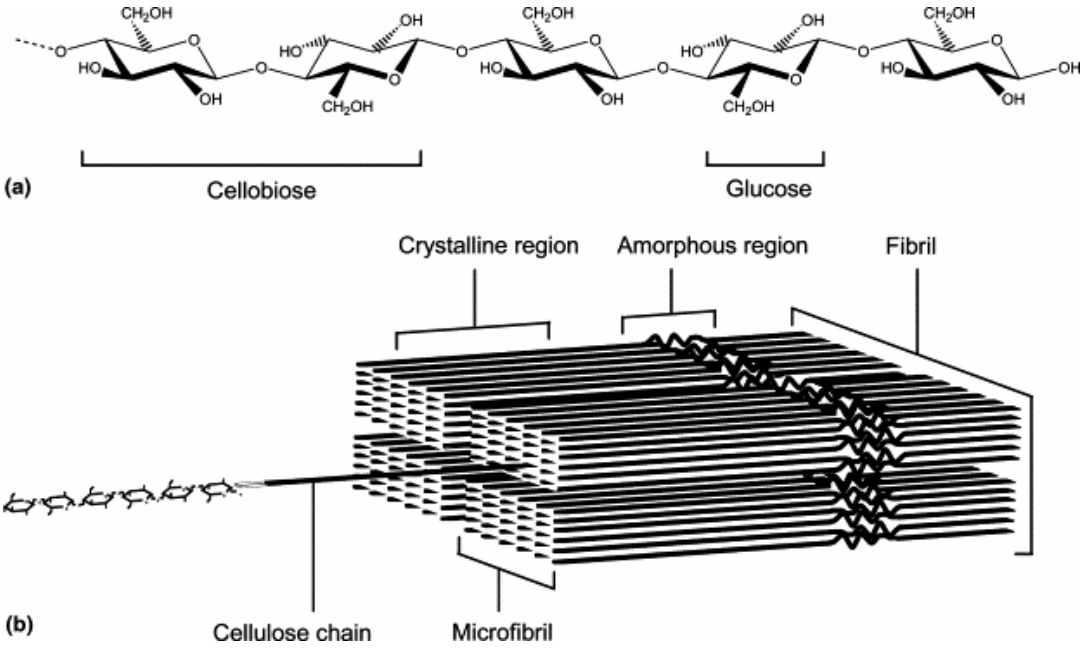

Figure 1.1 (a) The primary structure of cellulose. (b) A scheme of the structure of the cellulose fibril (Desvaux, 2005).

1.2 The cellulosome

Crystalline cellulose is chemically homogenous, although structurally complex, and no single enzyme is capable of hydrolysing it. The action of three enzymes is required for the complete hydrolysis of cellulose; the endoglucanases, the exoglucanases, and the β-glucosidases. Endoglucanases randomly cleave the intermonomer bonds found in cellulose, exoglucanases remove mono- and dimers from the end of the glucose chains, and β-glucosidase hydrolyses the glucose dimers (Malherbe and Cloete, 2002).

Aerobic microorganisms produce single enzyme components that are attached to binding modules. The enzymes are produced in high concentrations and act collectively (Schwartz, 2001). In aerobic microorganisms the cellulases that are produced arise as separate units (Boisset *et al.*, 1999). In contrast, some anaerobic microorganisms have developed a more energy efficient manner of producing cellulases and therefore the degradation of cellulose. The anaerobic microorganisms, *Clostridium*, *Acetivibrio*, *Bacteroides* and *Ruminococcus* have an elaborate extracellular multi-enzyme complex called the cellulosome (Doi *et al.*, 2003). Table 1.1 summarises the cellulosome-producing anaerobic bacteria.

Table 1.1 Cellulosome-producing anaerobic bacteria (Doi *et al.*, 2003)

Species	Optimal growth temp[a]	Source
Acetivibrio cellulolyticus	M	Sewage
Bacteroides cellulosolvens	M	Sewage
Clostridium acetobutylicum	M	Soil
Clostridium cellulovorans	M	Wood fermenter
Clostridium cellobioparum	M	Rumen
Clostridium cellulolyticum	M	Compost
Clostridium josui	M	Compost
Clostridium papyrosolvens	M	Paper mill
Clostridium thermocellum	T	Sewage soil
Ruminococcus albus	M	Rumen
Ruminococcus flavefaciens	M	Rumen
Ruminococcus succinogenes	M	Rumen

[a] M, mesophilic; T, thermophilic (above 50°C).

The cellulosome is a macromolecular machine that has components that interact in a synergistic fashion in order to catalyse the efficient degradation of cellulose (Bayer *et al.*, 1998b). They are possibly the largest protein/ enzyme complexes as they range from 650 000 Da to 2.5 MDa. Cellulosomes are cell protuberances that can degrade not only cellulose, but also hemicelluloses (which includes xylan and mannose) and pectin. Cellulase activity, however, is therefore not the only hydrolytic activity of the cellulosome as the nomenclature suggests. Cellulosomes are now thought to degrade hemicelluloses, chitin, xylan and pectin (Doi *et al.*, 2003). Cellulase activity is the bulk

activity of the cellulosome and it is made up of numerous kinds of cellulases and their related subunits. These are assembled to the nonenzymatic scaffoldin (Murashima *et al.*, 2002).

The cellulosome is made up of a scaffolding protein called either CbpA, CipA or CipC combined with a number of cellulosomal enzymes. The scaffolding proteins are large and nonenzymatic. They typically contain a number of cohesin domains (Coh) and cellulose binding domains (CBDs). The number of cohesins present on a cellulosomal scaffolding proteins varies, as figure 1.2 illustrates. The "scaffoldins" can also contain hydrophilic domains, dockerin II domains, the enzyme coding domain and a few other domains that haven't been identified (Doi *et al.*, 2003; Craig *et al.*, 2006).

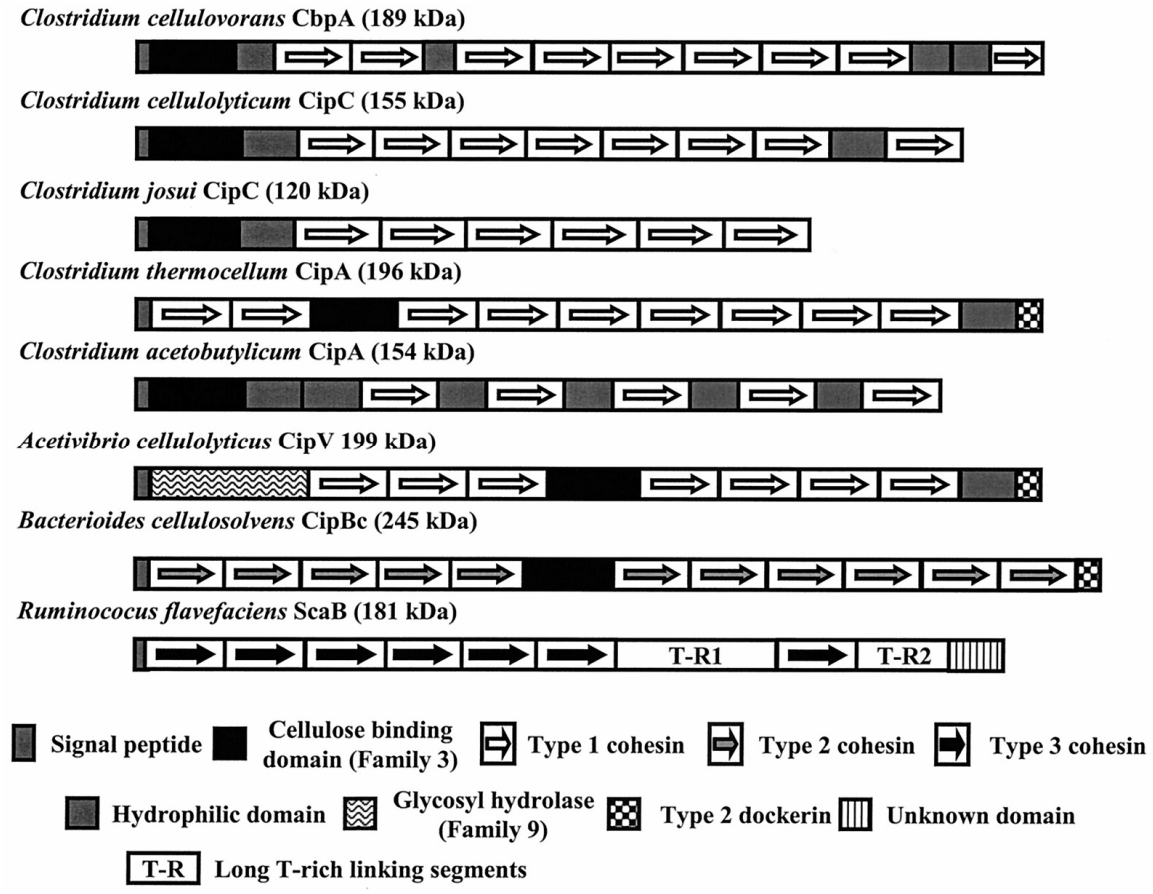

Figure 1.2 Structural properties of cellulosomal scaffolding proteins (scaffoldins) from various species (Doi *et al.*, 2003).

The cohesins are always present in scaffoldins and their function is to serve as binding sites for the dockerin domains of the cellulosomal enzymes as figure 1.3 illustrates. This interaction is key in the assembly of the cellulosome (Doi *et al.*, 2003). The CBD of scaffoldins facilitate in the strong binding of the cellulosome to cellulose (figure 1.3). It is evident that the CBD binds to the crystalline form of cellulose more readily than to amorphous cellulose (Doi *et al.*, 2003).

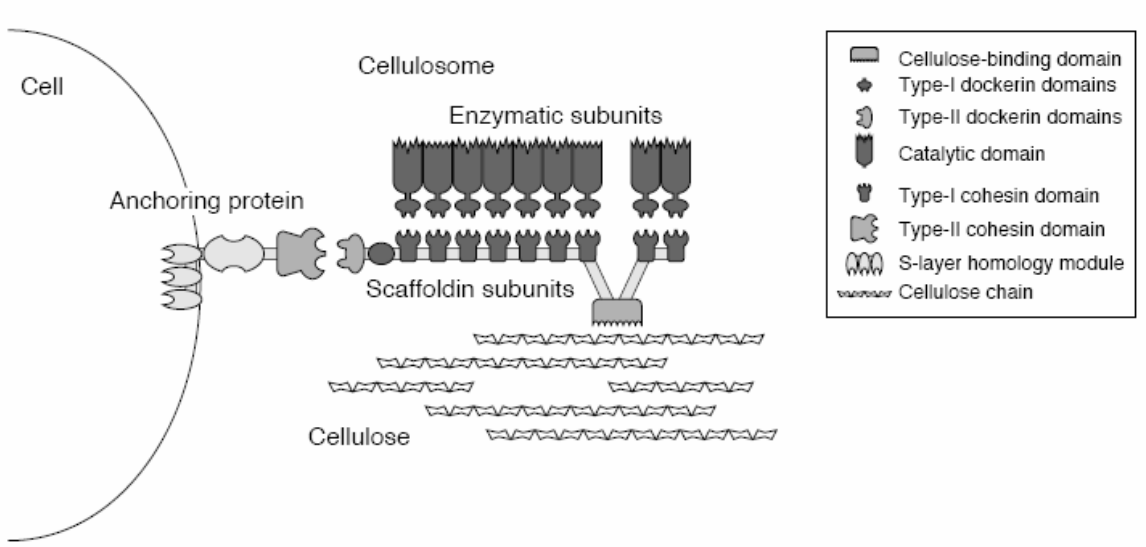

Figure 1.3 Simplified schematic view of the interaction between the *Clostridium thermocellum* cellulosome and its substrate, and its connection to the cell surface via an associated anchoring protein (Shoham *et al.*, 1999).

Initially the bulk of the cellulosomal research was conducted on *C. thermocellum* (Morag *et al.*, 1996; Boisset *et al.*, 1999; Adams *et al.*, 2004). Shoham *et al.* (1999) stated that the arrangement of cellulosomes on the cell surface of *C. thermocellum* was visualised using immonocytochemical labelling and electron microscopy. The cellulosome is arranged on the cell surface as polycellulosomal protuberance-like organelles, as illustrated in figure 1.4. When these organelles bind to the cellulosome, they undergo a dramatic comformational change to form elongated fibres between the cellulose and the cell surface (Shoham *et al.*, 1999).

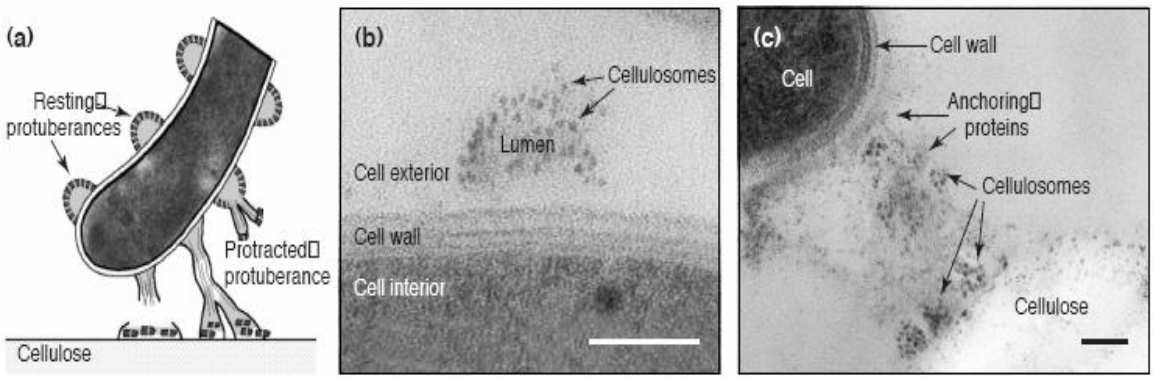

Figure 1.4 Ultrastructure of the *Clostridium thermocellum* cell surface. **(a)** Diagrammatic representation of a typical cell bound to cellulose. **(b)** Transmission electron micrograph of a resting polycellulosomal protuberance. **(c)** Transmission electron micrograph of a protracted polycellulosomal protuberance. The cellulosome is mainly associated with the cellulose surface and connected to the cell via extended fibrous material, believed to comprise the anchoring proteins. Scale bars = 100 nm (Shoham *et al.*, 1999).

The cellulosome may have several advantages for the effective hydrolysis of cellulose; this includes the optimisation of the synergism by the correct ratio between the components, which is determined by the composition of the complex. The prevention of non-productive adsorption is accomplished by the optimal spacing of components that work together in synergistic fashion is another advantage (Schwartz, 2001). The avoidance of competition in binding sites (due to a limited number of binding sites) is accomplished by binding the whole complex to a single site through a strong binding domain with low specificity. The avoidance to a halt in hydrolysis when one of the structural types of cellulose is depleted at the site of adsorption, by the presence of other enzymes that have different specificity, is another advantage (Schwartz, 2001).

1.3 The cellulases of the cellulosome

Cellulases hydrolyse the β-1,4 glucosidic bonds in cellulose. Cellulases are members of the glycosyl hydrolase family of enzymes; these enzymes hydrolyse oligosaccharides and polysaccharides (Bayer *et al.*, 1998b). Cellulases include endoglucanases or 1,4-β-D-

glucan 4-glucanohydrolases, which act randomly in the polymeric chain and produce new ends. Exoglucanases, include 1,4-β-D-glucan glucanohydrolases, which liberate D-glucose from β-glucan and cellodextrins and also include 1,4-β-D-glucan cellobiohydrolases, which liberate D-cellobiose from β-glucan in a processive way. The third set of cellulase enzymes are the β-glucosidases or β-D-glucoside glucohydrolases, which liberate D-glucose units from soluble cellodextrins and various glycosides (Schwartz, 2001). Figure 1.5 shows the schematic representation of the hydrolysis of amorphous and microcrystalline cellulose by noncomplexed 1.5 (A) and complexed 1.5 (B) cellulase systems.

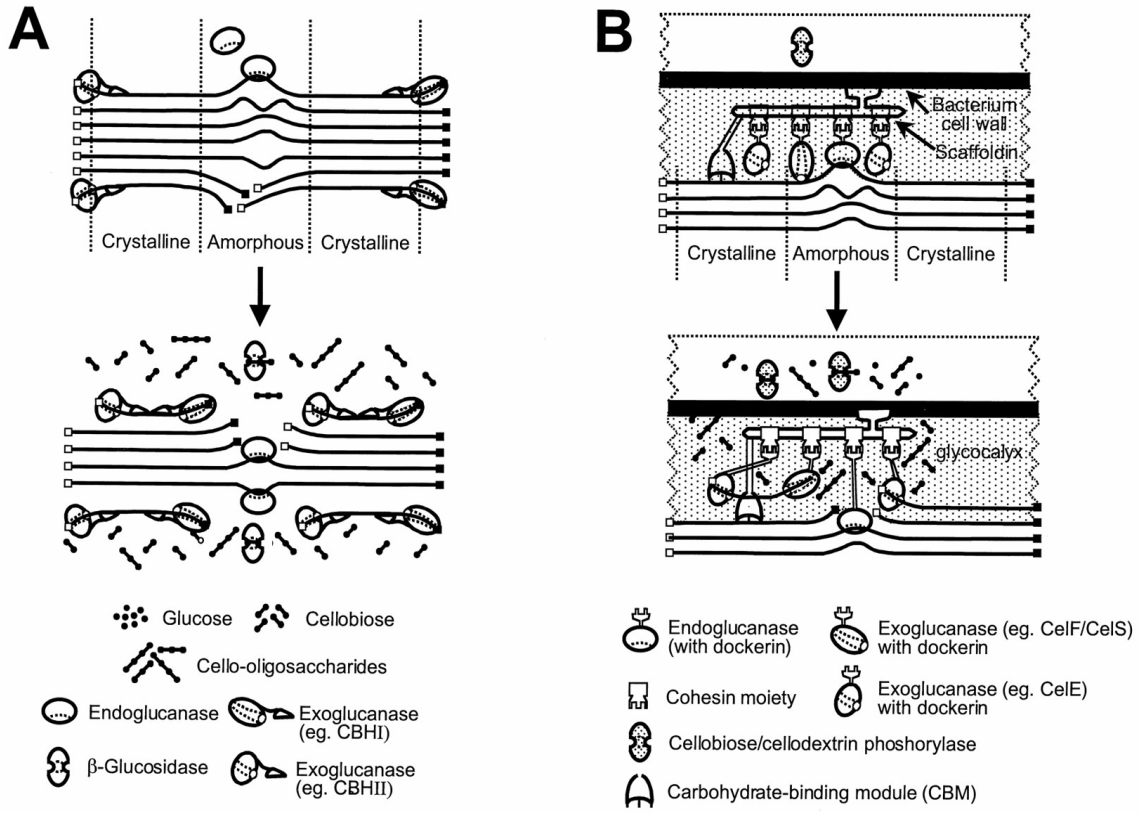

Figure 1.5 Schematic representation of the hydrolysis of amorphous and microcrystalline cellulose by noncomplexed (A) and complexed (B) cellulase systems. The solid squares represent reducing ends, and the open squares represent nonreducing ends. Amorphous and crystalline regions are indicated. Cellulose, enzymes, and hydrolytic products are not shown to scale (Lynd *et al.*, 2002).

Cellulases are modular where each module or domain is made up of a consecutive portion of the polypeptide chain and forms an independently folding, structurally and functionally distinct unit (Bayer *et al.*, 1998b). The three-dimensional structures of cellulases and related enzymes from 15 different families have been determined (figure 1.6).

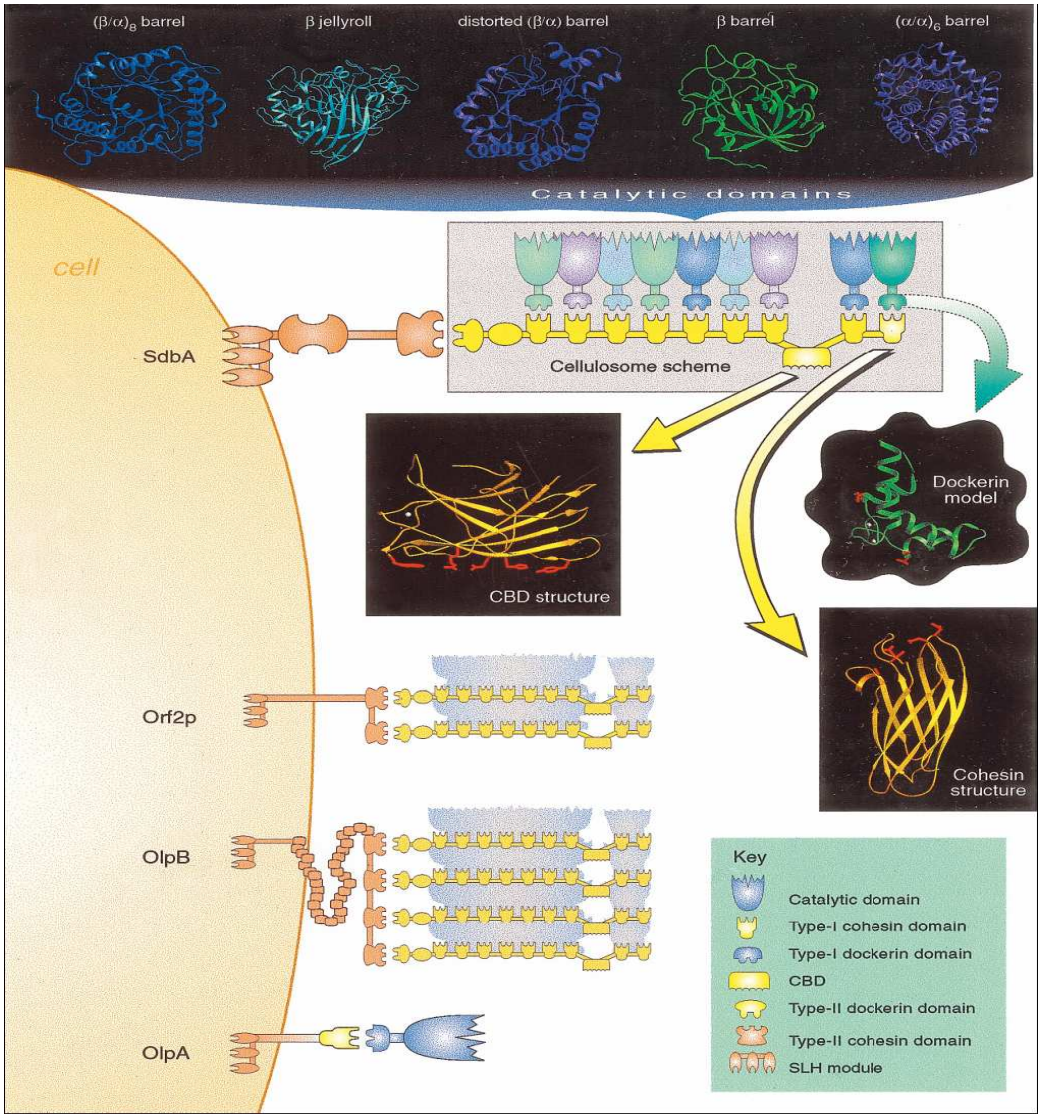

Figure 1.6 Schematic representation of cellulosome organisation and attachment to the *C. thermocellum* cell surface. The cellulosome and its associated anchoring proteins all comprise of modular components. The scaffoldin protein of *C. thermocellum*, shown in yellow, is composed primarily of nine copies oh cohesion module, a Family-IIIa CBD and a type-II dockerin domain.

It is generally accepted that the overall 3-D structure and stereo-specificity of hydrolysis are conserved within a family. The enzymes are complex and comprised of discrete modules; the catalytic domains are joined to non-catalytic modules, by linker regions (Schwarz, 2001). The cellulosomal enzyme subunits are not different from related free cellulases, as both contain common types of catalytic domains from the same collection of glycosyl hydrolase families. The major difference is that all cellulosomal enzymes contain a dockerin domain, which mediates its integration into the cellulosome. Instead of a dockerin domain, free enzymes usual contain at least one copy of a cellulose-binding domain (CBD) that targets a given catalytic domain to the substrate (Bayer *et al.*, 1998b).

1.4 Clostridium beijerinckii and its cellulosome

C. beijerinckii, previously known as *C. acetobutylicum* (Keis *et al.*, 1995), is a gram-positive anaerobe that forms spores and it has a rod shape (Sabathé and Soucaille, 2003). *C. beijerinckii* is able to break down polysaccharides into acids such as acetate and butyrate and also solvents including acetone, butanol and ethanol (Ezeji *et al.*, 2006). Previous studies by Sabathé *et al.* (2002) have shown that the cellulosome of C. *acetobutylicum* did not contain exoglucanase activity although it has the genes that code for the cellulosomal enzymes and the cellulosome (Doi *et al.*, 2003; Sabathé and Soucaille, 2003).

The cellulosomal cellulases that are produced by *C. beijerinckii* include one exoglucanase; CelF, seven endoglucanases; CelA, CelH, EngA, CelG, CelL, CelE and CAC3469; a mannanase, ManA and a sialidase, CAC0919 (Doi *et al.*, 2003). Table 1.2 summarises the cellulosomal subunits of a mesophilic *C. beijerinckii*.

Table 1.2 Cellulosomal subunits of *C. beijerinckii* (Doi *et al.*, 2003)

Cellulosomal enzyme	Function	Mol mass (kDa)	Modular structure[a]
CelF	Exoglucanase	81	GH48-DS1
CelA	Endoglucanase	54	GH5-DS1
CelH	Endoglucanase	80	GH9-CBM3-DS1
EngA	Endoglucanase	67	GH44-DS1
CelG	Endoglucanase	77	GH9-CBM3-DS1
CelL	Endoglucanase	60	GH9-DS1
ManA	Mannanase	47	GH5-DS1
CAC0919	Sialidase	91	GH74-DS1
CelE	Endoglucanase	96	CBM3-Ig-GH9-DS1
CAC3469	Endoglucanase	110	(SLH)3-GH5-X-DS1

[a] The modular structures of cellulosomal subunits are indicated by the following abbreviations: GH, glycosyl hydrolase; Ig, immunoglobulin-like module; DS1, dockerin domain type 1; X, unknown domain..

1.5 The effect of sulphide on hydrolases

Sulphide is a disulphide reducing agent and may act in reducing the disulphide bonds in enzymes, thereby stimulating or activating them. Studies performed by Sá-Pereira *et al.* (2002) found that the reducing agent dithiothreitol (DTT) enhanced xylanolytic activity of a *Bacillus subtilis* strain isolated from a hot-spring. Lamed *et al.* (1985) showed that the thiol containing compound cysteine activated cellulase and endoglucanase activities of the cellulosome of *C. thermocellum*. Sulphide may act in a similar way to these compounds, since it is both a reducing agent and thiol containing compound.

Watson and Pletschke (2006) found that sulphide activated the activities of α-glucosidases. Whiteley *et al.* (2002) found that sulphide activated β-glucosidase activities. Whiteley *et al.* (2003) determined that sulphide activated lipase activities. Futhermore, Watson *et al.* (2004) found that sulphide activated the activity of proteases. This showed that sulphide is able to activate various hydrolases including β-glucosidase, a cellulase enzyme.

1.6 Anaerobic digestion

Anaerobic digestion is a microbial process where the microorganisms degrade organic matter including cellulose. The final products of the degradation are methane (CH_4) and CO_2 (Desvaux, 2005). The process is not as uniquely a man-made process as for example industrial fermentation. The same reactions are carried out in nature, in soil, streams and the oceans. The objective of the biotechnologist or engineer is to confine the natural organisms in a man-made system and to optimise the rates and the extent of the natural reactions so that pollutants or organic matter is rapidly and completely degraded (Hobson and Wheatley, 1993).

The metabolic steps that are involved in anaerobic digestion have been identified and are illustrated in figure 1.7. As figure 1.7 illustrates, the first step is hydrolysis, this is carried out by fermentative bacteria including *Bacteriodes*, *Butyrivibrio*, *Clostridium*, and *Lactobacillus*. The products of this step include sugars, amino acids and peptides. This step is the focus of this study as the hydrolysis of complex polymeric substances is the rate-limiting step in anaerobic digestion (Mata-Alvarez *et al.,* 2000; Burrell *et al.,* 2004). These microorganisms hydrolyse organic compounds such as cellulose and hemicellulose into smaller compounds that are transported to the interior of cells and fermented into various products including ethanol, butyrate and acetate. They require CO_2 as an electron acceptor and an organic acid as a carbon source, ammonium as nitrogen source, cysteine or sulphide as sulphur sources and mineral salts (Novaes, 1986).

The second step is acidogenesis, where H_2, CO_2, acetate and other organic acids larger than acetate are formed. This step is also catalysed by the fermentative bacteria. The third step in anaerobic digestion is acetogenesis, where organic acids are produced and converted to H_2 and acetate. This step is catalysed by the acetogenic bacteria. H_2 and CO_2 are also converted to acetate by the homoacetogenic bacteria. In the fourth and fifth steps the methanogens reduce CO_2 to methane and the acetoclastic methanogenic bacteria convert acetate to methane (Novaes, 1986).

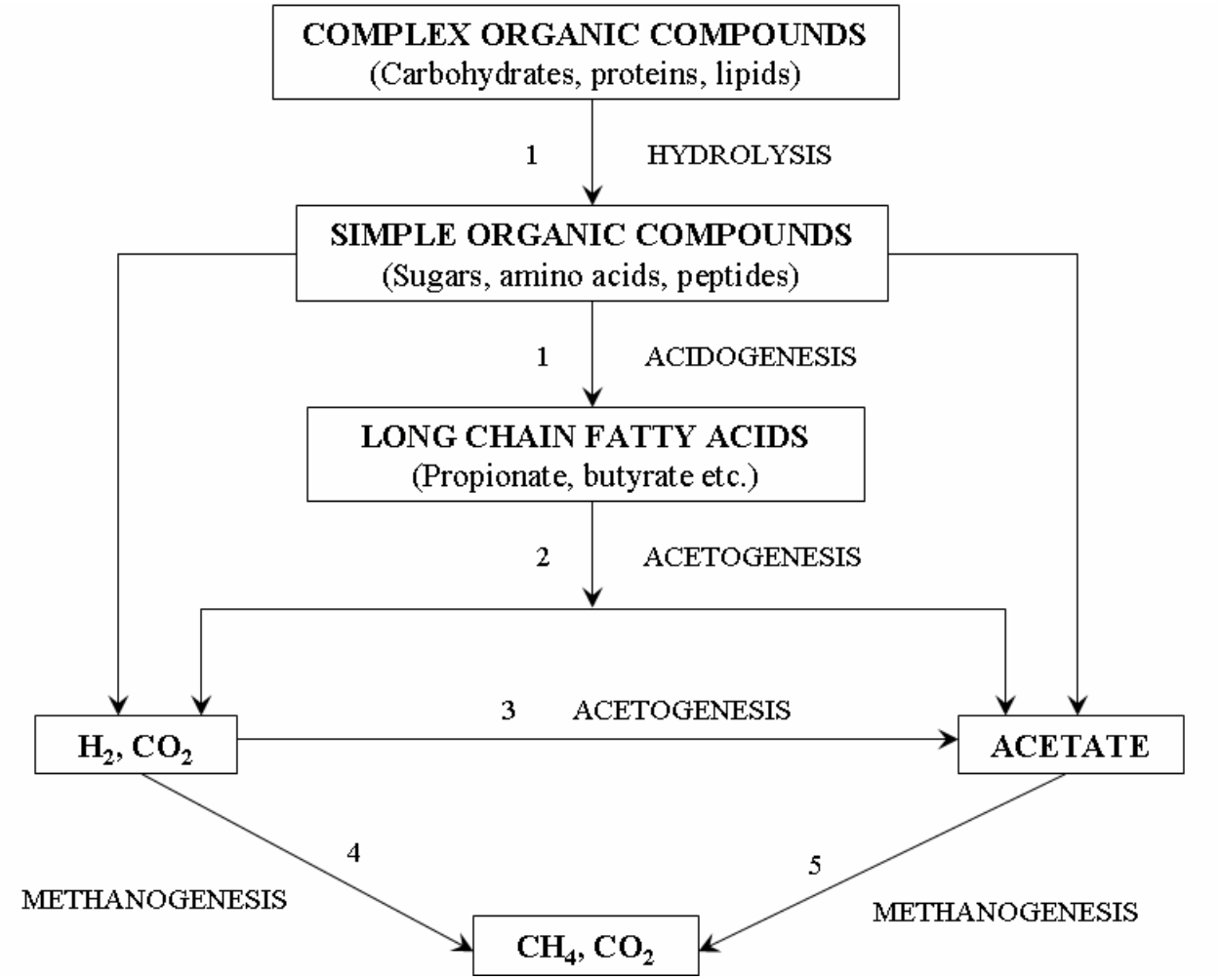

Figure 1.7 Metabolic steps and microbial groups involved in anaerobic digestion: 1) Fermentative bacteria; 2) H_2-producing acetogenic bacteria; 3) H_2-consuming acetogenic or homoacetogenic bacteria; 4) CO_2-reducing methanogenic bacteria; 5) Acetoclastic methanogenic bacteria (Novaes, 1986).

1.7 Biofuels and other applications of cellulosomes

Research into the cellulosome, particularly research on the activation of the cellulosome and therefore the development of a more efficient anaerobic digestion system is important. This is due to the applications of cellulosomal research to anaerobic digestion. The research can be applied to the biofuel sector, solid waste management sector, pulp- and paper- and the food industry, among others. Of particular interest is the production of biofuels. Since a revival in interest into renewable fuels has re-emerged due to the possible depletion of fossil fuels and the environmental impact of fossil fuels, research in the area is being re-emphasised (Chynoweth *et al.*, 2001).

A great challenge is to meet the growing demand for energy for transportation, heating and industrial processes, and to supply raw material for industries in a sustainable way. Security of the oil supply has increased concerns due to increasing oil prices, which in 2006, approached US$80 per barrel. Moreover, the future energy supply must also include a simultaneous substantial reduction of green house gas emissions (Hahn-Hägerdal *et al.*, 2006). Many governments are endorsing biofuels as the fuel of the future, for example in the USA, the *Energy Policy Act* of 2005 requires blending of 7.5 billion gallons of alternative fuels by 2012 (Hahn-Hägerdal *et al.*, 2006).

Ethanol has been introduced on a large scale in Brazil, the USA and some European countries and researchers anticipate it to be one of the leading renewable biofuels in the transport sector within the next 20 years. Ethanol can be mixed with petrol or it can be used alone in suitably designed engines, taking advantage of the higher octane number and higher heat of vaporisation. Ethanol is also a good option for future advanced flexi-fuel hybrid vehicles (Hahn-Hägerdal *et al.*, 2006).

The problem, however, is that ethanol for the fuel market is produced from sugar (Brazil) or starch (USA). This raw material base also has to be used for animal feed and human consumption and therefore it will not be adequate in meeting the increasing demand for fuel ethanol. These factors have led to the exploitation of lignocellulose feed-stocks,

such as agricultural and forest residues, as well as dedicated crops, or cellulose for the production of ethanol (Hahn-Hägerdal *et al.*, 2006).

Along with cellulosomal research being applied to ethanol production is the production of methane. In the USA's energy supply, methane composes about 20%. In the USA, there is also an extensive pipeline distribution system and a range of hardware that are in place for its domestic, municipal, and industrial use. However, this cannot be said for many other countries. Compared to fossil fuels, methane produces fewer atmospheric pollutants and produces less carbon dioxide per unit energy (Lundsford, 2000; Chynoweth *et al.*, 2001). Because methane is a comparatively clean fuel, there is a move towards its increased use for appliances, vehicles, industrial applications, and power generation. Other fuels such as methanol and hydrogen are commercially developed for production and use. They are also more difficult to produce from biomass (Chynoweth *et al.*, 2001). Ethanol is a popular biomass-derived fuel. However, even with its advantage of easy storage and transport, the fermentation process for its production requires feedstock pretreatment and pure culture maintenance. The energy requirements that arise with feed processing and product separation result in overall low process efficiencies. These problems, however, are not characteristic of processes for biological conversion of biomass to methane, that is, anaerobic digestion (Chynoweth *et al.*, 2001).

Millions of tons of solid waste are produced each year from municipal, industrial and agricultural sources. There is a large-scale contamination of land, water and air due to the uncontrolled decomposition of solid wastes. The methane and carbon dioxide released from landfills is a major concern for global warming (Yu *et al.*, 2002). However, utilising controlled anaerobic digestion of the biomass, where the biogas is captured, the methane emissions can be reduced and the energy from it can be used, thereby serving as a CO_2-neutral energy source (Møller *et al.*, 2004). There is a large interest in alternative waste management techniques, which can increase the rate of anaerobic decomposition of wastes. For instance, grass is a major organic component of the solid waste, it makes up about 14.6% of the total municipal solid waste produced and about 50% of the organic

fraction of the waste. Therefore using a method such as anaerobic digestion as a waste management option is an important option (Møller *et al.*, 2004; Rani and Nand, 2004).

1.8 Problem statement and motivation

It is generally accepted that the hydrolysis step in anaerobic digestion is the rate-limiting step in the anaerobic digestion of cellulosic material. An increase in the rate of hydrolysis would lead to an increase in the overall efficiency of the process. In this study, sulphide was used to increase or activate this step.

1.9 Hypothesis

Sulphide is able to accelerate the rate-limiting step in anaerobic digestion, by enhancing the cellulolytic activity of the cellulosome. This may be exploited for more rapid and complete degradation of organic matter.

1.10 Aims and Objectives

The aims and objectives were to:

a) isolate a *Clostridium* anaerobe from a biosulphidogenic bioreactor and to screen for the isolate with the highest cellulase activity
b) identify the anaerobe using 16S rDNA analysis
c) purify the cellulosome of the anaerobe
d) determine the subunits of the cellulosome
e) characterise the cellulosomal and non-cellulosomal fractions of the *Clostridium* by determining the effect of increased additions of sulphide, sulphate, cellobiose, glucose and acetate on cellulase and endoglucanase activities of the cellulosomal and non-cellulosomal fractions of the *Clostridium*
f) determine the pH and temperature optima of the cellulases and endoglucanases
g) determine the effect of sulphide on anaerobic digestion using the *Clostridium sp.* in anaerobic flasks/bioreactors.

CHAPTER 2

THE ISOLATION AND IDENTIFICATION OF *CLOSTRIDIUM BEIJERINCKII* sLM01 AND THE PURIFICATION OF ITS CELLULOSOME

2.1 INTRODUCTION

As mentioned previously, cellulosomes are large extracellular multi-enzyme complexes. Cellulosomes are produced by anaerobic microorganisms in order to hydrolyse cellulose, hemicelluloses and pectin among other polysaccharides. The cellulosomes degrade the plant cell walls in the environment of the anaerobe. These environments include soil, wood chip piles, sewage and rumens. Cellulosomes are cell protuberances that are possibly the largest complexes known as they range from 650 000 Da to 2.5 MDa (Doi *et al.*, 2003).

Clostridium beijerinckii is a gram-positive, spore-forming anaerobe that converts a variety of polysaccharides into acids and solvents. The anaerobe produces small quantities of the cellulosome of approximately 665 kDa (Perret *et al.*, 2004). Previous work performed by Sabathé *et al.* (2002) demonstrated that *C. acetobutylicum* ATCC 824 produced an inactive cellulosome, regardless of the evidence presented by the sequence analysis of its genome, where a cellulosomal gene cluster was present. Sabathé *et al.* (2002) found the presence of four major subunits in the cellulosome of *C. acetobutylicum* ATCC 824. These included the scaffolding protein CipA and they cellobiohydrolases Cel48A, Cel9X and Cel9C or Cel9E.

Various methods are used in purifying the cellulosome, including the affinity chromatography purification procedure (Lamed and Bayer, 1988) and the affinity digestion purification procedure (Morag *et al.*, 1992). The affinity chromatography purification method exploits the adsorption ability of the cellulosome to crystalline cellulose and elution of the cellulosome from the cellulose with triethylamine (TEA), while the affinity digestion method uses the adsorption of the cellulosome to amorphous

cellulose and digestion of cellulose and dialysis. Both methods were used in this study for the purification of the cellulosome from *C. beijerinckii*.

2.5 AIMS

The overall objective of this chapter was to purify the cellulosome of *C. beijerinckii* and to determine its cellulase and endoglucanase activities.

To achieve this objective several aims were addressed:

a) to isolate a *Clostridium* anaerobe from a biosulphidogenic bioreactor and screen for the isolate with the highest cellulase activity
b) to identify the anaerobe using 16S rDNA analysis
c) to purify the cellulosome of the anaerobe
d) to determine the subunits of the cellulosome.

2.6 MATERIALS AND METHODS

2.3.1 Isolation of a mesophilic Clostridium sp.

A sample from a biosulphidogenic bioreactor was taken for the subsequent culture and isolation of the microbe. The sample was streaked onto reinforced Clostridial medium (DIFCO) agar plates and was incubated at 28 ˚C for 48 hours. Colonies were picked and streaked onto fresh reinforced Clostridial medium agar plates at 28 ˚C for 48 hours. Anaerobic conditions were maintained using Anaerocult® A and anaerobic jars (Merck). All general reagents were supplied by Merck, unless otherwise stated.

2.3.2 Screening for cellulase activity

The anaerobic colonies were inoculated into anaerobic media that contained 0.5 % (w/v) Avicel, 1% L-cysteine, 0.05 % (w/v) agar and 0.005 % (w/v) yeast extract and were incubated at 28 ˚C for 48 hours. The cell suspension was used for the screening for cellulase activity. The colorimetric assay used for the determination of cellulase activity was the dinitrosalicyclic acid (DNS) method (Miller *et al.*, 1960). The DNS was prepared as described by Wood and Bhat (1988) where 2 g dinitrosalicyclic acid, 0.4 g phenol, 0.1 g sodium sulphite and 40 g Rochelle salt were dissolved in 100 ml of 2 % (w/v) NaOH solution and diluted to 200 ml with distilled water. Samples (100 µl) were incubated at 50 ˚C for 30 min with 50 µl 2 % (w/v) Avicel (for cellulase activity) substrate and 250 µl of 0.05 M citrate buffer, pH 4.8, after which 200 µl DNS was added and the samples were boiled for 5 min and put on ice for 10 min. The assay was performed in triplicates. Controls where no substrate was added were performed throughout the study. The absorbance at 540 nm was then determined. The samples with the highest activity were selected for further studies. Appendix A illustrates a typical glucose standard curve obtained.

2.3.3 Effect of sulphide on cellulase activity

The effect of sulphide on the isolated samples was determined. The samples (100 µl) were pre-incubated at room temperature in reaction tubes containing 200 µl of 0.05 M citrate buffer, pH 4.8 with a final concentration of sulphide of 60, 120, 240, 300, 480 and 600 mg l^{-1}, respectively for 15 min. A volume of 50 µl of 2 % (w/v) Avicel was then added to the reaction mixture. The experiments were performed in triplicates. Samples were incubated at 50 ˚C for 30 min after which 200 µl DNS was added and the samples were boiled for 5 min and put on ice for 10 min. The absorbance at 540 nm was then determined. The strain that exhibited high activity and was most affected by the additions of sulphide was selected for further studies.

2.3.4 Isolation of DNA and 16S rDNA sequence analysis

A single colony of the anaerobe that was selected in section 2.3.3 was inoculated into 5 ml reinforced Clostridial medium (DIFCO) and incubated with shaking (150 rpm) for 48 hours at 28 ˚C. Bacterial genomic DNA was isolated using the miniprep method of Ausubel *et al.* (2002). The 16S rRNA gene was amplified by the polymerase chain reaction (PCR) using a 35 µl reaction containing approximately 0.5 µg template DNA/35 µl, 1.5 mM $MgCl_2$, 1x PCR buffer, 200 µM of each deoxynucleotide, 0.57 µM of both primers; GM5 F (5' – cct acg gga ggc agc ag - 3') and 907 R (5' - cgc ccg ccg cgc ccc gcg ccc gtc ccg ccg ccc ccg ccc gcc gtc aat tcc ttt gag ttt - 3') incorporating a GC clamp and 1.75 U/35 µl Taq polymerase (Promega). PCR was performed on Hybaid PCR Sprint thermocycler using a touchdown procedure with initial denaturation at 95 °C followed by 28 cycles of denaturation at 94 °C for 30 s, annealing at 68 °C and decreasing every 4 cycles by 2 °C for 45 s, extension at 72 °C and followed by a final extension at 72 °C for 5 min. The PCR product was quantified on a 1 % agarose gel. The PCR amplification products were cloned into pGEM®-T Easy Vector Systems (Promega) and transformed into *Escherichia coli* JM 109 cells. The plasmids were purified from the transformed cells using a QAIprep® Spin Miniprep kit (Qiagen) and visualised in a 1 % agarose gel. The plasmids were digested with *Eco*RI (Roche) to confirm that the PCR product had

ligated onto the pGEM®-T vector, where 2 µl Sure/Cut buffer H, 2 µl plasmid DNA, 0.3 µl *Eco*RI and 16 µl double distilled water to the reaction tube and incubated at 37 ˚C for 2 hours and the digestion results were visualised on a 1 % agarose gel. Purified plasmids were prepared for cycle sequencing using ABI PRISM® BigDye V3.1 (Applied Biosystems). The primers used were universal sequencing primers T7 and SP6 (Promega). Protocols were followed as described by the suppliers' instructions. Samples were sequenced using a 3100 ABI Genetic analyser. The 16S rDNA sequences were entered into the nucleotide-nucleotide BLAST search to identify the anaerobe.

2.3.5 Scanning electron micrograph (SEM) of C. beijerinckii

C. beijerinckii was grown in culture medium based on that used by Lamed and Bayer (1988), described in detail in section 2.3.6. Two 1 ml cultures were centrifuged at 13 000 rpm for 5 min using the Heraeus Biofuge Pico microlitre centrifuge. The supernatant was decanted and 200 µl cold buffered fixative (2.5% (w/v) glutaraldehyde in 0.1 M phosphate buffer, pH 7.3) was added. The samples were allowed to fix for 12 hours at 4 ˚C. The fixative was decanted and 200 µl cold 0.1 M phosphate buffer, pH 7.3 was added to the wash for 10 min twice. The phosphate buffer was decanted and 200 µl of 30 % ethanol was added. The tubes were allowed to stand to dehydrate for 5 min. This step was repeated with each of the ethanol concentrations (50, 70, 80 and 90 % ethanol) with two changes of 100 % ethanol. The specimens were transferred into the critical point drying (CPD) baskets, then placed in 100 % ethanol, and the baskets were placed in basket holders and then in the CPD chamber and dried. After drying the samples were placed on stubs and coated with a thin film of gold.

2.3.6 Purification of the cellulosome from C. beijerinckii

C. beijerinckii was grown in a culture medium based on that used by Lamed and Bayer (1988) for the subsequent purification of the cellulosome. The medium consisted of 0.25 g $MgCl_2$, 0.65 g NH_4SO_4, 2.5 g yeast extract, 2.5 g Avicel and 0.25 g agar in 500 ml distilled water. The media was flushed with nitrogen gas and autoclaved. Prior to

inoculation, 1 ml (15 % w/v) autoclaved cysteine-HCl, pH 8.5 was added and then a single colony of *C. beijerinckii* was inoculated into the medium. The flasks were incubated at 28 ˚C with constant shaking (100 rpm) for 14 days.

The first cellulosome purification procedure used was based on that of Lamed and Bayer (1988) where 1 litre culture (500 ml culture was also used) of *C. beijerinckii* after incubation was centrifuged (Beckman Coulter Avanti® J-E centrifuge) at 1000 g for 5 min. The supernatant was decanted and 12 g Avicel (Fluka) was added. The suspension was stirred mechanically using a rocker (Bellco Biotechnology) for 1 hour at room temperature and then centrifuged at 1000 g for 2 min. The supernatant was discarded and the cellulose was washed with 250 ml 0.05 M Tris buffer, pH 7.7 and the cellulosome was eluted with 125 ml 1 % triethylamine. This was performed at 4 ˚C. The eluate was neutralised with 10 % acetic acid and concentrated with polyethylene glycol (PEG) 20 000 using dialysis tubing at 4 ˚C. The concentrated eluate was microcentrifuged at 3 000 rpm for 5 min and 500 µl of the supernatant was applied onto a Sepharose 4B column (15 x 1.0 cm). The major protein peaks (obtained from the A280 nm chromatogram) were pooled.

The second purification procedure was based on the protocol outlined by Morag *et al.* (1992) whereby the cellulosome was purified using the affinity digestion procedure. Amorphous cellulose was prepared as described by Lamed *et al.* (1985) prior to purification of the cellulosome. A volume of 1 litre of *C. beijerinckii* culture was grown as described previously in this section and then centrifuged at 1000 g for 5 minutes. The supernatant was used for the purification of the cellulosome and 0.5 g of amorphous (phosphoric acid swollen) cellulose was added to the supernatant. The mixture was incubated at 4 ˚C for 2 hours, and then centrifuged at 10 000 g for 5 min. The pellet was resuspended in 5 ml of 0.5 M Tris buffer pH 7.5 and dialysed versus 0.5 M Tris buffer, pH 7.5 at 37 ˚C overnight. The sample was microcentrifuged at 3000 rpm for 3 min and 1 ml of the supernatant was applied onto a Sepharose 4B column (30 x 1.5 cm). The major protein peaks (obtained from the A_{280nm} chromatogram) were pooled.

2.3.7 Determination of cellulase/ endoglucanase activity and protein concentration

The cellulase/ endoglucanase assays were performed as described in section 2.3.2 and the substrates 2 % (w/v) Avicel (for cellulase) and 2 % (w/v) CMC (for endoglucanase) were used. Protein concentration was determined using the Bradford method and bovine serum albumin (BSA) as the standard (Bradford, 1976). Appendix B shows a typical BSA standard curve obtained.

2.3.8 SDS-PAGE, zymograms and MALDI-TOF analysis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using a 10% polyacrylamide gel as described by Bollag *et al.* (1996). The samples were concentrated with acetone prior to loading onto the gel where 1 ml ice cold acetone was added to 200 µl sample, the mixture was vortexed briefly and incubated at -20 ˚C for 2 hours and microcentrifuged at 13 000 rpm for 15 min. The supernatant was decanted and the samples were air dried, followed by the resuspension of the pellet by adding one pellet volume of 0.05 M Tris buffer, pH 7.7 (first cellulosome purification protocol) or 0.05 M Tris buffer, pH 7.5 (second cellulosome purification protocol). The approximate molar amounts of the bands were determined using densitometry software (Uvitech). The molar amount of each band was expressed relative to the S1 (subunit 1) band, which was arbitrarily assigned a value of 1 (Murashima *et al.*, 2002). The zymogram for endoglucanase was performed using 0.1% (w/v) CMC incorporated into the polyacrylamide. Renaturing of the gels after SDS-PAGE was performed as described by Han *et al.* (2004). The zones of clearing, which corresponded to endoglucanase activity were visualized using a 0.3% (w/v) Congo red stain for 10 min and destained using 1 M NaCl (Han *et al.*, 2004). A 10 % SDS-PAGE of the purification fractions was performed again in order to obtain the bands of interest for MALDI-TOF analysis. The MALDI-TOF service of the Department of Molecular and Cell Biology of the University of Cape Town was employed. The Coomassie stained bands were excised, digested with trypsin and a tryptic peptide mass finger print was obtained for each band. Expasy tool

(PeptideMass) and the NCBI protein search were used to search and match the peptide mass to identify the origin of the enzymes.

2.7 RESULTS

2.4.1 Screening for cellulase activity of the isolated anaerobe and the effect of sulphide on cellulase activity

Mesophilic anaerobes were isolated from a biosulphidogenic bioreactor and screened for cellulase activity (figure 2.1) using Avicel. One unit (U) was defined as nmol glucose released min^{-1} ml^{-1} because of the low activities obtained. The strains with the highest cellulase activities, samples 3, 4 and 8, denoted cLM01, cLM02 and cLM03, respectively, were selected for further studies. The cLM03 strain contained the highest cellulase activity (0.034 nmol glucose released min^{-1} ml^{-1}), figure 2.1, and was the most dramatically stimulated by sulphide, figure 2.2, showing a 317 % increase in cellulase activity- 0.136 nmol glucose released min^{-1} ml^{-1}.

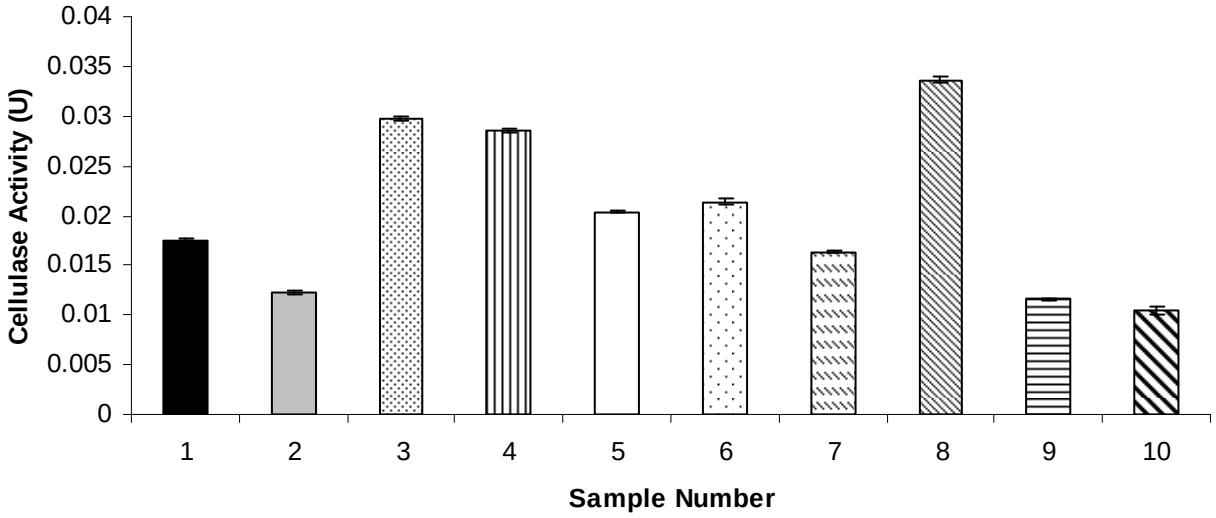

Figure 2.1 Cellulase activity screening of isolated anaerobes. Values are expressed as means ± SD (*n*=3).

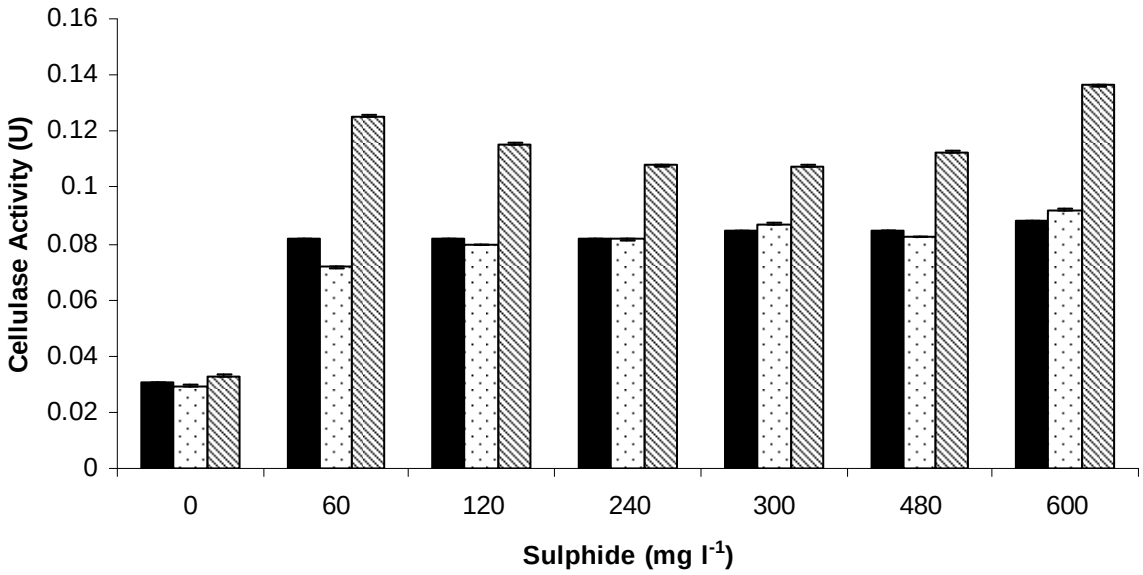

Figure 2.2 Effect of sulphide on cellulase activity of cLM1 (■), cLM2 (⊡) and cLM3 (▨). Values are expressed as means ± SD (*n*=3).

2.4.2 Isolation of DNA and 16S rDNA sequence analysis

The cLM03 strain was selected during the screening process, its DNA was isolated and the partial 16S rDNA region was PCR amplified (figure 2.3). The PCR amplified 16S rDNA region was ligated into a pGEM®- T Easy vector and transformed into competent *E. coli* JM109 cells. The plasmid was purified (figure 2.4) and digested with *Eco*RI to confirm ligation (figure 2.5). The 500 bp PCR amplified 16S rDNA region was sequenced and the sequence that was generated was entered into the nucleotide-nucleotide BLAST search and the resulting organism, with 99 % identity was identified putatively as *Clostridium beijerinckii* Accession number X68180.1 (figure 2.6). PCR was repeated using the 9F (5' - gat ttt gat cct ggc tca g – 3') and 1541R (5' – aag gag gtg atc cag cc – 3') primers, which amplified 1300 bp of the 16S rDNA region. Appendix E shows the results resulting from a BLAST search using the sequence obtained. A 98 % identity to *C. beijerinckii* (Accession number X68180.1) and putatively identified the isolated microorganism as *C. beijerinckii*.

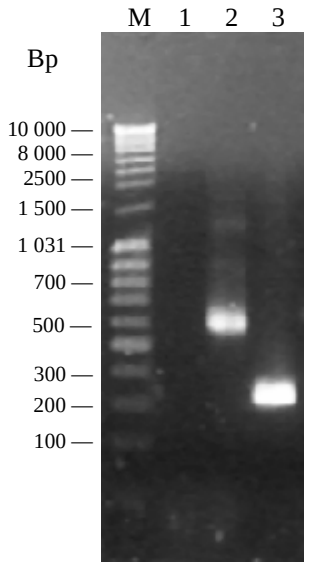

Figure 2.3 Agarose gel (1 %) showing PCR products. M; Molecular weight marker (MassRuller^{TM} DNA ladder mix # SM0403); 1; negative control; 2; PCR amplified 16S rDNA of isolated anaerobe; 3; Positive control (Promega).

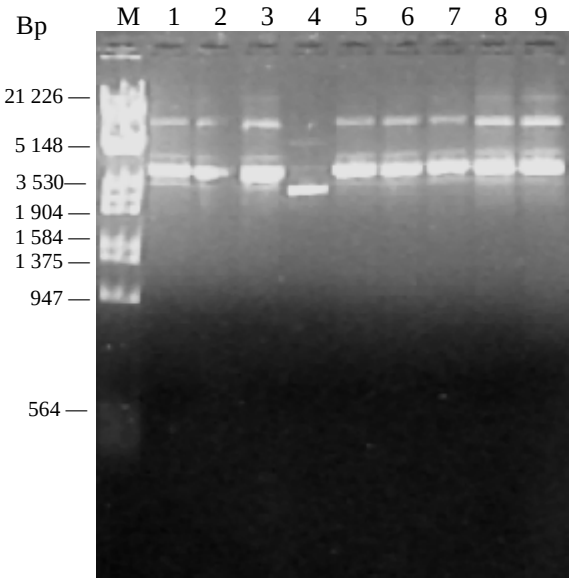

Figure 2.4 Agarose gel (1 %) of plasmid DNA isolated from transformed *E. coli* JM 109 cells. M; Molecular weight marker (λDNA/ *Eco*RI + *Hind*III); 1-9; purified plasmid DNA of screened transformants.

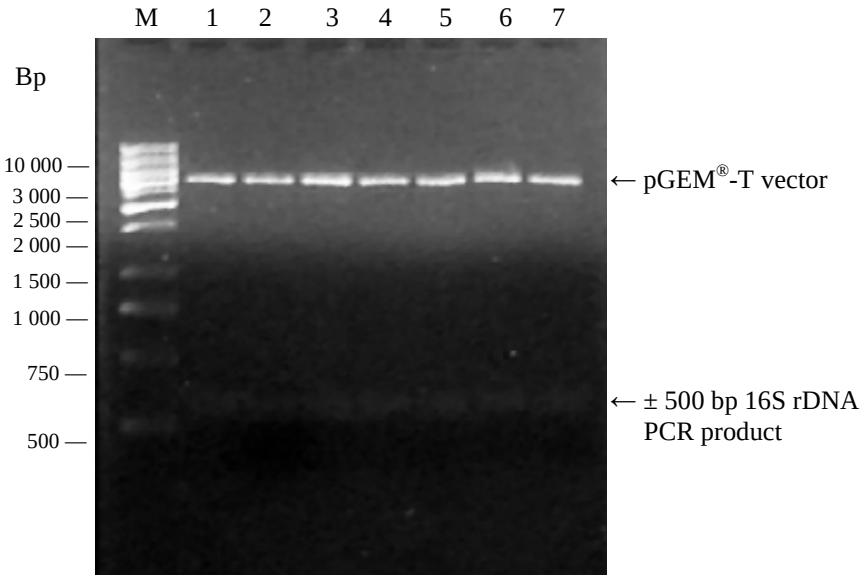

Figure 2.5 Agarose gel (1 %) showing the *Eco*RI digestion of the plasmid containing the 16S rDNA PCR product. M; Molecular weight marker (O'GeneRuler™ 1 kb DNA Ladder); 1-7; *Eco*RI digested vectors.

```
1     TCAATTCCTT TGAGTTTTAA TCTTGCGACC GTACTCCCCA GGCGGAATAC
      TTAATGCGTT AGCGGCGGCA CAGAGGTCAT GACAACCCCT ACACCTAGTA

101   TTCATCGTTT ACGGCGTGGA CTACCAGGGT ATCTAATCCT GTTTGCTCCC
      CACGCTTTCG AGCCTCAGTG TCAGTTACAG TCCAGAAAGT CGCCTTCGCC

201   ACTGGTATTC TTCCTAATCT CTACGCATTT CACCGCTACA CTAGGAATTC
      TACTTTCCTC TCCTGCACTC TAGATATCCA GTTTGGAATG CAGCACCCAG

301   GTTAGGCCCG AGTATTTCAC ATCCCACTTA AATATCCACC TACGCTCCCT
      TTACGCCCAG  TAAATCCGGA CAACGCTTGC CACCTACGTA TTACCGCGGC

401   TGCTGGCACG TAGTTAGCCG TGGCTTCCTC CTCAGGTACC GTCATTATCG
      TCCCTGAAGA CAGAGCTTTA CAATCCGAAG ACCGTCATCA CTCACGCGGC

501   GTTGCTGCAT CAGGGTTTCC CCCATTGNGC AATATTCCCC ACTGCTGCCT
      CCCGTAG
```

Figure 2.6 The 16S rDNA sequence of the isolated *Clostridial* anaerobe.

2.4.3 Scanning electron micrograph (SEM) of C. beijerinckii sLM01

The selected strain was grown on cellulose and visualised using SEM, the microorganism (as figure 2.7 illustrates) is rod shaped and acts on cellulose.

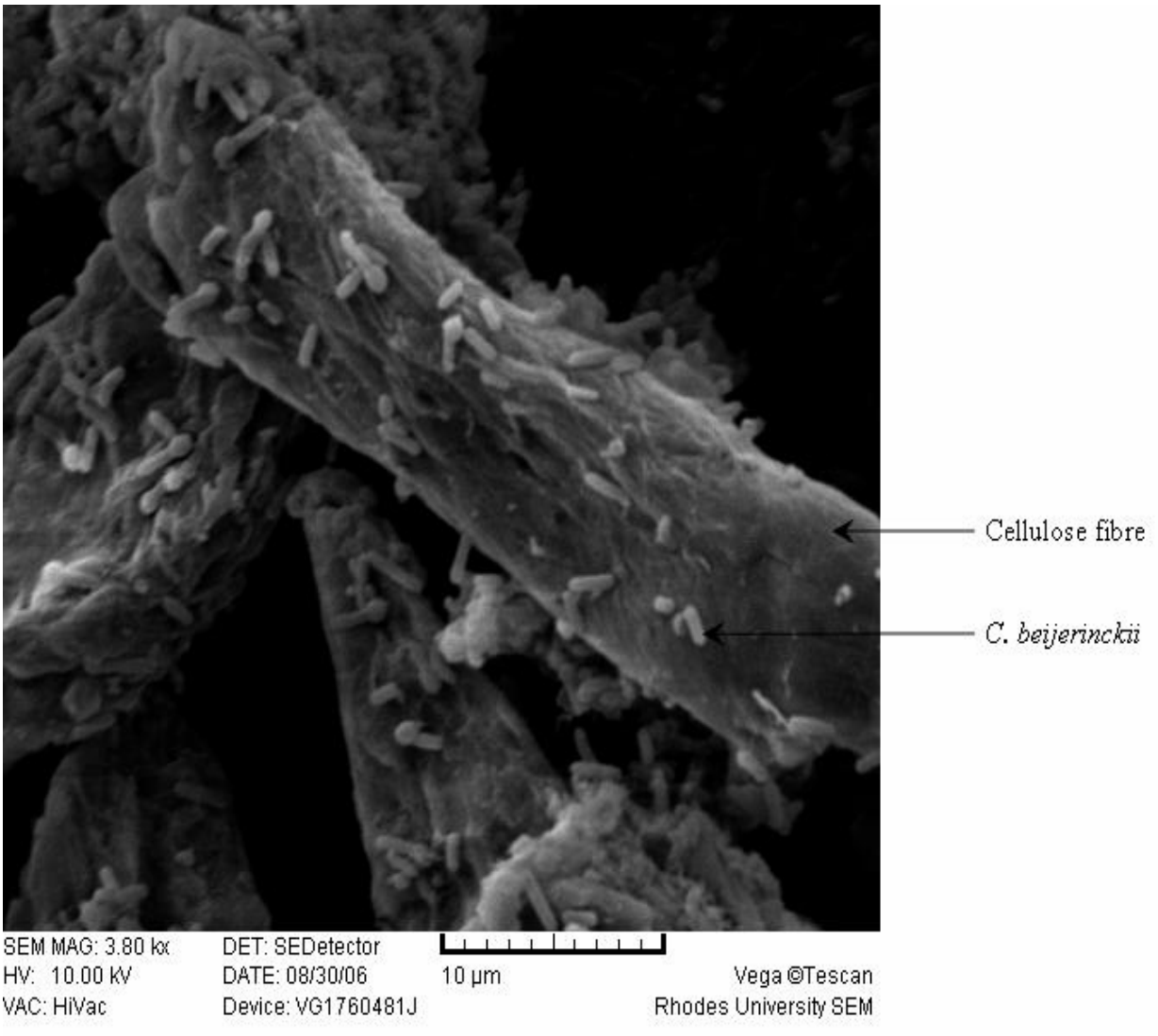

Figure 2.7 Scanning electron micrograph of *C. beijerinckii* sLM01 acting on cellulose.

2.4.4 Purification of the cellulosome from C. beijerinckii sLM01 using affinity chromatography

After identifying the microbe with 16S rDNA sequence analysis and visualising it on SEM, its cellulosome was purified. The first procedure used was the affinity chromatography purification procedure (Lamed and Bayer, 1988). Figure 2.8 shows the Sepharose 4B chromatogram obtained in the first purification. Five peaks resulted, which were not well resolved or separated. These were denoted S4B1-S4B5 (Sepharose 4B fraction 1-5). The fractions in each peak were pooled and cellulase and endoglucanase assays were performed and the protein concentration was determined.

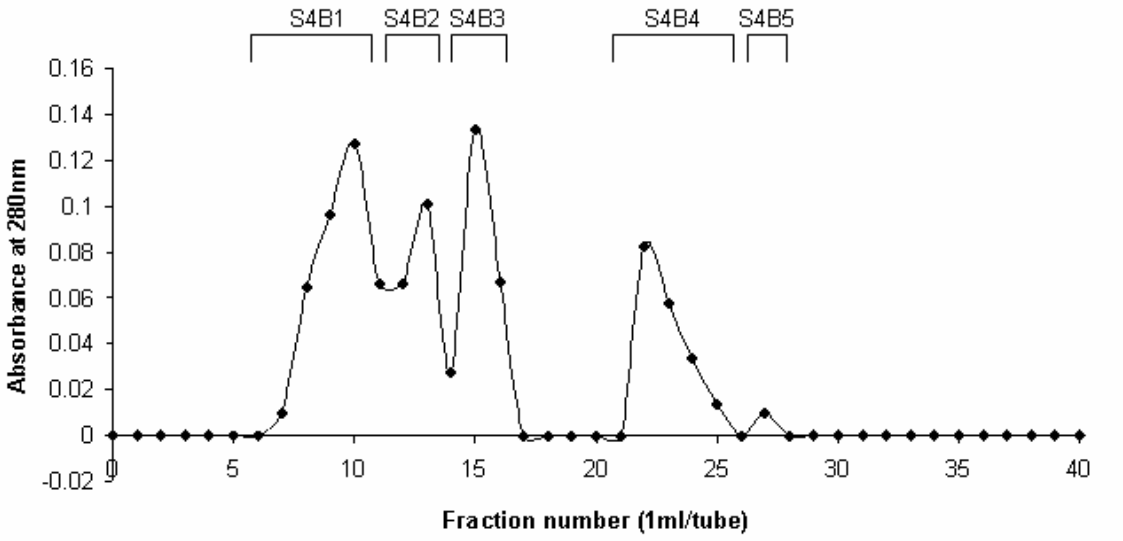

Figure 2.8 The first Sepharose 4B chromatogram of a cellulosome containing fraction from *C. beijerinckii*. Column dimensions, 15 x 1.0 cm and flow rate, 20 ml h^{-1}.

Table 2.1 shows the purification table for the purification of the cellulases of the *C. beijerinckii* cellulosome using the affinity chromatography purification procedure. The specific activities and the fold purification values of the Sepharose 4B fractions showed that the purification was successful as these values were higher than of the crude extract. Figure 2.8 shows that S4B3 had a larger peak than S4B5, however, table 2.1 shows that

S4B3 had a lower total protein value than S4B5. This may be due to a lower tyrosine or tryptophan content in the S4B5 fraction and therefore a lower A_{280nm} reading was obtained. The Bradford's assay was able to detect the protein present to a higher degree.

Table 2.1 Purification table for cellulases of the cellulosome of *C. beijerinckii* sLM01

Fraction Name	Total Volume[a]	Activity[b]	Protein Concentration[c]	Total Protein[d]	Total Activity[e]	Specific Activity[f]	% Yield	Fold Purification
Crude Extract	964	2.200	0.317	305.6	2131	6.940	100.0	1.00
Supernatant	900	1.260	0.331	297.9	1134	3.807	53.2	0.55
Supernatant after binding	412	0.787	0.276	113.7	324.1	2.851	15.2	0.41
Wash	670	0.605	0.153	102.5	405.4	3.954	19.0	0.57
Elution	181	0.748	0.081	14.66	135.4	9.235	6.4	1.33
Concentrated Elution	4.5	3.526	0.851	3.830	15.87	4.143	0.7	0.60
S4B1	27	0.847	0.043	1.162	22.87	19.70	1.1	2.82
S4B2	13.5	0.858	0.073	0.990	11.58	11.75	0.5	1.69
S4B3	22	1.601	0.081	1.782	35.22	19.77	1.6	2.84
S4B4	45	1.826	0.127	5.716	82.18	14.38	3.9	2.07
S4B5	20	1.744	0.144	2.880	34.88	12.11	1.6	1.74

[a] ml; [b] nmol glucose released min^{-1} ml^{-1}; [c] mg ml^{-1}; [d] mg; [e] nmol glucose released min^{-1}; [f] nmol glucose released mg^{-1} protein min^{-1}.

Table 2.2 shows the purification table for the endoglucanases of the *C. beijerinckii* cellulosome, using the affinity chromatography purification procedure. The endoglucanases were also purified successfully, as the specific activities and fold purification values were higher (except for S4B5) than that of the crude. Despite the fact that the total activity decreased substantially throughout the purification for both cellulases and endoglucanases (tables 2.1 and 2.2), the purification was fairly successful.

Table 2.2 Purification table for endoglucanases of the cellulosome of *C. beijerinckii* sLM01

Fraction Name	Total Volume[a]	Activity[b]	Protein Concentration[c]	Total Protein[d]	Total Activity[e]	Specific Activity[f]	% Yield	Fold Purification
Crude Extract	964	0.545	0.317	305.6	525.4	1.719	100.0	1.00
Supernatant	900	0.072	0.331	297.9	64.80	0.218	12.3	0.13
Supernatant after binding	412	0.044	0.276	113.7	18.13	0.159	3.5	0.09
Wash	670	0.055	0.153	102.5	36.85	0.359	7.0	0.21
Elution	181	0.149	0.081	14.66	26.97	1.840	5.1	1.07
Concentrated Elution	4.5	2.261	0.851	3.830	10.17	2.657	1.9	1.55
S4B1	27	0.132	0.043	1.161	3.564	3.070	0.7	1.79
S4B2	13.5	0.193	0.073	0.990	2.606	2.644	0.5	1.54
S4B3	22	0.561	0.081	1.782	12.34	6.926	2.3	4.03
S4B4	45	0.484	0.127	5.716	21.78	3.811	4.1	2.22
S4B5	20	0.160	0.144	2.880	3.200	1.111	0.6	0.65

[a] ml; [b] nmol glucose released min^{-1} ml^{-1}; [c] mg ml^{-1}; [d] mg; [e] nmol glucose released min^{-1}; [f] nmol glucose released mg^{-1} protein min^{-1}.

Figure 2.9 shows the 10 % SDS-PAGE results for the purification of the cellulosome of *C. beijerinckii* using the affinity chromatography purification procedure. Various bands were obtained, however these were not clear. The S4B1-S4B5 fractions did not result visible bands on the gel (S4B1 results shown on figure 2.9). The gel therefore did not show whether the cellulosomal proteins were present in the fractions. The purification was repeated in order obtain improved separation of the peaks in the chromatogram as well as to obtain a clearer SDS-PAGE result, with more distinct bands in the cellulosomal and non-cellulosomal fractions.

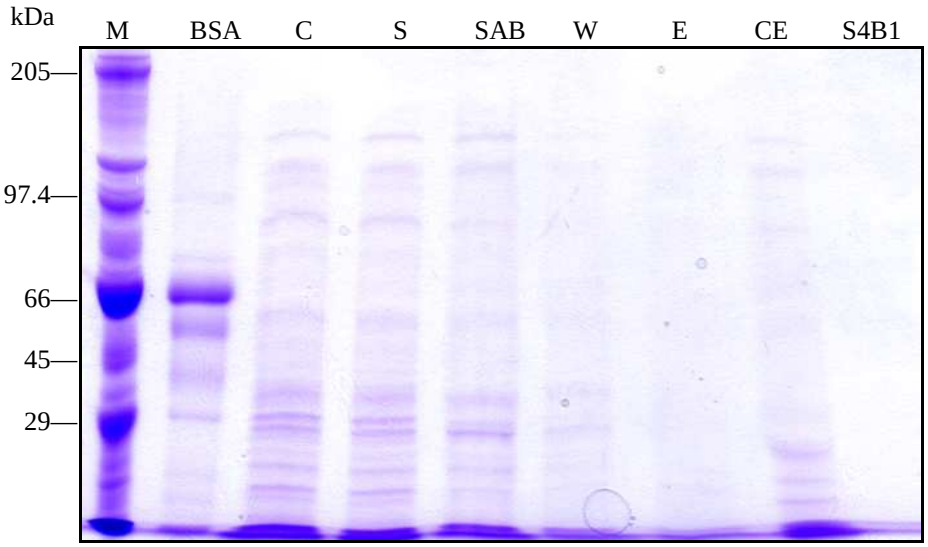

Figure 2.9 SDS-PAGE (10 %) results for the purification of the cellulosome of *C. beijerinckii* using the affinity chromatography purification procedure. M: Molecular weight marker (Sigma 29- 205 kDa); BSA: Bovine serum albumin; C: Crude extract; S: Supernatant; SAB: Supernatant after cellulose binding; W: Wash; E: Elution; CE: Concentrated elution; S4B1: Sepharose 4B fraction 1.

Figure 2.10 shows the Sepharose 4B chromatogram produced from a repeat of the purification procedure. Six peaks were observed, which were better resolved or separated than in the first purification (figure 2.8). These peaks were denoted S4B1-S4B6 (Sepharose 4B fractions 1-6). The fractions constituting each peak were pooled together and cellulase and endoglucanase assays were performed and the protein concentration was determined.

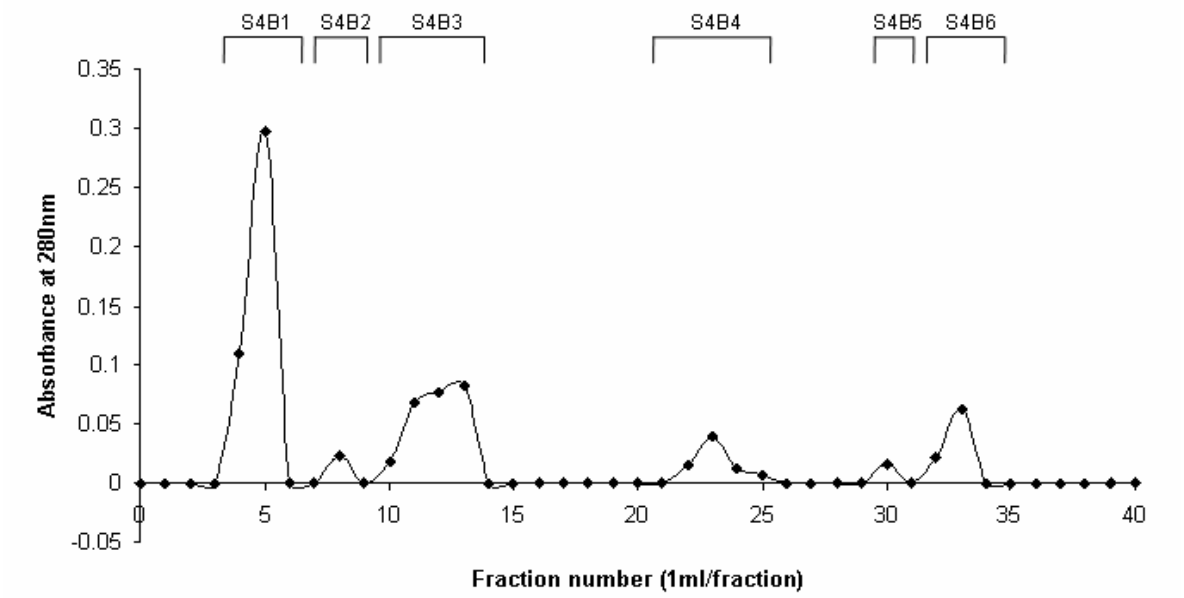

Figure 2.10 The second Sepharose 4B chromatogram of a cellulosome containing fraction from *C. beijerinckii*. Column dimensions, 15 x 1.0 cm and flow rate, 20 ml h^{-1}.

Table 2.3 shows the purification table for the cellulases of the *C. beijerinckii* cellulosome using the affinity chromatography purification procedure. The specific activities and fold purification values were dramatically higher in this purification than that of the previous purification. As it was found in the previous purification, S4B5 had a higher total protein value than S4B3. This was not expected as the A_{280nm} reading was higher for S4B3 than S4B5. This may be due to a lower tyrosine or tryptophan content in the S4B5 fraction and therefore a lower A_{280nm} reading was obtained. The higher total protein value indicates that the Bradford's assay was able to detect the protein present to a higher degree. The highest specific activity was 146.9 nmol glucose released mg^{-1} protein min^{-1} in this purification, while in the first purification it was 19.77 nmol glucose released mg^{-1} protein min^{-1} (table 2.1). The highest fold purification value was 14.19 fold for the second purification (table 2.3) and 2.84 fold for the first purification (table 2.1).

Table 2.3 Purification table for cellulases of the cellulosome of *C. beijerinckii* sLM01

Fraction Name	Total Volume[a]	Activity[b]	Protein Concentration[c]	Total Protein[d]	Total Activity[e]	Specific Activity[f]	% Yield	Fold Purification
Crude Extract	480	1.584	0.153	73.44	760.4	10.35	100.0	1.00
Supernatant	472	2.266	0.168	79.30	1070	13.49	140.7	1.30
Supernatant after binding	442	1.634	0.168	74.26	722.1	9.720	95.0	0.94
Wash	63	1.601	0.015	0.945	100.8	106.7	13.3	10.31
Elution	123	3.801	0.020	2.460	467.5	190.0	61.5	18.36
Concentrated elution	5	9.466	0.942	4.710	47.33	10.05	6.2	0.97
S4B1	35	1.144	0.050	1.750	40.04	22.88	5.3	2.21
S4B2	28	1.348	0.043	1.200	37.73	31.34	5.0	3.03
S4B3	30	1.260	0.023	0.880	37.79	54.77	5.0	5.29
S4B4	58	1.469	0.010	0.580	85.18	146.9	11.2	14.19
S4B5	39	1.991	0.028	1.090	77.66	71.11	10.2	6.87
S4B6	20	1.463	0.017	0.340	29.26	86.07	3.8	8.32

[a] ml; [b] nmol glucose released min^{-1} ml^{-1}; [c] mg ml^{-1}; [d] mg; [e] nmol glucose released min^{-1}; [f] nmol glucose released mg^{-1} protein min^{-1}.

Table 2.4 shows the purification table produced on repeating the purification procedure of the endoglucanases from *C. beijerinckii*. The specific activities and fold purification values were higher in this purification than that of the previous purification (table 2.2). The highest specific activity was 93.51 nmol glucose released mg^{-1} protein min^{-1} in this purification, while in the first purification it was 6.926 nmol glucose released mg^{-1} protein min^{-1} (table 2.2). The highest fold purification value was 26.54 fold for the second purification (table 2.4) and 4.03 fold for the first purification (table 2.1). The purification of cellulases and endoglucanases was therefore more successful in the second purification attempt.

Table 2.4 Purification table for endoglucanases of the cellulosome of *C. beijerinckii* sLM01

Fraction Name	Total Volume[a]	Activity[b]	Protein Concentration[c]	Total Protein[d]	Total Activity[e]	Specific Activity[f]	% Yield	Fold Purification
Crude Extract	480	0.539	0.153	73.44	258.7	3.523	100.0	1.00
Supernatant	472	0.319	0.168	79.30	150.6	1.899	58.2	0.54
Supernatant after binding	442	0.292	0.168	74.26	128.9	1.735	49.8	0.49
Wash	63	0.297	0.015	0.945	18.71	19.80	7.2	5.62
Elution	123	0.842	0.020	2.460	103.5	42.08	40.0	11.94
Concentrated elution	5	9.076	0.942	4.710	45.38	9.635	17.5	2.73
S4B1	35	4.675	0.050	1.750	163.6	93.51	63.2	26.54
S4B2	28	0.622	0.043	1.200	17.40	14.45	6.7	4.10
S4B3	30	0.567	0.023	0.880	17.00	24.63	6.6	6.99
S4B4	58	0.572	0.010	0.580	33.18	57.21	12.8	16.24
S4B5	39	0.506	0.028	1.090	19.74	18.07	7.6	5.13
S4B6	20	0.545	0.017	0.340	10.89	32.03	4.2	9.09

[a] ml; [b] nmol glucose released min^{-1} ml^{-1}; [c] mg ml^{-1}; [d] mg; [e] nmol glucose released min^{-1}; [f] nmol glucose released mg^{-1} protein min^{-1}.

SDS-PAGE results of the repeat of the purification of the cellulosome of *C. beijerinckii* using the affinity chromatography purification procedure were unsuccessful. There were no bands present in the gel in any of the fractions except the concentrated elution fraction. This was unexpected as the activities and specific activities during this purification were high. The second purification technique was employed in order to determine if this would improve the results, especially whether bands would be present in the Sepharose 4 B fractions on 10% SDS-PAGE.

2.4.5 Purification of the cellulosome from C. beijerinckii sLM01 using the affinity digestion procedure

The second purification procedure was based on a protocol outlined by Morag *et al.* (1992) whereby the cellulosome was purified using the affinity digestion procedure. In this procedure a larger column was used (30 x 1.5 cm), amorphous cellulose instead of crystalline cellulose was used during the purification procedure and there was an overnight digestion step (section 2.3.6). Figure 2.11 shows the chromatogram obtained from the affinity digestion procedure. The peaks were well resolved. Four peaks resulted, designated as S4B1-4 (Sepharose 4B fractions 1-4). The fractions in each peak were pooled and the cellulase and endoglucanase activities, and protein concentration was determined.

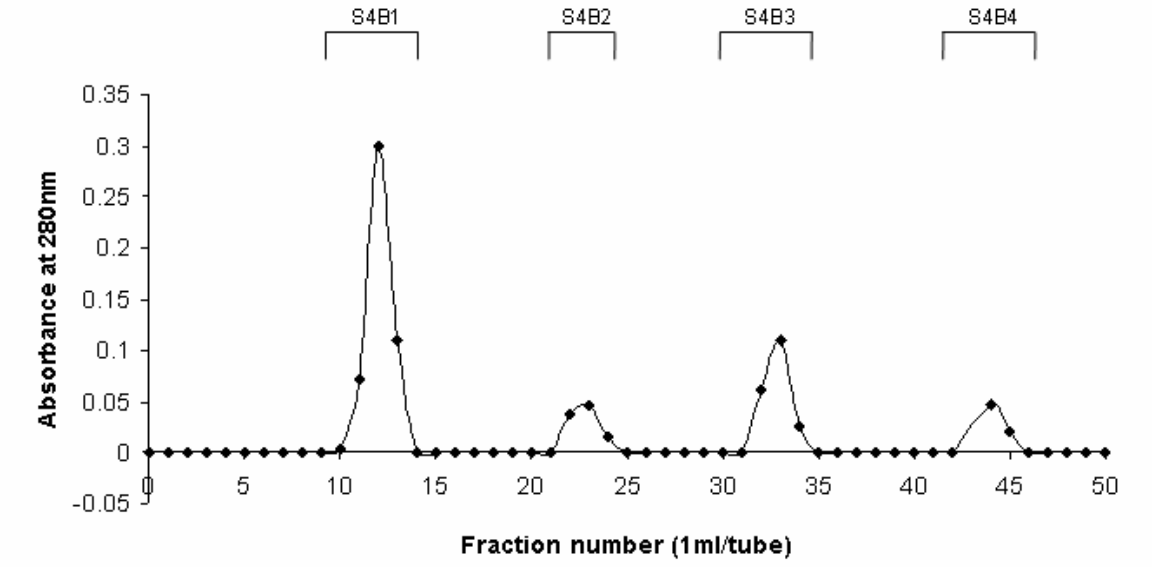

Figure 2.11 Sepharose 4B chromatogram of a cellulosome containing fraction from *C. beijerinckii*. Column dimensions, 30 x 1.5 cm and flow rate, 20 ml h^{-1}.

Table 2.5 shows the purification table for the cellulases of the *C. beijerinckii* cellulosome using the affinity digestion procedure. The specific activities and the fold purification values of the Sepharose 4B fractions showed that the purification was successful, as these values were significantly higher than of the crude extract.

Table 2.5 Purification table for cellulases of the cellulosome of *C. beijerinckii* sLM01

Fraction Name	Total Volume[a]	Activity[b]	Protein Concentration[c]	Total Protein[d]	Total Activity[e]	Specific Activity[f]	% Yield	Fold Purification
Crude Extract	961	0.770	0.056	53.82	740.0	13.75	100.0	1.00
Supernatant	818	0.704	0.028	22.90	575.9	25.14	77.8	1.83
Supernatant after binding	773	0.649	0.009	6.957	501.7	72.11	67.8	5.24
Digested and Dialysed	13	3.025	0.440	5.720	39.33	6.875	5.3	0.50
S4B1	74	0.583	0.007	0.518	43.14	83.29	5.8	6.06
S4B2	54	0.583	0.010	0.540	31.48	58.30	4.3	4.24
S4B3	62	0.611	0.014	1.166	37.85	43.64	5.1	3.17
S4B4	58	0.534	0.010	0.580	30.97	53.40	4.2	3.88

[a] ml; [b] nmol glucose released min^{-1} ml^{-1}; [c] mg ml^{-1}; [d] mg; [e] nmol glucose released min^{-1}; [f] nmol glucose released mg^{-1} protein min^{-1}.

Table 2.6 shows the purification table of the endoglucanases of the *C. beijerinckii* cellulosome using the affinity digestion procedure. The specific activity and the fold purification were low for all of the Sepharose 4B fractions. The activity decreased dramatically from 1.936 nmol glucose released min^{-1} ml^{-1} in the digested and dialysed fraction to 0.006 nmol glucose released min^{-1} ml^{-1}, indicating that most of the activity was lost during the Sepharose 4B chromatography step.

Table 2.6 Purification table for endoglucanases of the cellulosome of *C. beijerinckii* sLM01

Fraction Name	Total Volume[a]	Activity[b]	Protein Concentration[c]	Total Protein[d]	Total Activity[e]	Specific Activity[f]	% Yield	Fold Purification
Crude Extract	961	0.253	0.056	53.82	243.1	4.518	100.0	1.00
Supernatant	818	0.033	0.028	22.90	27.00	1.179	11.1	0.26
Supernatant after binding	773	0.017	0.009	6.957	12.76	1.834	5.2	0.41
Digested and Dialysed	13	1.936	0.440	5.720	25.17	4.400	10.4	0.97
S4B1	74	0.006	0.007	0.518	0.444	0.857	0.2	0.19
S4B2	54	0.012	0.010	0.540	0.648	1.200	0.3	0.27
S4B3	62	0.011	0.014	1.166	0.682	0.786	0.3	0.17
S4B4	58	0.012	0.010	0.580	0.696	1.200	0.3	0.27

[a] ml; [b] nmol glucose released min^{-1} ml^{-1}; [c] mg ml^{-1}; [d] mg; [e] nmol glucose released min^{-1}; [f] nmol glucose released mg^{-1} protein min^{-1}.

The two large peaks obtained in the chromatogram (see figure 2.11), that is, the first and the third peaks were denoted Sepharose 4B fraction 1 (S4B1)- the cellulosomal fraction and Sepharose 4B fraction 3 (S4B3)- the non-cellulosomal fraction, respectively. These peaks exhibited high cellulase specific activities; 83.29 nmol glucose released mg^{-1} protein min^{-1} and 43.64 nmol glucose released mg^{-1} protein min^{-1}, respectively and the highest endoglucanase specific activities obtained were also obtained in these fractions- 1.200 nmol glucose released mg^{-1} protein min^{-1} for each fraction. S4B1 and S4B3 were subsequently used for the characterisation of the cellulosomal and non-cellulosomal fractions of *C. beijerinckii*.

Figure 2.12 shows the SDS-PAGE results obtained for the purification of the cellulosome using the affinity digestion procedure. In the S4B1 fraction, there was one faint band present at approximately 50 kDa (not visible in figure 2.13). This band is more clearly shown in 2.14. However, obtaining only one band cannot confirm whether S4B1 is the cellulosomal fraction (therefore, the purification procedure was repeated).

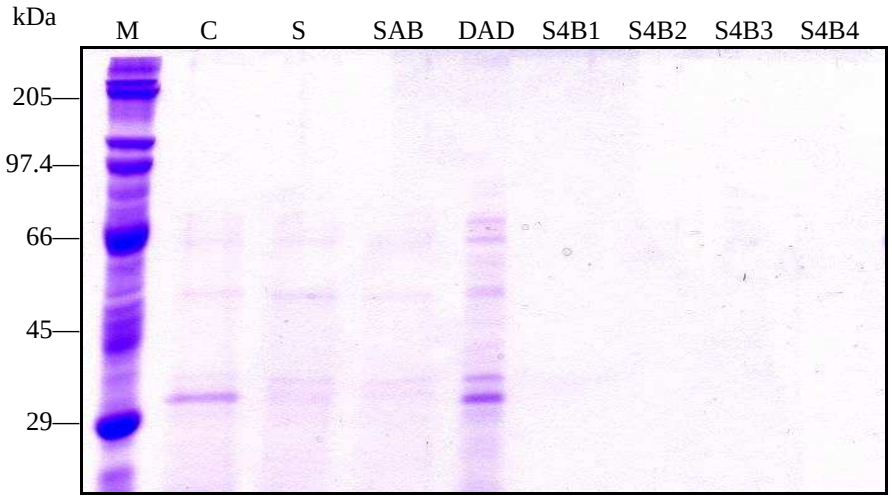

Figure 2.12 SDS PAGE (10 %) of purification of the cellulosome of *C. beijerinckii* M: Molecular weight marker (Sigma 29- 205 kDa); C: Crude extract; S: Supernatant; SAB: Supernatant after cellulose binding; DAD: Digested and dialysed; S4B1: Sepharose 4B fraction 1; S4B2: Sepharose 4B fraction 2; S4B3: Sepharose 4B fraction 3; S4B4: Sepharose 4B fraction 4.

The digested and dialysed fraction was the only fraction that exhibited clearing zones in the zymogram (data not shown), showing endoglucanase activity. This was because the fraction contained the highest endoglucanase activity (1.936 nmol glucose released min^{-1} ml^{-1}) as compared to 0.253 nmol glucose released min^{-1} ml^{-1} in the crude extract. The other fractions did not contain high enough endoglucanase activity for detection by the zymogram method. The bands were also not clear in the zymogram possibly due to denaturation by acetone; however, it did confirm that endoglucanase activity was indeed present.

After characterisation of the cellulosomal and non-cellulosomal fractions, the subunits obtained in the SDS-PAGE were identified. In order to do this, N-terminal sequencing or MALDI-TOF analysis can be employed. The purification was repeated in order to obtain fresh samples for analysis. Figure 2.13 illustrates the Sepharose 4B chromatogram obtained. Three peaks resulted, with the first peak, the cellulosomal fraction, being the most prominent.

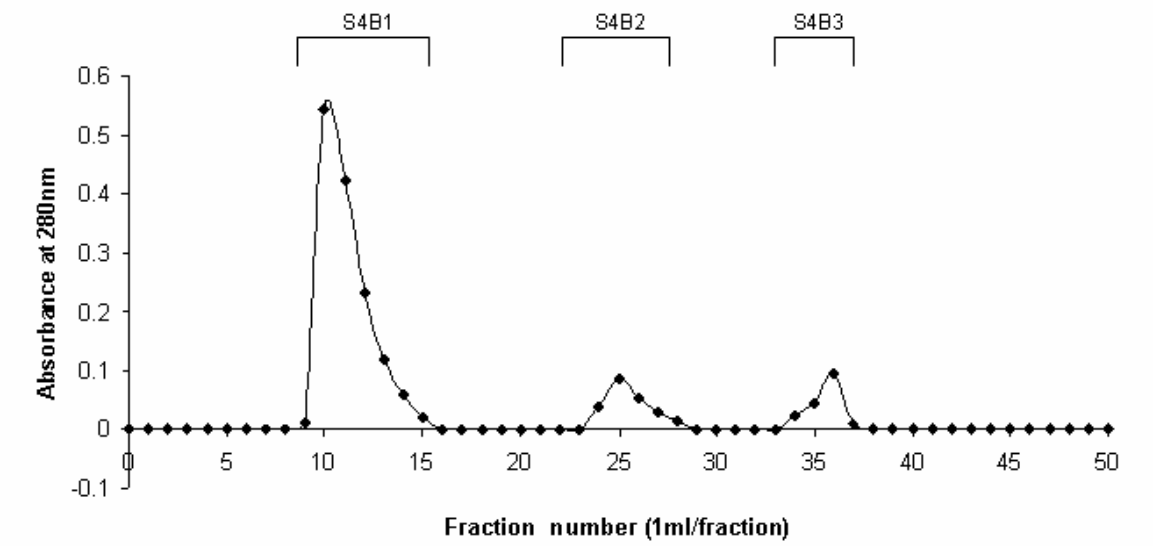

Figure 2.13 The second Sepharose 4B chromatogram of a cellulosome containing fraction from *C. beijerinckii*. Column dimensions, 30 x 1.5 cm and flow rate, 20 ml h^{-1}.

The cellulase and endoglucanase activities and protein concentration were determined in order to complete the purification tables for both cellulases (table 2.7) and endoglucanases (table 2.8). The purification was successful for the cellulases and as with the previous purification (table 2.6) the endoglucanase specific activities and fold purification values were rather low in comparison to previous purifications.

Table 2.7 Purification table for cellulases of the cellulosome of *C. beijerinckii* sLM01

Fraction Name	Total Volume[a]	Activity[b]	Protein Concentration[c]	Total Protein[d]	Total Activity[e]	Specific Activity[f]	% Yield	Fold Purification
Crude Extract	955	1.309	0.091	86.91	1250	14.38	100.0	1.00
Supernatant	863	1.023	0.055	47.47	882.8	18.60	70.6	1.29
Supernatant after binding	789	0.501	0.022	17.36	395.3	22.77	31.6	1.58
Digested and Dialysed	8	3.702	0.652	5.216	29.62	5.678	2.4	0.39
S4B1	64	0.699	0.101	6.464	44.74	6.920	3.6	0.48
S4B2	49	0.847	0.020	0.980	41.50	42.35	3.3	2.95
S4B3	36	0.737	0.012	0.432	26.53	61.42	2.1	4.27

[a] ml; [b] nmol glucose released min^{-1} ml^{-1}; [c] mg ml^{-1}; [d] mg; [e] nmol glucose released min^{-1}; [f] nmol glucose released mg^{-1} protein min^{-1}.

Table 2.8 Purification table for endoglucanases of the cellulosome of *C. beijerinckii* sLM01

Fraction Name	Total Volume[a]	Activity[b]	Protein Concentration[c]	Total Protein[d]	Total Activity[e]	Specific Activity[f]	% Yield	Fold Purification
Crude Extract	955	0.446	0.091	86.91	425.5	4.896	100.0	1.00
Supernatant	863	0.050	0.055	47.47	43.15	0.909	10.1	0.19
Supernatant after binding	789	0.006	0.022	17.36	4.734	0.273	1.1	0.06
Digested and Dialysed	8	2.250	0.652	5.261	18.45	3.451	4.3	0.70
S4B1	64	0.072	0.101	6.464	4.574	0.713	1.1	0.15
S4B2	49	0.006	0.020	0.980	0.271	0.300	0.06	0.06
S4B3	36	0.006	0.012	0.432	0.198	0.500	0.05	0.10

[a] ml; [b] nmol glucose released min^{-1} ml^{-1}; [c] mg ml^{-1}; [d] mg; [e] nmol glucose released min^{-1}; [f] nmol glucose released mg^{-1} protein min^{-1}.

SDS-PAGE results showed clear defined bands (see figure 2.14). The cellulosomal fraction (S4B1) contained three bands, at about 95 kDa, 60 kDa and 40 kDa, respectively.

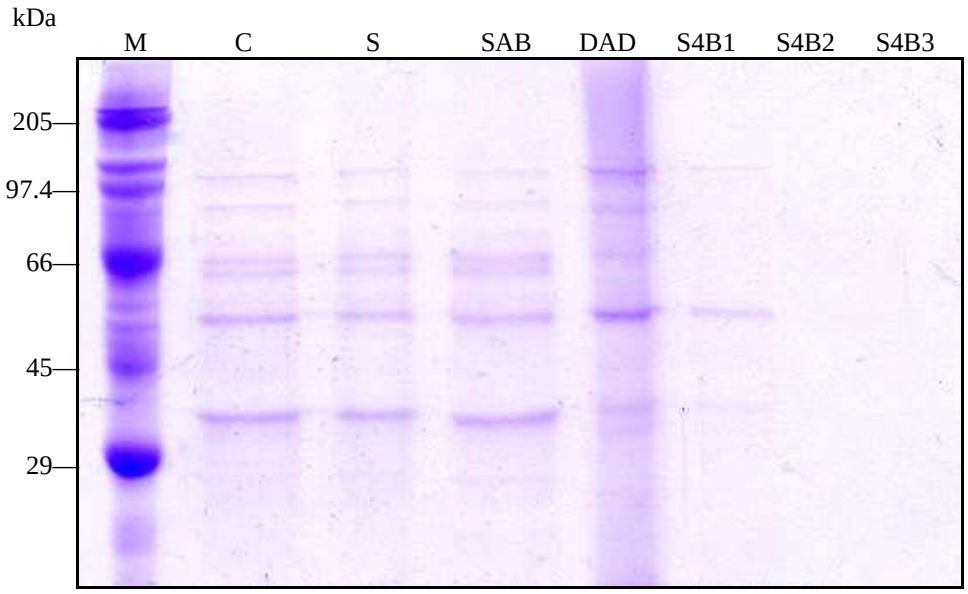

Figure 2.14 SDS PAGE (10 %) of purification of the cellulosome of *C. beijerinckii* M: Molecular weight marker (Sigma 29- 205 kDa); C: Crude extract; S: Supernatant; SAB: Supernatant after cellulose binding; DAD: Digested and dialysed; S4B1: Sepharose 4B fraction 1; S4B2: Sepharose 4B fraction 2; S4B3: Sepharose 4B fraction 3.

2.4.6 MALDI-TOF analysis

The samples that were sent for MALDI-TOF analysis were subunits 1-6 (figure 2.15) of the digested and dialysed (DAD) fraction (figure 2.14). Figures 2.16-2.18 illustrate the MALDI-TOF mass spectra of a tryptic digest of subunit 2, subunit 4 and subunit 5, respectively. MALDI-TOF analysis for the other subunits was not successful.

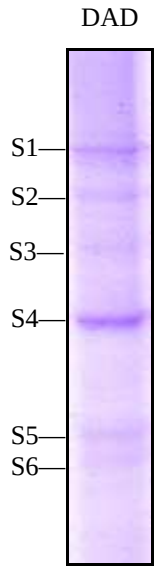

Figure 2.15 Subunits analysed with MALDI-TOF. S1-S6 denotes subunits 1-6.

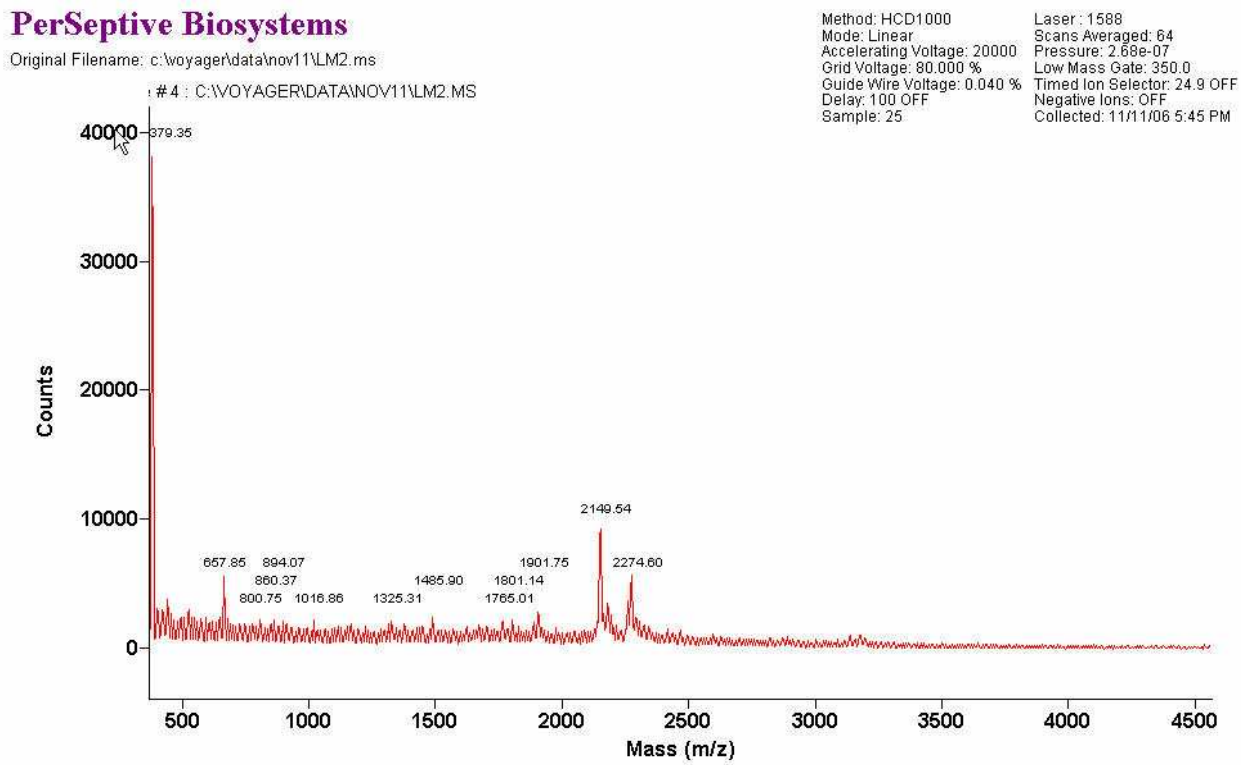

Figure 2.16 MALDI-TOF mass spectrum of a trypic digest of subunit 2

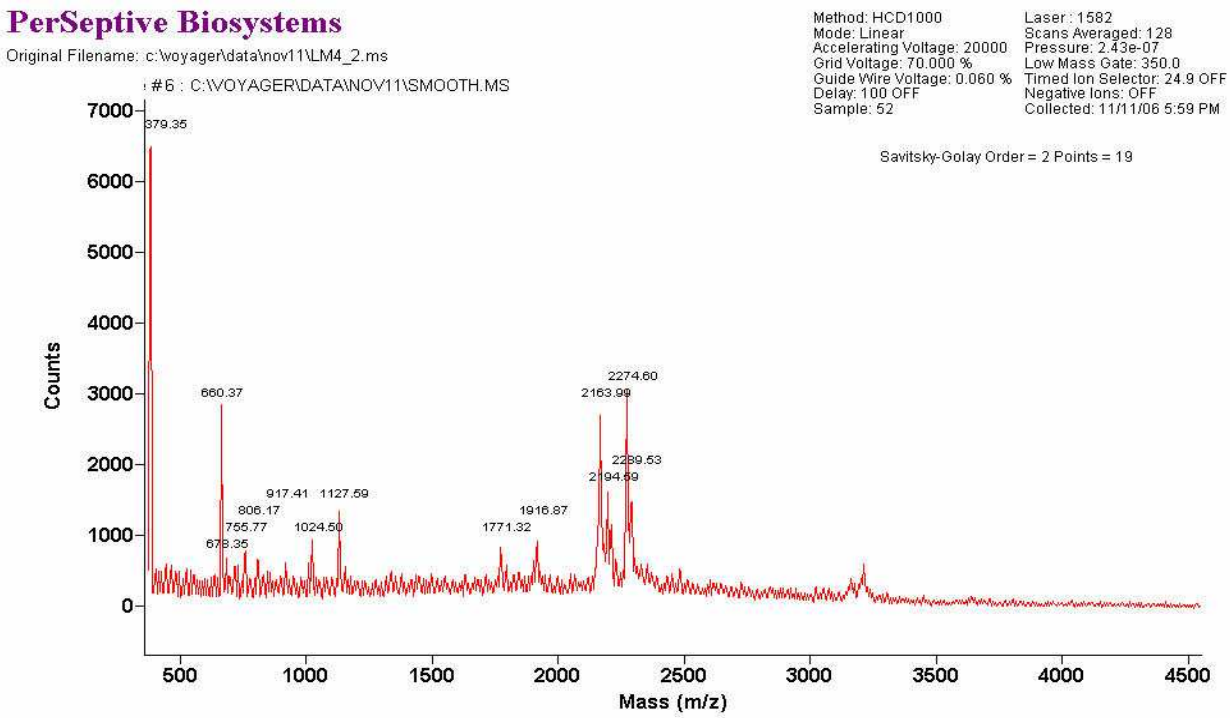

Figure 2.17 MALDI-TOF mass spectrum of a trypic digest of subunit 4

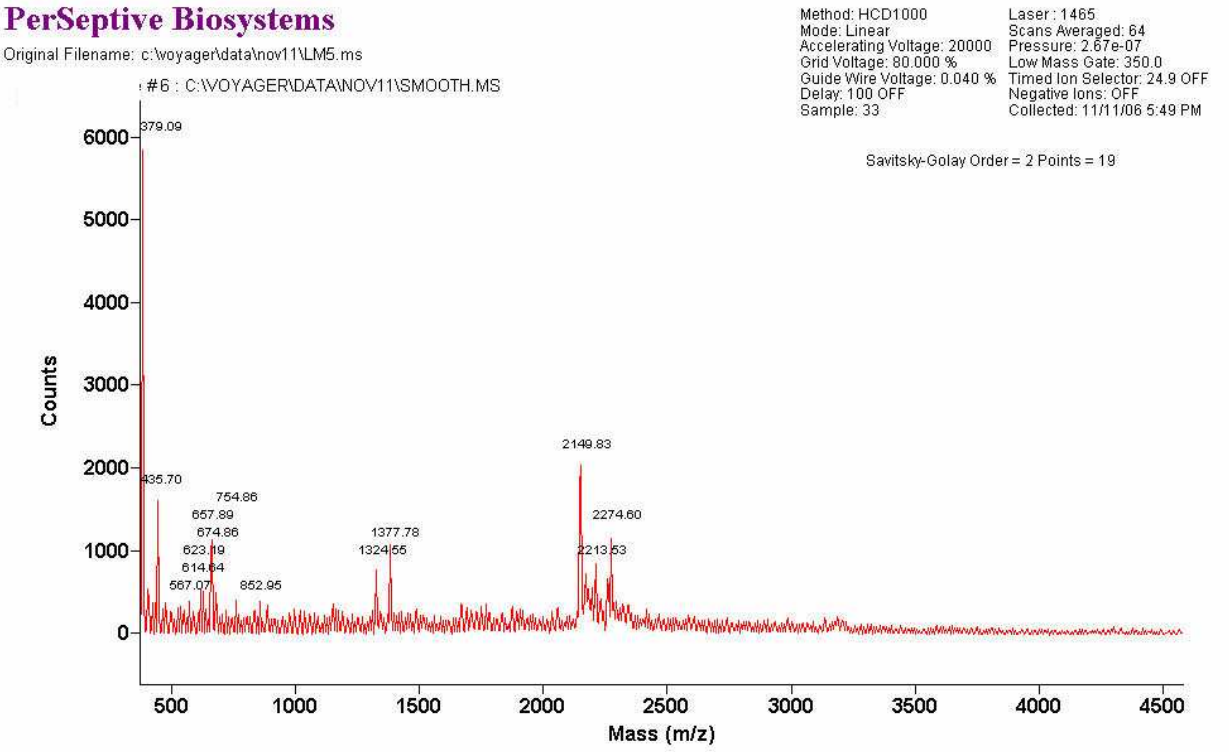

Figure 2.18 MALDI-TOF mass spectrum of a trypic digest of subunit 5

For the MALDI-TOF analysis, the NCBI protein search was employed in order to obtain the protein sequence of trypsin, exoglucanases and endoglucanases in FASTA format, these were then entered into the PeptideMass Expasy tool and the masses (m/z) produced after digestion with trypsin were produced. Since trypsin also cleaves itself, its masses would also be present in the MALDI-TOF mass spectra and this was taken into account. The exoglucanases that were produced by the NCBI website protein search belonged to *C. thermocellum*, *C. josui* and *C. cellulovorans*. Exoglucanases belonging to *C. acetobutylicum* or *C. beijerinckii* were not available and therefore the exoglucanases of the mesophiles (*C. josui* and *C. cellulovorans*) were used. The masses of all three subunits were compared with those of the exoglucanases and subunit 2 had the highest percentage match with the 80 kDa exoglucanase of *C. josui*, which has the same modular structure as *C. acetobutylicum* (Doi *et al.*, 2003). Endoglucanases were searched using the NCBI protein search and various endoglucanases produced by *C. acetobutylicum* were obtained. The masses of all three subunits were compared with those of the endoglucanases and subunit 4 had the highest percentage match with the 60 kDa endoglucanase from the endoglucanase family 9. Subunit 5 had the highest percentage match with the 41 kDa endoglucanase family 5.

For subunit 2, of the 13 masses produced (figure 2.17), 46.2 % matched with those of the exoglucanase, 38 % matched with trypsin and the rest of the masses did not match with either. Trypsin digested 80 kDa exoglucanase of *C. josui* produces a total of 41 masses ranging from 549.26 to 7013.26 (PeptideMass), and the produced masses matched with 14.6 % of the total known masses of the exoglucanase.

For subunit 4, of the 14 masses produced in figure 2.18, 57.1 % matched with those of the 60 kDa endoglucanase of *C. acetobutylicum*, 28.5 % matched to trypsin and the rest did not match with either. The trypsin digested endoglucanase contained a total of 31 masses ranging from 603.30 to 7873.52 (PeptideMass), and the produced masses matched with 25.8 % of the total known masses of the endoglucanase.

For subunit 5, of the 13 masses produced in figure 2.19, 46.2 % matched with those of the 41 kDa endoglucanase of *C. acetobutylicum*, 15.4 % matched to trypsin and the rest did not match with either. The endoglucanase contained a total of 25 masses ranging from 577.30 to 4102.99, the produced masses therefore matched with 24.0 % of the total known masses of the endoglucanase.

Figure 2.19 illustrates the relative molar amounts of the subunits of the cellulosome (figure 2.15) isolated from *C. beijerinckii* sLM01. The molar amount of each band was expressed relative to the S1 (subunit 1) band, which was arbitrarily assigned a value of 1. As the graph shows, subunit 4 had the highest relative molar amount and therefore was the most dense or concentrated subunit.

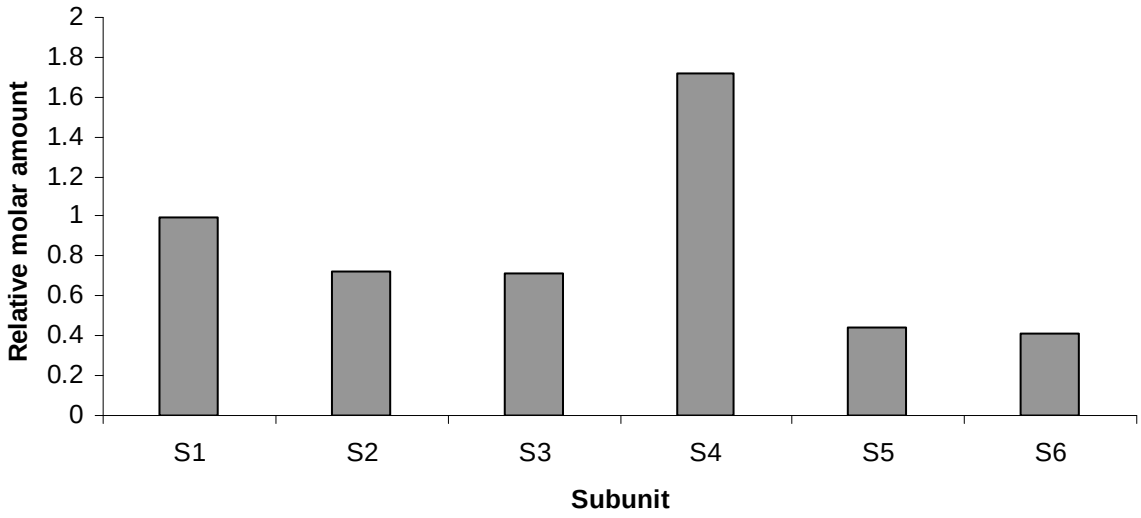

Figure 2.19 Relative molar amounts of the subunits isolated from the cellulosome of *C. beijerinckii*

2.5 DISCUSSION

A clostridial strain that contained the highest cellulase activity and was most affected by increased additions of sulphide was selected for subsequent purification of the cellulosome. The anaerobe was identified using partial 16S rDNA sequencing, where 500 bp of the region was PCR amplified, cloned into pGEM-t® Easy and subsequently sequenced. This was repeated using different primers in order to obtain the complete 16S rDNA region of about 1500 bp and a sequence of about 1300 bp was produced (see Appendix E). The sequences that were generated were entered into the nucleotide-nucleotide BLAST search and the resulting organism, with 99 % identity and 98 % identity for the 500 bp and 1300 bp, respectively were identified putatively as *Clostridium beijerinckii* (Accession number X68180.1). Further identification (e.g. performing metabolic studies) is required in order to confirm the identity of the organism. Scanning electron micrograph (SEM) was performed in order to visualise the isolated anaerobe.

After screening and identification, the organism's cellulosome was purified using two methods, the affinity chromatography purification procedure (Lamed and Bayer, 1988) and the affinity digestion purification procedure (Morag *et al.*, 1992). The affinity chromatography purification method exploits the adsorption ability of the cellulosome to crystalline cellulose, which is complex in structure. This is followed by an elution of the cellulosome from the cellulose using triethylamine (TEA), which elutes the proteins from the cellulose. The eluent is concentrated and applied to a Sepharose 4 B column.

The cellulose affinity step in the affinity digestion purification (Morag *et al.*, 1992) exploits the ability of the cellulosome of *C. beijerinckii* to adsorb to amorphous (phosphoric acid-swollen) cellulose. This ensures that proteins that do not hydrolyse cellulose are separated from those that are able to bind to the amorphous cellulose. The next step in the procedure was the digestion of cellulose by the cellulosome, which was performed in order to clarify the cellulose suspension and prevent the cellulosome from binding to the cellulose thereby allowing the cellulosome to be free in the suspension.

The suspension was centrifuged and the supernatant was applied onto a Sepharose 4B column. Cellulosomes are known to be macromolecular structures that range from 650 000 Da to 2.5 MDa (Doi *et al.*, 2003). The Sepharose 4B column has a fractionation range between 60 000 Da and 20 MDa and is often used in the purification of cellulosomes (Morag *et al.*, 1992; Boisset *et al.*, 1999).

The highest specific activities obtained for the purified cellulases and endoglucanases were 146.9 and 93.51 nmol glucose released mg^{-1} protein min^{-1}, respectively, for the affinity chromatography purification procedure, and 83.29 and 1.200 nmol glucose released mg^{-1} protein min^{-1}, respectively, for the affinity digestion purification procedure. Both methods were successful in purifying cellulases (which includes exoglucanases and endoglucanases). The affinity digestion purification procedure, however, was not as successful in the purification of endoglucanases as the affinity chromatography purification procedure. In particular, most of the endoglucanase activity was lost after the Sepharose 4B step; this was possibly due to dilution of the enzyme and leaching off the column. Another reason that the endoglucanase activity was low could be the fact that a larger column was used in the second purification and this could have led to the dilution of the endoglucanases. Cellulase activity, however, was retained, indicating that Sepharose 4B chromatography was a sufficient size exclusion medium for these enzymes. The affinity digestion purification procedure produced better resolved peaks in the chromatograms (figures 2.11 and 2.13) than that of the affinity chromatography purification procedure (figures 2.8 and 2.10). SDS-PAGE results were more clearly visible in the affinity digestion purification procedure (possibly due to a higher yield of protein) and there were also bands evident in the S4B1 fraction using this procedure (see figure 2.14).

Sabathé *et al.* (2002) stated that *C. acetobutylicum* or *C. bejeirinckii* do not have cellulase or exoglucanase activity, while the strain that was isolated in this study (*C. beijerinckii* sLM01) showed high cellulase activity. Perret *et al.* (2004) indicated that *C. acetobutylicum* has very low endoglucanase activity, again this contradicts our findings as the endoglucanase activity, before Sepharose 4B chromatography was fairly high.

Throughout the purification procedures, high cellulase and endoglucanase activities were observed. What is interesting is that Sabathé *et al.* (2002) and Perret *et al.* (2004) state that *C. acetobutylicum* contains a large cellulosomal gene cluster and hence has the genes that are required to produce cellulose-degrading enzymes. These genes are similar to those found in *C. cellulolyticum*, which has been shown to contain both exoglucanase and endoglucanase activity (Doi *et al.*, 2003; Desvaux, 2005). What Sabathé *et al.* (2002) have postulated is that maintenance of the ATCC 824 strain in laboratory conditions for several years, without a selective advantage for cellulose hydrolysis, could have led to the cellulosome defect. The *C. beijerinckii* strain that was used in this study was obtained from environmental samples, which could help to explain the difference in findings.

Due to problems with the equipment the MALDI-TOF data was not as reliable as expected. The analysed isolated subunits produced percentage matches ranging from 46.2- 57.1 %. These percentages do not conclusively indicate that the subunits obtained are exoglucanases and endoglucanases of *C. beijerinckii* as they are not high enough to indicate certainty. However, the molecular weights of subunits 2, 4 and 5 corresponded with those of the exoglucanase and endoglucanases that they obtained the highest percentage match with. To improve the results, a different MALDI-TOF matrix could be used that is suitable for both low and high masses. Also concentrating the protein further may improve the results as subunit 4, which had the highest relative molar amount, had the highest percentage match. Alternatively, to produce more accurate results the N-terminal sequencing method of identification of the proteins should rather be employed. This method has been used widely in the cellulosome field (Kosugi *et al.*, 2001; Murashima *et al.*, 2002).

2.6 CONCLUSIONS

C. beijerinckii sLM01 was isolated from a biosulphidogenic bioreactor and identified using 16S rDNA analysis. The cellulosome was purified using affinity chromatography purification and affinity digestion purification methods. Both methods purified the cellulosomal and non-cellulosomal enzymes successfully; however, the affinity digestion purification procedure resulted in very low endoglucanase activities in the cellulosomal and non-cellulosomal fractions. The affinity digestion method had better resolved and separated peaks than that of the affinity chromatography purification method and it also produced more defined bands on SDS-PAGE. The MALDI-TOF results were not conclusive due in the error of the system used. The results did, however, appear to indicate that the subunits were a 80 kDa exoglucanase, a 60 kDa endoglucanase and a 41 Da endoglucanase.

CHAPTER 3

CHARACTERISATION OF THE CELLULOSOME OF *CLOSTRIDIUM BEIJERINCKII* sLM01

3.1 INTRODUCTION

C. beijerinckii sLM01 was isolated from a biosulphidogenic bioreactor and because of this environment, the characterisation of the cellulases and endoglucanases of the cellulosomal and non-cellulosomal fractions of *C. beijerinckii* included determining the effect of increased additions of sulphide, sulphate, cellobiose and glucose on these enzymes. In a biosulphidogenic bioreactor or environment sulphate is reduced by the sulphate reducing prokaryotes to sulphide (Mizuno *et al.*, 1997). Cellobiose and glucose are hydrolysis products obtained from the hydrolysis of cellulose (Malherbe and Cloete, 2002) and their effect on the cellulosome facilitates is therefore relevant. Further characterisation of the cellulases and endoglucanases in the cellulosomal and non-cellulosomal fractions included a determination of their pH and temperature optima. These two physico-chemical parameters and their effect on the activity of the enzymes is very relevant to bioreactor design and process optimisation.

3.2 AIMS

The overall objective of this chapter was to characterise the cellulosomal and non-cellulosomal fractions of *C. beijerinckii*.

In order to achieve this, the following aims were addressed:

a) to determine the effect of increased additions of sulphide, sulphate, cellobiose, glucose and acetate on the cellulase and endoglucanase activities of the cellulosomal and non-cellulosomal fractions of *C. beijerinckii*, and
b) to determine the pH and temperature optima of the cellulases and endoglucanases of the cellulosomal and non-cellulosomal fractions of *C. beijerinckii*.

3.3 MATERIALS AND METHODS

3.3.1 Effect of additions of suitable amounts of sulphide, sulphate, cellobiose, glucose and acetate on cellulase and endoglucanase activities of the cellulosomal and non-cellulosomal fractions

The pooled protein fractions obtained in section 2.4.5, which contained the highest cellulase activity, were used to determine the effect of sulphide, sulphate, cellobiose, glucose and acetate on the cellulase and endoglucanase activities of the cellulosome. These fractions were denoted S4B1 (Sepharose 4B fraction 1- the cellulosomal fraction) and S4B3 (Sepharose 4B fraction 3- the non-cellulosomal fraction). The samples (100 µl) were pre-incubated at room temperature in reaction tubes containing 200 µl of 0.05 M citrate buffer, pH 4.8 with a final concentration of sulphide 0-600 mg l^{-1} (0-18.7 mM), sulphate 0-600 mg l^{-1} (0-6.25 mM), cellobiose 0-600 mg l^{-1} (0-1.75 mM), glucose 0-600 mg l^{-1} (0-3.33 mM) and acetate 0-600 mg l^{-1} (0-9.99 mM), for 15 minutes. A volume of 50 µl Avicel (for cellulase) and 50 µl CMC (endoglucanase) substrates were added to their respective tubes and the assays were performed using the DNS method (Miller *et al.*, 1960). Samples were incubated at 50 ˚C for 30 min after which 200 µl DNS was added and the samples boiled for 5 min and put on ice for 10 min. The absorbance at 540 nm was then determined.

3.3.2 Effect of sulphide and acetate on pH

The effect on pH of increased sulphide (Merck) and acetate (SIGMA) additions of a final concentration of 0, 60, 120, 240, 300, 480 and 600 mg l^{-1} in 0.05 M citrate buffer (pH 4.8) was determined, where the final volume used was 5 ml. The pH was measured for each concentration and the difference in pH between 0 mg l^{-1} sulphide and 60, 120, 240, 300, 480 and 600 mg l^{-1} was determined.

3.3.3 pH and temperature optima determination of the cellulase and the endoglucanase activities in the cellulosomal and non-cellulosomal fractions of C. beijerinckii sLM01.

The effect of pH and temperature on cellulase and endoglucanase activities was determined using fractions S4B1 and S4B3. In order to determine the pH optimum of each fraction, the assays were performed as described in section 2.3.2 using Avicel and CMC as substrates and buffers of varying pH. The following range of 0.05 M buffers were used; citrate buffer (pH 3-6) and phosphate buffer (pH 6-8). Temperature optima studies were performed on the fractions using the pH optima obtained and incubating the reaction at varying temperatures (15-50 ˚C).

3.4 RESULTS

3.4.1 Effect of additions of suitable amounts of sulphide, sulphate, cellobiose, glucose and acetate on cellulase and endoglucanase activities of cellulosomal and non-cellulosomal fractions

Fractions S4B1 and S4B3 (section 2.4.5, figure 2.11) were used to determine the effect of sulphide, sulphate, cellobiose, glucose and acetic acid on the cellulase and endoglucanase activities of the cellulosomal and non-cellulosomal fractions of *C. beijerinckii* sLM01, respectively. The activity, U, was expressed as nmol glucose released min^{-1} ml^{-1}. Figures 3.1 and 3.2 illustrate the effect of sulphide and sulphate, and cellobiose and glucose, respectively on the cellulase activity of the cellulosomal fraction. As figure 3.1 illustrates, sulphide activated the cellulase activity, there was a 60 % increase in activity at 120 mg l^{-1} of sulphide and then a decrease in activity occurred. At 480-600 mg l^{-1} of sulphide, the cellulase activity was stimulated by sulphide. As figure 3.1 indicates, increased additions of sulphate activated the cellulase activity; however, not to the same extent as additions of sulphide, as there was only a 12.5 % increase in activity at 600 mg l^{-1} of sulphate.

Increased additions of cellobiose and glucose (figures 3.2) inhibited cellulosomal cellulase activity. Higher additions of glucose inhibited the cellulase activity more so than cellobiose, at an addition of 240 mg l^{-1} cellobiose there was activity observed while at 240 mg l^{-1} glucose no cellulase activity was detected. The higher inhibition by glucose was due to the fact that glucose is a less complex molecule than cellobiose and it is the final product of cellulose hydrolysis. The products of hydrolysis of cellulose inhibit the activity. At higher concentrations than 240 mg l^{-1} of cellobiose, the enzyme was inhibited completely and no further activity was detected.

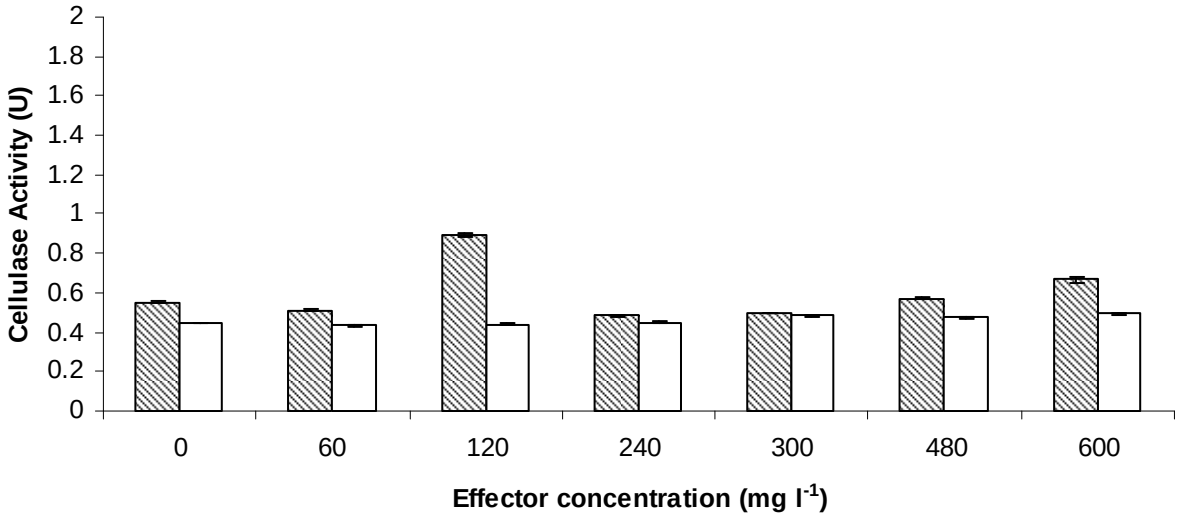

Figure 3.1 The effect of increased additions of sulphide (▨) and sulphate (□) on cellulosomal cellulase activity of *C. beijerinckii*. Values are expressed as means ± SD (*n*=3).

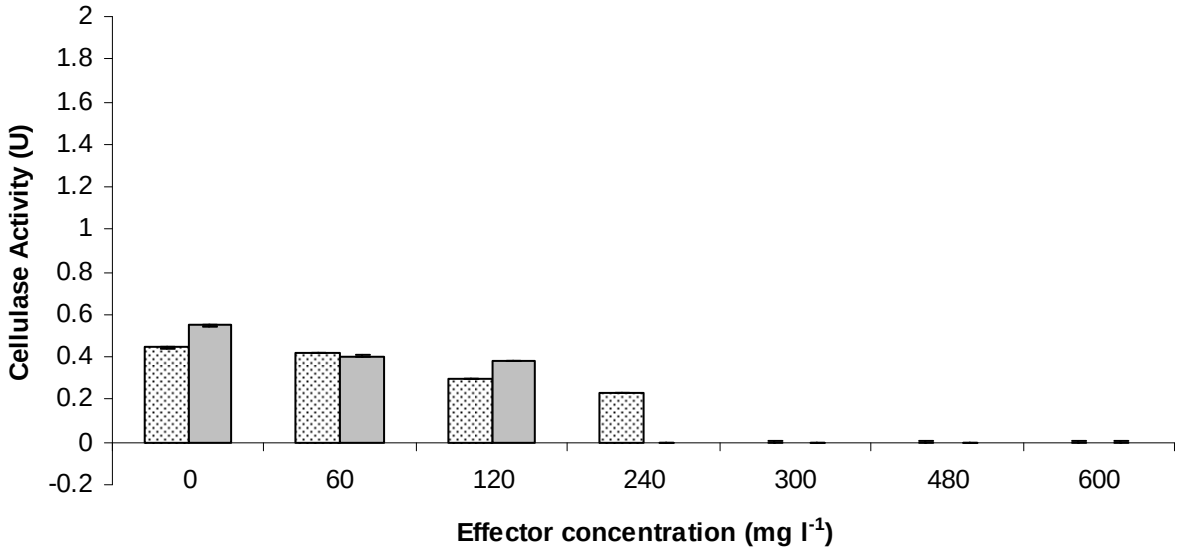

Figure 3.2 The effect of increased additions of cellobiose (⊡) and glucose (▭) on cellulosomal cellulase activity of *C. beijerinckii*. Values are expressed as means ± SD (*n*=3).

Figures 3.3 and 3.4 illustrate the effect of sulphide and sulphate, and cellobiose and glucose, respectively on non-cellulosomal cellulase activity. As figure 3.3 illustrates, increased additions of sulphide activated non-cellulosomal cellulase activity. There was a dramatic increase between 240 mg l^{-1} sulphide and 300 mg l^{-1} sulphide. The activity was at its highest at 300 mg l^{-1} sulphide resulting in a 202 % increase in non-cellulosomal cellulase activity. The activity remained high and began to decrease at 600 mg l^{-1} sulphide; however the activation of the enzyme was still 127 % percent higher than at 0 mg l^{-1} of sulphide. Figure 3.3 illustrates that sulphate inhibited the non-cellulosomal cellulase activity; at 600 mg l^{-1} sulphate, the decrease in activity was 30 %. Increased additions of cellobiose and glucose also inhibited the cellulase activity as shown in figure 3.4. The concentration at which no cellulase activity was detected was 240 mg l^{-1} and 480 mg l^{-1} for cellobiose and glucose, respectively.

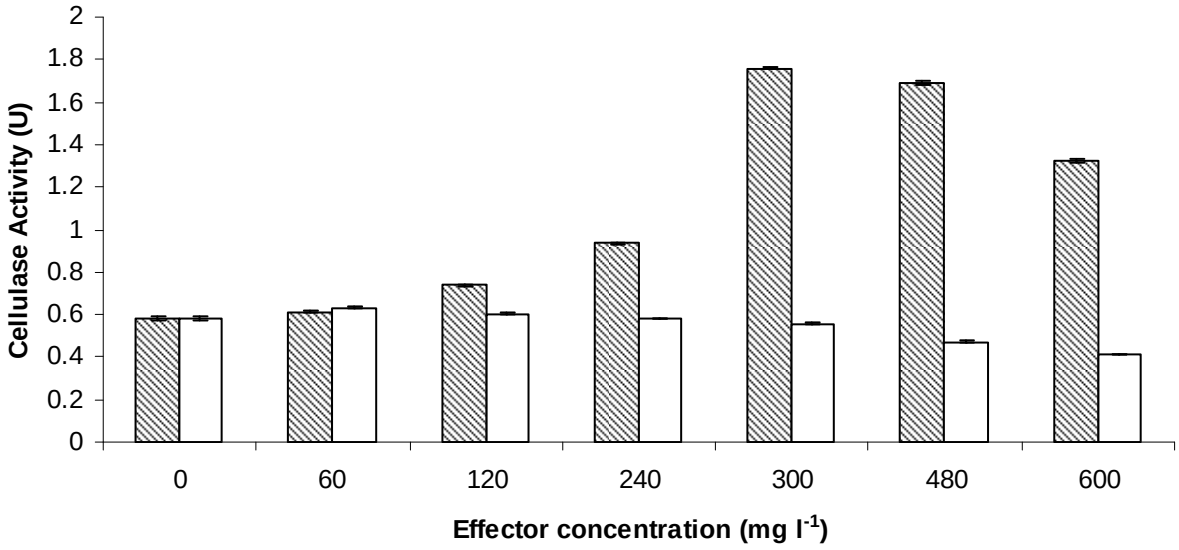

Figure 3.3 The effect of increased additions of sulphide (▨) and sulphate (□) on non-cellulosomal cellulase activity of *C. beijerinckii*. Values are expressed as means ± SD (*n*=3).

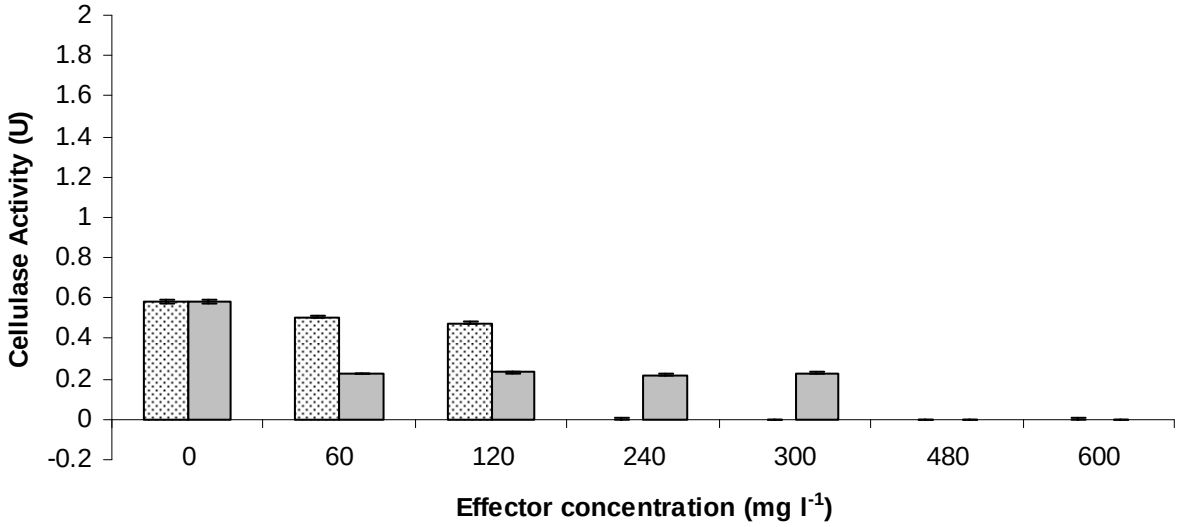

Figure 3.4 The effect of increased additions of cellobiose (▩) and glucose (▩) on non-cellulosomal cellulase activity of *C. beijerinckii*. Values are expressed as means ± SD (*n*=3).

Figure 3.5 illustrates that increased additions of acetate inhibited the cellulosomal and non-cellulosomal cellulase activities to some degree. The inhibition was slight and complete inhibition of the enzyme did not occur. The cellulase activity was decreased by 18.6 % and 12.3 % at 600 mg l^{-1} for the cellulosomal and non-cellulosomal fractions, respectively. This shows that acetate has a higher inhibition effect on cellulosomal cellulases than non-cellulosomal cellulases.

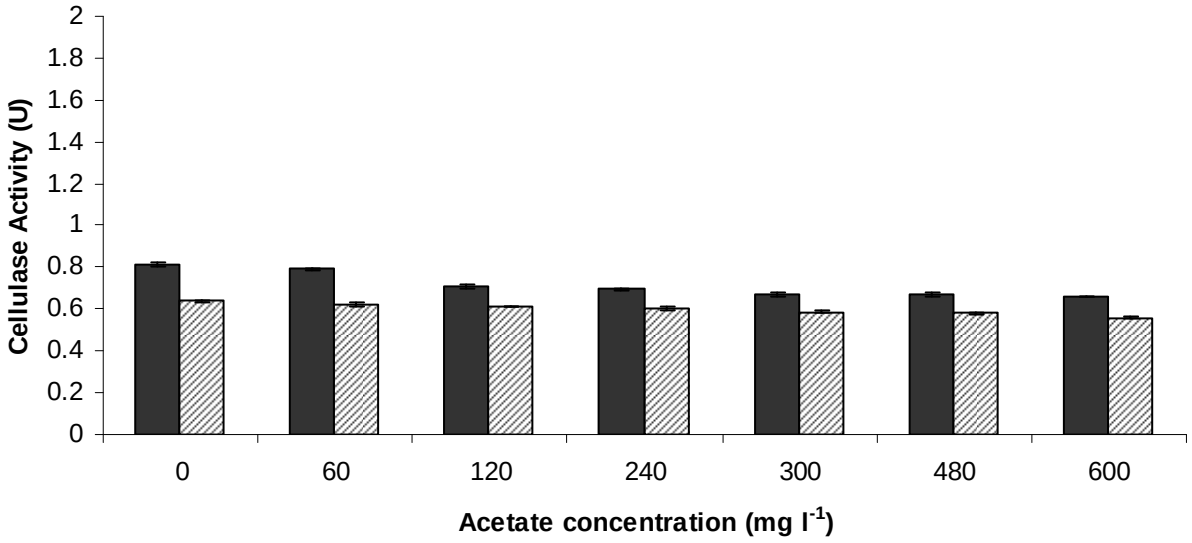

Figure 3.5 The effect of increased additions acetate on the cellulosomal (■) and non-cellulosomal (▨) cellulase activities of *C. beijerinckii*. Values are expressed as means ± SD (*n*=3).

Figure 3.6 shows that sulphide activated cellulosomal endoglucanase activity. The effect was dramatic, a 700 % increase in endoglucanase activity was evident at 300 mg l^{-1} sulphide, which was the highest activity obtained, 0.044 U. Sulphate also increased activity (figure 3.6), albeit not as dramatically as sulphide. The maximal activity at 600 mg l^{-1} was 0.011 U and that constituted a 50 % increase in endoglucanase activity.

As figure 3.7 shows, both cellobiose and glucose strongly inhibited the cellulosomal endoglucanase activities, at 60 mg l^{-1} cellobiose concentrations and higher and at 120 mg l^{-1} glucose concentrations and higher, no further activity was observed.

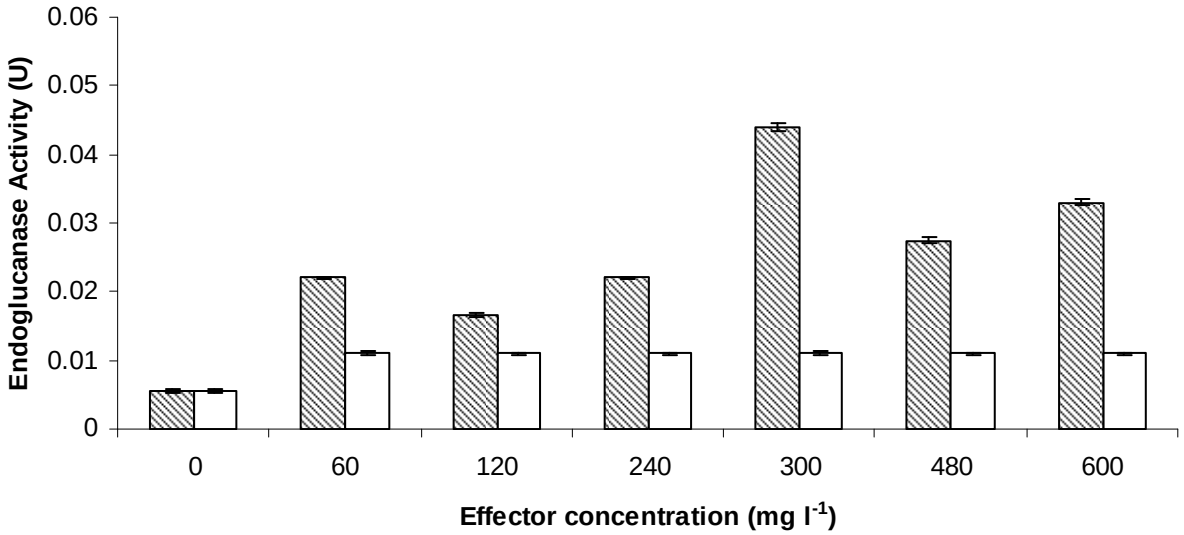

Figure 3.6 The effect of increased additions of sulphide (▨) and sulphate (□) on cellulosomal endoglucanase activity of *C. beijerinckii*. Values are expressed as means ± SD (*n*=3).

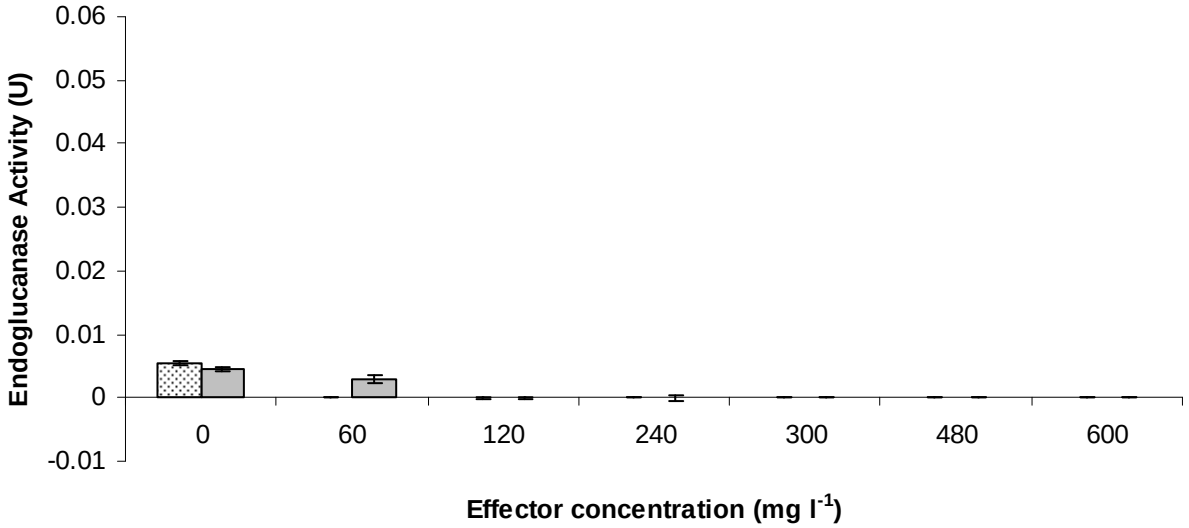

Figure 3.7 The effect of increased additions of cellobiose (▨) and glucose (▨) on cellulosomal endoglucanase activity of *C. beijerinckii*. Values are expressed as means ± SD (*n*=3).

Figure 3.8 shows that sulphide activated non-cellulosomal endoglucanase activity. There was a steady increase in activity with an increase of sulphide and the highest activity was observed at 600 mg l^{-1} sulphide (56.6 % increase in activity). Figure 3.8 illustrates that increased additions of sulphate inhibited non-cellulosomal endoglucanase activity dramatically, where no endoglucanase activity was detected at sulphate concentrations ≥ 120 mg l^{-1}. Cellobiose and glucose inhibited the non-cellulosomal endoglucanase activity in this fraction as well (figure 3.9). Figure 3.10 illustrates that increased additions acetate inhibited the cellulosomal and non-cellulosomal endoglucanase activities to some degree. The endoglucanase activities were decreased by 16.7 % and 10.4 % at 600 mg l^{-1} for the cellulosomal and non-cellulosomal fractions, respectively. Acetate also has a higher inhibition effect on cellulosomal endoglucanases than non-cellulosomal endoglucanases.

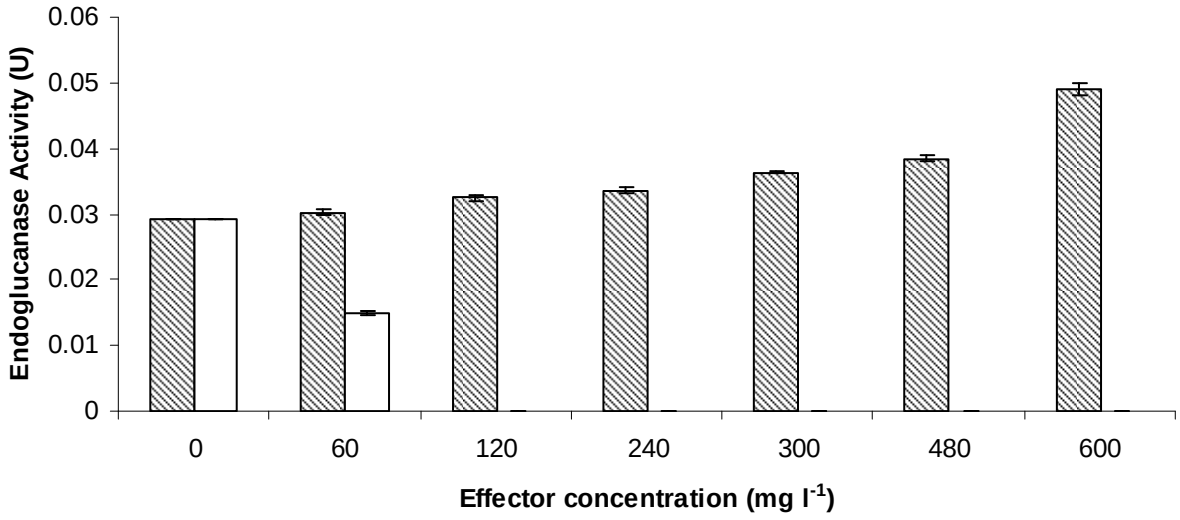

Figure 3.8 The effect of increased additions of sulphide (▨) and sulphate (□) on non-cellulosomal endoglucanase activity of *C. beijerinckii*. Values are expressed as means ± SD (*n*=3).

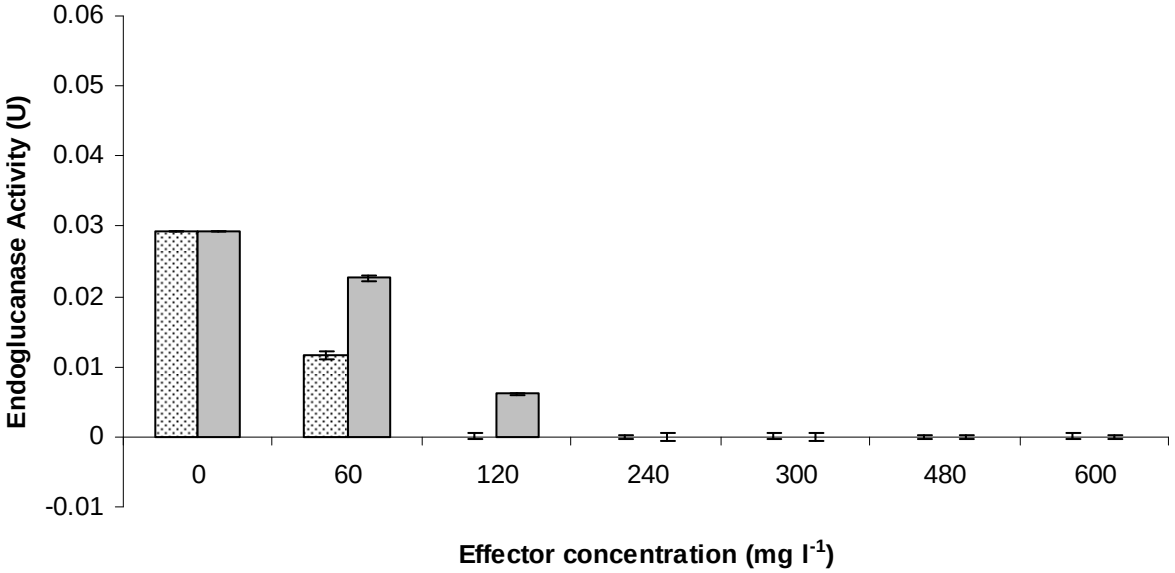

Figure 3.9 The effect of increased additions of cellobiose (⊡) and glucose (▭) on non-cellulosomal endoglucanase activity of *C. beijerinckii*. Values are expressed as means ± SD (*n*=3).

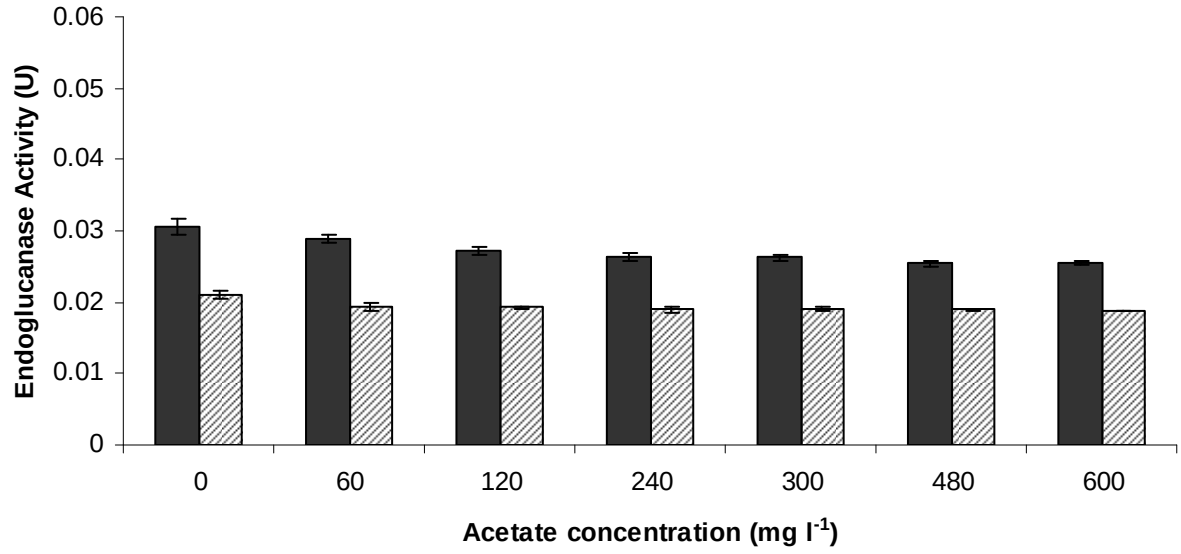

Figure 3.10 The effect of increased additions acetate on the cellulosomal (■) and non-cellulosomal (▨) endoglucanase activities of *C. beijerinckii*. Values are expressed as means ± SD (*n*=3)

3.4.2 Effect of sulphide and acetate on pH

The pH values of the 0.05 M citrate buffer (pH 4.8) containing final concentrations of 0, 60, 120, 240, 300, 480 and 600 mg l^{-1} of sulphide were 4.76, 4.93, 5.05, 5.40, 5.47, 5.96 and 6.45, respectively. The difference in pH between 0 mg l^{-1} sulphide and 60, 120, 240, 300, 480 and 600 mg l^{-1} was 0.17, 0.29, 0.64, 0.71, 1.20 and 1.69 pH units, respectively.

The pH values of the 0.05 M citrate buffer (pH 4.8) containing final concentrations of 0, 60, 120, 240, 300, 480 and 600 mg l^{-1} of acetate were 4.81, 4.78, 4.77, 4.76, 4.74, 4.73 and 4.72, respectively. The difference in pH between 0 mg l^{-1} acetate and 60, 120, 240, 300, 480 and 600 mg l^{-1} was -0.03, -0.04, -0.05, -0.07, -0.08 and -0.09 pH units, respectively.

3.4.3 pH and temperature optima determination of cellulase and endoglucanase activities in the cellulosomal and non-cellulosomal fractions of C. beijerinckii.

Figure 3.11 shows the pH optimum profiles obtained for cellulase activity for the cellulosomal and the non-cellulosomal fractions. Figure 3.11 illustrates that the observed pH optima of cellulase activity of the cellulosomal fraction were pH 5.0 and pH 7.5, respectively (indicating that it possibly has heterogeneous enzyme composition) and in contrast, a single pH optimum for the non-cellulosomal fraction was observed at pH 7.5. Figure 3.12 shows that the pH optima of endoglucanase activity of the cellulosomal and non-cellulosomal fractions were at pH 5.0 and pH 7.5, respectively.

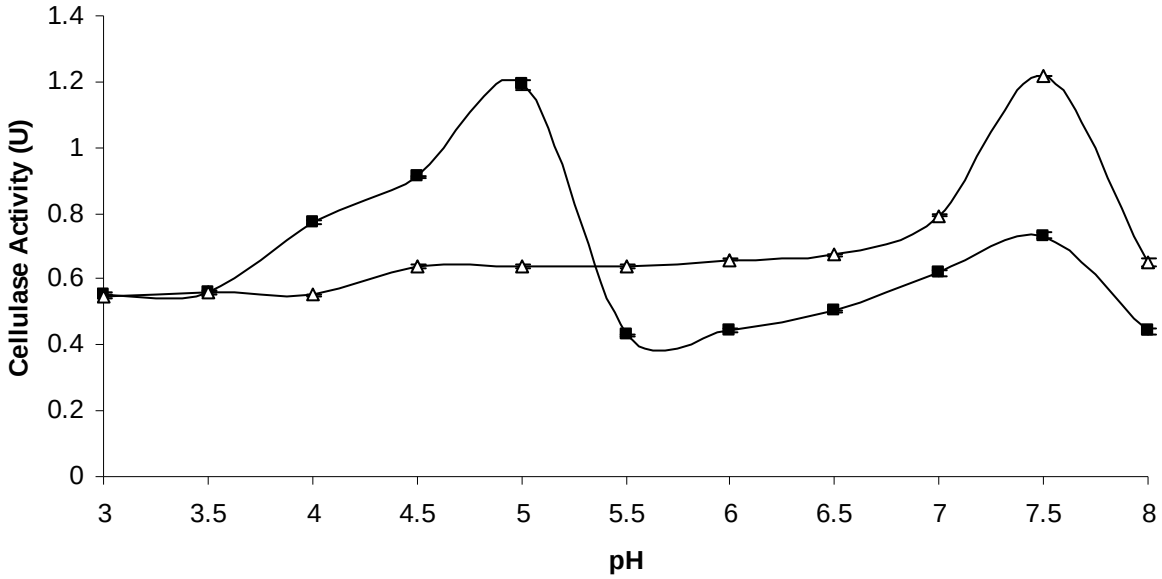

Figure 3.11 pH profiles of cellulosomal (■) and non-cellulosomal (△) cellulase activities. Values are expressed as means ± SD (*n*=3).

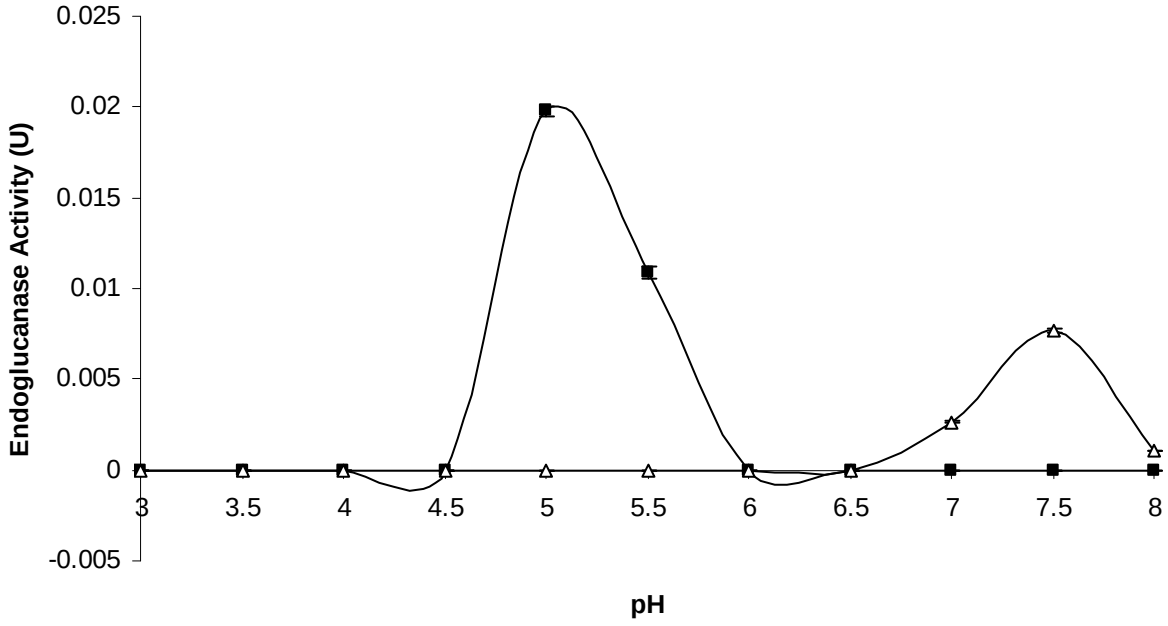

Figure 3.12 pH profiles of cellulosomal (■) and non-cellulosomal (△) endoglucanase activities. Values are expressed as means ± SD (*n*=3).

For temperature optima determination, cellulases and endoglucanases containing fractions S4B1 and S4B3 were incubated at their respective pH optima. Figure 3.13 shows the temperature optimum profiles obtained for the cellulases for the cellulosomal and non-cellulosomal fractions. Figure 3.14 shows the temperature optimum profiles obtained for the endoglucanases for the cellulosomal and non-cellulosomal fractions. The temperature optimum obtained for each enzyme of each fraction was 30 ºC.

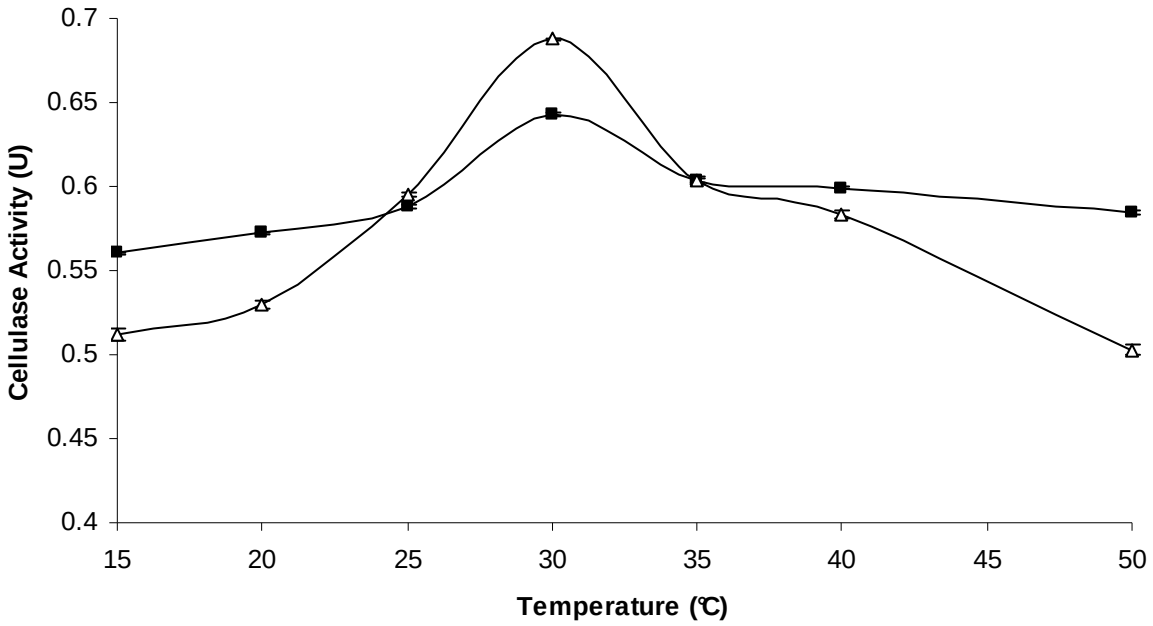

Figure 3.13 Temperature profiles of cellulosomal (■) and non-cellulosomal (△) cellulase activities. Values are expressed as means ± SD (*n*=3).

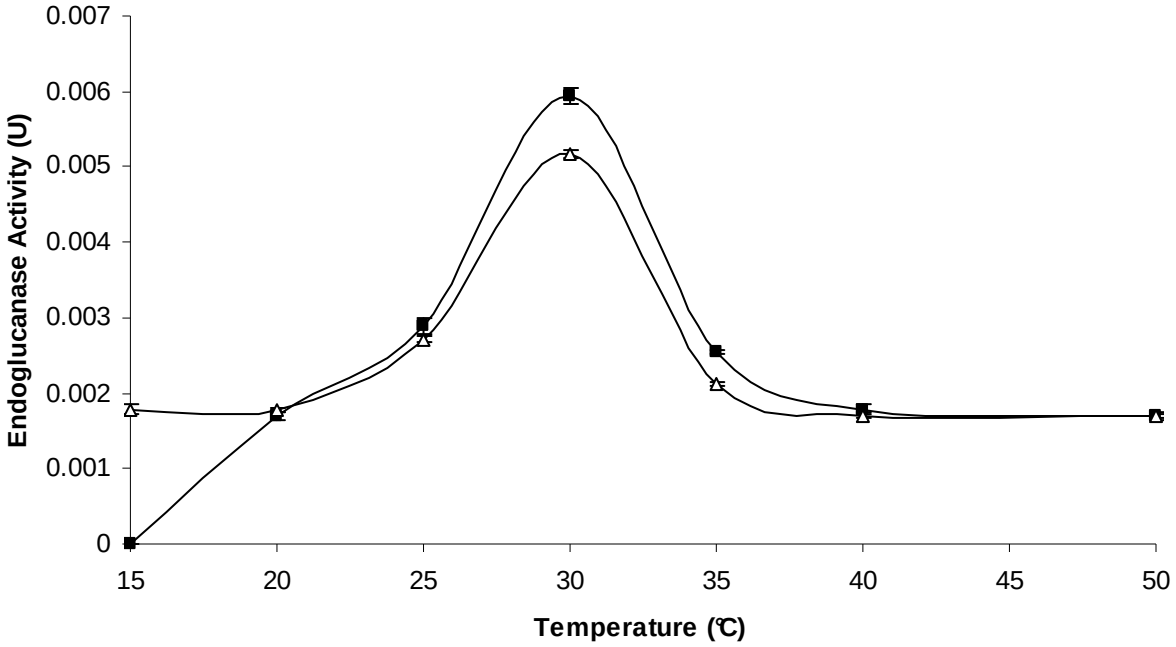

Figure 3.14 Temperature profiles of cellulosomal (■) and non-cellulosomal (△) endoglucanase activities. Values are expressed as means ± SD (*n*=3).

3.5 DISCUSSION

Sulphide activated the cellulase and endoglucanase activities in both the cellulosomal and non-cellulosomal fractions (S4B1 and S4B3). The effect was dramatic- for example cellulosomal endoglucanase activity was activated by 700 % at a level of 300 mg l^{-1} of sulphide. Work performed by Watson *et al.* (2004) and Whiteley *et al.* (2002) have also shown that increased additions of sulphide are able to activate the activity of hydrolases.

Whiteley *et al.* (2002) studied β-glucosidases, which are not present in the cellulosome complex; however, they do hydrolyse the hydrolytic product of cellulose hydrolysis by the cellulosome, cellobiose, once it is taken up by the cell (Coughlan, 1991). β-glucosidases are one set of enzymes that are encompassed in the term "cellulases" and therefore the results of the Whiteley *et al.* (2002) study are relevant to our findings.

Watson and Pletschke (2006) reported activation of α-glucosidase activity with increased additions of sulphide, they also found inhibition of the enzyme with increased additions of sulphate. This coincides with what this study found with the non-cellulosomal fraction where the cellulase and endoglucanase activities were activated by increased additions of sulphide and inhibited by increased additions of sulphate. The cellulosomal fraction, S4B1, however, showed a slight increase in activity of the enzymes with increased sulphate concentration, suggesting that the two fractions are different in regards to the sensitivity to sulphate. The characterisation of the cellulosome in terms of activation or inhibition by sulphide and sulphate is a novel observation.

It was expected that cellobiose, a product of cellulose hydrolysis by the cellulosome, and glucose, the hydrolytic product of cellobiose hydrolysis by β-glucosidase, would inhibit the cellulase and endoglucanase activities of *C. beijerinckii*. Lamed *et al.* (1985) also found that cellobiose inhibited the activity of the enzymes in *C. thermocellum*. This is because the hydrolysis products act through a feedback inhibition.

C. beijerinckii breaks down polysaccharides into acids such as acetate and butyrate and also solvents including acetone, butanol and ethanol (Ezeji *et al.*, 2006); hence the effect of acetate was determined. It was determined that increased additions of acetate slightly inhibited the activities of cellulosomal and non-cellulosomal cellulases and endoglucanases. This was possibly due to the slight decrease in pH that resulted from the additions of acetate, however, given that the pH decrease was only 0.09 pH units at 600 mg l^{-1} the pH effect is not significant. The DNS cellulase assay is performed at pH 4.8 (Miller *et al.*, 1960), which was close to optimal pH of 5 that was determined for cellulosomal cellulases and endoglucanases. The decrease in pH therefore moved away from the optimal range.

Since sulphide may exert its effect via a change in pH, the pH of sulphide at a final concentration of 0-600 mg l^{-1} was determined. The results showed that there was slight increase in pH as the 0.05 M citrate buffer maintained the pH within a narrow range (pH change of 1.69 pH units at 600 mg l^{-1} was observed). This slight change suggests that pH is a potentially contributing factor in the activation of the enzymes. Sulphide is also a

reducing agent and this may be another way sulphide is able to activate these enzymes. Sulphide, like dithiothreitol (DTT) is a reducing agent and may act in reducing the disulphide bonds in the enzymes, thereby stimulating or activating their activities. Studies performed by Sá-Pereira *et al.* (2002) found that the reducing agent, DTT, enhanced xylanolytic activity of a *Bacillus subtilis* strain isolated from a hot-spring 2.5 fold. Lamed *et al.* (1985) showed that the thiol containing compound, cysteine, activated cellulase and endoglucanase activities of the cellulosome of *C. thermocellum*. This correlates with the findings in this study; however, the change in pH after addition of cysteine was not monitored by these reports.

The cellulosomal and non-cellulosomal fractions (S4B1 and S4B3) also differ with regards to their pH optima. The cellulosomal fraction had pH optima of 5.0 and 7.5 for cellulase activity and pH 5.0 for endoglucanase activity, while both sets of enzymes in the non-cellulosomal fraction exhibited a pH optimum of 7.5. Hobson and Wheatley (1993) indicated that previous studies on *Clostridia* have shown that they grow over a pH range of 6-8 and that the optima were about pH 7 to 7.5. This correlates with the determined pH optimum of 7.5. The temperature optimum for both fractions and enzymes was 30 ˚C. This was expected as *C. beijerinckii* is a mesophilic organism (Doi *et al.*, 2003), which was cultivated at 28 ˚C in our laboratory.

The standard pH for the cellulase assay is pH 4.8 and at this pH the cellulases and endoglucanases of the cellulosomal fraction were performing in their optimal range, while those of the non-cellulosomal fraction were not. This is due to the fact that the pH optima of the cellulosomal fraction for the cellulases were pH 5.0 and pH 7.5 and the pH optimum for endoglucanase activity was pH 5.0. The cellulases and endoglucanases of the non-cellulosomal fraction were not performing at their optimal pH as this was pH 7.5. In the following chapter the pH of the anaerobic bioreactor used was pH 8 (a variation of pH 7-8 was observed) and in this case the cellulase and endoglucanase enzymes with an optimal pH of 7.5 were operating in their optimal range. These include the cellulosomal cellulases, non-cellulosomal cellulases and non-cellulosomal endoglucanases.

3.6 CONCLUSIONS

The cellulosomal and non-cellulosomal fractions were characterised with regards to their pH, temperature and sensitivity to a variety of key metabolites. Increased additions of sulphide activated the cellulase and endoglucanase activities up to 700 % (cellulosomal endoglucanase activity), while increased additions of sulphate either increased the activity slightly or inhibited it dramatically, depending on the cellulosomal and non-cellulosomal fractions. Cellobiose and glucose both inhibited cellulase and endoglucanase activities. Increased additions of acetate slightly decreased the cellulase and endoglucanase activities of the cellulosomal and non-cellulosomal fractions. pH optima of 5.0 and 7.5 were observed for cellulosomal cellulases and 5.0 for cellulosomal endoglucanases. The non-cellulosomal fraction exhibited a pH optimum of 7.5 for both cellulase and endoglucanase activities. Both fractions exhibited a temperature optimum of 30 ˚C for both cellulases and endoglucanases.

CHAPTER 4

THE EFFECT OF SULPHIDE ON THE RATE-LIMITING STEP IN ANAEROBIC DIGESTION

4.1 INTRODUCTION

In chapter 3, it was established that sulphide activates the cellulases and endoglucanases of the cellulosomal and non-cellulosomal fractions of *C. beijerinckii* sLM01. In this chapter, this knowledge was applied to anaerobic digestion using serum bottles and subsequently using bench scale bioreactors. The degradation of cellulose in anaerobic digestion comprises the rate-limiting step (Mata-Alvarez *et al.*, 2000) and in this chapter sulphide is used in an attempt to accelerate this step. This addressed the overall hypothesis that sulphide is able to increase the rate-limiting step. The previous chapter served to prove this fundamentally and this chapter builds on this by utilising an anaerobic digestion system. In other words, this constitutes the application of the fundamental knowledge gained thus far.

Two studies were performed, a serum bottle study and a bioreactor study. The serum bottle study served as the preliminary study for the bioreactor study. This was done in order to optimise the parameters for the bioreactor study. Various changes in the operating parameters were made from the information obtained from the serum bottle study in order to improve the running of the anaerobic digester or bioreactor.

The parameters that were determined in each study were the same, that is: cellulase and endoglucanase activities, reducing sugar concentrations (glucose equivalents), chemical oxygen demand (COD), sulphide concentrations, pH and volatile fatty acid (VFA) levels. Various tests and controls were conducted and each parameter was included to determine the exact effect of sulphide on the anaerobic bioreactor.

4.2 AIMS

The overall objective of this chapter was to determine the effect of sulphide on anaerobic digestion containing putative *C. beijerinckii* sLM01.

To achieve this objective, the aims were to determine and monitor the following parameters during the course of each study:

a) cellulase and endoglucanase activities
b) reducing sugar concentrations
c) chemical oxygen demand (COD)
d) sulphide concentrations
e) pH, and
f) volatile fatty acids (VFAs)

4.3 MATERIALS AND METHODS

4.3.1 Set-up of serum bottles for serum bottle study

Four sets of duplicate serum bottles were set up. Grass was selected as the substrate since it's a major constituent of solid wastes. One set contained 5 % (w/v) milled and dried Kikuyu grass (*Pennisetum clandestinum*), which was the substrate, 0.1 M phosphate buffer, pH 8 and 300 mg l^{-1} final concentration of sulphide. The 300 mg l^{-1} final concentration of sulphide was selected because in the fundamental studies in chapter 3 maximal activation of cellulase and endoglucanase activities were at this concentration of sulphide or greater. The serum bottles were bubbled with nitrogen, sealed and autoclaved. A culture of *C. beijerinckii* sLM01 was added using a sterile syringe to a final concentration of 10 % (v/v). The second set of serum bottles contained 5 % (w/v) milled grass and 0.1 M phosphate buffer, pH 8, the bottles were bubbled with nitrogen, sealed and autoclaved. The *C. beijerinckii* sLM01 culture was inoculated under sterile conditions to a final concentration of 10 % (v/v). The third set contained 5 % (w/v)

milled grass and 300 mg l^{-1} final concentration of sulphide. The fourth set contained only 5 % (w/v) milled grass. These were then bubbled with nitrogen, sealed and autoclaved.

The final total volume in all the bottles was 125 ml. As figure 4.1 illustrates, the bottles were covered in foil in order to prevent photosynthesis from occurring within the serum bottles during the study. Sterile syringes were placed on the top of the bottles and set at 1 ml in order to measure any gas emissions during the study. The serum bottles were incubated at 28 ºC for 13 days with shaking at 100 rpm on an orbital shaker.

Figure 4.1 Serum bottles used in preliminary studies. **(A)** Syringe; **(B)** Serum bottle containing 5 % (w/v) milled grass, *C. beijerinckii* and 300 mg l^{-1} sulphide (test), or 5 % (w/v) milled grass and *C. beijerinckii* (control 1), or 5 % (w/v) milled grass and 300 mg l^{-1} sulphide (control 2), or 5 % (w/v) milled grass (control 3). All were made in 0.1 M phosphate buffer, pH 8 and in duplicate.

4.3.2 Set-up of bench scale bioreactors for bioreactor study

Due to the results obtained in the serum bottle study sections 4.4.1 to 4.4.6, several changes were made. Three bench scale bioreactors were set up. One contained 2 % (w/v) milled and dried Kikuyu grass, 0.1 M phosphate buffer, pH 8 and 600 mg l^{-1} final concentration of sulphide. The bioreactor was bubbled with nitrogen, sealed and autoclaved. A culture of *C. beijerinckii* was added under sterile conditions to a final concentration of 10 % (v/v). The second bioreactor contained 2 % (w/v) milled grass, and 0.1 M phosphate buffer, pH 8, it was bubbled with nitrogen, sealed and autoclaved. *C. beijerinckii* culture was added under sterile conditions to a final concentration of 10 % (v/v). The third bioreactor contained 2 % (w/v) milled grass and 0.1 M phosphate buffer, pH 8, bubbled with nitrogen, sealed and autoclaved. The final volume in all bioreactors was 2 l. Figure 4.2 shows that the bioreactors were covered in foil and connected to a zinc acetate trap (5 % ZnAc, w/v) in order to collect any release of hydrogen sulphide.

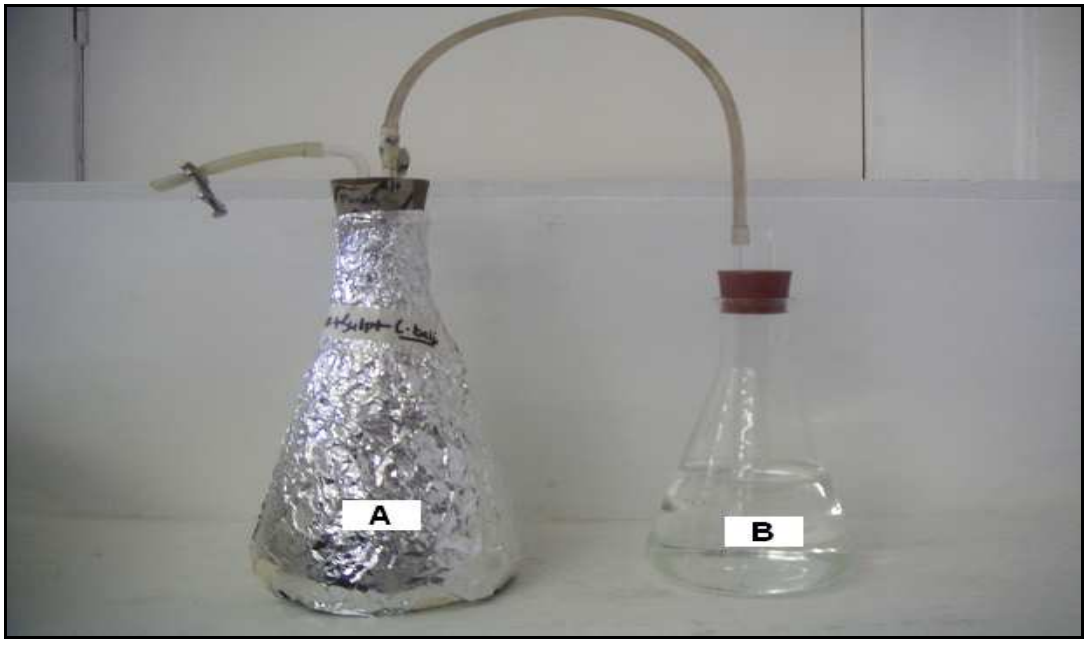

Figure 4.2 Representative bioreactor set up for degradation studies. **(A)** Bioreactor with 2 % (w/v) milled grass, *C. beijerinckii* and 600 mg l^{-1} sulphide, 2 % (w/v) milled grass and *C. beijerinckii* (control 1), or 2 % (w/v) milled grass (control 2). All bioreactor components were made in 0.1 M phosphate buffer, pH 8. **(B)** Zinc acetate trap.

4.3.3 Cellulase and endoglucanase assays

Serum bottle or bioreactor samples (100 µl) from each day of the studies were added reaction tubes containing 0.05 M citrate buffer, pH 4.8 and were incubated at 50 ˚C for 30 min with 50 µl of 2 % (w/v) Avicel (for cellulase) and 2 % (w/v) CMC (for endoglucanase). The experiments were conducted in triplicates. DNS (200 µl) was added and the samples were boiled for 5 min and put on ice for 10 min. The absorbance at 540 nm was then determined. Controls where no substrate was added were performed and the reducing sugars present were taken into account. Appendix A illustrates a typical glucose standard curve.

4.3.4 Reducing sugars determination

DNS was added to each sample obtained from the study and these were boiled for 5 min and put on ice for 10 min. The absorbance at 540 nm was then determined.

4.3.5 Chemical Oxygen Demand (COD) determination

COD determines the amount of organic compounds present. The COD Spectroquant® test was performed as described by the Spectroquant® kit. The measuring range that was used was 500- 10 000 mg l^{-1} COD. The COD readings were read using a Spectroquant® reader.

4.3.6 Sulphide concentration determination

The sulphide test was performed using N,N-dimethyl-p-phenylene diamine dihydrochloride solution, where 1 g of this salt was dissolved in 250 ml concentrated HCl. Ferric chloride solution was made by dissolving 4 g in 250 ml 6.0 M HCl. Zinc acetate solution was made by adding 5.22 g of zinc acetate to 500 ml distilled water. The sulphide stock solution was made by adding 0.150 g sodium sulphide nonahydrate in 200 ml distilled water to give a final concentration of 1.0 g l^{-1}. The standards used were 0,

0.2, 0.4, 0.6, 0.8 and 1.0 mg ml^{-1} sulphide. The assay was carried out as described by Rees *et al.* (1971) where 100 µl zinc acetate was added into a test tube, a diluted sample of final volume 5 ml was added, followed by 500 µl of ferric chloride solution, 500 µl of N,N-dimethyl-ρ-phenylenediamine dihydrochloride solution was added and the reaction mixture was allowed to stand for 1 hour and the absorbance at 670 nm was determined. Appendix C illustrates a typical sulphide standard curve.

4.3.7 pH determination

The pH of each sample during the two studies was measured.

4.3.8 Volatile fatty acids (VFAs) determination

A spectrophotometric determination of VFAs was employed. A 50 % (v/v) sulphuric acid solution was made where sulphuric acid was added slowly with constant stirring to 100 ml distilled water and it was allowed to cool. A 18 % (w/v) sodium hydroxide solution was made by dissolving 45 g sodium hydroxide in 200 ml distilled water, it was cooled and diluted to 250 ml with distilled water. Acidic ethane diol reagent was prepared by mixing 30 ml ethane diol with 4 ml of 50 % (v/v) sulphuric acid. This reagent was prepared freshly each day. A 10 % (w/v) hydroxyammoniun sulphate solution was made by adding 10 g hydroxyammonium sulphate to 80 ml of distilled water and then diluted to 100 ml with distilled water. This reagent was stored at 4 ºC. The hydroxylamine reagent was made by mixing 20 ml of 18 % (w/v) sodium hydroxide with 5 ml of 10 % (w/v) hydroxyamonnium sulphate. This reagent was prepared freshly. Acidic ferric chloride reagent was made by dissolving 10 g ferric chloride hexahydrate in 250 ml distilled water, 10 g sulphuric acid was added and diluted with water to 500 ml and stored at 4 ºC. The stock acetic acid solution was made by adding 2 g glacial acetic acid to about 100 ml distilled water and diluted to 200 ml using distilled water to give a final concentration of 10 000 mg l^{-1} acetic acid. The standards used were 0, 250, 500, 1000, 2500, 5000 and 10 000 mg l^{-1} acetic acid.

For the analysis of the samples and standards, 500 µl of the samples were pipetted into dry test tubes, then 1.7 ml acidic ethane diol reagent was added and mixed. The tubes were heated in a boiling water bath for 3 min and cooled immediately in cold water. Then 2.5 ml hydroxylamine reagent was added and mixed. The tubes were left to stand for 1 min and 1.88 ml of each sample was pipetted into fresh test tubes and 4 ml acidic ferric chloride reagent was added, this was made up to 10 ml by adding 4.12 ml distilled water. The tubes were shaken vigorously and allowed to stand for 5 min with the stopper removed. The absorbance at 500 nm was determined. Appendix D shows a typical acetic acid standard curve.

4.4 RESULTS

Preliminary studies of the serum bottle study

4.4.1 Cellulase and endoglucanase activities in the serum bottle study

The activity, U, was expressed as nmol glucose released min^{-1} ml^{-1}. Figures 4.3 and 4.4 show the cellulase activities in the serum bottles that contained 5 % (w/v) substrate, *C. beijerinckii* and 300 mg l^{-1} sulphide and 5 % (w/v) substrate and *C. beijerinckii*, respectively during the 13 day incubation period. The activities decreased as time progressed in both cases. The activity in the serum bottles that contained 5 % (w/v) substrate, *C. beijerinckii* and 300 mg l^{-1} sulphide was slightly higher than that containing the 5 % (w/v) substrate and *C. beijerinckii* after day 7. As shown in chapter 3, sulphide activated cellulase activity and this was also evident here. This, however, wasn't true for days 1 and 3 where the activity in the serum bottles containing 5 % (w/v) substrate and *C. beijerinckii* was higher than in the bottles containing *C. beijerinckii* and 300 mg l^{-1} sulphide.

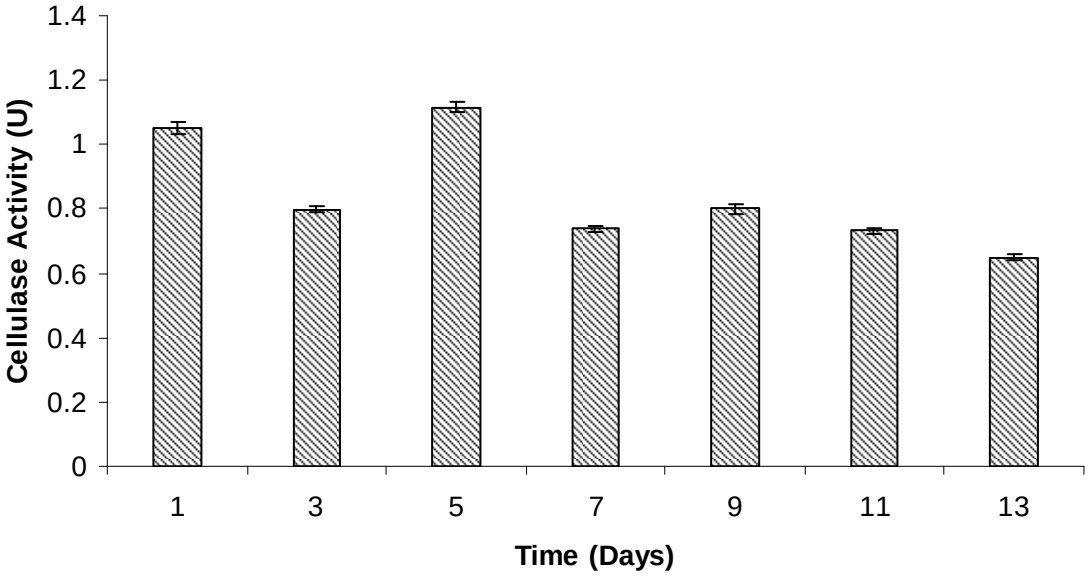

Figure 4.3 Cellulase activity in the serum bottles that contained 5 % (w/v) substrate, *C. beijerinckii* and 300 mg l^{-1} sulphide during the 13 day incubation period. Values are expressed as means ± SD (*n*=3).

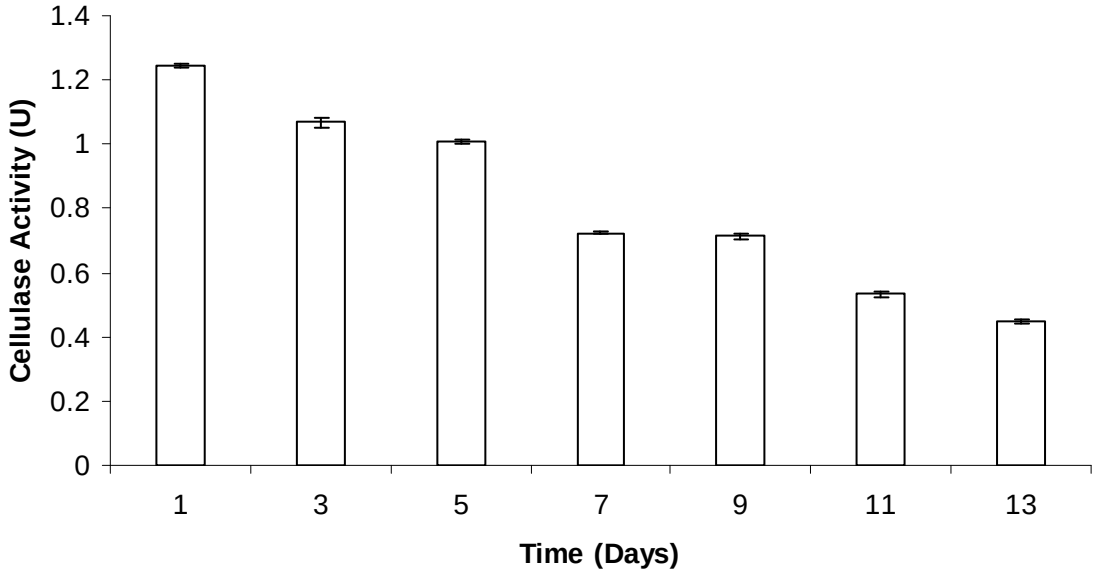

Figure 4.4 Cellulase activity in the serum bottles that contained 5 % (w/v) substrate and *C. beijerinckii* during the 13 day incubation period. Values are expressed as means ± SD (*n*=3).

As expected, the serum bottles that contained only 5 % (w/v) substrate and 300 mg l^{-1} sulphide and 5 % (w/v) substrate did not exhibit cellulase activity. Figures 4.5 and 4.6 illustrate the endoglucanase activities in the serum bottles that contained 5 % (w/v) substrate, *C. beijerinckii* and 300 mg l^{-1} sulphide and 5 % (w/v) substrate and *C. beijerinckii*, respectively during the 13 day incubation period. Initially, the serum bottles that contained 5 % (w/v) substrate and *C. beijerinckii* exhibited higher endoglucanase than that containing 5 % (w/v) substrate, *C. beijerinckii* and 300 mg l^{-1} sulphide (days 1-5). From days 9 to 13 the serum bottles that contained 5 % (w/v) substrate, *C. beijerinckii* and 300 mg l^{-1} sulphide exhibited endoglucanase activity while the serum bottles with 5 % (w/v) substrate and *C. beijerinckii* exhibited no endoglucanase activity. The inhibition of endoglucanase activity was due to the presence of cellobiose and glucose, the products of cellulose hydrolysis. The inhibition effects of cellobiose and glucose on endoglucanase activity were highlighted in chapter 3 and this may help to explain the inhibition of the enzyme at day 7.

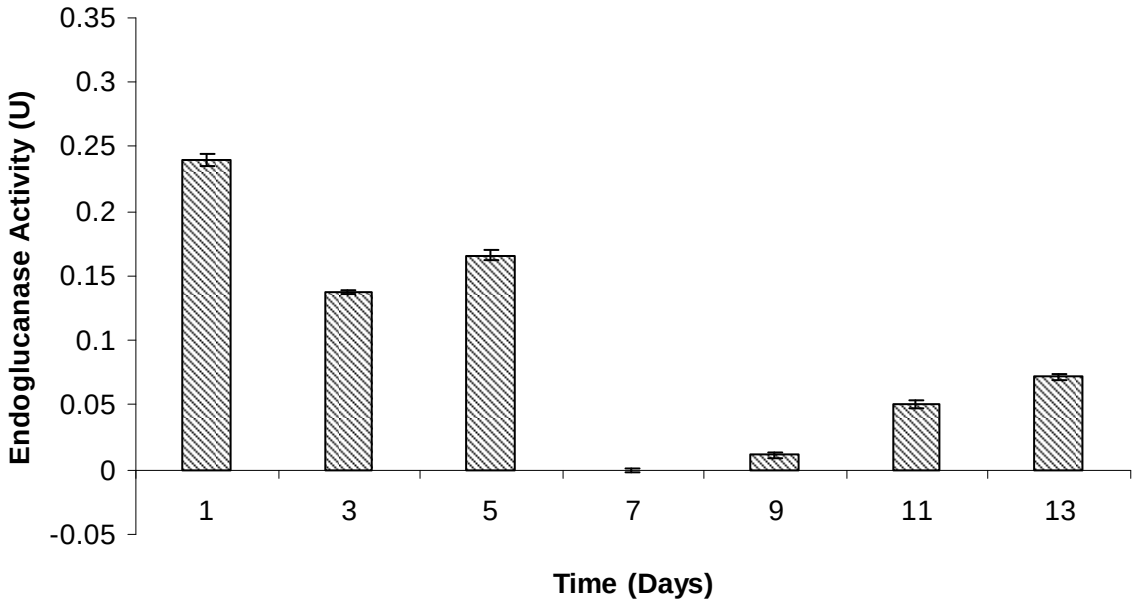

Figure 4.5 Endoglucanase activity in the serum bottles that contained 5 % (w/v) substrate, *C. beijerinckii* and 300 mg l^{-1} sulphide during the 13 day incubation period. Values are expressed as means ± SD (*n*=3).

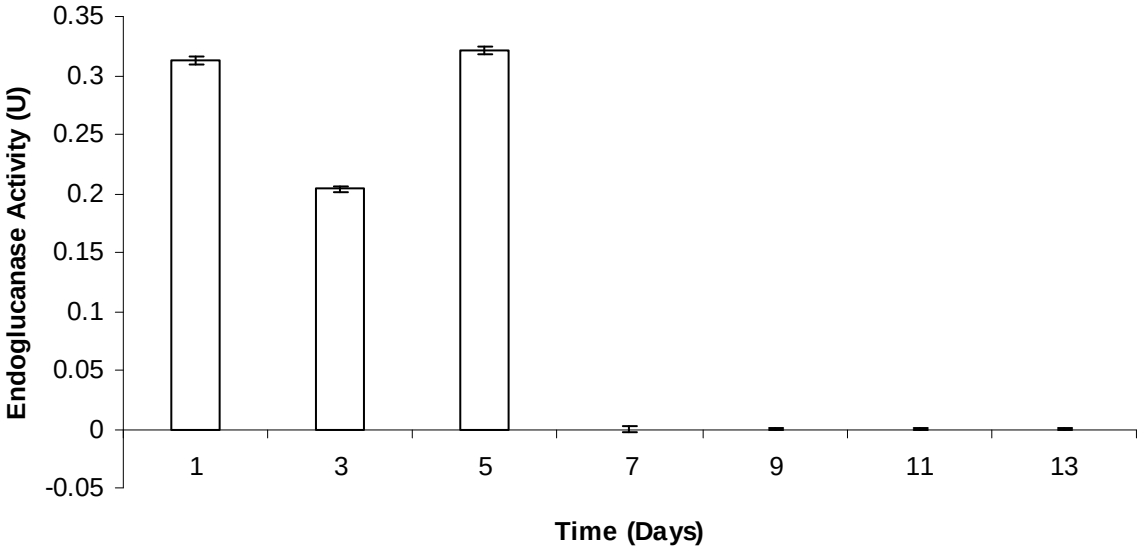

Figure 4.6 Endoglucanase activity in the serum bottles that contained 5 % (w/v) substrate and *C. beijerinckii* during the 13 day incubation period. Values are expressed as means ± SD (*n*=3).

The serum bottles that contained only 5 % (w/v) substrate and 300 mg l^{-1} sulphide and 5 % (w/v) substrate did not exhibit endoglucanase activity during the 13 day incubation period.

4.4.2 Reducing sugar concentrations in the serum bottle study

The concentration of reducing sugars found in serum bottles that contained 5 % (w/v) substrate, *C. beijerinckii* and 300 mg l^{-1} sulphide (figure 4.7) were similar to those found in the serum bottles that contained 5 % (w/v) substrate and *C. beijerinckii* (figure 4.8) during the 13 day incubation period. The serum bottles that contained only 5 % (w/v) substrate and 300 mg l^{-1} sulphide (figure 4.9) and the serum bottles that contained 5 % (w/v) substrate (figure 4.10) exhibited a similar trend to those of figures 4.7 and 4.8. However, the concentrations found in these serum bottles (figures 4.9 and 4.10) were lower. This indicated that more reducing sugars were released during the incubation period in the serum bottles that contained *C. beijerinckii*. As figures 4.7-4.10 indicate there is a decrease in reducing sugars during days 3 to 5 of the study. This decrease is more pronounced in figures 4.7 and 4.8, which are the bioreactors that contained 5 % (w/v) substrate, *C. beijerinckii* and 300 mg l^{-1} sulphide and 5 % (w/v) substrate and *C. beijerinckii*, respectively. This could be due to the *Clostridium* sp. utilising the reducing sugars at a faster rate than producing them. However, figures 4.9 and 4.10 also show this trend, albeit not as dramatically.

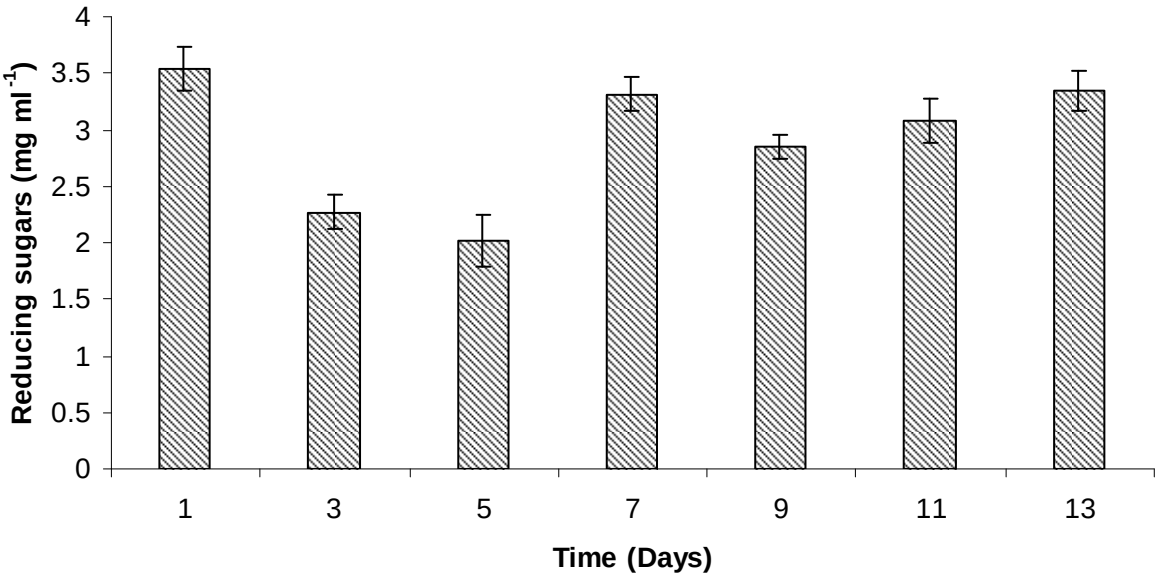

Figure 4.7 Reducing sugars present in the serum bottles that contained 5 % (w/v) substrate, *C. beijerinckii* and 300 mg l^{-1} sulphide during the 13 day incubation period. Values are expressed as means ± SD (*n*=3).

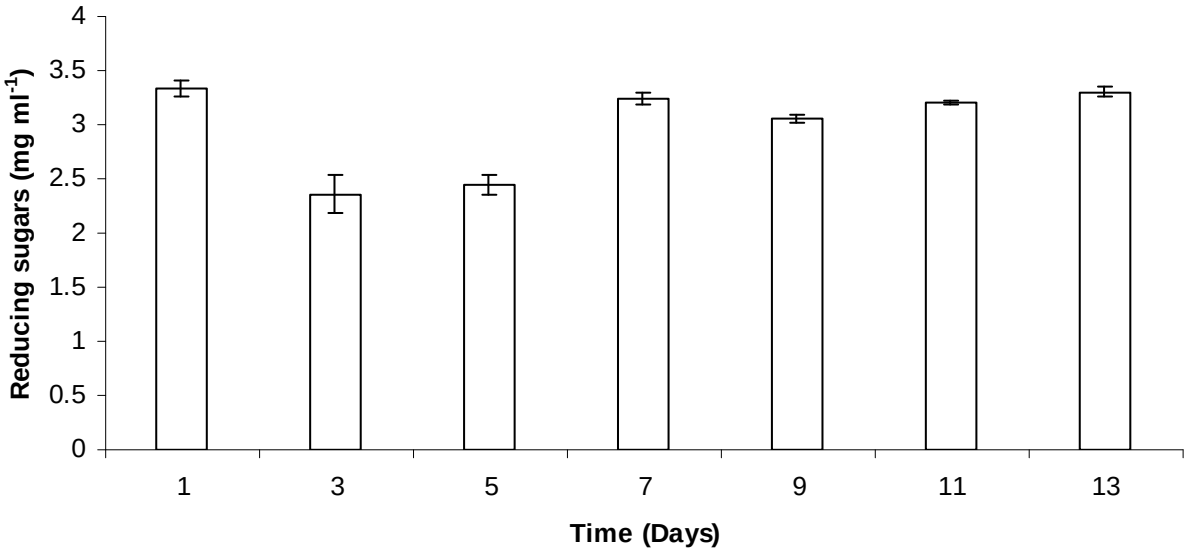

Figure 4.8 Reducing sugars present in the serum bottles that contained 5 % (w/v) substrate and *C. beijerinckii* during the 13 day incubation period. Values are expressed as means ± SD (*n*=3).

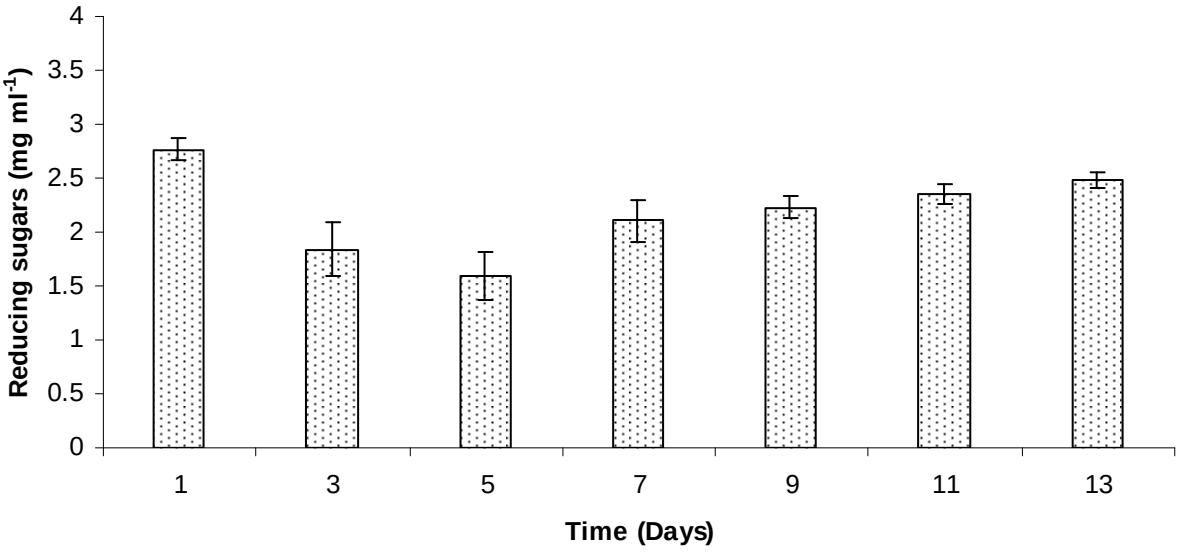

Figure 4.9 Reducing sugars present in the serum bottles that contained 5 % (w/v) substrate and 300 mg l^{-1} sulphide during the 13 day incubation period. Values are expressed as means ± SD (*n*=3).

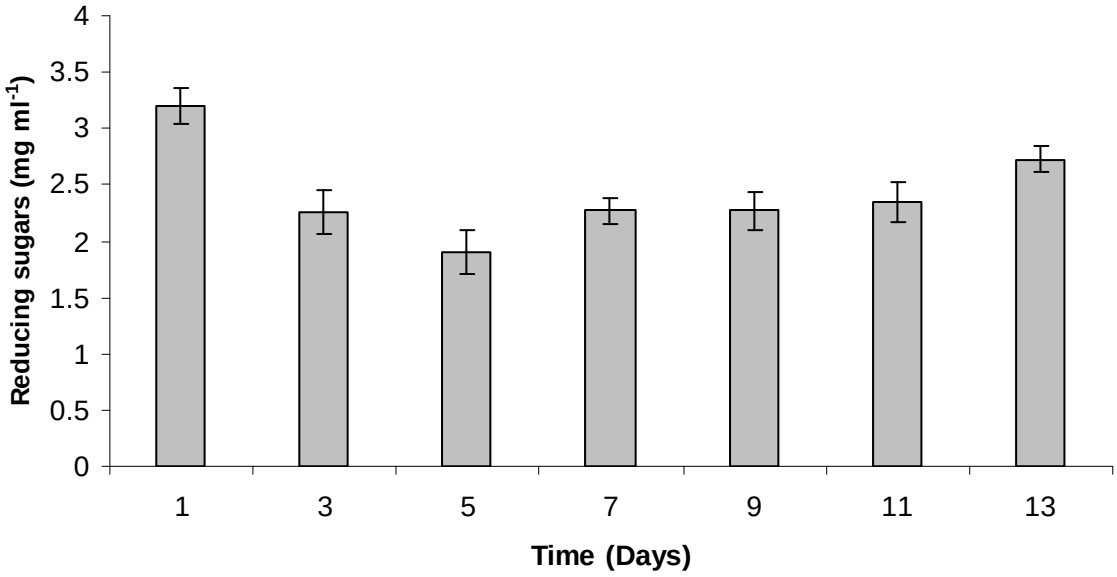

Figure 4.10 Reducing sugars present in the serum bottles that contained 5 % (w/v) substrate during the 13 day incubation period. Values are expressed as means ± SD (*n*=3).

4.4.3 Chemical oxygen demand (COD) removal in serum bottle study

Figure 4.11 shows the removal of COD from each set of serum bottles during the 13 day study and table 4.1 shows this in % removal of COD. As both figure 4.11 and table 4.1 show, there was higher COD removal from the serum bottle that contained 5 % (w/v) substrate, *C. beijerinckii* and 300 mg l^{-1} sulphide than that containing 5 % (w/v) substrate and *C. beijerinckii*. As section 4.4.1 shows, sulphide activated cellulase and endoglucanase activities and this was confirmed previously in the partial characterisation of the cellulosomal and non-cellulosomal fractions of *C. beijerinckii* (chapter 3). There was no COD removal evident in the serum bottles that contained 5 % (w/v) substrate, and 300 mg l^{-1} sulphide and 5 % (w/v) substrate. There was a higher COD reading in the 5 % (w/v) substrate, *C. beijerinckii* and 300 mg l^{-1} sulphide, probably due to the COD content of the growth media that was used to grow the microorganism.

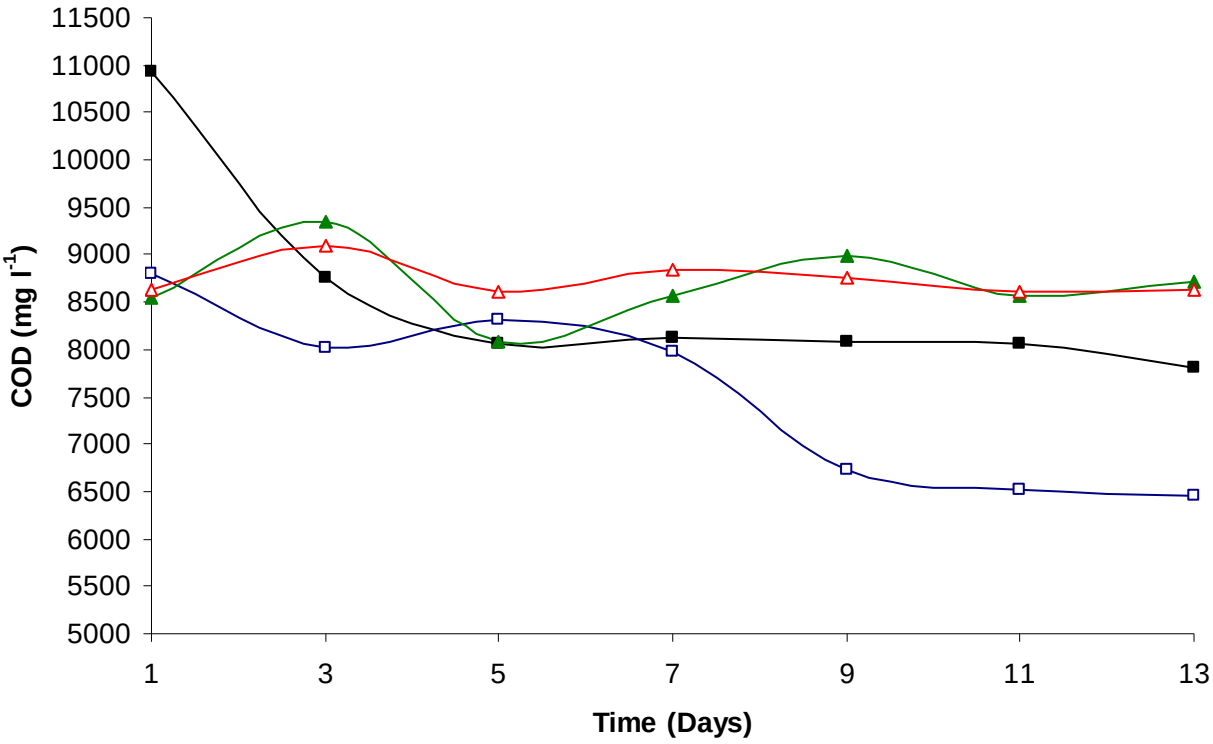

Figure 4.11 COD removal in the serum bottles over the 13 day incubation period. Substrate + sulphide + *C. beijerinckii* (■); substrate + *C. beijerinckii* (▫); substrate + sulphide (▲); substrate (△).

Table 4.1 Percentage COD removal in the serum bottle study

Sample	% COD removal
Substrate + sulphide + *C. beijerinckii*	28.5
Substrate + *C. beijerinckii*	26.6
Substrate + sulphide	0
Substrate	0

4.4.4 Sulphide concentration in serum bottles study

Sulphide concentrations were much lower than expected (figure 4.12), where the concentrations varied from 5-13 mg l^{-1}, where 300 mg l^{-1} sulphide was added. The colour of the sample was a dark brown and this may have interfered with the colorimetric assay. Due to this, the amount of sulphide added in the bioreactor studies was increased to a final concentration of 600 mg l^{-1} sulphide. No sulphide was evident in the serum bottles that did not contain sulphide (as expected).

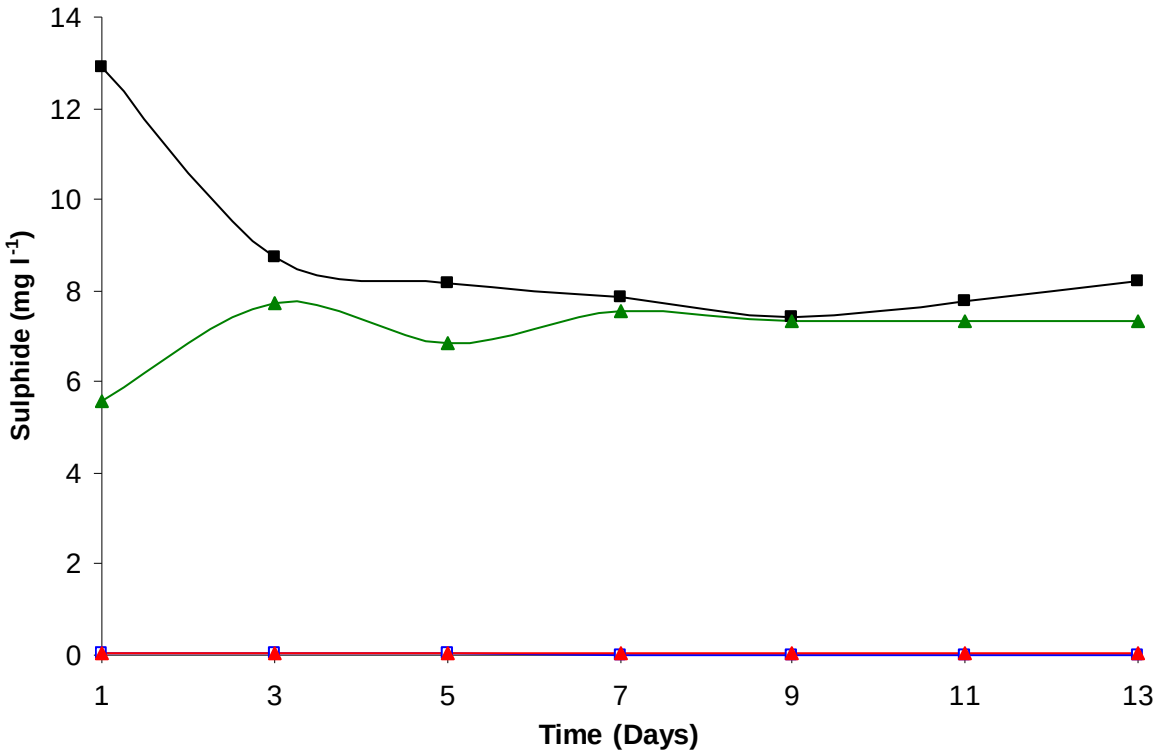

Figure 4.12 Sulphide concentrations in the serum bottles during the 13 day incubation period. Substrate + sulphide + *C. beijerinckii* (■); substrate + *C. beijerinckii* (▫); substrate + sulphide (▲); substrate (△).

4.4.5 pH in the serum bottle study

The pH of each serum bottle was determined and figure 4.13 shows the variation in pH during the 13 day incubation period of the study. The 0.1 M phosphate buffer was able to buffer the pH in the serum bottles effectively during the study.

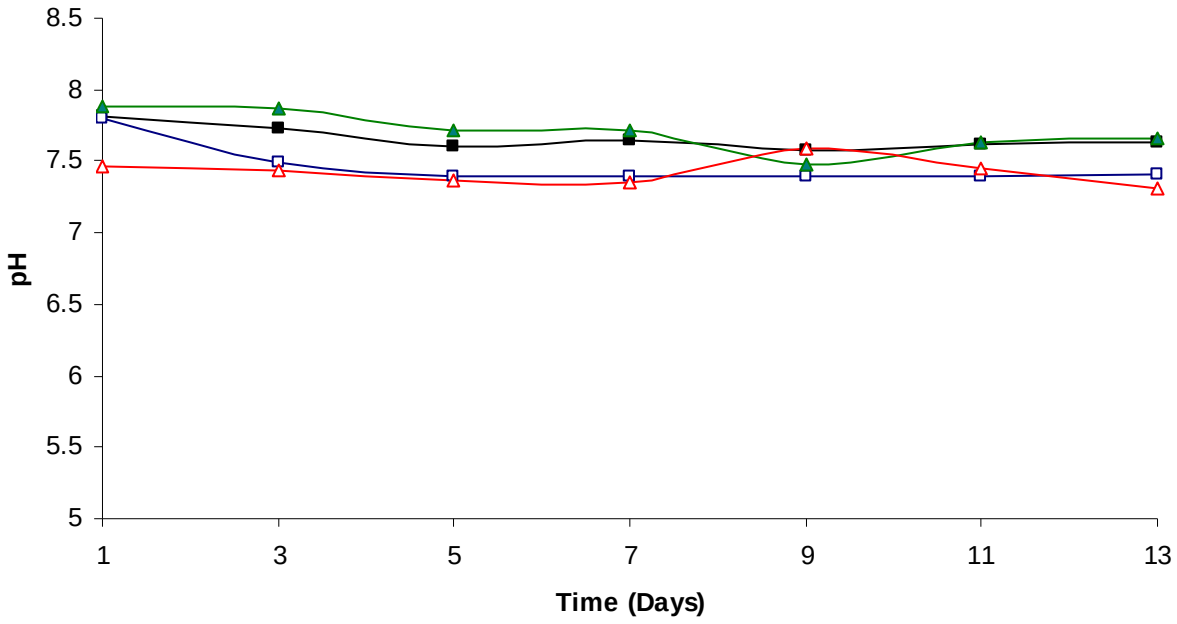

Figure 4.13 pH in the serum bottles during the 13 day incubation period. Substrate + sulphide + *C. beijerinckii* (■); substrate + *C. beijerinckii* (▫); substrate + sulphide (▲); substrate (△).

4.4.6 Volatile fatty acids in the serum bottle study

Figure 4.14 shows the volatile fatty acids (VFAs) present in the serum bottles during the study. There was no definite trend, however, the results show that there were VFAs present in all the serum bottles (including the controls that contained no *C. beijerinckii*). This indicated that there were residual VFAs present in the grass or substrate.

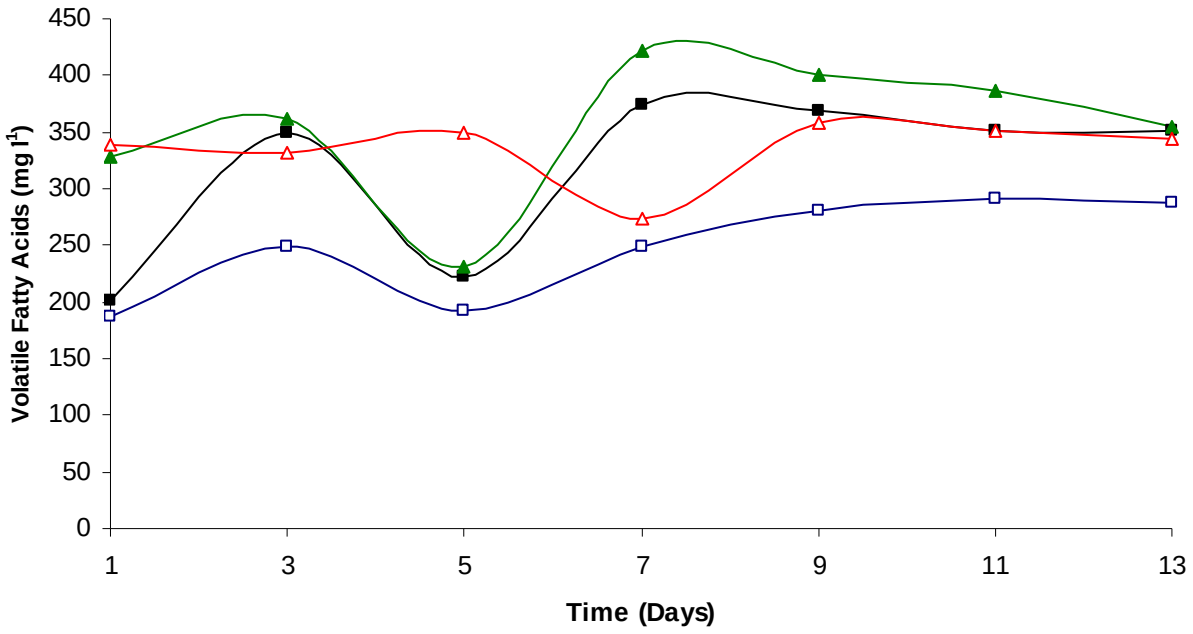

Figure 4.14 Volatile fatty acid concentrations in the serum bottles during the 13 day incubation period. Substrate + sulphide + *C. beijerinckii* (■); substrate + *C. beijerinckii* (▫); substrate + sulphide (▲); substrate (△).

Bioreactor studies

The bioreactor studies were performed over 28 days, 2 % (w/v) substrate instead of 5 % (w/v) was used and a final concentration of 600 mg l^{-1} sulphide was used where appropriate. At 5 % (w/v) substrate, the majority of the substrate formed clumps and was not well suspended in the medium, therefore, the substrate concentration was decreased to 2 % (w/v) in the bioreactor study. The increase from 300 mg l^{-1} to 600 mg l^{-1} sulphide was due to the low concentrations of sulphide determined in section 4.4.4. The control including sulphide and no *C. beijerinckii* was eliminated in this study as it behaved much like the control that contained only substrate.

4.4.7 Cellulase and endoglucanase activities in the bioreactor study

The activity, U, was expressed as nmol glucose released min^{-1} ml^{-1}. Figures 4.15 and 4.16 show the cellulase activities in the bioreactors that contained 2 % (w/v) substrate, *C. beijerinckii* and 600 mg l^{-1} sulphide and 2 % (w/v) substrate and *C. beijerinckii*, respectively during the 28 day incubation period. The activities varied during the period of the study. The activity observed in the bioreactor that contained 2 % (w/v) substrate, *C. beijerinckii* and 600 mg l^{-1} sulphide was higher than that with the 2 % (w/v) substrate and *C. beijerinckii* as figures 4.15 and 4.16 indicate. Figures 4.15 and 4.16 also show a trend where the activity increased at day 2 and then decreased at about day 6. This may have been due to the inhibition of the cellulase enzymes of *C. beijerinckii* by the hydrolysis products (e.g. glucose and cellobiose). At this stage the microorganisms take up the reducing sugars and then the cellulase activity increases at about day 13 in order to use more of the remaining substrate.

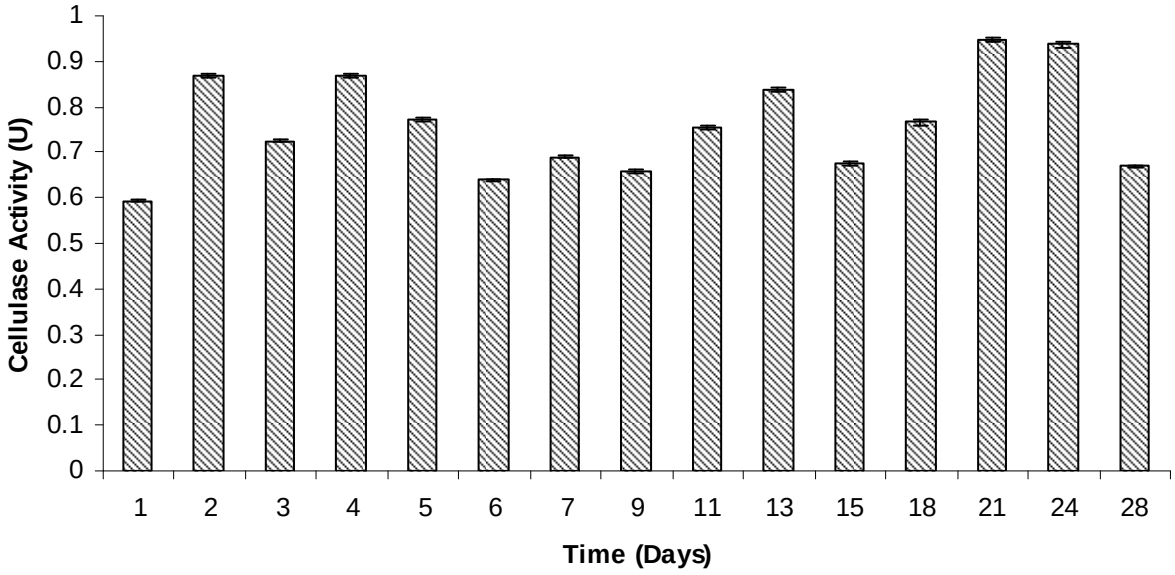

Figure 4.15 Cellulase activity in the bioreactor that contained 2 % (w/v) substrate, *C. beijerinckii* and 600 mg l^{-1} sulphide during the 28 day incubation period. Values are expressed as means ± SD (*n*=3).

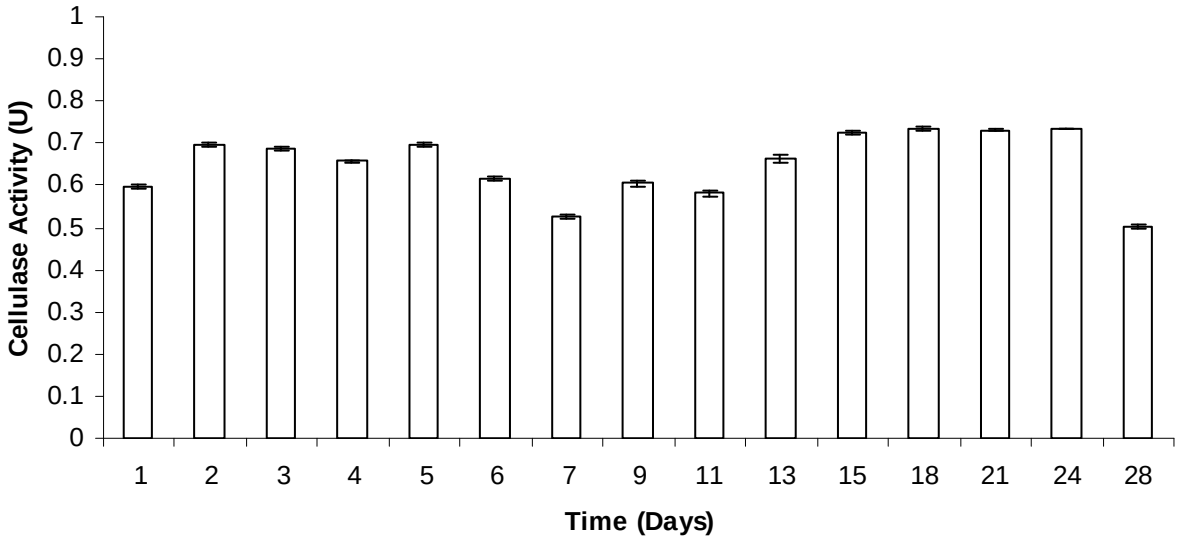

Figure 4.16 Cellulase activity in the bioreactor that contained 2 % (w/v) substrate and *C. beijerinckii* during the 28 day incubation period. Values are expressed as means ± SD (*n*=3).

There was no cellulase activity evident in the bioreactor that contained only 2 % (w/v) substrate. Figures 4.17 and 4.18 illustrate the endoglucanase activities evident during the 28 day bioreactor study. The bioreactor that contained 2 % (w/v) substrate, *C. beijerinckii* and 600 mg l^{-1} sulphide (figure 4.17) contained higher endoglucanase activities than the bioreactor that contained 2 % (w/v) substrate and *C. beijerinckii* (figure 4.18). The bioreactor that contained 2 % (w/v) substrate and *C. beijerinckii* exhibited no endoglucanase activities until day 15 of the study. This indicates the extent to which sulphide acts on the endoglucanases. In chapter 3, figure 3.5 showed that sulphide dramatically activated endoglucanase activity. No endoglucanase activity was detected in the bioreactor that contained only 2 % (w/v) substrate.

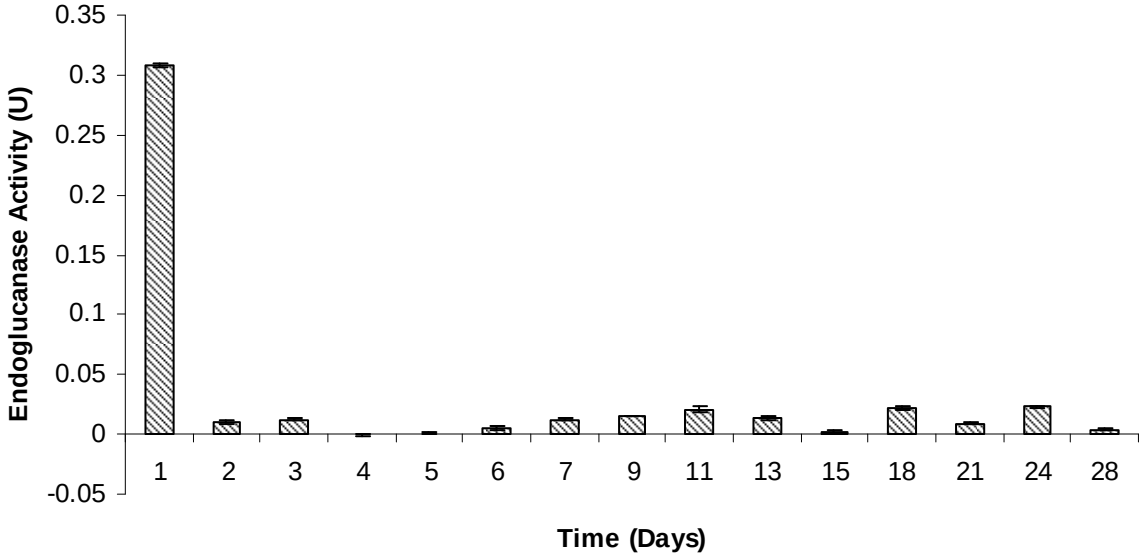

Figure 4.17 Endoglucanase activity in the bioreactor that contained 2 % (w/v) substrate, *C. beijerinckii* and 600 mg l^{-1} sulphide during the 28 day incubation period. Values are expressed as means ± SD (*n*=3).

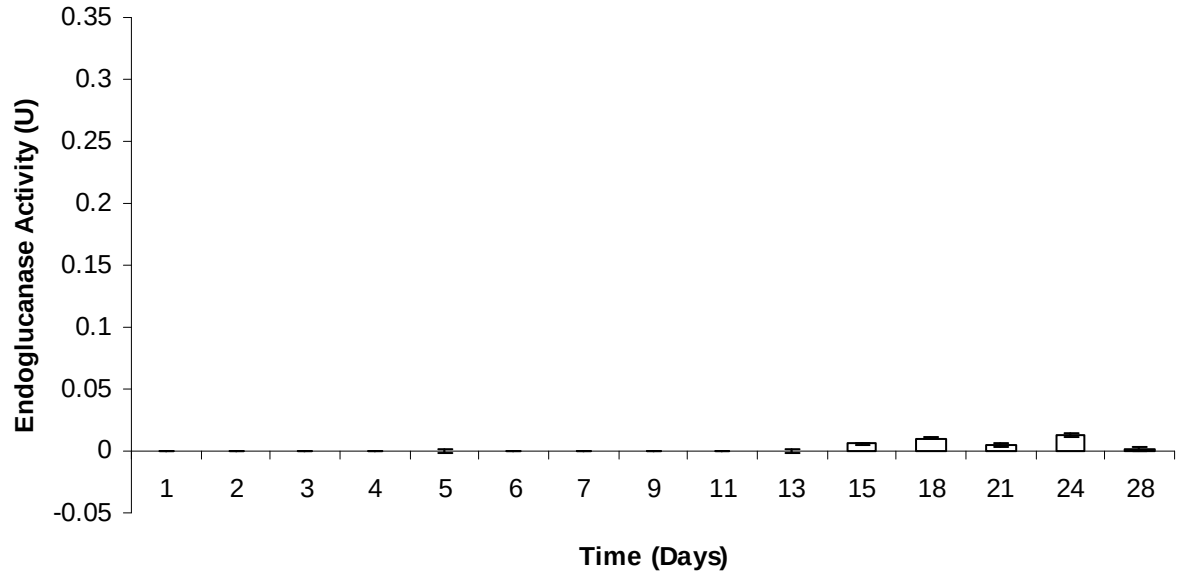

Figure 4.18 Endoglucanase activity in the bioreactor that contained 2 % (w/v) substrate and *C. beijerinckii* during the 28 day incubation period. Values are expressed as means ± SD (*n*=3).

4.4.8 Reducing sugars in the bioreactor study

Figures 2.19, 2.20 and 2.21 illustrate the concentrations of sugars present in the bioreactor that contained 2 % (w/v) substrate, *C. beijerinckii* and 600 mg l^{-1} sulphide, 2 % (w/v) substrate and *C. beijerinckii* and 2 % (w/v) substrate, respectively. A higher level of reducing sugars was evident in the bioreactor that contained 2 % (w/v) substrate, *C. beijerinckii* and 600 mg l^{-1} sulphide than the bioreactor that contained 2 % (w/v) substrate and *C. beijerinckii*. The bioreactor that contained the substrate only (figure 2.21), showed a stable concentration of reducing sugars throughout the study.

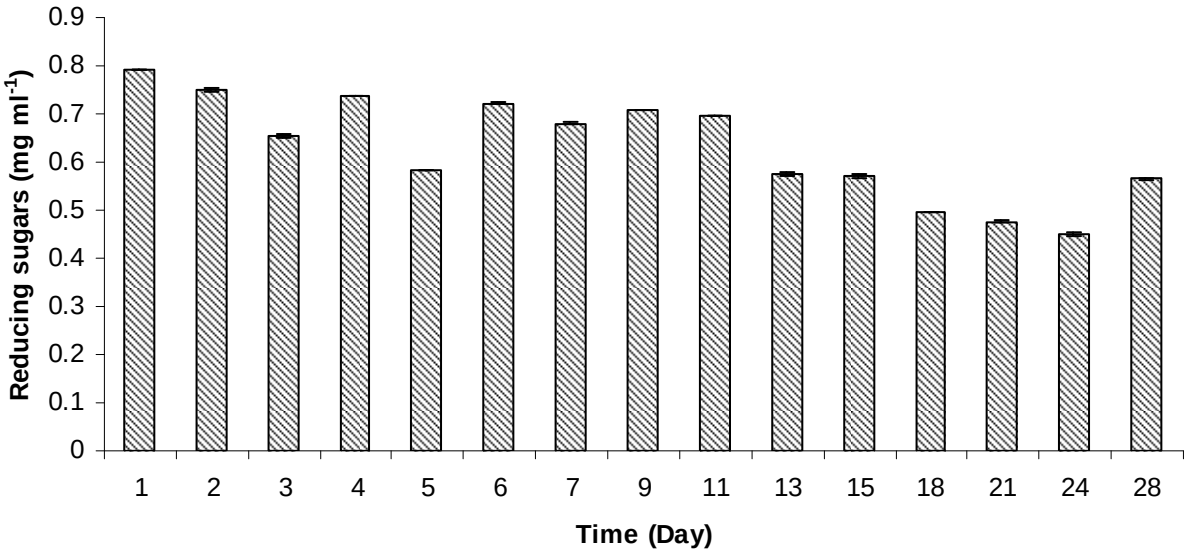

Figure 4.19 Reducing sugars present in the bioreactor that contained 2 % (w/v) substrate, *C. beijerinckii* and 600 mg l^{-1} sulphide during the 28 day incubation period. Values are expressed as means ± SD (*n*=3).

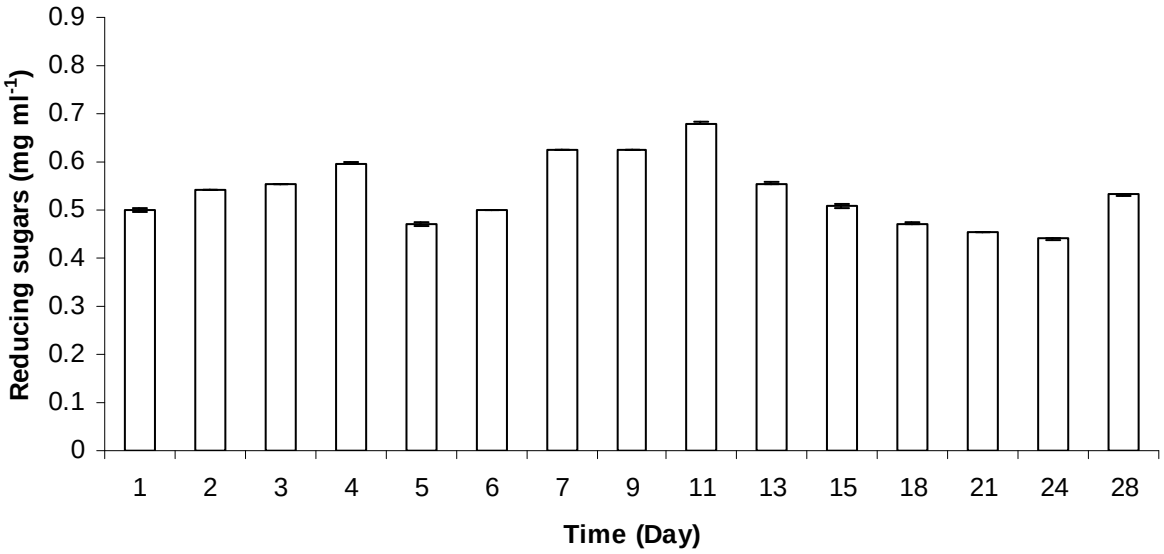

Figure 4.20 Reducing sugars present in the bioreactor that contained 2 % (w/v) substrate and *C. beijerinckii* during the 28 day incubation period. Values are expressed as means ± SD (*n*=3).

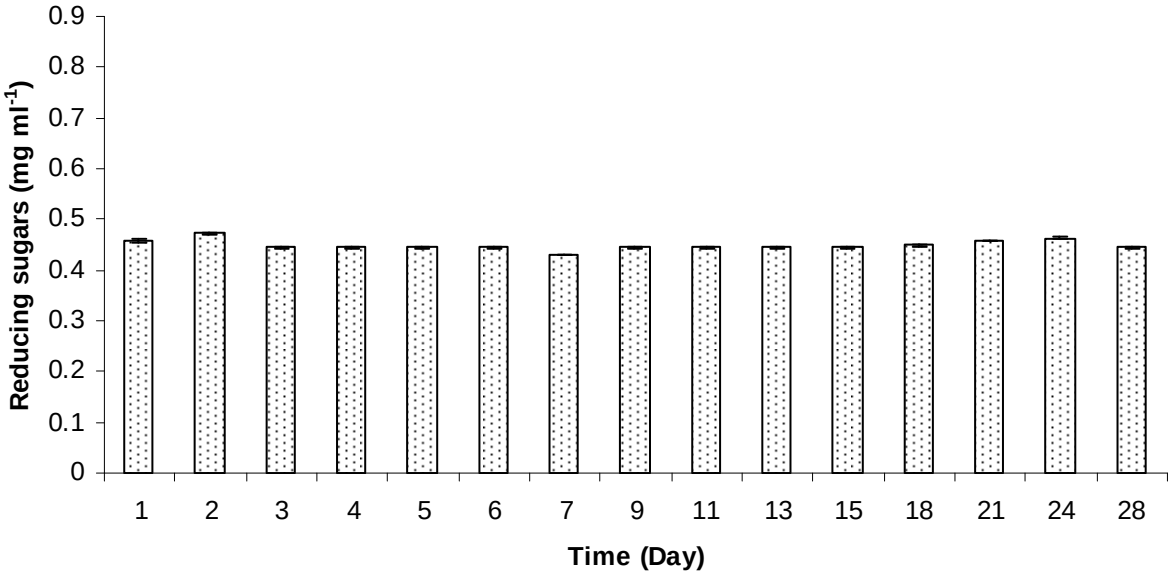

Figure 4.21 Reducing sugars present in the bioreactor that contained 2 % (w/v) substrate during the 28 day incubation period. Values are expressed as means ± SD (*n*=3).

To give a clearer indication of the reducing sugars present in the bioreactors during the study the control values, i.e. the bioreactor that contained substrate only, were subtracted from the values of the bioreactors that contained 2 % (w/v) substrate, *C. beijerinckii* and 600 mg l^{-1} sulphide and 2 % (w/v) substrate and *C. beijerinckii*. As figure 4.22 illustrates, there were higher concentrations of reducing sugars in the bioreactor that contained 2 % (w/v) substrate, *C. beijerinckii* and 600 mg l^{-1} sulphide than the bioreactor with 2 % (w/v) substrate and *C. beijerinckii*.

One trend that is evident in figure 4.22 is that a high concentration of reducing sugars was released on day 1 and then there was a gradual decrease in reducing sugars during the 28 day incubation period in the bioreactor that contained 2 % (w/v) substrate, *C. beijerinckii* and 600 mg l^{-1} sulphide. This bioreactor always had a higher concentration of reducing sugars than the bioreactor with 2 % (w/v) substrate and *C. beijerinckii* and this was more pronounced on days 1-6.

The second trend observed was that the reducing sugars released in the bioreactor that contained 2 % (w/v) substrate and *C. beijerinckii*, where there is a gradual increase of reducing sugars, which peaked at day 11 and decreased for the rest of the incubation period. The cellulases therefore gradually hydrolysed the substrate, releasing reducing sugars and then their activities were inhibited by the high concentrations of reducing sugars and therefore the decrease was due to *C. beijerinckii* assimilating the reducing sugars.

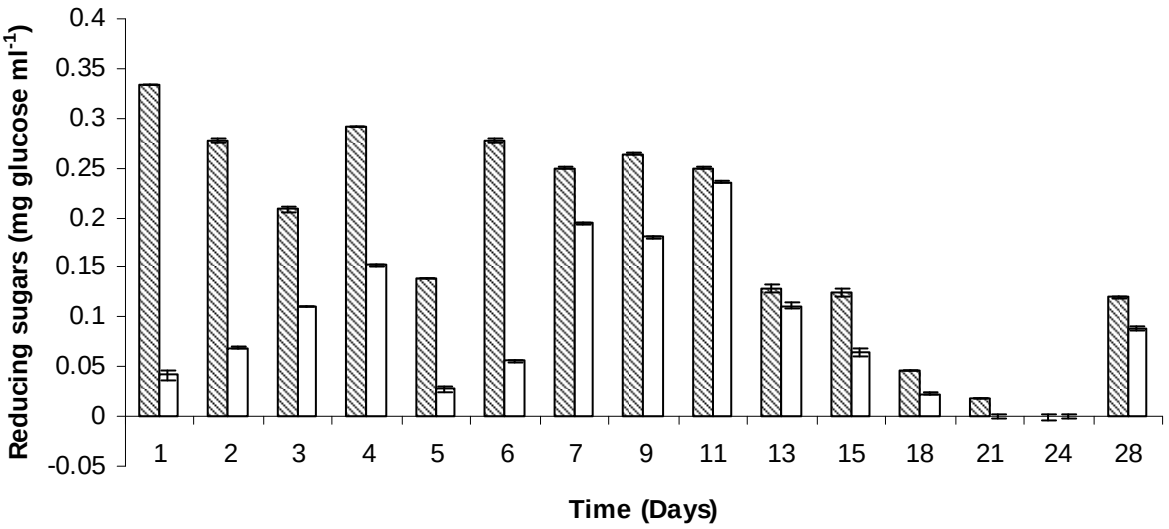

Figure 4.22 Actual reducing sugar concentrations present in the bioreactor that contained 2 % (w/v) substrate, *C. beijerinckii* and 600 mg l^{-1} sulphide (▨) and 2 % (w/v) substrate and *C. beijerinckii* (□) during the 28 day study. Values are expressed as means ± SD (*n*=3).

4.4.9 Chemical oxygen demand (COD) removal in the bioreactor study

Figure 4.23 shows the removal of COD from each bioreactor during the 28 day study and table 4.2 shows this in % removal of COD. As both figure 4.23 and table 4.2 show, there was a higher COD removal from the bioreactor that contained 2 % (w/v) substrate, *C. beijerinckii* and 600 mg.l^{-1} sulphide than that containing 2 % (w/v) substrate and *C. beijerinckii*. There was a larger difference between the % COD removal rates between the two bioreactors compared to the previous preliminary study (section 4.4.3). In the preliminary study the serum bottles that contained 5 % (w/v) substrate, *C. beijerinckii* and 300 mg.l^{-1} sulphide had a 1.9 % higher % COD removal rate than the serum bottles that contained 5 % (w/v) substrate and *C. beijerinckii*. In the bioreactor study the bioreactor that contained 2 % (w/v) substrate, *C. beijerinckii* and 600 mg.l^{-1} sulphide exhibited a 17.7 % higher % COD removal rate than the bioreactor that contained only 2 % (w/v) substrate and *C. beijerinckii*. There was no COD removal evident in the bioreactor that contained only 2 % (w/v) substrate.

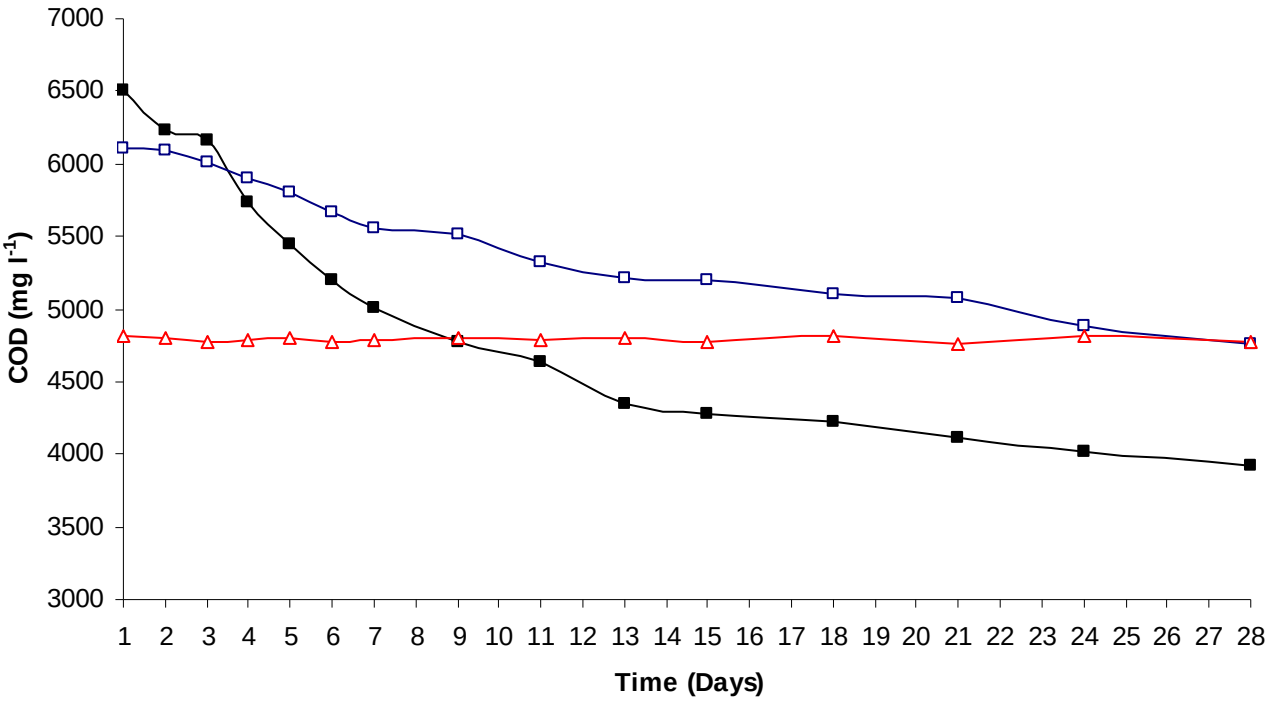

Figure 4.23 COD removal in the bioreactors over the 28 day incubation period. Substrate + sulphide + *C. beijerinckii* (■); substrate + *C. beijerinckii* (▫); substrate (△).

Table 4.2 Percentage COD removal in the bioreactor study

Sample	% COD removal
Substrate + sulphide + *C. beijerinckii*	39.7
Substrate + *C. beijerinckii*	22.0
Substrate	0

4.4.10 Sulphide concentration in the bioreactor study

Figure 4.24 illustrates the sulphide concentrations present in the bioreactors during the study. The sulphide concentrations were not as low as in section 4.4.4 as 600 mg l^{-1} sulphide was used instead of 300 mg l^{-1} sulphide. As expected no sulphide was evident in the bioreactors that contained only 2 % (w/v) substrate and *C. beijerinckii* and 2 %

(w/v) substrate. The bioreactor that contained 2 % (w/v) substrate, *C. beijerinckii* and 600 mg l^{-1} sulphide contained sulphide concentrations varying from 250-300 mg l^{-1} sulphide. This was about half of the sulphide that was added into the bioreactor, this indicates that the assay is not sensitive or that there are inhibitors of the assay present in the bioreactors.

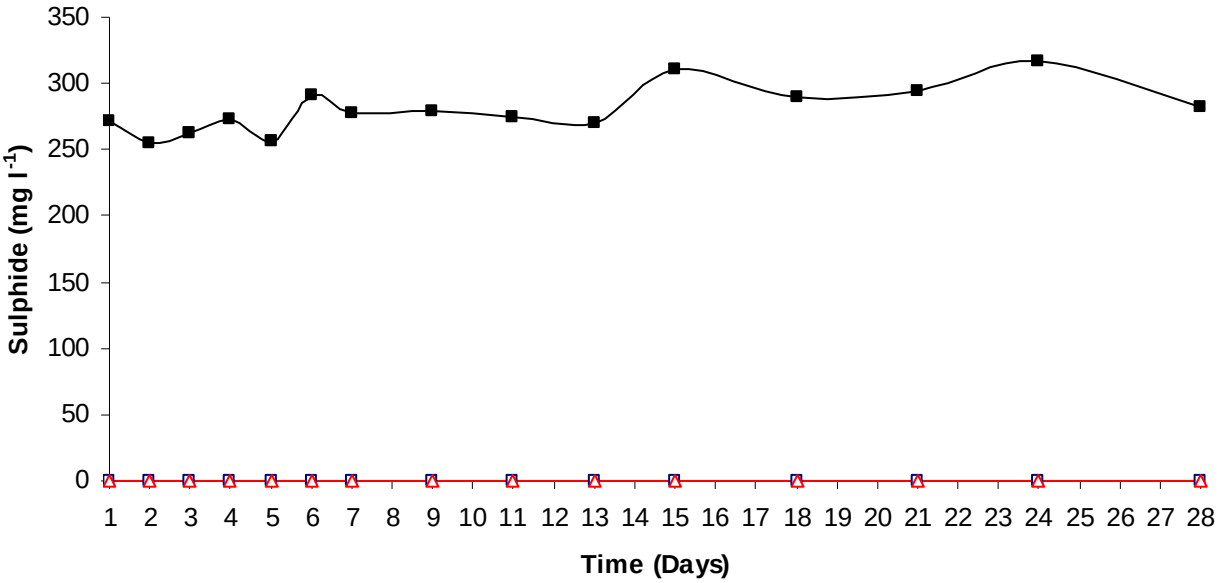

Figure 4.24 Sulphide concentrations in the bioreactors during the 28 day incubation period. Substrate + sulphide + *C. beijerinckii* (■); substrate + *C. beijerinckii* (▫); substrate (△).

4.4.11 pH determination in the bioreactor study

The pH of each bioreactor was determined and figure 4.25 shows the pH during the 28 day study. The 0.1 M phosphate buffer was able to buffer the bioreactors effectively during the study; however, the bioreactor that contained 2 % (w/v) substrate, *C. beijerinckii* and 600 mg l^{-1} sulphide had pH values of about 1 pH unit higher than the other bioreactors. This is due to the pH effect of sulphide as described in section 3.4.2.

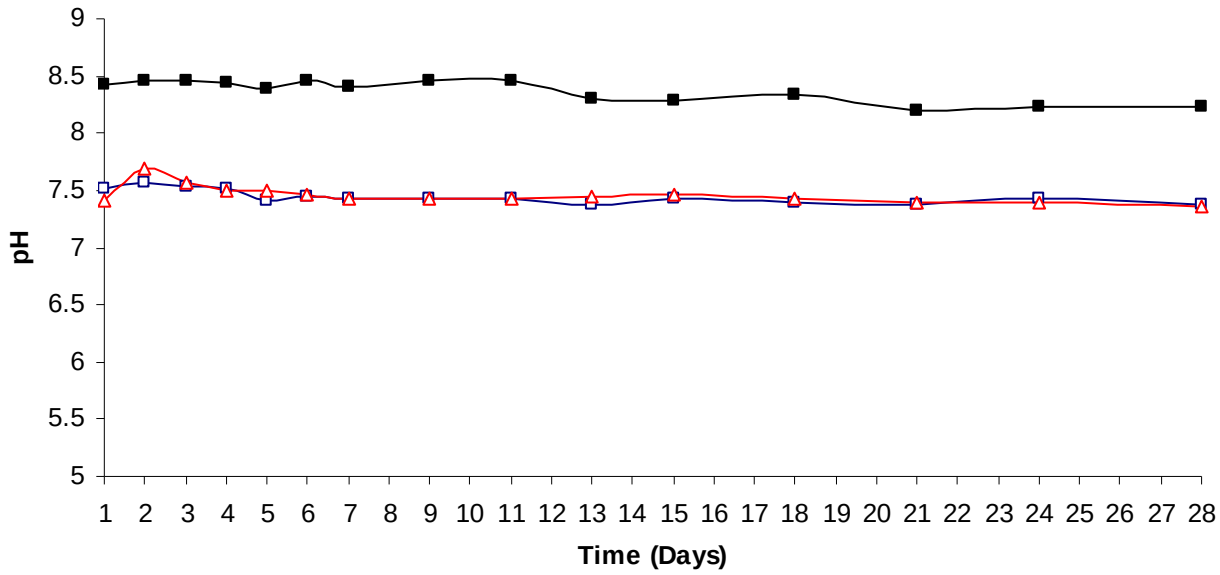

Figure 4.25 pH in the bioreactors during the 28 day incubation period. Substrate + sulphide + *C. beijerinckii* (■); substrate + *C. beijerinckii* (▫); substrate (△).

4.4.12 Volatile fatty acids in the bioreactor study

Figure 4.26 shows the levels of volatile fatty acids (VFAs) during the bioreactor study. The bioreactor that contained 2 % (w/v) substrate contained the baseline VFA concentration, that is, the residual VFAs that were initially present. The difference between the VFAs concentrations determined in this bioreactor to the other two bioreactors were the VFAs produced by *C. beijerinckii*. Slightly higher concentrations of VFAs were produced in the bioreactor that contained 2 % (w/v) substrate, *C. beijerinckii* and 600 mg l^{-1} sulphide than the bioreactor that contained 2 % (w/v) substrate and *C. beijerinckii*.

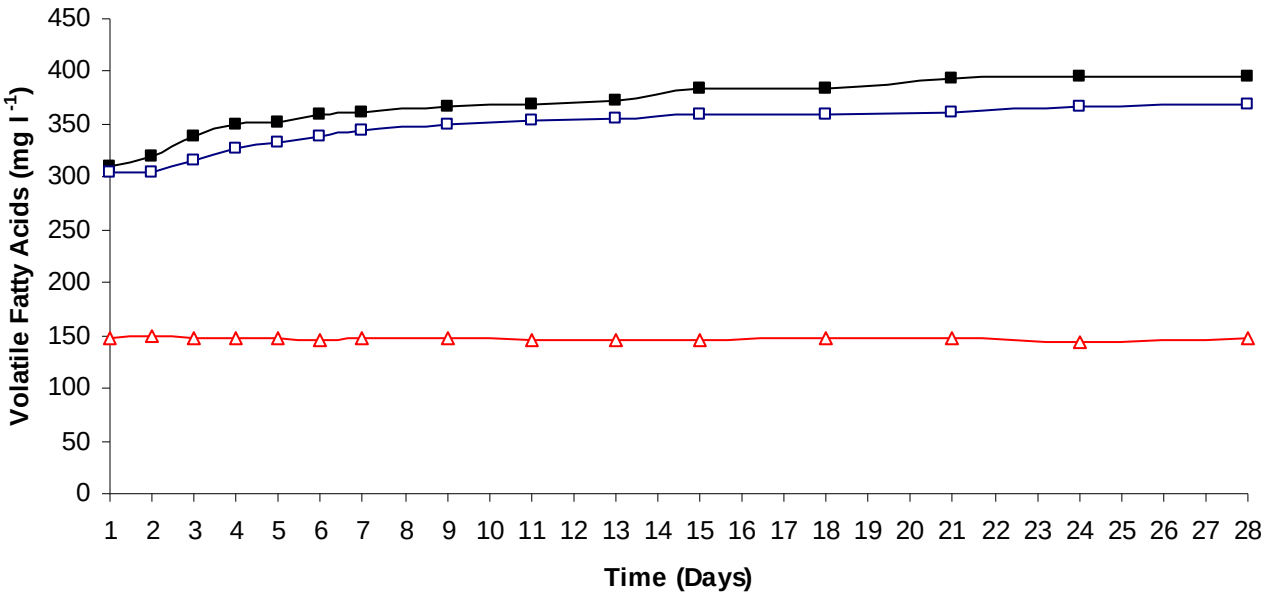

Figure 4.26 Volatile fatty acid concentrations in the bioreactors during the 28 day incubation period. Substrate + sulphide + *C. beijerinckii* (■); substrate + *C. beijerinckii* (▫); substrate (△).

4.5 DISCUSSION

Most anaerobic digestion studies are performed with a consortium of microorganisms that are typically present, such as the study performed by Yu *et al.* (2002). These include fermentative bacteria, acidogens, acetogens, sulphate reducing bacteria and methanogens (Novaes, 1986). In this study, however, since the interest was in the activation of the hydrolases produced by the fermentative bacteria, namely the cellulases and endoglucanases, no other microorganism was added. This study therefore focuses on the first step, the rate-limiting step, of anaerobic digestion.

In the serum bottle study, the serum bottles that contained 5 % (w/v) substrate, *C. beijerinckii* and 300 mg l^{-1} sulphide had higher cellulase activities than the serum bottles that contained 5 % (w/v) substrate and *C. beijerinckii* during the 13 day incubation period. Sulphide activated the cellulase activities in the serum bottles; this was also shown by the fundamental studies in chapter 3. The activities decreased over time in both cases, possibly due to more reducing sugars being released. Data in chapter 3 showed that cellobiose and glucose inhibited the cellulase and endoglucanase activities and this was possibly what occurred. The negative controls, that is, the serum bottles that contained only 5 % (w/v) substrate and 300 mg l^{-1} sulphide and 5 % (w/v) substrate did not exhibit cellulase activity. This was expected, as there were no microorganisms available to produce the enzymes present in the serum bottles. Sulphide also activated the endoglucanase activities during the study. The negative controls exhibited no endoglucanase activities.

Higher concentrations of reducing sugars were released during the incubation period in the serum bottles that contained 5 % (w/v) substrate, *C. beijerinckii* and 300 mg l^{-1} sulphide and 5 % (w/v) substrate and *C. beijerinckii* than the negative controls. This was as a result of the enzyme activity exhibited by the *Clostridium* sp. in the serum bottles that contained the microorganism.

Since sulphide activated the activities of the cellulases and endoglucanases in the serum bottles, the COD removal rate was higher in the serum bottles that contained 5 % (w/v) substrate, *C. beijerinckii* and 300 mg l^{-1} sulphide than that containing 5 % (w/v) substrate and *C. beijerinckii*. The percentage removal rate however, was only 1.9 % higher. This could have been due to the fact that at 5 % (w/v) substrate, the availability of the substrate within the medium was insufficient. Hence, the substrate concentration was decreased to 2 % (w/v) in the bioreactor study. As expected, no COD removal was evident in the negative controls.

Sulphide concentrations were much lower than expected and therefore more sulphide was added in the bioreactor studies to a final concentration of 600 mg l^{-1} sulphide and also because this was the level of sulphide observed in bioreactors used previously (Watson and Pletschke, 2006). The pH range of all the serum bottles was around 7.5-8.0. Hydrogen sulphide has a p_{Ka} of 6.89 and a pH range of 1-7, therefore at pH values $\leq$ 7, sulphide is converted to hydrogen sulphide (Duan *et al.*, 2006). That is why pH 8.0 was selected, to ensure that sulphide was not lost as hydrogen sulphide during the study. Hobson and Wheatley (1993) stated that clostridia grow over a pH range of 6-8 and that the optimum growth conditions were about pH 7.0 to 7.5. The bioreactor pH employed in this study fell within this range. The VFAs results indicated that there were residual VFAs present in the grass used, perhaps from microbes that had been growing on the grass prior to use in the study.

The bioreactor studies were performed over 28 days (which was a longer period compared to the above study) 2 % (w/v) substrate was used instead of 5 % (w/v) and a final concentration of 600 mg l^{-1} sulphide was used where appropriate. The cellulase activities in the bioreactor that contained 2 % (w/v) substrate, *C. beijerinckii* and 600 mg l^{-1} sulphide were higher than that exhibited with 2 % (w/v) substrate and *C. beijerinckii*, as was found in the preliminary serum bottle study. The control exhibited no cellulase activity. The activation of endoglucanases in the bioreactor that contained 2 % (w/v) substrate, *C. beijerinckii* and 600 mg l^{-1} sulphide was evident as it contained much higher endoglucanase activity than the bioreactor that contained 2 % (w/v) substrate and *C.*

beijerinckii. The bioreactor that contained 2 % (w/v) substrate and *C. beijerinckii* exhibited no endoglucanase activities until day 15 of the study. The control did not exhibit endoglucanase activities as expected, since no microorganism was inoculated into the medium. There was a higher concentration of reducing sugars in the bioreactor that contained 2 % (w/v) substrate, *C. beijerinckii* and 600 mg l^{-1} sulphide than the bioreactor that contained 2 % (w/v) substrate and *C. beijerinckii*. This was due to the higher cellulase and endoglucanase activities in the former, due to the activation effect of sulphide.

There was higher COD removal from the bioreactor that contained 2 % (w/v) substrate, *C. beijerinckii* and 600 mg l^{-1} sulphide than that containing 2 % (w/v) substrate and *C. beijerinckii*. The former bioreactor had 17.7 % higher % COD removal rate than the bioreactor that contained 2 % (w/v) substrate and *C. beijerinckii*. There was no COD removal evident in the control. This % COD removal rate was 15.8 % higher than that obtained in the serum bottle study. This was due to several factors; the study was performed for 28 days instead of 13, the substrate was at a lower concentration therefore facilitating a more homogenous suspension and the sulphide concentration was higher than that of the serum bottle study with a range of 250-300 mg l^{-1} sulphide (as determined by the sulphide assay). It is recommended that in future experiments to measure biomass or cell numbers during the serum bottle and bioreactor studies. The determination of cell numbers can be compared with the COD removal rate in order to provide a more complete assessment of the studies. Hobson and Wheatley (1993) stated that clostridia have been found to grow over a pH range of 6-8, the 2 % (w/v) substrate, *C. beijerinckii* and 600 mg l^{-1} sulphide had pH values of about 8.5 throughout the study. This was about 1 pH unit higher than the other bioreactors due to the addition of sulphide to a final concentration of 600 mg l^{-1}, however this is only 0.5 pH units above the recommended range and therefore the pH did not appear to have an inhibitory effect on *C. beijerinckii*.

C. beijerinckii produced VFAs as the results indicated (figure 4.32). Slightly higher concentrations of VFAs were produced in the bioreactor that contained 2 % (w/v) substrate, *C. beijerinckii* and 600 mg l^{-1} sulphide than the bioreactor that contained 2 %

(w/v) substrate and *C. beijerinckii*. *C. beijerinckii* or *C. acetobutylicum* converts sugars and polysaccharides to acids (acetate and butyrate) and solvents (acetone, butanol and ethanol) (Sabathé *et al.*, 2002; Perret *et al.*, 2004) and this would explain the production in VFAs during the study. It was shown in chapter 3 that increased additions of acetate slightly inhibited the cellulosomal and non-cellulosomal cellulase and endoglucanase activities. The 0.1 M phosphate buffer was able to buffer the bioreactors effectively during the study and therefore there was no decrease in pH as was seen in chapter 3. Figures 4.19 and 4.20 show that there was no decrease in cellulase activity during the bioreactor study and although figures 4.22 and 4.23 indicate a decrease in endoglucanase activity during the study, this cannot be attributed to the production of butyrate and acetate alone. Other factors such as the level of reducing sugars present could be inhibiting the enzymes.

4.6 CONCLUSIONS

Both the preliminary serum bottle study and the bioreactor study showed that sulphide stimulated the rate-limiting step, the hydrolysis step. Sulphide activated the cellulases and endoglucanases produced by *C. beijerinckii* sLM01. There was also a higher concentration of reducing sugars present in the bioreactor that contained sulphide, substrate and the microorganism. Sulphide therefore stimulated COD removal, where the COD removal rate was higher in the bioreactor containing sulphide, substrate and the microorganism. This effect was more pronounced in the bioreactor study. The 0.1 M phosphate buffer buffered the bioreactor efficiently in both studies. Higher volatile fatty acids concentrations were evident in the bioreactor containing sulphide, substrate and the microorganism.

CHAPTER 5

OVERALL CONCLUSIONS AND FUTURE RECOMMENDATIONS

5.1 OVERALL CONCLUSIONS

This study set out to determine the effect of sulphide on the cellulases and endoglucanases of cellulosomal and non-cellulosomal fractions of putative *Clostridium beijerinckii* sLM01 and the effect of sulphide on the rate-limiting step of anaerobic digestion. This was done by purifying the cellulosome of *C. beijerinckii* and determining its cellulase and endoglucanase activities. This was done by isolating the *Clostridium* anaerobe from a biosulphidogenic bioreactor and then screening for the isolate with the highest cellulase activity. Identification of the anaerobe was then performed using 16S rDNA analysis. The purification of the cellulosome of the anaerobe was performed.

The next objective was to partially characterise the cellulosomal and non-cellulosomal fractions of *C. beijerinckii*. This was accomplished by determining the effect of increased additions of sulphide, sulphate, cellobiose, glucose and acetate on cellulase and endoglucanase activities of cellulosomal and non-cellulosomal fractions of *C. beijerinckii*. The pH and temperature optima of the cellulases and endoglucanases of cellulosomal and non-cellulosomal fractions of *C. beijerinckii* were also determined. This provided the fundamental evidence required for the design and analysis of the bioreactor study.

The next objective was to determine the effect of sulphide on anaerobic digestion where *C. beijerinckii* was the microorganism used. The parameters that were determined during this study were cellulase and endoglucanase activities, reducing sugar concentrations, chemical oxygen demand (COD), sulphide concentrations, pH and volatile fatty acid (VFA) levels.

5.1.1 Isolation of C. beijerinckii sLM01, purification of its cellulosome and determination of cellulase and endoglucanase activities

C. beijerinckii was isolated from a biosulphidogenic bioreactor and identified using16S rDNA analysis. The partial 16S rDNA sequence was of a 1300 bp region of the 1500 bp gene. The cellulosome was purified using affinity chromatography purification and the affinity digestion purification procedures. These two methods were used in order to find the most suitable method for purification of the cellulosome of *C. beijerinckii*. Both methods purified cellulases successfully. The highest specific activities for cellulases were 146.9 and 83.29 nmol glucose released mg^{-1} protein min^{-1}, for the affinity chromatography purification procedure and the affinity digestion purification procedure, respectively. The affinity digestion purification procedure resulted in very low endoglucanase activities in the cellulosomal and non-cellulosomal fractions; the highest specific activity observed was 0.786 nmol glucose released mg^{-1} protein min^{-1} compared to 83.29 nmol glucose released mg^{-1} protein min^{-1} of the affinity chromatography purification procedure. The affinity digestion purification procedure yielded better resolved peaks than that of the affinity chromatography purification and it also yielded more defined and clearer bands on SDS-PAGE. The MALDI-TOF analysis of the isolated subunits indicated that the subunits were a 80 kDa exoglucanase, a 60 kDa endoglucanase and a 41 Da endoglucanase, these results were not conclusive, however. N-terminal sequencing should be performed in order to obtain an accurate and reliable identification of the proteins.

5.1.2 Characterisation of cellulosomal and non-cellulosomal fractions of C. beijerinckii sLM01

The cellulosomal and non-cellulosomal fractions were characterised with regards to their sensitivity to sulphide, sulphate, cellobiose, glucose and acetate, and the pH and temperature optima were determined. Increased additions of sulphide activated the cellulase activity of the cellulosome and non-cellulosomal fractions up to 700 %, while increased additions of sulphate either increased the activity slightly or inhibited it

dramatically. Sulphate slightly activated the cellulase and endoglucanase activities of the cellulosomal fraction, while it inhibited the cellulase and endoglucanase activities of the non-cellulosomal fraction.

Cellobiose and glucose both inhibited the cellulase and endoglucanase activities of both the cellulosomal and non-cellulosomal fractions. This was expected as these compounds are the hydrolytic products of cellulases (i.e., exoglucanases, endoglucanases and β-glucosidases). Increased acetate levels slightly inhibited the cellulosomal and non-cellulosomal cellulases and endoglucanases; this was possibly due to the decrease in pH caused by the addition of the acids.

pH optima of 5.0 and 7.5 for cellulosomal cellulase and 5.0 for cellulosomal endoglucanase were observed. The non-cellulosomal fraction exhibited a pH optimum of 7.5 for both cellulase and endoglucanase activities. Both fractions and enzymes exhibited a temperature optimum of 30 ˚C, this was expected since the anaerobe is a mesophile.

5.1.3 The effect of sulphide on anaerobic digestion

Two studies were performed; the preliminary serum bottle study and the bioreactor study. Both studies showed that sulphide stimulated the rate-limiting step, i.e. the hydrolysis step. This is because sulphide activated the cellulases and endoglucanases produced by *C. beijerinckii* sLM01. The fundamental studies from chapter 3 showed that sulphide activated the cellulosomal and non-cellulosomal fractions of *C. beijerinckii* sLM01 and the effect of sulphide was confirmed when it was applied to the bioreactors.

There was also a higher concentration of reducing sugars in the bioreactor that contained sulphide, substrate and the microorganism. This was due to higher enzyme activities due to the action of sulphide. Sulphide also stimulated COD removal, where the COD removal rate was higher in the bioreactor with sulphide, substrate and the microorganism than the one with substrate and the microorganism. This effect was more pronounced in the bioreactor study where the difference in % COD removal rates was much higher than

that of the serum bottles study. This was due to several factors that were optimised after the serum bottles study such as the incubation period (this was increased from 13 to 28 days), substrate concentration (this was decreased from 5 % (w/v) to 2 % (w/v)), and sulphide concentration (increased from 300 to 600 mg l^{-1} final concentration). Higher volatile fatty acids concentrations were evident in the bioreactor containing sulphide, substrate and the microorganism, indicating that the microorganism was growing at a faster rate, possibly due to the activated enzymes producing higher concentrations of the hydrolytic product.

5.1.5 Summary

The hypothesis that sulphide increases the rate-limiting step of anaerobic digestion was validated. This can be applied into various industries that utilise glucose or cellobiose, as cellulose will be utilised at more efficient rates. Since cellulose is a renewable resource, there is unlimited potential in the applications of the findings of this study to industries such as the bioethanol, methane, solid waste management, pulp- and paper- and the food industry. Since sulphide can be removed, the products would still be utilisable.

5.2 FUTURE RECOMMENDATIONS

In this study, the cellulosomal and non-cellulosomal fractions of *C. beijerinckii* were purified and the cellulases and endoglucanases were partially characterised. Future recommendations therefore would be to characterise the other hydrolytic enzymes present in the cellulosome. These include cellulosomal and non-cellulosomal hemicellulases (xylanases and mannanases) and pectin lyases (Doi *et al.*, 2003). Characterisation of these enzymes will include the determination of their pH and temperature optima, as well as determining the effect of sulphide on their activities. In this manner, a more complete picture of the effect that sulphide has on the cellulosomal and non-cellulosomal fractions of *C. beijerinckii* will be established. Although cellulose is the most abundant

polysaccharide on earth (Bayer *et al.*, 1998a) it is important to study all the enzymes associated with the cellulosome.

Studies on pectin lyases (Tamaru and Doi, 2001) and particularly on xylanases have been conducted (Kosugi *et al.*, 2001; Murashima *et al.*, 2003; Han *et al.*, 2004), however, the effect of sulphide on the enzymes has not been investigated.

Determining the mechanism of action of sulphide on the enzymes is also important. Along with pH effects, determining whether the reducing properties of sulphide are a contributing factor in the activation of the enzymes should be established. Lamed *et al.* (1985) showed that the thiol containing compound cysteine activated cellulase and endoglucanase activities of the cellulosome of *C. thermocellum*. Cysteine is also a reducing agent and like sulphide contains a thiol group. One method of determining whether the reducing properties of sulphide lead to activation of the enzymes is by comparing it with other reducing agents such as dithiothreitol (DTT). For instance studies performed by Sá-Pereira *et al.* (2002) found that the reducing agent, DTT, enhanced the xylanolytic activity of a *Bacillus subtilis* strain.

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APPENDICES

APPENDIX A

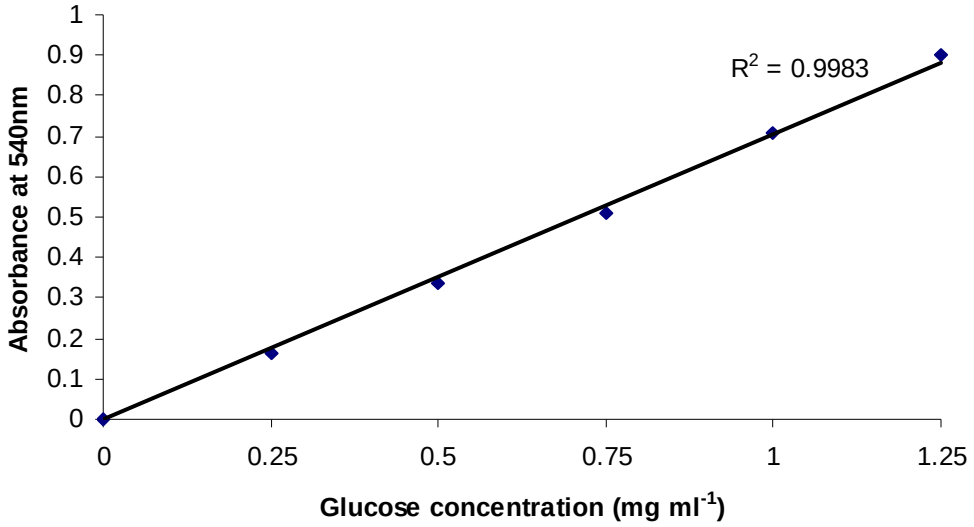

Figure A Glucose standard curve

APPENDIX B

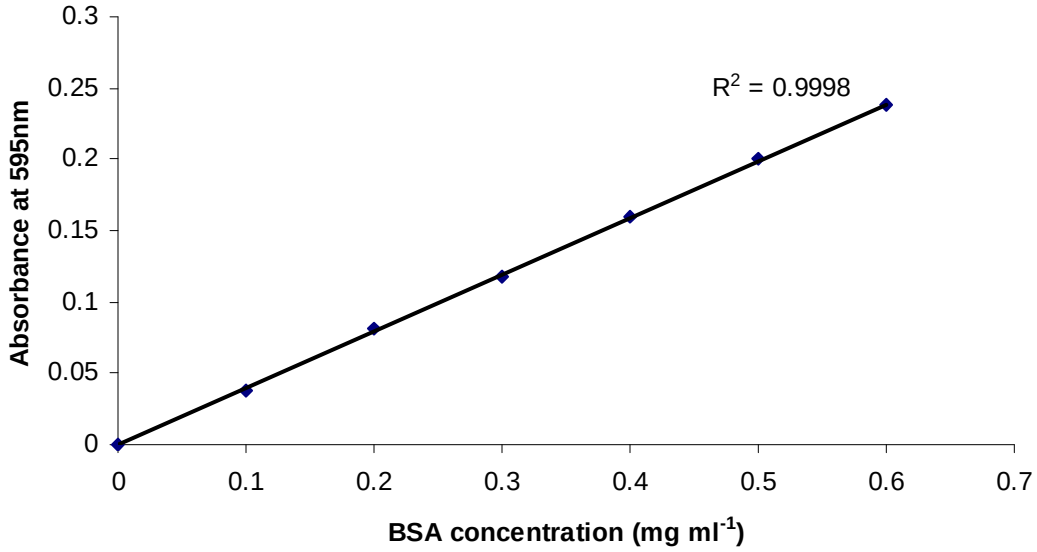

Figure B Bradford's assay standard curve

APPENDIX C

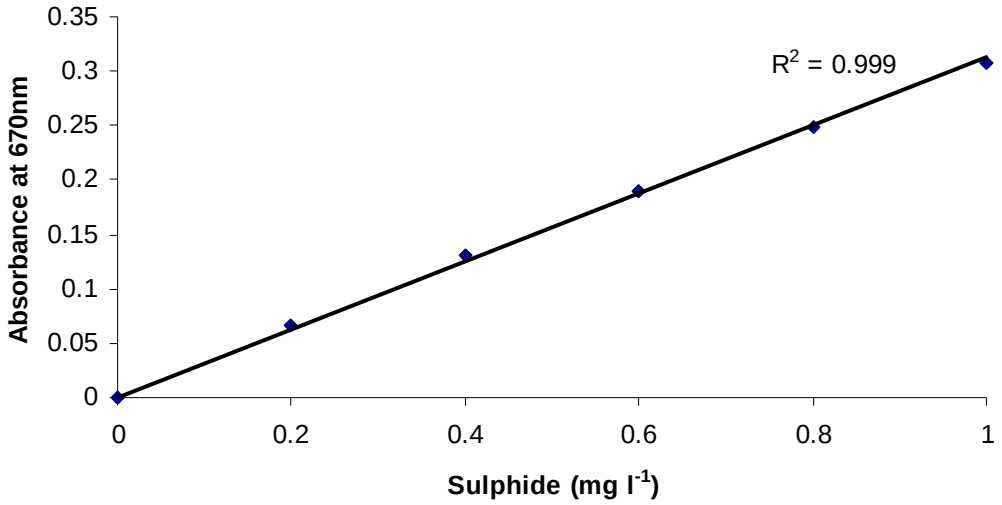

Figure C Sulphide standard curve

APPENDIX D

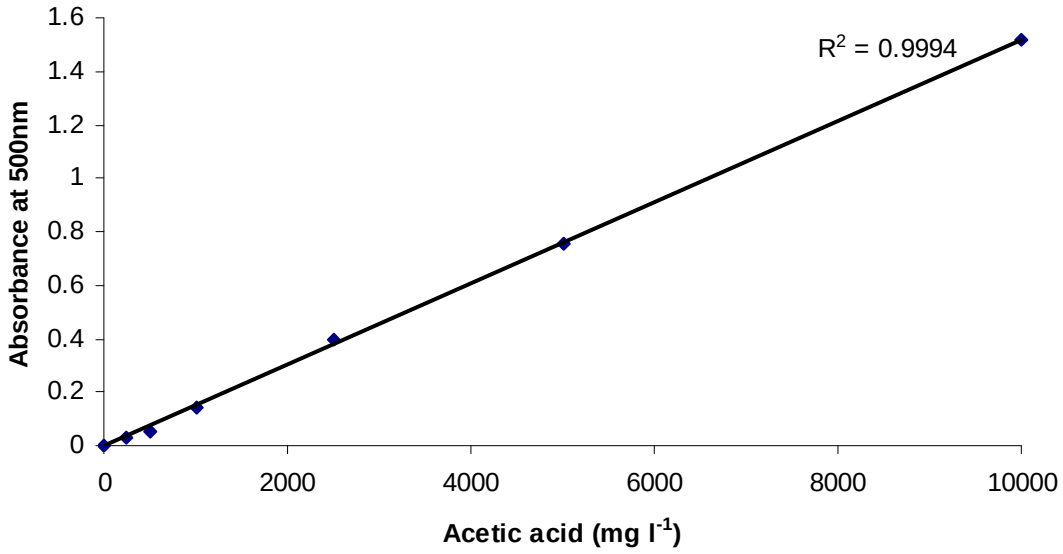

Figure D Acetic acid standard curve

Appendix E

Table E.1 shows the volumes and final concentrations used for each reagent in the PCR reaction. Table E.2 shows the PCR conditions that were used.

Table E.1 Volumes added and final concentrations of reagents used in PCR reaction

Reagent/component*	Volume (µl)	Final Concentration
$MgCl_2$, 25mM	2	1.5 mM
10x buffer	3.5	1x
PCR nucleotide mix (10mM each)	0.7	200 µM each
9F primer (10mM)	2	0.57 µM
1541R primer (10mM)	2	0.57 µM
Template DNA	1	0.5 µg/35µl
Nuclease free distilled water to final vol. 35µl	23.55	
Taq polymerase	0.25	1.75 U/35µl

* All reagents were purchased from Promega except for the primers, which were purchased from Inqaba Biotech.

Table E.2 PCR conditions used for 16S rDNA amplification

Conditions	Temperature (ºC)	Duration (min)
Initial denaturation	94	2
Denaturation	94	1
Annealing	55	1
Extension	72	1.5
Final extension	72	10

The number of PCR cycles performed using the Sprint PCR machine were 30.

Figure E.1 illustrates the 1500 bp 16S rDNA region that was PCR amplified.

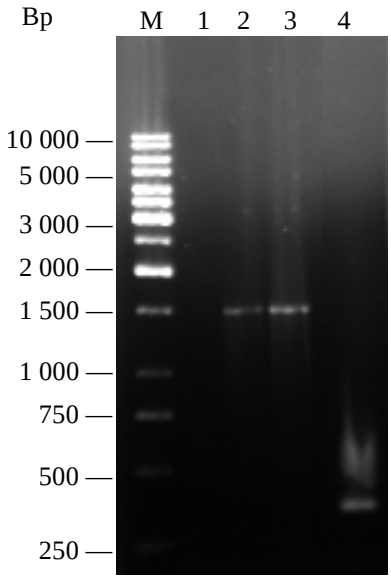

Figure E.1 Agarose gel (1 %) showing PCR products. M; Molecular weight marker (O'GeneRuler^{TM} 1 kb DNA ladder); 1; negative control; 2; Sample 1 PCR amplified 16S rDNA of isolated anaerobe; 3; Duplicate PCR amplified 16S rDNA of isolated anaerobe; 4; Positive control (Promega).

Figure E.2 shows the 16S rDNA sequence produced.

```
1     TTTGATCCTG GCTCAGGACG AACGCTGGCG GCGTGCTTAA CACATGCAAG
      TCGAGCGATG AATCTCCTTC GGAAGTGGAT TAGCGGCGGA CGGGTGAGTA

101   ACACGTGGGT AACCTGCCTC ATAGAGGGGA ATAGCCTTTC GAAAGGAAGA
      TTAATACCGC ATAAGATTGT AGTGCCGCAT GGCATAGCAA TTAAAGGAGT

201   AATCCGCTAT GAGATGGACC CGCGTCGCAT TAGCTAGTTG GTGAGGTAAC
      GGCTCACCAA GGCGACGATG CGTAGCCGAC CTGAGAGGGT GATCGGCCAC

301   ATTGGGACTG AGACACGGCC CAGACTCCTA CGGGAGGCAG CAGTGGGGAA
      TATTGCACAA TGGGGGGGAC CCTGATGCAG CAACGCCGCG TGAGTGATGA

401   CGGTCTTCGG ATTGTAAAGC TCTGTCTTCA GGGACGATAA TGACGGTACC
      TGAGGAGGAA GCCACGGCTA ACTACGTGCC AGCAGCCGCG GTAATACGTA

501   GGTGGCAAGC GTTGTCCGGA TTTACTGGGC GTAAAGGGAG CGTAGGTGGA
      TATTTAAGNG GGATGTGAAA TACTCNGGCT TAACCTGGGN GCTGCATTCC

601   AAACTGGATA TCTAGAGTGC ANGAGAGGAA AGTAGAATTC CTAGTGTAGC
      GGNGGAAATG CGTANAGATT AGGAAGAAAA CCAGTGGCGA AGGCGACTTT

701   CTGGGACTGN AACTGACACT GAGGCTCGAA AGCGNGGGGA GCAAACAGGA
      TTAGATACCC TGGTAGTCCA CGCCGTAAAC GATGAATACT AGGTGTAGGG

801   GTTGTCATGA CCTCTGTGCC GCCGCTAACG CATTAAGTAT TCCGCCTGGG
      GANTACGGTC GCAAGATTAA AACTCAAAGG AATTGACGGG GGCCCGCACA

901   AGCAGCGGAG CATGTGGTTT AATTCGAAGC AACGCGAAGA ACCTTACCTA
      GACTNGACAT CTCCTGAATT ACCCTTAATC GGGGAAGCCC TTCGGGGCAG

1001  GAAGACAGGT GGTGCATGGT TGTCGTCAGC TCGTGTCGTG AGANGTTGGG
      TTAAGTCCCG CAACGAGCGC AACCCTTATT GTTAGTTGCT ACCATTTAGT

1101  TGAGCACTCT AGCGAGACTG CCCGGGTTAA CCNGGAGGAA GGTGGGGATG
      ACGTCAAATC ATCATGCCCC TTATGTCTAG GGCTACACAC GTGCTACAAT

1201  GGCTGGNACA GAGAGATGCT AAACCGCNAG GTGGAGCCAA ACTTNAAAAC
      CAGTCTCAGT TCGGATTGNA GGCTGAAACT CGCCTACATG AAGCTGGAGT

1301  TGCTAGTAAT
```

Figure E.2 The 16S rDNA sequence of the isolated *Clostridium* anaerobe.