CHARACTERIZATION OF A NOVEL AEROBIC CELLULOLYTIC THERMOPHILIC BACTERIUM AND ITS CELLULASES

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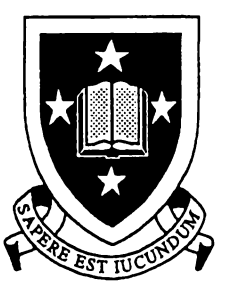

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ABSTRACT

A taxonomic position was established for a thermophilic, cellulolytic bacterium strain COMP.A2 on the basis of both phenotypic and phylogenetic characteristics. The bacterium was a Gram-positive, aerobic endospore-forming rod. Growth was observed between 60 and 70°C, with an optimum at 65°C. The optimum pH for growth was between 6.5 and 7.5. A wide range of substrates including Avicel and Sigmacell 20 could be utilised as sole carbon and energy source at 70°C. Lactate and acetate were produced when grown on cellobiose or amorphous cellulose medium. The organism was very sensitive to antibiotics such as penicilin G, streptomycin, and neomycin. Growth was inhibited by low concentrations of agar or agarose. A preference for uptake of cellobiose over glucose as the substrate was observed in a mixture of glucose and cellobiose medium. The bacterium was unable to grow autotrophically using hydrogen and carbon dioxide. The lack of alicyclic fatty acid in the cell membrane and the neutral growth pH indicates that the strain is not a member of the genus *Alicyclobacillus*. The G+C content of the DNA was 62.4 mol%. The 16S rRNA sequences showed that strain COMP.A2 is phylogenetically distinct from other members of genus *Bacillus* and forms a cluster with the genus *Alicylobacillus* and two facultatively chemolithoautotrophilic hydrogen-oxidizing *Bacillus* species, *B. tusciae* and *B. schlegelii*. Strain COMP.A2 is tentatively proposed as a new species of a new genus, namely *Caldibacillus cellulovorans* gen. nov., sp. nov. on the basis of its physiological and phylogenetic characters.

Two CMCases (CMCase A and CMCase B) and one ß-glucosidase have been purified from the culture filtrate of strain COMP.A2 by Phenyl-Sepharose chromatography, ion exchange chromatography and chromatofocusing. CMCase A and ß-glucosidase were shown to be electrophoretically homogeneous on PAGE and SDS-PAGE. CMCase B was partially purified.

The molecular weight of CMCase A was determined to be 85.1 kDa by SDS-PAGE and 174 kDa by size-exclusion chromatography. The isoelectric point of CMCase A was 4.12. With an assay time of 10 min, CMCase A displayed highest activity at 80°C. An optimum pH for activity was in the range of pH 6.5 to 7.0. An active energy was 46.6 KJ mol^{-1}. The enzyme did not lose activity over one hour at 70°C and still possessed 83% of the original activity after three hours incubation at 70°C. The half-life at 80°C was 32 min and the thermostability was increased by the addition of bovine serum albumin. The enzyme activity was inhibited by heavy metal ions (Zn^{2+} and Hg^{2+}) and the thiol-specific inhibitor *p*-chloromercuribenzenesulphonic acid (pCMPS). The CMCase A showed greatest hydrolytic activity on CMC and low activity on Avicel and xylan. CMCase A hydrolyses CMC or amorphous cellulose in an exo-model of action following initial and random attack. The Km value of the enzyme for CMC was 3.4 mg ml^{-1}. The enzyme cleaved cellopentaose to cellotetraose and glucose or cellotriose and cellobiose. Cellotetraose was cleaved to cellobiose. Cellotriose and cellobiose were not degraded.

The ß-glucosidase was largely cell-associated. The enzyme has a molecular weight of 52.5 kDa as determin by SDS-PAGE and an isoelectric point of 4.43. With an assay time of 15 min, ß-glucosidase displayed highest activity at 75°C. The enzyme

showed optimum activity in the range of pH 5.5 to 7.0. An active energy was 35.4 KJ mol^{-1}. The enzyme was inhibited by the thiol-specific inhibitor pCMPS and heavy metal ions (Cd^{2+}, Hg^{2+}, Ag^{+}). The thermostability of ß-glucosidase was increased by the addition of trehalose. The half-life of ß-glucosidase was less than 5 minutes at 80°C and 2.5 hours at 70°C in comparison with 24 minutes at 80°C and 11.75 hours at 70°C in the presence of trehalose. The enzyme was competitive inhibited by gluconolactone. The ß-glucosidase showed very broad substrate specificity and was active against sophorose, cellobiose and pNPG with Km values of 2.0 mM, 3.4 mM and 0.36mM, respectively.

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LIST OF ABBREVIATIONS

Bicine	*N, N*-bis(2-hydroxyethyl)glycine
Bis-Tris	bis(2-hydroxyethyl)imino-tris(hydroxymethyl)methane
Bis-Tris propane	1,3-bis(tris(hydroxymethyl)methylamino)propane
BSA	bovine serum albumin
CAPS	3-(cyclohexylamino)-1-propanesulphonic acid
CHES	(2-[N-Cyclohexylamino]-ethanesulfonic acid)
CMC	carboxymethylcellulose
CMCase	carboxymethylcellulase
CTC	copper-tartrate-carbonate
DEAE	diethylaminoethyl
DOC	sodium deoxycholate
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EPPS	*N*-(2-hydroxyethyl)piperazine -*N*-(3-propanesulphonic acid)
HEPES	(N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid])
IEF	isoelectric focusing
MES	2(*N*-morpholino)ethanesulphonic acid
MOPS	3(*N*-morpholino)propanesulphonic acid
MU	4-methylumbelliferone
MUC	4-methylumbelliferyl ß-D-cellobioside
MUCase	4-methylumbelliferyl ß-D-cellobiosidase
pNP	*p* -nitrophenol
pNPC	*p* -nitrophenyl ß-D-cellobioside
pNPG	*p* -nitrophenyl ß-D-glucopyranoside
pNPL	*p* -nitrophenyl ß-D-lactopyranoside
PAHBAH	*p* -hydroxybenzoic acid hydrazide
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SSC buffer	0.15 M NaCl and 0.015 M trisodium citrate at pH 8.0
TCA	trichloroacetic acid
TE buffer	10 mM Tris and 1.0 mM EDTA at pH 8.0
Tris	tris(hydroxymethyl)aminomethane

CHAPTER ONE

INTRODUCTION

Cellulose, which accounts for 50% of the dry weight of plant biomass, is the most abundant organic material in the world. The potential of cellulose as a source of energy and chemical feedstock has led to increasing interest in cellulose-decomposing microorganisms. Most cellulolytic microorganisms are fungi and bacteria. The cellulases from the fungus *Trichoderma reesei* and the thermophilic anaerobic bacterium *Clostridium thermocellum* have been intensively studied. They produce cellulase systems which degrade crystalline cellulose.

Most thermophilic, cellulolytic bacteria reported to date belong to the spore-forming anaerobic clostridia. The most extensively researched species include *Clostridium thermocellum* (Ng et al. 1977) and *Clostridium stercorarium* (Madden, 1983). Some asporogenous, extremely thermophilic, anaerobic rod-shaped bacteria capable of degrading crystalline cellulose from thermal springs have been described by Sissons et al. (1987) and Taya et al. (1988).

Relatively few aerobic cellulolytic, thermophilic bacteria have been reported. The actinomycetes *Thermomonospora curvata* (Stutzenbenger, 1971) and *Thermomonospora fusca* (Hagerdal et al., 1978) were reported to grow on crystalline cellulose at 55°C. An acidophilic, thermophilic bacterium, designated *Acidothermus cellulolyticus*, was reported by Mohagheghi et al. (1986). Recently a marine thermophilic bacteria *Rhodothermus marinus* has been reported to produce thermostable cellulases and hemicellulases (Hreggvidsson et al., 1996; Nordberg et al., 1997; Gomes and Steiner, 1998). Members of the genus *Bacillus* are producers of extracellular enzymes including amylases, proteinases, and polysaccharide hydrolases (Priest, 1977). Although some of them have been reported to produce carboxymethylcellulase (CMCase) activity (Chan and Au, 1987; Robson and Chambliss, 1984; Fukumori et al., 1985), there are only a few reports of *Bacillus* species capable of degrading crystalline cellulose (Jorgensen and Hansen, 1990; Kim, 1995; Han et al., 1995). A thermophilic aerobic spore-forming bacterium, designated as strain COMP.A2, was isolated from artificial compost in our laboratory. The isolate can utilise Avicel, a microcrystalline cellulose, as sole carbon and energy source for growth at high temperature. This suggests that the strain COMP.A2 may produce endo-, and exo-glucanases to degrade microcrystalline cellulose.

The object of this thesis was to determine the taxonomic position of this new isolate, strain COMP.A2, by comparing phenotypic and phylogenetic characters with related microorganisms. Another purpose was to identify and characterize the components of the cellulase system of strain COMP.A2. Conventional chromatographic techniques combined with fast protein liquid chromatography were used to purify extracellular cellulases produced from strain COMP.A2. We hope that the study in this thesis will lead us to know more about cellulose hydrolysis in aerobic cellulolytic, thermophilic bacteria.

CHAPTER TWO

LITERATURE REVIEW

2.1 THERMOPHILIC BACTERIA

2.1.1 Definition and terminology

A thermophile is defined by Brock (1986) as an organism capable of living at or near the maximum temperature for its taxonomic group. The maximum temperatures are about 50°C for insects, vertebrates and plants, about 55°C for eucaryotic algae and about 60°C for the fungi. Thermophilic bacteria are those capable of growing at temperatures of 55°C or higher (Brock, 1986). Thermophilic microorganisms were also classified by their cardinal growth temperatures (Table 1).

Table 1 Classification of thermophiles by cardinal temperatures

	Cardinal temperature (°C)		
	Minimum	Optimum	Maximum
Thermotolerants	_	<45	>50
Thermophiles	>25	>45	>50
Extreme thermophiles	>45	>65	>70
Barothermophiles*	?	>100	>100

Data from Wiegel (1990). *Stetter et al. (1990) used the term hyperthermophile.

2.1.2 Diversity of thermophilic microorganisms

Thermophilic bacteria are found in a variety of thermal and non-thermal environments. Thermal environments include areas of geothermal activity, solar-heated soils and ponds, and biological self-heating habitats such as compost, hay, straw and manure. Natural geothermal areas are widely distributed in the world, associated mainly with tectonic activity. The well-studied geothermal areas are in Iceland, U. S. A., New Zealand, Japan, Italy, and Russia.

Thermophilic bacteria are found in most bacterial metabolic groups, but have a limited species composition in each group. Kristjansson and Stetter (1992) listed 50 species of

thermophilic bacteria and 41 species of thermophilic archaea which belong to 26 and 22 genera, respectively. Most of the thermophilic eubacteria have an optimum growth temperature below 75°C. *Aquifex pyrophilus* and *Thermotoga maritima* show the highest growth temperatures among eubacteria with T_{max} at 95°C and 90°C, respectively (Stetter, 1996). Thermophilic archaea can grow up to and above the boiling point of water. Species of the genus *Pyrodictium* and *Pyrolobus fumarii* are the organisms with the highest growth temperatures of 110°C and 113°C, respectively (Stetter et al. 1990; Blochl et al., 1997). The diversity of thermophilic bacterial species is not only affected by high temperature but also by other environmental factors, such as pH, nutrients, available energy and ionic strength etc. For example, the diversity of thermophilic bacterial species in thermal acid environments is more limited than in neutral habitats (Zeikus, 1979).

2.2 TAXONOMIC STUDIES OF THERMOPHILIC BACILLI

There are two major genera of thermophilic aerobic bacteria, *Bacillus* (spore-forming rods) and *Thermus* (nonspore-forming thin rods and filaments). The taxonomic studies of thermophilic bacilli are discussed in this section. Reviews about other thermophilic aerobic bacteria are given by Williams (1992), Aragno (1992), Kristjansson and Alfredsson (1992).

The genus *Bacillus* is a phenotypically heterogeneous collection of aerobic or facultatively anaerobic, Gram-positive, rod-shaped, endospore-forming bacteria (Claus and Berkeley, 1986). Thermophilic bacilli have been isolated from a wide range of thermophilic and mesophilic environments. The first thermophilic *Bacillus* strain was isolated from the river Seine in 1888 (Sharp et al., 1992). Since then many thermophilic *Bacillus* species have been reported with T_{max} at or above 60°C. Based on the systematic study of 216 thermophilic cultures carried out by Gordon and Smith in 1949, only *B. stearothermophilus* and *B. coagulans* were considered to be thermophilic species of *Bacillus* in the 7th edition of Bergey's Manual (Sharp et al, 1988). Walker and Wolf (1971) examined the biochemical and physiological properties of 230 thermophilic, aerobic spore-forming strains. All the strains were classified into three major groups with minor sub-groups. One unique characteristic of Group 1 was the formation of gas from nitrate under anaerobic conditions. This was not found in the other two groups. Group 3 consisted of strains from *B. calidolactis*, *B. stearothermophilus*, and *B. thermoliquefaciens*.

Sharp et al. (1980) reported the results of extensive biochemical and genetic studies of some thermophilic species of *Bacillus*. Ten strains of thermophilic bacilli could be

classified into the three major groups as suggested by Walker and Wolf (1971). Two *B. stearothermophilus* strains, *B. caldotenax*, *B. caldovelox*, and *B. caldolyticus* were in the group 1. The other two groups contained different *B. stearothermophilus* strains. However, the classification into three groups based on phenotypic and genotypic characteristics did not always concur. Only six of the strains remained in the same group when characterized on several different criteria, such as DNA hybridization results, G+C mol%, esterase analysis, and Walker & Wolf's classification (1971).

Seventeen thermophilic members of the genus *Bacillus,* isolated from a variety of environments, were listed in a recent review by Sharp et al. (1992). Three thermophilic bacilli, *B. caldotenax*, *B. caldovelox*, and *B. caldolyticus*, were isolated from Yellowstone National Park in the USA (Heinen, 1971; Heinen and Heinen. 1972). They were different from *B. stearothermophilus* in temperature optima for growth, fatty acid composition, and submicroscopic structure. The isolation of an acidophilic, thermophilic strain *B. acidocaldarius* was reported by Darland and Brock (1971) and grew at temperatures between 45°C and 70°C with pH values from 2.0 to 6.0. The high G+C mol% and living in thermal acid environments suggest that *B. acidocaldarius* is not closely related to *B. coagulans* or *B. stearothermophilus*. Two strains of thermophilic, endospore-forming, facultatively chemolithoautotrophic bacteria were described by Schenk and Aragno (1979). They were isolated from the surface layer of the sediment of a small neutrophic lake in Switzerland and oxidised hydrogen in the presence of O_2 and CO_2. The isolates, which were designated as *B. schlegelii,* grew optimally at 70°C and had 67-68 mol% G+C. Bonjour and Aragno (1984) reported the isolation of a thermoacidophilic, facultatively autotrophic sporeformer from ponds in the geothermal area of Italy. The optimum pH and temperature for growth of the organism was 4.2-4.8 and 55°C, respectively. The low DNA:DNA homologies between these isolates and *B. acidocaldarius* or *B. schlegelii* indicated that they were quite different, and they were subsequently placed in a new species and the name of *Bacillus tusciae* was proposed. Other thermophilic bacilli include *B. acidoterrestris* and *B. cycloheptanicus* (Deinhard et al., 1987a,b), *B. thermoglucosidasius* (Suzuki et al. 1983), *B. pallidus* and *B. thermocloaceae* (Scholz et al., 1987; Demharter and Hensel, 1989), *B. thermoleovorans* (Zarilla and Perry, 1987), *B. kaustophilus*, *B. thermocatenulatus*, *B. thermodenitrificans*, *B. flavothermus*, *B. coagulans*, and *B. thermoruber* (Sharp et al., 1992). All these can grow above 55°C.

The application of nucleic acid sequencing and hybridization studies had an important influence on classification of the genus *Bacillus*. Stackebrandt et al. (1987) reported the presence of at least three main clusters based on 16S rRNA cataloguing studies of nine *Bacillus* species and several related non-*Bacillus* taxa. *B. stearothermophilus* was

clustered with *Thermoactinomyces vulgaris* and branched early from the other two groups. Ash et al. (1991) determined the 16S rRNA sequences of 51 *Bacillus* species, most of them mesophilic. Comparative analyses of these sequences revealed five phylogenetically distinct clusters. The largest cluster included *B. subtilis* and 27 other *Bacillus* species. The thermophilic *Bacillus* species *B. stearothermophilus*, *B. kaustophilus* and *B. thermoglucosidasius* clustered together to form group 5. They were quite distinct from other mesophilic *Bacillus* species. The thermophilic acidophile *B. cycloheptanicus* exhibited very low sequence similarities with all other strains examined and formed a separate line of descent.

Wisotzkey et al. (1992) further investigated the primary sequence and secondary structure of the 16S rRNA of the three thermophilic acidophiles *Bacillus acidocaldarius*, *Bacillus acidoterrestris* and *Bacillus cycloheptanicus*. The 16S rRNA sequences of these three species were determined and compared with other *Bacillus* species and genera in the *Bacillaceae*. The results showed that *Bacillus acidocaldarius*, *Bacillus acidoterrestris* and *Bacillus cycloheptanicus* were specifically related to one another and separated from all other *Bacillus* species including the traditional *Bacillus* thermophile *B. stearothermophilus*. The three thermoacidophilic *Bacillus* species also contained unique alicylic fatty acids as the major membrane lipid components; these have not been found in any other *Bacillus* species. A new genus *Alicyclobacillus* was proposed for these thermoacidophiles (Wisotzkey et al., 1992). None of the members of the genus *Alicyclobacillus* are capable of utilizing cellulose as carbon and energy source.

The 16S rRNA sequences from 16 thermophilic strains of the genus *Bacillus* were recently determined and compared with the existing database for the genera *Bacillus* and *Alicyclobacillus* by Rainey et al. (1994a). Five major rDNA groups were obtained. Seven of the 15 strains were found to cluster with *B. stearothermophilus* and the related species which formed group 5 described by Ash et al. (1991). *B. caldovelox*, *B. caldolyticus* and *B. caldotenax* formed a cluster with *B. kaustophilus* and *B. thermoleovorans* and had almost the same 16S rRNA sequence. The second group of closely related thermophilic bacilli includes *B. pallidus*, *B. thermoalkalophilus* and *Bacillus* sp. DSM 2349. *B. thermocloaceae* was found to be phylogenetically isolated from other groups. The low similarity of *B. tusciae* and *B. schlegelii* with other *Bacillus* species and their high DNA G+C mol% suggested that they may belong to the genus *Alicyclobacillus*. All the studies show that thermophilic bacilli are not only phenotypically heterogeneous but also phylogenetically heterogeneous. The members of *Bacillus* species should be split into multiple genera.

2.3 CELLULOSE AND BIOLOGICAL DEGRADATION OF CELLULOSE

2.3.1 Structure of cellulose

Cellulose is a high molecular weight linear polymer made of anhydroglucose residues joined by 1,4-ß-D-glucoside bonds. Each glucose residue is rotated 180° relative to its neighbouring residue, so the basic repeating unit is cellobiose. The degree of polymerization (D.P.) of the glucose units varies according to the source of cellulose. There are approximately 14000 glucose residues in native plant cellulose, 3470 in bacterial cellulose such as that produced by *Acetobacter xylinum* (Marx-Figini, 1982) and 50 to 5000 in commercial celluloses (Ljungdahl and Eriksson, 1985).

Native cellulose fibres are made of microfibrils. Each microfibril contains several elementary fibrils in which the cellulose chains are bound together by intra- and inter-molecular hydrogen bonds. The microfibrils consist of highly ordered crystalline regions and less ordered amorphous regions. There are at least four crystalline cellulose forms, designated as cellulose I, II, III, and IV on the basis of difference in X-ray and/or electron diffraction patterns (Coughlan, 1990). Cellulose I, which is found in nature, can be converted into cellulose II by alkali treatment. Recent results show that type I celluloses are composed of two slightly different forms named Iα and Iß. The former is predominant in algal and bacterial celluloses whereas the latter is predominant in higher plant celluloses (Tomme et al., 1995).

The degree of crystallinity within fibres varies with the source of cellulose and pretreatment, and ranges from 0% for amorphous, acid-swollen cellulose, to nearly 100% for the cellulose isolated from *Valonia macrophysa* (Beguin and Aubert, 1994). Cellulose is the most abundant polysaccharide in plant cell walls and cellulose fibres are usually found in close association with hemicellulose, lignin, and other polysaccharides (Coughlan, 1985).

2.3.2 Cellulolytic microorganisms

Cellulolytic microorganisms are ubiquitous in nature and are found in a variety of environments where cellulosic wastes accumulate, many of which are under conditions of extreme pH and temperature. These habitats include swamps, soils, marshes, river, lake and sea water sediments, rotting grasses, leaves and wood, cotton bales, sewage sludge, compost heaps, muds and decaying vegetable matter in hot springs, acid springs, and alkaline springs (Coughlan and Mayer, 1992).

Cellulolytic microorganisms normally do not exist alone in nature and are associated with other microorganisms including non-cellulolytic species. These associations lead to the complete degradation of cellulose. Ljungdahl and Eriksson (1985) listed 56 different species of cellulolytic fungi. The important genera are *Penicillium*, *Aspergillus*, *Trichoderma*, *Fusarium*, and *Chaetomium*. Aerobic fungi including white-rot, brown-rot and soft-rot species are the principal decomposers of wood. The white-rot fungus *Sporotrichum pulverulentum* and the mold *Trichoderma reesei* are well studied and can produce enzyme systems to degrade crystalline cellulose (Ljungdahl and Eriksson, 1985). Anaerobic cellulolytic fungi have been isolated from the rumen of a number of animals. The flagellates *Neocallimastix frontalis, Piromonas communis* and *Sphaeromonas communis* were found to be species of anaerobic phycomycete fungi (Coughlan, 1990). They produced extracellular cellulases with activity against crystalline cellulose as well as CM-cellulose and short chain cellooligosaccharides (Wood, 1989).

In 1992, at least 97 cellulolytic bacterial species were described ranging from aerobic to anaerobic, mesophilic to thermophilic (Coughlan and Mayer, 1992). The thermophilic cellulolytic bacteria are reviewed in sections 2.4 and 2.5 of this thesis. Some of the mesophilic cellulolytic bacteria are briefly discussed here. *Acetivibrio cellulolyticus*, isolated from municipal sewage sludge, is a Gram-negative non spore-forming obligate anaerobe and forms capsules (Saddler and Khan, 1979; Patel et al., 1980). It grows at pH 6.5-7.7 (optimum pH 7) and at temperatures from 20 to 40°C (optimum temperature 35°C). It only utilizes cellulose, cellobiose, and the glucoside salicin among 30 polysaccharides tested. *Bacteroides succinogenes* (now called *Fibrobacter succinogenes*), *Butyrivibrio fibrisolvens*, *Ruminococcus albus*, and *Ruminococcus flavefaciens* are the important rumen cellulolytic bacteria (Coughlan and Mayer, 1992). *F. succinogenes*, and some cellulolytic *Bacteroides* strains have been isolated from the bovine rumen, rat caecum, pig intestine, buffalo rumen, anaerobic marine environments and municipal sewage sludge (Ljungdahl and Eriksson, 1985). *F. succinogenes* is an obligately anaerobic, mesophilic, non sporeforming, Gram-negative rod. It ferments cellulose, starch, dextrin, trehalose, maltose, cellobiose and glucose to succinate and acetate. Scanning electron microscopy revealed that cellulose fibres were covered with *F. succinogenes* cells (Groleau and Forsberg, 1981). The endoglucanase activity present in supernatant fluid was largely associated with sedimentable membrane fragments, and cellulosome-like protuberances were found on the surface of the bacteria. *Ruminococcus albus*, and *Ruminococcus flavefaciens*, isolated from the rumen of many animals, are anaerobic, mesophilic Gram-positive cocci which ferment cellulose, xylan and cellobiose (Bryant, 1986). Both *Ruminococcus* species produce high-molecular-weight cellulase complexes as well as low-molecular-weight cellulases

(Ljungdahl and Eriksson, 1985). A large number of cellulolytic aerobic bacteria have been isolated from soil. They include members of the genera *Bacillus, Cellulomonas, Cytophaga, Pseudomonas, Streptomyces, Sporocytophaga, Thermoactinomyces, and Thermomonospora* (Coughlan and Mayer, 1992). Multiple endoglucanases are produced by these bacteria. The *Cellulomonas* species are Gram-variable, mesophilic, and non sporeforming rods and can grow aerobically or anaerobically (Ljungdahl and Eriksson, 1985). They degrade a variety of celluloses including crystalline cellulose. The cellulase systems in the *Cellulomonas* species appears to be similar to that of *Clostridium thermocellum* (Ljungdahl et al., 1988). Cellobiose is the main product of cellulose hydrolysis by the extracellular cellulases from *Cellulomonas* (Rapp and Beermann, 1991). Two mechnisms of cellobiose cleavage exist in the *Cellulomonas* species: one is hydrolytic cleavage of cellobiose by ß-glucosidase and the other is phosphorolytic cleavage of cellobiose by cellobiose phosphorylase.

2.3.3 Cellulose degradation in aerobic and anaerobic environments

Cellulolytic microorganisms, which are usually associated with non-cellulolytic species, are regarded as "primary microorganisms". The microorganisms which rely on primary microorganisms for a supply of free sugars are called "secondary microorganisms".

Most of the celluloses in nature are eventually oxidized into carbon dioxide by aerobic microorganisms. About 10% cellulose is converted to methane by anaerobic microorganisms (Coughlan and Mayer, 1992). Cellulose is first degraded into cellobiose and glucose by the cellulolytic microorganisms. These sugars are utilized by primary and secondary microorganisms for cell growth and maintenance. Removal of the excess of sugar by the secondary microorganisms facilitates cellulose degradation by the primary microorganism. The mixed culture *Sporocytophaga myxococcoides* and *Flavobacterium* sp. is an example of stable association between primary and secondary microorganisms (Ljungdahl and Eriksson, 1985). This mixed culture isolated from a sewage treatment plant was superior in degrading cellulose than the cellulolytic bacterium *S. myxococcoides* alone.

Cellulose degradation in anaerobic environments is considered as two interrelated processes: hydrogen production and hydrogen utilization (Ljungdahl and Erikasson, 1985). The primary cellulolytic species (e.g. *Clostridium thermocellum*) convert cellulose to cellobiose and glucose which is then fermented to lactate, acetate, ethanol, CO_2 and H_2 by primary and secondary bacteria (Wiegel and Ljungdahl, 1981; Wiegel et al. 1979; Wiegel et al. 1981). Ancillary bacteria (e.g., *Desulfotomaculum nigrificans*) can convert lactate and ethanol arising from the primary and secondary bacteria to

acetate, CO_2, and H_2 (Ljungdahl and Eriksson, 1985). This is the first stage, in which the bacteria are hydrogen-producing. In the second stage the hydrogen-utilizing bacteria assimilate the hydrogen produced from the above and use it for the reduction of CO_2 to acetate or methane, sulfate to H_2S or nitrate to ammonia or N_2. For example, CO_2 is reduced to methane by the methanogenic bacteria (Zeikus, 1977).

2.4 ANAEROBIC THERMOPHILIC CELLULOLYTIC BACTERIA

Anaerobic thermophilic cellulolytic bacteria have been found in a wide variety of habitats, such as manure, compost, hay, straw, anaerobic digesters, and hot springs. The spore-forming clostridial strains, which can be isolated from thermophilic and non-thermophilic habitats, are the best-known cellulolytic, thermophilic bacteria (Duong et al., 1983). However, recent work has resulted in a great diversity of thermophilic cellulolytic bacteria which include non-spore forming rods and spirochetes being described.

2.4.1 Thermophilic cellulolytic spore-forming strains

2.4.1.1 *Clostridium thermocellum*

The first report of thermophilic fermentation of cellulose was in 1896 by MacFayden and Blaxall. Viljoen reported the isolation of *Clostridium thermocellum* from stacked manure in 1926, and pure cultures were obtained in 1948 by McBee using an anaerobic incubation technology developed by Hungate (Lamed and Bayer, 1991). Another thermophilic cellulolytic anaerobic bacterium, designated as *Clostridium thermocellulaseum*, was isolated and described by Enebo in the late 1940s (Duong et al., 1983). The main difference between *C. thermocellulaseum* and *C. thermocellum* was the substrate utilization pattern. Unfortunately, *C. thermocellulaseum* was lost. No *C. thermocellum* strains were described in the literature until 1975 when Lee and Blackburn isolated a thermophilic cellulolytic *Clostridium* strain M-7 from manure. The strain M7 was similar to *C. thermocellulaseum* in cell morphology and fermentative abilities. Subsequently numerous *C. thermocellum* strains have been isolated from diverse environments, such as LQR1 from sewage sludge (Lamed and Zeikus, 1980), JW20 from cotton bales (Ljungdahl et al., 1981; Freier et al., 1988), YS from soil (Bayer et al., 1983), and YM4 from volcanic soils in Japan (Coughlan and Mayer, 1992). *C. thermocellum* is an obligately anaerobic thermophilic spore-forming bacterium with an optimum growth temperature in the range 60-64°C. Cellulose and cellobiose are fermented into ethanol, acetate, lactate, carbon dioxide and hydrogen (Ng et al. 1977). The substrate utilisation pattern varies widely with different strains of *C.*

thermocellum. For example, *C. thermocellum* strain M7 grew on a variety of mono-, di- and polysaccharides as well as cellulose (Lee and Blackburn, 1975), while three strains of *C. thermocellum* (LQ8, N1 and H1) fermented cellulose and cellulose derivatives, but not glucose, xylose, or other sugars (Ng et al. 1977). However, Patni and Alexander (1971) found that *C. thermocellum* could ferment glucose with improved conditions. Caution must be taken for glucose utilization of *C. thermocellum* because it may be attributable to a stable mixed culture (Lamed and Bayer, 1991), or that an adaptation time is required for *C. thermocellum* to grow on glucose. Bender et al. (1985) compared *C. thermocellum* strains obtained from various sources and found all exhibited similar characteristics of *C. thermocellum* and were unable to grow on xylose. Strain JW20 can grow on cellulose and xylan, but not on xylose (Wiegel et al., 1985). Lamed and Bayer (1991) pointed out that the inability to utilize xylose might be a distinguishing feature for *C. thermocellum*.

The growth of *C. thermocellum* on cellulose has attracted a great deal of interest. *C. thermocellum* secretes a yellow affinity substance (YAS) that binds to the cellulose fibres during growth on cellulose (Ljungdahl et al. 1983). The production of YAS precedes the synthesis of cellulase. YAS is a water insoluble, carotenoid-like substance. YAS-cellulose has a higher affinity for cellulases than cellulose itself. YAS-cellulose has been used as an affinity matrix in columns for the purification of the cellulase complexes (Ljungdahl et al. 1988).

Johnson et al. (1982) compared the cellulase system of *C. thermocellum* to that of *Trichoderma reesei*. In the presence of calcium ions and dithiothreitol, the extracellular cellulases produced by *C. thermocellum* can completely hydrolyse native and derived forms of cellulose (cotton, filter paper, and Avicel) at a rate and an extent comparable with *T. reesei* cellulase. Similar yields of cellulase activity were found in culture supernatants of *C. thermocellum* and of *T. reesei*, but the cellulases of *C. thermocellum* have a much higher specific activity than those of *T. reesei*. The studies of *C. thermocellum* strain YS by Lamed et al. (1983a, 1983b) and Bayer et al. (1983) led to the discovery that the majority of cellulases are aggregated into a high molecular weight, multi-functional, multiple-enzyme complex which they termed the cellulosome.

The cellulosome was found to consist of at least 14 different polypeptides, many of which were shown to possess endoglucanase activities. At least one exoglucanase called CelS, which is a major component of the cellulosome, has been found in the *C. thermocellum* cellulosome (Wang et al., 1994; Kruus et al., 1995). The molecular weight of the cellulosome in the strain YS was about 2.1×10^6 Da. (Lamed et al. 1983a). The cellulolytic activity of the cellulosome was enhanced by calcium ions and

thiols and inhibited by cellobiose. The cellulosomes originate at the surface of the cells, where they mediate attachment of the bacterium to the substrate, the cellulosic fibers. They are released from the cell and the substrate as cultivation proceeds (Coughlan and Ljungdahl, 1988). The protuberant structures were visualized by specific immunolabeling and by a general staining procedure using cationized ferritin to demonstrate their cell surface structure of *C. thermocellum* by transmission electron microscopy (Bayer et al., 1985). The cellulosomes are attached to the exterior of the protuberances (Lamed and Bayer, 1991). Cellulosome-like complexes have also been found in other clostridial species, some anaerobic bacteria and fungi (Bayer et al., 1998a). More discussion on structure and function of the cellulosome will be presented in section 2.8.

2.4.1.2 *Clostridium stercorarium*

Another important thermophilic cellulolytic *Clostridium* species was isolated from a compost heap. This isolate was classified as *C. stercorarium* and was different from *C. thermocellum* in not producing yellow pigmentation around cellulose hydrolyzing colonies, growing slowly on cellulose and fermenting hemicellulose (Madden, 1983). *C. stercorarium* is an obligately anaerobic, sporeforming thermophile with optimum growth temperature of 65°C at pH 7.3. The cells are gram-negative straight rods, and have oval terminal endospores. The fermentation products from glucose and cellulose are ethanol, acetate, lactate, hydrogen and carbon dioxide. One endoglucanase, one exoglucanase and one ß-glucosidase from *C. stercorarium* has been purified and characterized (Creuzet and Frixon, 1983; Creuzet et al., 1983; Bronnenmeier and Staudenbauer, 1988a,b). The genes encoding for the thermostable xylanase and Avicelase of *C. stercorarium* have been sequenced and expressed in *E. coli* recently (Fukumura et al., 1995; Bronnenmeier et al., 1997)

2.4.1.3 Other thermophilic cellulolytic clostridial strains

Other clostridial cellulolytic thermophiles, which have been isolated recently, include strain Tx from compost (Taya et al., 1984), *Clostridium thermolacticum* from methanogenic enrichment cultures (LeRuyet et al., 1985), *Clostridium josui* from a Thai compost (Sukhumavasi et al., 1988), *Clostridium thermocopriae* from camel faeces, compost soil and hot spring water in Japan (Jin and Toda, 1988), *Clostridium thermopapyrolyticum* from a riverside mud sample in Brazil (Mendez et al., 1991), *Clostridium cellulosi* and *Clostridium cellulofermentans* from a cow manure compost and soil of a dairy farm in China (He et al., 1991) are all assigned to the genus *Clostridium* on the basis of their sporogenous nature and anaerobic growth.

2.4.2 Anaerobic non sporeforming cellulolytic bacteria

The cellulolytic species of *Clostridium* described above had temperature optima around 60-65°C except *Clostridium josui* with a optimum temperature of 45°C (Sukhumavasi et al., 1988). Ljungdahl et al (1981) first reported the existence of cellulolytic, extremely thermophilic bacteria in a culture inoculated from an Icelandic hot pool. Since then many new anaerobic, nonsporeforming, cellulolytic bacteria with temperature optima of 70°C and above have been reported.

2.4.2.1 *Caldocellulosiruptor saccharolyticus*

Eight anaerobic cellulolytic cultures were obtained from New Zealand thermal pool samples by culturing at 75°C with microcrystalline cellulose as growth substrate (Sissons et al. 1987). All of these isolates stained Gram-negatively and were obligately anaerobic non sporeforming rods. Five of these isolates degraded cellulose and lignocellulose to different extents at 70°C (Donnison et al. 1989). The cellulolytic activities from three isolates that grew optimally at 72°C were compared with *C. thermocellum* (Reynolds et al. 1986). DNA-DNA hybridization studies, the absence of terminal spores and G+C mol% proved that the new isolates were different from *C. thermocellum* and *C. stercorarium* and it was suggested that they belonged to a new genus (Donnison et al. 1988). The strain Tp8.T.6.3.3.1 had the most active and most stable cellulase activity, and was tentatively named *Caldocellum saccharolyticum*, but was subsequently designated *Caldicellulosiruptor saccharolyticus* gen. nov., sp. nov. (Rainey et al. 1994b). The growth temperature range of *Cs. saccharolyticus* is 45-80°C with an optimum of 70°C. Glucose, cellobiose, xylose, xylan and cellulose can be utilized as carbon sources by *Cs. saccharolyticus*. The main fermentation end products on cellobiose are lactate and acetate. The majority of extracellular Avicelase and CMCase activities in *Cs. saccharolyticus* were bound to the cellulose in the medium. Genes of thermostable cellulases from *Cs. saccharolyticus* have been cloned and expressed in *E.coli*. (Luthi et al., 1990, 1991).

Mladenovska et al. (1995) recently isolated an extremely thermophilic, cellulolytic, anaerobic bacterium from an alkaline hot spring in Iceland. The cells of isolate strain 6A are asporogenous rods and are similar to *Cs. saccharolyticus* in growth temperature and pH, the major fermentation end products and G+C mol%. 16S rDNA sequence analysis also showed that strain 6A was phylogenetically related to *Cs. saccharolyticus*. Mladenovska et al. (1995) proposed that the isolated bacterium strain 6A was a new species of the genus *Caldicellulosiruptor* and that it be named *Caldicellulosiruptor lactoaceticus* sp. nov.

2.4.2.2 Strain NA 10

A cellulolytic, anaerobic, extreme thermophile designated as strain NA 10 was isolated from an alkaline hot spring in Japan (Taya et al., 1988). Cells of this strain were Gram-negative staining, asporogeneous, flagellated rods and grew optimally at 75°C and pH 8.0-8.3. Strain NA 10 degraded cellulose to produce acetic acid, hydrogen and carbon dioxide as the main end products. The characteristics of this strain were compared with those of *C. thermohydrosulfuricum*, *C. thermocellum*, *Thermoanaerobacter ethanolicus* and strain Tp8.T.6.3.3.1. It was found that strain NA 10 was similar to *C. thermocellum* in cellulose utilization, but different in sporulation, optimum growth temperature and pH (Taya et al., 1988). Recently, genes encoding for a multidomain cellulase and β-glucosidase have been sequenced and expressed in *E. coli* (Miyake et al., 1998; Sota et al., 1994).

2.4.2.3 Strain H173

An extremely thermophilic anaerobic cellulolytic strain was isolated from a hot spring algal mat in New Zealand by Hudson et al. (1990a). The isolate, designated as strain H173, was capable of utilising cellobiose, Avicel, xylan, and starch as carbon source, and fermented Avicel to acetate, lactate, glucose, and cellobiose at 70°C. Cells of strain H173 were Gram-negative staining short rods. The optimum growth temperature and pH were 70°C and pH 7.0. The taxonomic position of this organism is not yet established, but it was suggested that it is different from NA10 (Taya et al., 1988) and other New Zealand strains.

2.4.2.4 *Spirochaeta thermophila*

Two obligately anaerobic, polysaccharolytic, extremely thermophilic spirochetes were isolated and described by Rainey et al. (1991), Svetlichny et al (1990) and Aksenova et al. (1992). The strain RI 19.B1, isolated from a thermal spring on Raoul Island in New Zealand, and strain Z-1203 isolated from a marine hot spring on Shiashkotan Island in the Soviet Far East, grew optimally at 65°C. A variety of mono-, di-, and polysaccharides, including cellulose and xylan, were utilised as energy sources by both strains. On the basis of their extremely thermophilic, polysaccharolytic nature, obligately anaerobic metabolism, being free living and having a morphology typical of *Spirochaeta* species, it was proposed that the two strains should be included in the genus *Spirochaeta* as a new species, *Spirochaeta thermophila*, with strain Z-1203 (=DSM 6578) as the type strain. These strains are the most thermophilic and the only cellulolytic members described in the family Spirochaetales.

2.4.2.5 *Thermotoga* species.

Several ***Thermotoga*** species have been reported to produce thermostable xylanases, cellulases, mannanases, and galactosidases (Simpson et al., 1991; Bronnenmeier et al., 1995; Duffard et al., 1997; Bok et al., 1998). A *Thermotoga* sp. strain FjSS3-B.1 was isolated from a hot spring in Fiji (Huser et al, 1986). The strain grew on carboxymethylcellulose, glucose, xylose, starch, and maltose, but not on insoluble or amorphous cellulose. The optimum growth temperature is 80-85°C at pH 7.0. Thermostable cellobiohydrolase, xylanase, ß-glucosidase and ß-xylosidase have been purified from this isolate (Ruttersmith and Daniel, 1991, 1993, Simpson, et al., 1991). A marine extreme-thermophile *Thermotoga neapolitana* produced highly thermostable endoglucanases, when grown in a medium containing cellobiose as the carbon source (Bok et al., 1998). Two purified enzymes showed optimum temperature for activity at 95 and 106°C, respectively.

2.4.2.6. Other isolates

Eleven pure cultures of freshwater anaerobic, rod-shaped, asporogenous bacteria were isolated from the hot springs of the Soviet Far East by enrichment cultures with micro-crystalline cellulose as the sole carbon and energy source (Svetlichny et al., 1990). They were morphologically and physiologically similar to the extremely thermophilic, cellulolytic anaerobes *Anaerocellum thermophilum* and *Dictyoglomus* sp. Phylogenetic analysis showed that *Anaerocellum thermophilum* strain Z-1320 was closely related to *Caldicellulosiruptor saccharolyticus* (Rainey et al., 1993). Twelve strains of marine anaerobic, extremely thermophilic, asporogenous, cellulolytic eubacteria were also isolated from the shallow coastal marine hydrotherms. These isolates were identified as strains of *Thermotoga maritima* and *Thermotoga neapolitana* and grew optimally at 80-85°C. In the same poster, Svetlichny et al (1990) reported the isolation of a marine, obligately anaerobic, cellulolytic, thermophilic spirochete which degraded cellulose at 68°C and pH 7.5. The study showed that extremely thermophilic, cellulolytic bacteria are widely distributed in freshwater and marine hot springs.

A survey of cellulolytic anaerobic thermophiles from hot springs in New Zealand and Azores was carried out using a variety of media, pH values and enrichment temperatures (Hudson et al. 1990b). A *Clostridium thermocellum* -like strain was isolated once. All other isolates obtained were anaerobic asporogenous rods with optimum growth temperatures above 60°C and fermented cellulose to lactic acid and acetic acid. The isolates were similar to *Caldocellum saccharolyticum* and the strain NA 10 (Taya et al., 1988) and grew at near neutral pH.

An endoglucanase was identified recently from the hyperthermophilic archaeon *Pyrococcus furiosus* (Bauer et al., 1999). This is the first report of archaea which could produce endoglucanase to degrade the β–1,4 bonds of β–glucans and cellulose. However the lack of growth on cellulose suggests that *P. furiosus* is different from other cellulolytic microorganisms.

2.5 AEROBIC THERMOPHILIC CELLULOLYTIC BACTERIA

Most of our understanding about cellulose degradation comes from the study of the mesophilic fungi and anaerobic thermophilic cellulolytic bacteria. Very few aerobic thermophilic cellulolytic bacteria have been reported in comparison with anaerobic thermophilic cellulolytic bacteria. Some members of actinomycetes and some *Bacillus* species (Stutzenberger, 1991) received the most attention for aerobic cellulose degradation at high temperatures until the isolation of the aerobic acidophilic cellulolytic bacterium *Acidothermus cellulolyticus* (Mohagheghi et al, 1986). Recently a marine thermophilic bacteria has been reported to produce thermostable glycosyl hydrolases, including a cellulase (Hreggvidsson et al., 1996), a xylanase (Nordberg Karlsson et al., 1997), and a mannanase (Gomes and Steiner, 1998).

2.5.1 *Acidothermus cellulolyticus*

Several isolates of aerobic, acidophilic cellulolytic thermophiles from acidic hot springs at the upper Norris Geyser basin area of Yellowstone National Park were Gram-variable, non-pigmented, non-flagellated, and non-sporulating aerobic slender rods or long slender filaments (Mohagheghi et al, 1986). Some characteristics of the isolates were similar to those of *Thermus* species, but they differed from *Thermus* species in DNA mol% G+C, antibiotic sensitivity, carbon/energy sources supporting growth, and pH and temperature optima for growth. The isolates, designated as *Acidothermus cellulolyticus* strains by Mohagheghi et al. (1986), grew at temperature and pH ranges of 37-70°C and pH 3-7. The optimum temperature and pH were 50 to 60°C and pH 5.0, respectively. A variety of mono-, di-, and polysaccharides including xylan, cellulose, and starch could be utilized as sole source of carbon and energy.

Cellulases were produced by all three isolates when grown on microcrystalline or amorphous cellulose medium. Cellulases from *A. cellulolyticus* were released during late logarithmic and early stationary growth phases, and could completely degrade crystalline cellulose. Glucose and cellobiose were found to be the products of cellulose hydrolysis (Mohagheghi et al, 1986). The optimum temperatures for total cellulase and endoglucanase activities were 75°C and 83°C, respectively (Seltzer, 1987). Two

cellulase components with molecular weights of 188 kDa and 30 kDa showed both filter paper and CMC activities.

2.5.2 *Bacillus* species

The genus *Bacillus* is composed of a large number of spore-forming aerobic or facultatively anaerobic species. Although most members of the genus are mesophiles, some thermophilic species have been described. Large amounts of commercial proteinases and amylases are produced by *Bacillus* species. Cellulolytic activity has been reported in a number of species of the genus *Bacillus* including *B. brevis, B. firmus, B. licheniformis, B. pumilus, B. subtilis, B. polymyxa, B. cereus* (Coughlan and Mayer, 1992). They produced enzymes with activity against acid-swollen cellulose or CMC, not crystalline cellulose. Several *Bacillus* species including *B. circulans, B. lautus* PL236, and *Bacillus* sp. D04 have been reported to produce cellulases to degrade crystalline cellulose (Jorgensen and Hansen, 1990; Kim, 1995; Han et al., 1995).

A cellulolytic *Bacillus* strain DLG related to *B. subtilis* was isolated from a heat-shocked soil sample (Robson & Chamblis, 1984). The isolate DLG is a gram-positive, spore-forming, and strictly aerobic bacterium. It produced ß-1,4-glucanase which was capable of degrading both carboxymethyl cellulose and trinitrophenyl carboxymethyl cellulose, but not crystalline cellulose. The optimum temperature and pH of the enzymes was 58°C and pH 4.8. The production of the endoglucanase was not repressed by glucose or cellobiose. In contrast, a variety of sugars including sucrose, maltose, lactose, cellobiose and glucose stimulated the production of trinitrophenyl carboxymethyl cellulose degrading activity. The gene encoding the endoglucanase in *B. subtilis* DLG was cloned and expressed in *E. coli* C600SF8 and *B. subtilis* PSL1 (Robson and Chambliss, 1986). All the ß-1,4-glucanases in *E. coli* C600SF8 transformants were found to be intracellular or cell associated. In contrast, *B. subtilis* PSL1 transformants secreted 95% or more of the ß-1,4-glucanases they produced. The largest form of ß-1,4-glucanase (50.5 kDa) was found in the cytoplasmic fractions of both *E. coli* and *B. subtilis* transformants and was considered to be the unprocessed primary translation product of the ß-1,4-glucanase gene (Robson and Chambliss, 1986). The DNA sequence of the ß-1,4-glucanase gene from *B. subtilis* DLG was determined by Robson and Chambliss (1987). The result showed that the gene has a coding capacity for a 55 kDa protein. The purified mature exocellular ß-1,4-glucanase was found to be only 35.2 kDa. This suggests the ß-1,4-glucanase precursor has undergone post-translational modifications to form the mature exocellular ß-1,4-glucanase (Robson & Chambliss, 1987).

The *Bacillus* strain AU-1 was obtained from *B. subtilis* T-14 by treatment with N-nitrosoguanidine (Chan and Au, 1987). The strain AU-1 produced more CMCase activity than that of the wild type strain *B. subtilis* T-14 which was isolated from cotton waste compost. CMCase activity was produced by strain AU-1 when grown on a variety of carbohydrates as the major carbon source. Cell growth of strain AU-1 was poor in the medium containing filter paper or Sigmacell as the carbon source. Maximum CMCase production was obtained when *Bacillus* strain AU-1 grew in a liquid medium containing 0.2% raffinose as the major carbon source at pH of 6.0 at 50°C. The synthesis of CMCase in *B. subtilis* AU-1 was induced by cellobiose at low concentration, but repressed at high concentration. One endoglucanase was purified and characterized from *B. subtilis* AU-1 (Au and Chan, 1987). The purified enzyme was active up to 70°C and stable at 65°C for 60 min.

A cellulolytic, thermophilic *Bacillus* strain PDV isolated from soil produced constitutive endoglucanase and xylanase (Sharma et al., 1987). The optimum temperature of the purified endo-1,4-ß-glucanase was 60°C. An endoglucanase gene from the *Bacillus* strain PDV was cloned and expressed in *E. coli*. Recently, a sporulating, aerobic *Bacillus* strain was isolated from a Zimbabwe alkaline hot spring (Zvauya and Zvidzai, 1995). The isolate grew on various carbohydrates and produced endoglucanase constitutively. The endoglucanase was produced optimally at 50°C and an initial pH of 6.0. The production of a bacterial alkaline CMCase has been reported from the alkalophilic *Bacillus* sp. N-4 (Horikoshi et al., 1984), *Bacillus* sp. no. 1139 (Fukumori et al., 1985), and neutrophilic *Bacillus* strain, KSM-522 (Kawai et al., 1987).

The mesophilic *B. lautus* PL236 was isolated from compost and could produce cellulases to degrade crystalline cellulose (Jorgensen and Hansen, 1990). One cellulase gene *celB*, which was cloned and expressed in *E. coli* and *B. subtilis*, was closely related to *celE* of *C. thermocellum*, but distantly related to other *Bacillus cel* genes.

Bacillus sp. D04 had the ability to hydrolyze crystalline cellulose (Han et al., 1995). A purified cellulase from this strain showed both endo- and exoglucanase activity. This is quite unusual and has not been observed in other *Bacillus* species. Similar activity, which was observed in a recombinant cellulase, eliminated the possibility that the purified cellulase might have a minor contaminating protein involved in catalyzing either the endo- or the exoglucanase.

Another *Bacillus* species, *B. circulans*, was also able to grow on crystalline cellulose (Kim, 1995). One important finding from Kim is that *B. circulans* produced extracellular cellulase complexes with an extraordinary capacity to degrade crystalline

cellulose. This appears to be the first report of cellulase complexes produced from *Bacillus* species.

Some thermophilic *Bacillus* species have been reported to produce thermostable xylanases. They include a thermophilic alkalophilic *Bacillus* strain NCIM 59 (Dey et al., 1992), a thermophilic acidophilic *Bacillus* strain 11-1S (Uchino and Nakane, 1981), and a thermophilic neutrophilic *Bacillus stearothermophilus*-like strain (Gruninger and Fiechter, 1986). The *Bacillus* strain 11-1S isolated from a Japanese hot spring grew optimally at 65°C and pH 3.5-4.0. The xylanase from this isolate had a maximum temperature for activity at 80°C and also exhibited activity against CMC and cellulose. The activity of the xylanase in the *B. stearothermophilus*-like strain was stable for more than 5 days at 68°C. The temperature for maximum activity of xylanase was 78°C at pH 7.5.

2.5.3. *Rhodothermus marinus*

Rhodothermus marinus was isolated from alkaline submarine hot springs and grew optimally at 65°C, pH 7.0 and 2% NaCl (Alfredsson et al., 1988). Sequence analysis of a 16S rRNA gene showed that *Rhodothermus marinus* is closely allied to the *Flexibacter-Cytophaga-Bacteroides* group (Andresson and Fridjonsson, 1994). It produced several thermostable polysaccharide-hydrolyzing enzymes, including a cellulase (Hreggvidsson et al., 1996), a xylanase (Nordberg Karlsson et al., 1997), a mannanase (Gomes and Steiner, 1998), and amylases (Hreggvidsson et al., 1992). The endoglucanase purified from *Rhodothermus marinus* had a half-life of 3.5 hours at 100°C (Hreggvidsson et al., 1996). The enzyme appears to be one of the most thermostable cellulases reported so far. The genes encoding the thermostable cellulase, xylanase, and laminarinase from *Rhodothermus marinus* have been cloned and expressed in *E. coli* (Krah et al., 1998; Halldorsdottir et al., 1998; Karlsson et al., 1997).

2.5.4 Actinomycetes

The actinomycetes are a morphologically, physiologically and ecologically diverse group of bacteria and can be isolated from a variety of soil types and heated plant biomass such as hay, bagasse, timber wastes, and composts (Stutzenberger, 1991). Many actinomycetes produced amylases, cellulases, hemicellulases, pectinases and lignolytic enzymes (Stutzenberger, 1990). The cellulolytic abilities of three actinomycete genera *Streptomyces, Microbispora*, and *Thermomonospora* have been well studied.

2.5.4.1 *Streptomyces* species

Very few thermophilic cellulolytic *Streptomyces* species have been reported. One such strain identified as *Streptomyces thermodiastaticus* was isolated from soil by Crawford and McCoy in 1972. An extracellular endoglucanase was produced at 42°C and pH 7.3 in mineral salts medium. The optimum enzyme activity on CMC was at 55°C and at pH 6.5. A mixture of glucose, cellobiose and oligosaccharides were obtained by hydrolysis of CMC at 55°C (Crawford and McCoy, 1972). Another thermophile *S. thermovulgaris* produced endoglucanases, exoglucanases, and cellobiase during growth on crystalline cellulose or CMC (Stutzenberger, 1990).

2.5.4.2 *Microbispora bispora*

Waldron et al. (1986) collected soil samples from hot springs, streams, pools, composts, volcanic fumeroles, and geysers in China, Japan, New Zealand, Portugal, the United States and the West Indies. Forty different strains of thermophilic actinomycetes were obtained using solid enrichment procedures at 60°C. Only eight of these strains produced readily detectable extracellular cellulase in liquid culture. One notably stable isolate produced cellulase in good yield and was identified as *Microbispora bispora* by its habit of producing mycelia and typical bispores and by comparison with known *Microbispora* species. It grew well on cellulose medium at 60°C and produced extracellular endoglucanases, and a cell-associated ß-glucosidase (Waldron et al, 1986). *M. bispora* ß-glucosidase retained 65%, 34%, and 21% of its original activity in the presence of 10%, 20%, and 30% glucose syrups, respectively. The endoglucanase from this strain was stable for more than a week at 60°C. It retained more than 50% activity after 15 minutes at 100°C (Waldron et al , 1986).

2.5.4.3 *Thermomonospora* species

Many thermophilic, lignocellulose-degrading *Thermomonospora* species have been found in manures, soil, and composts (McCarthy, 1989). Two species, *Thermomonospora curvata* and *Thermomonospora fusca*, have been widely investigated for cellulose degradation. Stutzenberger and his colleagues worked with *T. curvata* strain CUB 993 which was isolated from municipal solid waste compost (Stutzenberger et al., 1970, Stutzenberger, 1971). The optimum pH and temperature for cellulase production by *T. curvata* was pH 7.5-8.0 and 45-55°C. Cotton was the best substrate to induce synthesis of cellulase when compared to other carbohydrate sources (Stutzenberger, 1972). *T. curvata* secreted three endoglucanases with M_r values of 23 kDa, 46 kDa and 146 kDa during early exponential growth. This endoglucanase pattern

changed during stationary phase to include endoglucanases with Mr values of 52 kDa, 114 kDa, and 106 kDa (Lupo and Stutzenberger, 1988). The extracellular cellulase complex was inactivated by heating at 65°C at acidic pH, but a 50% enhancement in activity was effected by heating at 65°C at pH 8 (Stutzenberger and Lupo, 1986). One important finding was that *T. curvata* strongly preferred the uptake of cellobiose to that of glucose (Stutzenberger and Kahler, 1986). Glucose was not utilized until the cellobiose concentration dropped at very low level. Cellulosome-like structures were observed on the cell surface during cellulase induction (Bonner & Stutzenberger, 1988). No such structures were found when *T. curvata* grew on glucose.

Thermomonospora fusca strain 190Th was reported to produce cellulases at 55°C by Crawford and McCoy (1972). The morphological and physiological characterisation was first carried out by Crawford in 1975. The strain 190Th grew optimally at 50-55°C and utilized a variety of carbohydrates as carbon and energy sources (Crawford, 1975). *Thermomonospora fusca* strain YX produced extracellular CM-cellulase and Avicelase as well as intracellular ß-glucosidase when grown on microcrystalline cellulose at 55°C (Hagerdal et al. 1978). The *T. fusca* strain YX originally was identified as a *Thermoactinomyces* sp. and was reclassified as *Thermomonospora* sp. later on the basis of cell wall analysis and other characteristics (Ferchak et al. 1980). Treated cotton was hydrolysed to 43% during 11 days incubation at 55°C with culture filtrate of the strain YX (Ferchak et al , 1980). Two endoglucanases from the supernatant of *T. fusca* strain YX were purified and characterized by Calza et al. (1985). Both enzymes were monomeric glycoproteins and were inhibited by cellobiose but not by glucose. A mutant strain, *T. fusca* ER-1 with no proteinase activity, was isolated by Wilson and his colleagues (Wilson, 1988). Five endoglucanases were isolated and characterized from the culture supernatant of ER-1 cells grown on cellulose. The activity against filter paper exhibited by a mixture of all five enzymes showed twice that predicted from the sum of the individual activities (Wilson, 1988). Recombinant DNA studies on the enzyme system and regulation of cellulase synthesis in *T. fusca* have widely been investigated by Wilson and colleagues (Hu and Wilson, 1988; Ghangas and Wilson, 1987,1988; Lin and Wilson, 1988a, 1988b).

2.6 CELLULOLYTIC ENZYMES

2.6.1 Cellulose hydrolysing enzymes and terminology

The enzymatic hydrolysis of cellulose is a complex process requring the participation of several enzymes. Three main types of enzymes involved in the hydrolysis of cellulose are endoglucanases, exoglucanases, and ß-glucosidases (Rapp and Beermann, 1991). Phosphorolytic enzymes in some bacteria and oxidative enzymes in some fungi are believed to be important in cellulose hydrolysis (Coughlan, 1985).

1. Endoglucanases (1,4-ß-D-glucan glucanohydrolase, EC 3.2.1.4) randomly hydrolyse ß-1,4-glucosidic linkages to produce a rapid change in degree of polymerization. A rapid decrease in the degree of polymerization, as shown by a rapid decrease in the viscosity of a solution of CMC, couples with a slow increase in reducing sugars. The enzymes are inactive against cellobiose but hydrolyses alkali- or acid-swollen (amorphous) cellulose, soluble derivatives of cellulose such as CMC (carboxymethyl cellulose), and cellooligosaccharides (Wood, 1989). Activity decreases as the degree of polymerization of cellooligosaccharides decreases. Some endoglucanases show transglycosylation activity. The term "CMCase" is generally regarded as synonymous with endoglucanase in this thesis and other literature.

2. Exoglucanases (1-4-ß-D-glucan cellobiohydrolase EC 3.2.1.91 and 1,4-ß-D-glucan glucohydrolases EC 3.2.1.74) act by removing cellobiose from the reducing or nonreducing end of the cellulose chain or cellooligosaccharides. The product released is inverted to α-configuration. Cellobiohydrolases (CBHs) are active against amorphous cellulose and cellooligosaccharides but inactive against cellobiose or CMC. The term "Avicelase" in the literature is commonly regarded as synonymous with exoglucanase or CBH (Wood and Bhat, 1988). This definition is also applied in this thesis. However, there were some reports of inconsistencies with CBH behaviours (Bronnenmei and Staudenbauer, 1990; Kim, 1995).

3. ß-glucosidases (ß-D-glucoside glucohydrolase, EC 3.2.1.21) are inactive against crystalline or amorphous cellulose but catalyse the hydrolysis of cellobiose and cellooligosaccharides to glucose. The activity against cellooligosaccharides decreases with increasing degree of polymerization (Wood and Bhat, 1988). The enzymes from different microbial sources exhibit a high degree of variability with respect to substrate specificity. Most ß-glucosidases can hydrolyse aryl-ß-glucosides and oligosaccharides (including cellobiose), but some prefer either aryl-ß-glucosides or oligosaccharides.

According to I.U.B. recommendations, cellulase is regarded as an endoglucanase. In this thesis and most literature, cellulase represents the complete cellulase system and all its components. The term "cellulase complex" or "cellulase system" refers to the complex of three enzymes including endoglucanase, exoglucanase, and ß-glucosidase.

The classification of cellulases can not be applicable to all cellulolytic systems (Meinke et al., 1995). There have been some reports of cellulases which showed both endo and exo modes of action on cellulose (Tomme et al., 1996; Han et al., 1995; Li et al., 1998; Zverlov et al., 1998b). Tomme et al. (1996) recently suggested that it is not appropriate to classify β-1,4-glucanases in two mutually exclusive groups, endoglucanases and exoglucanases. They proposed that cellulose is hydrolyzed by processive, semiprocessive, and nonprocessive cellulases. A processive cellulase is the enzyme that "initiates attack at a reducing end and continues by removing many successive cellobiosyl residues from the same chain before jumping to a new site nearby". While a semiprocessive cellulase is the enzyme that "initiates attack at an internal β-1,4-glucosidic bond and removes only a few successive cellobiosyl residues from the new reducing end before jumping to another site", and a nonprocessive cellulase is the enzyme that jumps to a new site after each attack". From the combined use of capillary viscometry, reducing end-group analysis (disodium 2,2' -bicinchoninate measurement), and size exclusion chromatography methods, the endoglucanases were arranged into four groups with similar ratios of viscometric to reductometric activity (Vlasenko et al., 1998). Group I had a higher preference for hydrolysis of the bonds near the chain end, while group IV was designated as the 'true' endoglucanase.

The cellulase classification system above is mainly based on substrate specificity. Cellulases are also classified into glycosyl hydrolase families according to amino acids sequences. Further discussion is in section 2.7.

2.6.2 Multiplicity of cellulases

Most cellulolytic microorganisms produce multiple forms of cellulases which may arise from the expression of different genes or from post-translational modification by proteolysis or glycosylation inside or outside the cell (Gilbert and Hazlewood, 1993). Other possible causes of multiplicity include microheterogeneity which is due to the complexing of cellulolytic components with other proteins or polysaccharides, and macroheterogeneity, the formation of multienzyme aggregates (Coughlan, 1990).

The genes encoding for fifteen endoglucanases, two xylanases, and two ß-glucosidases were detected in DNA from *C. thermocellum* (Hazlewood et al., 1988). There is a

cluster of twelve contiguous genes encoding enzymes involved in xylan, cellulose and mannan hydrolysis in the anaerobic thermophilic bacterium *Caldicellulosiruptor saccharolyticum* (Bergquist et al., 1993).

Thermomonospora fusca YX produced a high level of cellulase, xylanase and protease activity. It has been shown that the protease in the culture supernatant modified the different cellulases without altering their cellulase activity (Calza et al., 1985). Wilson and his colleagues have purified five endoglucanases from the culture supernatant of a mutant strain of *T. fusca*, ER-1 that produced no detectable extracellular protease activity and two enzymes were glycoproteins among five endoglucanases (Wilson, 1988). The two cellulose-bound cellulases from *Cellulomonas* sp. were found to be glycosylated (Beguin and Eisen, 1978). The affinity of these two enzymes for Sephadex has been utilized for their purification. The types of cellulases produced by *Cellulomonas fimi* varied with the age of the culture, the growth substrate used and storage of the supernatants (Langsford et al., 1984). The variation of cellulase profiles in culture supernatant was thought to be due to proteolysis and de-glycosylation (Langsford et al., 1984). The later results showed that two glycosylated cellulases from *Cellulomonas fimi* were resistant to proteolysis (Langsford et al., 1987). Glycosylated cellulases have also been reported from culture supernatants of *Clostridium thermocellum* (Ait et al., 1979; Ng and Zeikus, 1981), and a *Bacillus* sp. (Paul and Varma, 1992).

The reasons for such multiplicity are the subject of some debate. Cellulose does not exist in pure form in nature and is always associated with a variety of other polysaccharides, such as lignin and hemicellulose, so multiple glucanases having different substrate specificities may be required for degradation of cellulose. Wood (1981) proposed that, for stereochemical reasons, at least two types of endoglucanases and two exocellobiohydrolases would be required for the hydrolysis of cellulose.

2.7 STRUCTURE AND FUNCTION OF CELLULASES

Sequence analysis of cellulases and xylanases, combined with biochemical characterization of wild-type and truncated enzymes, have shown that these enzymes are composed of distinct domains which have many possible arrangements. Most cellulases and xylanases consist of a catalytic domain and one or more ancillary domains joined by linker sequences. Ancillary domains include cellulose-binding domains (CBDs), the "duplicated segments", S-layer-like modules, and the fibronectin-type III (Fn3) modules (Tomme et al., 1995). The majority of fungal endoglucanases and cellobiohydrolases contain a catalytic domain and a C-terminal CBD joined by a

linker rich in proline and hydroxy amino acid residues. The bacterial cellulases exhibit much more diversity and range from small single domain cellulases to very large multidomain enzymes connected by various linker sequences (Tomme et al., 1995).

2.7.1 Catalytic domain

The classification of 21 cellulolytic and xylanolytic enzymes into six families was introduced by Henrissat et al. (1989), based on hydrophobic cluster analysis. Three more families were added to this classification by Gilkes et al. (1991). Now the classification has been extended to include a variety of glycosyl hydrolases which are grouped into more than 50 families of related amino acid sequences (Henrissat and Bairoch, 1993, 1996; Tomme et al. 1995; Warren, 1996). Among these, 18 families are cellulolytic and heteroxylanolytic hydrolases (Clarke, 1997). Members of Families D and L are all bacterial enzymes and Family C is a fungal enzyme (Tomme et al., 1995). Several families such as A, B, F, G, H and K contain both fungal and bacterial enzymes, while family E contains prokaryotic and plant enzymes. This suggests that lateral transfer of ß-1,4-glucanase genes has occurred (Gilkes et al. 1991). Furthermore, the relationship between enzymes in the same family is not in accordance with the phylogenetic relationship between the organisms that produce them. Enzymes of a given family share the same stereoselectivity, but they do not always show the same substrate specificity. For example, family A contains ß-1,4-glucanases, ß-1,3-glucanases, ß-1,4-mannanases, cellodextrinases and one ß-1,4-xylanase (Tomme et al. 1995). Three-dimensional structures of some cellulases and xylanases have been determined, and the structures support the view that cellulases and xylanases hydrolyse glycosidic bonds through an acid-base catalytic mechanism involving acidic amino acids (Beguin and Aubert, 1994).

2.7.2 Cellulose-binding domain

Many cellulases contain non-catalytic cellulose-binding domains (CBDs). The majority of CBDs are usually located at the N- or C-terminus of enzymes and are often separated from the catalytic domains by glycosylated Pro/Thr/Ser-rich linker segments. Most cellulose-binding domains can be classified into five families based on amino acid sequence similarities (Tomme et al., 1995). Family I CBDs are all from fungal cellulases and have highly conserved sequences of about 30 residues. Family II CBDs have been found in a variety of bacterial enzymes including cellulases, xylanases, an arabinofuranosidase and a chitinase. Family III CBDs are 130-170 amino acids long and are found in enzymes and the cellulose-binding proteins from *Bacillus* species, *Clostridium* species and *Cs. saccharolyticum* (Tomme et al., 1995).

All cellulases that are active against crystalline cellulose are found to have CBDs or are associated with cellulose-binding proteins (Beguin and Aubert, 1994). This suggests that the presence of a CBD will enhance the cellulase activity against crystalline cellulose. It has been shown that removal of the CBD will affect cellulase activity on various cellulosic substrates. Removal of the CBDs from *T. reesei* cellobiohydrolases I and II reduced the activity on Avicel by about 85% and 50% respectively, but activity towards soluble substrates remained unchanged (Tomme et al., 1988). Removal of the CBD from *C. fimi* CenA reduced the activity of enzyme on Avicel by 20% and increased the activity on CMC and acid-swollen cellulose by 50% (Gilkes et al., 1988). Grafting of the CBD of *T. fusca* endoglucanase E2 on to the catalytic domain of *Prevotella ruminicola* endoglucanase increased the activity of *P. ruminicola* endoglucanase on acid-swollen cellulose and ball-milled cellulose (Maglione et al. 1992). It was found that desorption of CBDs from cellulose required different conditions. Some CBDs were disassociated from cellulose easily with water, while in other cases protein denaturants, such as guanidinium hydrochloride, urea, and SDS, or organic solvents such as polyethylene glycol, and triethylamine, were required to effect dissociation of the CBD from the cellulose (Gilbert and Hazlewood, 1993).

2.7.3. Multidomain cellulases and xylanases

Cellulases and xylanases are usually composed of a catalytic domain and a cellulose-binding domain. However multidomain cellulases and hemicellulases have been found in *Cs. saccharolyticum* (Saul et al., 1989, 1990; Gibbs et al., 1992), *Clostridium thermocellum* (Zverlov et al., 1998a), *Fibrobacter succinogenes* (Paradis et al., 1993), *Clostridium cellulolyticum* (Gal et al., 1997), *Neocallimastix patriciarum* (Xue et al., 1992), *Ruminococcus flavefaciens* (Flint et al., 1993; Zhang and Flint, 1992), *Anaerocellum thermophilum* (Zverlov et al., 1998b), *Cellulomonas fimi* (Tomme et al., 1996), and *Bacillus* sp. D04 (Han et al., 1995). The polysaccharidase-encoding genes in the extremely thermophilic bacterium *Cs. saccharolyticum* were well studied by Bergquist's group at the University of Auckland in New Zealand. A cluster of 12 contiguous genes, all in the same orientation, encodes enzymes involved in xylan, cellulose, and mannan utilization (Bergquist et al., 1993). Each of the proteins encoded by the *celA-manA-celB-celC* gene cluster in *Cs. saccharolyticum* contains two independently functioning catalytic domains (Gibbs et al., 1992; Saul et al., 1990). The catalytic domains are at the N- and C-termini of enzymes and are separated by cellulose-binding domains. Two CBDs are found in CelA, ManA and CelC, but only one CBD is found in CelB. The domains are connected to each other by PT linkers (proline-threonine-rich regions) which act as flexible hinges. Two catalytic domains in CelB have endoglucanase and cellobiohydrolase activities, respectively (Saul et al.,

1990). The catalytic domains in ManA are a ß-mannanase and an endoglucanase (Gibbs et al. 1992). In CelA, the N-terminal catalytic domain shows the endoglucanase activity, but the C-terminal domain has no activity on cellulose and xylan substrates (Te'o et al. 1995). The bifunctional cellulases have also been purified and characterized from native hosts, such as *Anaerocellum thermophilum* (Zverlov et al., 1998b) and *Bacillus* sp. D04 (Han et al., 1995). All this suggests that multidomain cellulases and hemicellulases are not unusual in anaerobic and aerobic microorganisms.

2.8 MECHANISM OF ENZYMATIC HYDROLYSIS OF CELLULOSE

Most of our understanding of enzymatic hydrolysis of cellulose comes from studies of the cellulase systems of mesophilic fungal species. It is generally accepted that three main types of enzymes including endoglucanases, exoglucanases and ß-glucosidases act synergistically to degrade crystalline cellulose (Coughlan and Ljungdahl, 1988). Endoglucanases first hydrolyse the amorphous regions of cellulose fibres. This exposes reducing ends that permit cellulose hydrolysis at these sites by exoglucanases (cellobiohydrolases) which also continue to release cellobiose from crystalline regions of the cellulose. The ß-glucosidases cleave the cellobiose and prevent the disaccharide from inhibiting cellobiohydrolases (Coughlan and Ljungdahl, 1988). This model was first proposed in the late seventies from the study of cellulase systems of *Phanerochaete chrysosporium* and *Trichoderma koningii* (Beguin and Aubert, 1994). The model is assumed to apply to other aerobic fungi, such as *T. reesei* and *Penicillium pinophilum*, and several bacteria, such as *M. bispora*, and *T. fusca*. The endoglucanase from *Acidothermus cellulolyticus* and *Thermomonospora fusca* showed synergism with a fungal cellobiohydrolase in the saccharification of microcrystalline cellulose, respectively (Baker et al., 1994). However, some results show that this model is probably an oversimplification. The synergism between cellobiohydrolases from *Penicillium pinophilum* was observed (Wood and McCrae, 1986a,b). Further experiments on *P. pinophilum* showed that cotton fibre was degraded to a significant degree only when cellobiohydrolase I and cellobiohydrolase II and some specific endoglucanases existed together (Wood, 1989). Warren (1996) recently proposed that at least two exoglucanases are needed for hydrolysis of cellulose; one acts at the reducing ends of molecules, and another at the nonreducing ends. There is also evidence that some endoglucanases and cellobiohydrolases show limited exo- and endo-activity, respectively (Coughlan, 1990) as well as multidomain cellulases and hemicellulases.

The degradation of cellulose in cellulolytic bacteria seems different from that in fungi. All cellulolytic bacteria produce a variety of endoglucanases, but exoglucanases have

been found only in a few species of bacteria, for example *Clostridium thermocellum* (Wang et al., 1994; Zverlov et al., 1998a), *Clostridium stercorarium* (Creuzet and Frixon, 1983), *Ruminococcus albus* (Ohmiya and Schimizu. 1988a), *Cellulomonas uda* (Nakamura and Kitamura, 1988), and *Microbispora bispora* (Yablonsky et al., 1988). Some cellulases have exhibited both endo- and exoglucanase activity (Tomme et al., 1996; Han et al., 1995; Li et al., 1998; Zverlov et al., 1998). Remarkable cellulase (celA) activity on Avicel suggested that the enzyme could be regarded as a fusion protein of the two enzymes effecting cellulose hydrolysis of *C. stercorarium* (Zverlov et al., 1998b). Some bacteria do not produce ß-glucosidase. Cellobiose and cellodextrin are hydrolysed by cellobiose phosphorylases (EC 2.4.1.20) and cellodextrin phosphorylase (EC 2.4.1.49), respectively. Cellobiose phosphorylase was found in *Ruminococcus flavefaciens* (Ayers, 1959) and *Clostridium thermocellum* (Alexander, 1968). Cellodextrin phosphorylase found in *C. thermocellum* (Sheth and Alexander, 1969) catalyses the reversible phosphorylytic cleavage of cellodextrins ranging from cellotriose to cellohexose.

Also in several cellulolytic bacteria, such as *Clostridium thermocellum* (Lamed et al., 1983a), *Clostridium* strain C7 (Cavedon et al., 1990), *Clostridium cellulovorans* (Shoseyov and Doi, 1990), *Ruminococcus albus* (Wood et al., 1982), *Bacteroides cellulosolvens* (Lamed et al., 1991), and *Bacillus circulans* (Kim, 1995), the various cellulase components are organized into an elaborate multifunctional supramolecular complex, whose components interact in a synergistic manner to catalyze the efficient degradation of cellulose. The most thoroughly studied enzyme complex is the *C. thermocellum* cellulosome, which contains about 50 polypeptide molecules (Mayer et al., 1987). Rapid development of molecular biology techniques led to the cloning, sequencing, and expressing of the cellulosome enzymes. So far, 18 cellulases and hemicellulases from the *C. thermocellum* cellulosome have been described (Bayer et al., 1998b). Cellulase components of the cellulosome are organized around a large, noncatalytic glycoprotein CipA (for cellulosome integrating protein) that acts both as a scaffolding component and a cellulose-binding factor. CipA comprises a series of functional domains, amongst which is a single cellulose-binding domain and nine cohesin domains (Gerngross et al., 1993). The bond of cellulosome to the cellulosic substrate is accomplished by the cellulose-binding domain of CipA. CipA enhanced crystalline cellulose hydrolysis by anchoring endo- or exoglucanases to the cellulose surface (Kataeva et al., 1997; Wu and Demain, 1988). A complementary domain, termed the dockerin domain, is located on each enzymatic subunit and interacts with cohesin domains. The cohesin-dockerin interaction is thought to be very important for complex formation in the cellulosome and the degradation of crystalline cellulose (Shimon et al., 1997; Garciacampayo and Beguin, 1997).

The cellulose degradation in cellulosomes was first described by Mayer et al. (1987) as a simultaneous multicutting of the cellulose chain by adjacent enzymes and involved only endoglucanases. Demonstration of at least two exoglucanase CelS and CbhA in the *Clostridium thermocellum* cellulosome (Wang and Wu, 1993; Zverlov et al., 1998a) suggests that cellulose is hydrolysed synergistically by both endo- and exoglucanase. We need to realize that our understanding of cellulose hydrolysis by cellulosomes mainly comes from the study of isolated components of cellulosomes. New techniques are needed to study intact cellulosomes and their role in cellulose degradation.

CHAPTER THREE

CHARACTERIZATION OF AN AEROBIC, THERMOPHILIC, CELLULOLYTIC BACTERIUM

3.1 INTRODUCTION

The genus *Bacillus* is a phenotypically heterogeneous collection of aerobic or facultatively anaerobic, Gram-positive, rod-shaped, endospore-forming bacteria (Claus and Berkeley, 1986). Some members of the genus *Bacillus* have been reported to produce carboxymethylcellulase (CMCase) activity (Chan and Au, 1987; Robson and Chambliss, 1984; Fukumori, 1985). Only a few *Bacillus* species have been reported to be capable of degrading crystalline cellulose (Jorgensen and Hansen, 1990; Kim, 1995; Han et al., 1995). These *Bacillus* species are all mesophiles and produce less thermostable cellulases.

A thermophilic aerobic spore-forming bacterium strain COMP.A2 isolated from artificial compost is capable of utilizing crystalline cellulose as the sole carbon and energy source. In this chapter, the characterization of strain COMP.A2 and the determination of its taxonomic position are described.

3.2 MATERIALS AND METHODS

3.2.1 Chemicals

All chemicals were analytical grade, except that on some occasions reagent grade salts were used for making media. Antibiotics and agarose were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Pure agar was purchased from Oxoid Ltd, England.

3.2.2 Source of strains

The isolates COMP.A2 and COMP.A2.1 in this study were from the culture collection of the Thermophile Research Unit of the University of Waikato. They were designated as TG 523 and TG 616, respectively, in the culture catalogue. The COMP.A2.1 is a stable mixed culture containing strain COMP.A2 and a contaminating non-cellulolytic spore-forming *Bacillus* strain. The isolate COMP.A2 is a pure culture of the cellulolytic component of this mixed culture.

B. schlegelii (DSM 2000) was obtained from Deutsche Sammlung von Mikroorganismen (Gottingen, Germany) and stored in our culture collection as TG 408.

3.2.3 Media and culture condition

The basal growth and maintenance medium for COMP.A2 and COMP.A2.1 contained (g/litre) KH_2PO_4, 0.75; $Na_2HPO_4.12H_2O$, 2.1; $(NH_4)_2SO_4$, 0.5; MOPS buffer, 10; and Wolfe's mineral solution 50 ml. The composition of Wolfe's mineral solution is given in Appendix 1. The medium was adjusted to pH 7.3 at room temperature prior to autoclaving which corresponded to a pH of 6.8 at 70°C. On occasions, as specified, the medium was used with another buffer replacing the MOPS buffer routinely used. All media were autoclaved at 120°C for 15 or 20 minutes unless noted otherwise. Soluble substrates were added to the autoclaved media from filter-sterilised stock solutions (20% w/v). Insoluble carbon sources were added directly to media before autoclaving.

The strain COMP.A2 and mixed culture COMP.A2.1 were enriched and isolated by Dr Hudson in the Thermophile Research Unit of the University of Waikato. Medium ATC and M2ATC were used as enrichment and isolation media. Medium ATC contained (g/litre) KH_2PO_4, 0.053; $Na_2HPO_4.12H_2O$, 0.219; NH_4Cl, 1.0; $MgSO_4.7H_2O$, 0.2; $CaCl_2$ $2H_2O$, 0.1; and Wolfe's mineral solution 50 ml. Medium M2ATC contained (g/litre) NH_4Cl, 0.5; MOPS buffer, 5.0; Na-ß-glycerophosphate, 0.5; and Wolfe's mineral solution 50 ml.

Two solid media were prepared from the broth medium previously described. Solid agar medium was made by the addition of 15 g/litre purified agar (Oxoid, England). Solid Gelrite medium was made by adding 1.1 g/litre Gelrite (Merck, USA) and 1.0 g/litre $MgCl_2$ (Hudson et al., 1990b). Gelrite plates were poured as bilayers in which the lower layer had no carbon addition.

B. schlegelii was maintained on DSM 260 medium, containing (g/litre) $Na_2HPO_4.2H_2O$, 4.5; KH_2PO_4, 1.5; NH_4Cl, 1.0; $MnSO_4.H_2O$, 0.01; $MgSO_4.7H_2O$, 0.2; $CaCl_2.2H_2O$, 0.01; sodium pyruvate, 1.5; ferric ammonium citrate, 5.0; trace element solution SL-6, 3.0 ml. The medium was adjusted to pH 7.1 at room temperature prior to autoclaving.

An autotrophic medium (AM 1) (Kristjansson et al., 1985) was prepared as a 10-fold concentrated solution of the following composition (g/litre): $(NH_4)_2SO_4$, 1.3; $K_2HPO_4.3H_2O$, 0.47; $MgSO_4.7H_2O$, 0.25; $CaCl_2$, 0.07; and 1 ml of the trace element solution of Badzoing et al. (1978). The composition of the trace element solution of

Badziong is given in Appendix 1. The medium was stored at a pH of 4.0 to prevent precipitation. The pH was readjusted to 7.8 at room temperature with NaOH after dilution to working strength. Twenty millilitre of AM 1 in 100 ml serum bottles were prepared and autoclaved at 120°C for 15 minutes, and then the headspace was then pressurised to 2 atmospheres with filter-sterilized H_2/CO_2 (75%/25%, New Zealand Industrial Gases Ltd).

Another autotrophic medium (AM 2) had the following composition (g/litre): $(NH_4)_2SO_4$, 1.3; $K_2HPO_4.3H_2O$, 0.47; $MgSO_4.7H_2O$, 0.25; $CaCl_2$, 0.07; sodium thiosulphate, 5; and 1 ml of the trace element solution of Badzoing et al. (1978). The pH was adjusted to 7.3. Fifty ml of AM 2 in 250 ml conical flasks or 20 ml of AM 2 in 100 ml serum bottles was prepared and autoclaved at 120°C for 15 minutes. Serum bottles containing AM 2 medium had the air headspace pressurised to 2 atmospheres with filter-sterilized CO_2 (New Zealand Industrial Gases Ltd). Phenol red was added as pH indicator, at a concentration of 0.0024%.

Incubation of COMP.A2 was generally carried out at 70°C or 65°C in an orbital incubator operating at 120 rpm. *B. schlegelii* was incubated at 65°C.

Amorphous cellulose was prepared as follows: 25 g Avicel (Asahi Chemical Industry Company Ltd, Japan) was dried overnight in a vacuum dessicator over silica gel. Zinc chloride (700g) was added to 300 ml of water with constant stirring. The Avicel was added at room temperature to the zinc chloride solution gradually with vigorous stirring to avoid clumping. The suspension was stirred until all of the Avicel was in solution. Amorphous cellulose was formed by adding the Avicel solution to 4 litre of stirred water. The reprecipitated cellulose was recovered by centrifugation and washed 3 times with water. After the final wash the cellulose was transferred to a beaker containing 15 g of Na_2EDTA, made up to 1500 ml with water and stirred at 4°C overnight. The cellulose was then washed 4 times by resuspending in distilled water and centrifuging. The final amorphous cellulose pellet contained about 94.5% water by weight. The washed amorphous cellulose was stored as a slurry at 4°C.

3.2.4 Light microscopy

An Olympus BH-2 microscope with phase contrast optics was used for routine examination of culture purity and growth. Phase contrast photomicrographs were taken using the same microscope with an Olympus SC 35 camera and FP4-135 film (Ilford, Cheshire, UK). Cells were immobilized for photomicroscopy by mixing one drop of culture with one drop of 0.5% agar solution on a warm glass slide in a 70°C incubator.

A cover slip was added and the agar was allowed to set at room temperature and the slide examined by phase-contrast microscopy.

Photomicrographs of colonies of the isolate COMP.A2 on Gelrite plates were taken using a Nikon stereo microscope (Nikon SMZ-U).

3.2.5 Electron microscopy

3.2.5.1 Scanning electron microscopy

Fifty ml of basal medium with 0.4% (w/v) Sigmacell 20 in 250 ml conical flasks was prepared and, after autoclaving, was inoculated with 0.1 ml of an overnight culture of strain COMP.A2. The flasks were incubated at 70°C for two days in an orbital incubator at 100 rpm. Culture was centrifuged at 8000 × g and washed twice with basal medium. Then, two ml of culture was filtered through a sterile 0.2 µm Millipore filter and the filter fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer. After washing with buffer, filters were dehydrated through a series of ethanol solutions of 75%, 95% and 100% (three changes) and dried using a critical point dryer (Polaron, England). The filters were mounted on stubs sputter-coated with gold palladium and examined with an Hitachi S 4000 field emission SEM.

3.2.5.2 Transmission electron microscopy

Strain COMP.A2 was grown on 50 ml of the basal medium with 0.4% (w/v) Avicel in 250 ml conical flask. Flasks were incubated at 70°C for two days in an orbital incubator. The culture was centrifuged at 8000 × g and washed twice with basal medium. Two ml of culture was fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer at pH 7.2. After washing in buffer the sample was post-fixed in 1% osmium tetroxide for 30 minutes, washed in water and dehydrated through a series of ethanol solutions of 75%, 95% and 100% (three changes). The samples were infiltrated and embedded in Spurr's epoxy resin. Ultrathin sections were cut with a diamond knife, stained with uranyl acetate and lead citrate and examined with a Philips EM400 electron microscope at an accelerating voltage of 80KV.

3.2.6 Inhibition of growth

Aliquots of 50 ml basal medium in 250 ml Erlenmyer flasks were supplemented with either agar, agarose, sodium chloride or sodium azide before autoclaving. Filter sterilized cellobiose was also added to all the media after autoclaving to a final

concentration of 0.4% (w/v). All flasks were inoculated with 0.1 ml volume of an overnight culture of COMP.A2 and incubated at 70°C for 24 hours or longer in an orbital incubator at 100 rpm. Growth was estimated by measuring absorbance at 650 nm. Medium with no inhibitor additions was inoculated with COMP.A2 and run at the same time as control.

Inhibition of growth by antibiotics was determined at 65°C. Five ml of basal medium in 20 ml test tubes was prepared. Filter sterilized cellobiose and antibiotic were added to the autoclaved medium from stock solutions. The final cellobiose concentration was 0.4% (w/v) and the final antibiotic concentrations were 1.25, 2.5, 5, 10 or 20 μg/ml. The test tubes were inoculated with an overnight culture of COMP.A2 and incubated at 65°C in a stationary incubator. Growth was examined by phase-contrast microscopy. Control cultures with no antibiotics were incubated at the same time.

3.2.7 Assay methods

3.2.7.1 Reducing sugar determination by PAHBAH method

Reducing sugars were determined by the PAHBAH (*p*-hydroxybenzoic acid hydrazide) method of Lever (1973). Stock solutions of 0.5 M trisodium citrate, 1.0 M Na_2SO_3, 0.2 M $CaCl_2$ and 5.0 M NaOH were prepared and the first two solutions were stored in brown bottles. A working PAHBAH reagent was made by mixing 10 ml of each stock solution, in the order they are listed, with mixing between each addition. Distilled water (50 ml) and 1.52 g *p*-hydroxybenzoic acid hydrazide were added and the volume made up to 100 ml. The working PAHBAH reagent was prepared fresh each day and stored in a dark bottle.

When determing the amount of reducing sugar in a sample, 500 μl of the sample was added to 1 ml of PAHBAH reagent in a 1.5 ml Eppendorf tube, mixed, and boiled for 6 minutes. After cooling, the absorbance was read at 420 nm. A standard curve was prepared at the same time, using 500 μl of glucose standards over the concentration range 0.0 to 0.1 mM glucose.

3.2.7.2 Total protein determination

Total culture protein was determined by incubating 0.5 ml of culture broth with 0.5 ml of 1.0 M NaOH at 100°C for 15 minutes. After neutralization with 0.5 ml of 1.0 M HCl, 0.1 ml of 0.15% sodium deoxycholate (DOC) was added and the sample allowed to stand for 10 min. The protein was then precipitated by the addition of 100 μl of 72%

trichloroacetic acid (TCA) and centrifuged at maximum speed in a benchtop centrifuge (Microfuge E, Beckman, USA) for 10 min. The supernatant was decanted, and the protein content of the precipitate determined by the Folin Phenol method of Peterson (1983). Reagent A was prepared by mixing 1 part of copper-tartrate-carbonate (CTC) reagent with 2 parts 5% sodium dodecyl sulfate (SDS) and one part 0.8 M NaOH. Reagent B was made by the addition of 1 part 2N Folin-Ciocalteu phenol reagent (Sigma) to 5 parts distilled water. The sample (0.5 ml) was mixed with 0.5 ml of reagent A and held at room temperature for 10 min. Then, 0.25 ml of reagent B was added and mixed. After 30 min, the absorbance at 750 nm was read. Bovine serum albumin of 97.0% purity (Sigma) was used to construct a standard curve which was linear from 0.0 to 0.25 mg/ml. A minimum of duplicate assays was carried out for each sample.

3.2.7.3 High performance liquid chromatography

The utilization of soluble substrates and the accumulation of end products of growth were determined by high performance liquid chromatography (HPLC) using an Aminex HPX-87H organic analysis column (Bio-Rad, USA). The column was operated at 50°C. The mobile liquid phase consisted of 0.01 N H_2SO_4 at a flow rate of 0.5 ml/min. One litre of 0.01N H_2SO_4 was prepared by mixing 10 ml 1N H_2SO_4 with 990 ml Milli Q water, filtered through a 0.45 μm filter and vaccum degassed. The eluted peaks were monitored with a refractive index detector (Erma Optical Works Ltd, Japan). Data was collected by series 3000 Chromatography Data System (Nelson Analytical Inc, USA) using an Exzel XT computer. Standard curves were prepared for a mixed solution of cellobiose, glucose, xylose, ethanol, sodium acetate and sodium lactate. The linear ranges were from 3.12 to 25 mM for all compounds. For analysis, 20 μl volumes of standard solutions and cell-free supernatants were injected onto the column using a 50 μl glass syringe (Hamilton, USA) and a Rheodyne 7125 injection valve (Rheodyne, USA).

3.2.8 Growth and cellulose degradation

Aliquots of 200 ml basal medium was supplemented with either amorphous cellulose (2.5% w/v) or Sigmacell 20 (0.5% w/v) and inoculated with either strain COMP.A2 or the mixed culture COMP.A2.1. The flasks were incubated at 70°C at 120 rpm. Samples were withdrawn over a time course and assayed for residual cellulose, reducing sugars, and cell growth. One uninoculated flask of each medium type was incubated at the same time as control.

Residual cellulose was determined by the following method: Whatman membrane filters (with a mean pore size of 3 μm) were dried overnight in vacuum desiccators over silica gel. Each filter was weighed, and the weight recorded. A 5 ml sample of well stirred cell culture was filtered through the membrane filter and washed twice with 5 ml of distilled water. The membrane filter was placed in a petri dish, and was dried at 75°C overnight. The filters were re-weighed and any increase in weight attributed to residual cellulose.

Growth on soluble substrates was estimated by measuring the absorbance at 650 nm in a Varian DMS 80 spectrophotometer. Total culture protein was used as an estimation of growth on insoluble substrates. Reducing sugars were determined by the PAHBAH method and end products of growth were analysed by HPLC using an Aminex HPX-87H organic analysis column.

3.2.9 Growth and end product formation

The basal medium (100 ml) with 0.4% (w/v) cellobiose or 3% amorphous cellulose in 250 ml conical flasks was prepared and inoculated with strain COMP.A2. Flasks were incubated at 70°C with shaking at 120 rpm. The samples were withdrawn and assayed for cell growth and end products.

3.2.10 Utilization of cellobiose and glucose

The strain COMP.A2 was inoculated in 100 ml of the basal medium containing a mixture of 10 mM cellobiose and 10 mM glucose in 250 ml conical flasks. Flasks were incubated at 70°C with shaking at 120 rpm. The samples were withdrawn over the time course. The absorbance was read at 650 nm and residual substrates were assayed by HPLC.

3.2.11 Temperature and pH optimum determination

Aliquots of 50 ml basal medium supplemented with cellobiose (0.4% w/v) were used for determining the effect of temperature on growth. Basal medium (50 ml) with MOPS or where MOPS was replaced by EPPS and MES at 50 mM was adjusted to the various pH values at 65°C and supplemented with cellobiose (0.4% w/v). Flasks were inoculated with 0.5 ml of an overnight COMP.A2 culture and incubated in an orbital incubator at 120 rpm. Growth was measured by absorbance at 650 nm. The incubation temperature was 65°C for the optimum pH determination experiment. Each pH or temperature trial was run in duplicate flasks.

3.2.12 Cell membrane lipids analysis

For lipid analysis strain COMP.A2 was grown overnight on basal medium supplemented with 0.4% (w/v) maltose at 70°C. The cells were harvested by centrifugation at 8000 × g for 15 min, and washed twice in basal medium. The cell pellet was freeze dried and sent to CSIRO Marine Laboratories (Australia) for lipid analysis by the following method. Total lipid was extracted from the sample by the modified one-phase chloroform, methanol, water Bligh and Dyer method (1959). After phase separation, the lipids were recovered from the lower chloroform layer, concentrated using a rotary evaporator, sealed under N_2 and stored at -20°C. The upper aqueous layer was collected for cell residue analysis. The total lipid was fractionated into neutral-, glyco- and phospho-lipid using 1 g of activated 100-200 mesh Unisil silicic acid (chloroform 10 ml, acetone 20 ml, methanol 10 ml). The phospholipid fatty acid fraction was treated with 3 ml of methanol:concentrated hydrochloric acid:chloroform (10:1:1, v/v/v) and heated at 100°C for 60 minutes to produce the corresponding fatty acid methyl esters which were then extracted with hexane:chloroform (4:1, v/v).

The normal and hydroxy fatty acids from the aqueous layer were recovered following acidification of the lipid extract cell residue with 50 ml of 1.0 M hydrochloric acid which was then heated under reflux for three hours. After cooling the sample was extracted with chloroform and the fatty acids were methylated using the same procedure as for the phospholipids.

Fatty acid analysis was performed using a Hewlett Packard 5890 gas chromatograph (GC) fitted with a 50 m × 0.32 mm i.d. HP1 cross-linked methyl silicone fused-silica, capillary column with H_2 as the carrier gas. The GC was fitted with a flame ionisation detector set at 310°C and a purged split/splitless injector set at 290°C. Fatty acids methyl esters and sterols were quantified by chromatography software (DAPA). Identifications were confirmed by gas chromatography-mass spectrometry (GC-MS, Fisons MD800) and by the comparison of retention time and mass spectral data with data obtained from authentic and laboratory standards.

3.2.13 G+C mol%

DNA from the isolate COMP.A2. was extracted and purified by the modified Marmur method (1961). COMP.A2 cells were grown at 70°C for 31 hours in one litre of basal medium with 0.4% cellobiose as carbon source and harvested by centrifugation. Cells were washed twice with 0.1 M EDTA / 0.15 M NaCl, resuspended in 25 ml of water

containing 10 mg of lysozyme and incubated for 45 min at 37°C. Two ml of 25% SDS was added and incubated for three hours until cell lysis was complete as determined by phase-contrast microscopy. The suspension was then heated for 10 min at 60°C. An equal volume of chloroform:phenol:isoamyl alcohol 24:24:1 (v/v/v) was added and the flask was shaken for 30 min at room temperature. The liquid was transferred to centrifuge tubes and centrifuged at 10,000 g for 5 min. The top aqueous layer was transferred to a beaker and overlaid with twice the volume of ice-cold ethanol. The DNA was collected by slowly stirring and spinning a glass rod in the mixture. The DNA was re-dissolved at 1.0×SSC buffer (1.0×SSC buffer contains 0.15 M NaCl and 0.015 M trisodium citrate, pH 8.0) and precipitated once more with cold ethanol. The DNA wound on the glass rod was washed with 70% ethanol and dried in air, and then dissolved in 3 ml of TE buffer (which contains 10 mM Tris and 1.0 mM EDTA at pH 8.0). To the purified DNA in 3 ml TE buffer was added 10 µl of RNAase A (Sigma) at a concentration of 20 mg/ml in TE buffer and incubated 30 min at 37°C. 12 µl of Proteinase K (Sigma) at a concentration 5 mg/ml in TE buffer was added and incubation continued for a further hour. DNA solution in 20 ml TE buffer was re-extracted with an equal volume of chloroform:phenol:isoamyl alcohol 24:24:1 (v/v/v) with shaking for 30 min at room temperature. The liquid was transferred to centrifuge tubes and centrifuged at 10,000 g for 5 min. The top aqueous layer was transferred to a beaker and overlaid with twice the volume of ice-cold ethanol. The DNA was collected by slowly stirring and spinning a glass rod in the mixture. The DNA wound on the glass rod was washed with 70% ethanol and dried in air, and then dissolved in 3 ml of TE buffer. DNA sample was stored in a freezer before determintion of G+C mol%. The purity of the DNA sample was obtained by comparing the OD 280/260 ratio of a sample resuspended in TE buffer.

The G+C mol% value was determined by the thermal denaturation method (Mandel and Marmur, 1973). The DNA was dissolved in 5 ml of 0.5×SSC buffer and then was dialyzed against 500 ml 0.5×SSC buffer for 24 hours using a spectra/por dialysis membrane tubing (molecular weight cut off 6000-8000 Da). The purified DNA was diluted with 0.5×SSC buffer in a 1 ml quartz cuvette to give a final absorbance value of 0.426 at 260 nm. The temperature of a spectrophotometer quartz cuvette was measured directly by sealing a thermocouple probe (Omega, USA) into the cuvette containing 0.5 SSC. This reference cuvette was placed next to the experimental sample cuvette in a heated cuvette holder of a Lambda 3B spectrophotometer (Perkin-Elmer, USA) equipped with a digital temperature controller. The increase in OD 260 nm was recorded as the temperature increased at 1°C per minute. The value for G+C mol% was determined according to equation 2 from Mandel and Marmur (1973). *E.coli* strain B

DNA (Sigma, USA) was used as a standard, and its G+C mol% determined under the same conditions as the sample.

3.2.14 16S rRNA analysis

Cells of isolate COMP.A2 were grown overnight at 70°C in 50 ml of the basal medium supplemented with 0.4% (w/v) maltose as carbon source. The cells were harvested by centrifugation at 8000 × g for 15 min, and washed twice in basal medium. The cell pellet was freeze dried and sent to Dr F. Rainey (DSM, Germany) for 16S rRNA analysis. Genomic DNA extraction, PCR amplification of 16S rDNA, and 16S rDNA sequence analysis were performed using procedures described by Rainey et al (1992, 1994a). The 16S rDNA sequences were aligned to the existing database for the members of the genus *Bacillus* and *Alicyclobacillus*. Pairwise evolutionary distance was computed using the correction of Jukes and Cantor (1969). The least squares distance method of De Soete (1983) was used in the construction of the phylogenetic dendrogram from distance matrices.

3.3 RESULTS

3.3.1 Isolation of strain COMP.A2

The strain COMP.A2 was enriched and isolated by Dr Hudson in the Thermophile Research Unit of the University of Waikato. The enrichment and isolation procedures are described here for completeness. Subsamples (1 g) from an artificial compost maintained at 70°C were added to medium ATC containing 1 sheet of paper tissue (cut into strips) per 100 ml medium. Flasks were either unsupplemented or contained in addition 0.05% (w/v) cellobiose, 0.05% (w/v) yeast extract, or both. All flasks also contained 1 ml of filter-sterilised vitamins to give a final concentration of 2 mg/l of pyriodoxamine HCl, *p*-aminobenzoic acid, biotin and vitamin B_{12}. All flasks showed cellulose hydrolysis after 2-3 days. Subcultures were made to medium containing tissue paper but no cellobiose or yeast extract. These subcultures were successful and cellulose hydrolysis was indicated by the formation of small (5 mm) spherical pellets in the broths, which were not found in uninoculated media, and growth indicated by a drop in the pH of the medium. A pure culture of COMP.A2 was finally obtained after extensive and repetitive plating onto medium M2ATC solidified using Gelrite.

Culture COMP.A2.1 is a stable co-culture containing strain COMP.A2 and a contaminating non-cellulolytic spore-forming *Bacillus* strain. Strain COMP.A2 is a thin spore-forming cell and is very easy to distinguish from the contaminating *Bacillus* strain

whose cells are much larger. When the project started, the co-culture COMP.A2.1 was used in initial experiments. The isolation of strain COMP.A2 from the co-culture was not successful despite numerous attempts to isolate strain COMP.A2 from co-culture. These attempts included using series dilution, Gelrite-plate separation, and the use of various antibiotics to inhibit the growth of the non-cellulolytic strain. It is not clear how strain COMP.A2 was contaminated with the non-cellulolytic *Bacillus* strain. It may be contaminated in the late preparation.

3.3.2 Morphological characteristics

Cells of the strain COMP.A2 were thin rods measuring 3-10 μm long by 0.6 μm in diameter during growth on basal medium with 0.4% (w/v) maltose as the substrate. Terminal oval endospores were formed with swollen sporangia (Figure 3.1). More endospores were observed in the cells when strain COMP.A2 grew on crystalline cellulose, compared to cells grown on amorphous cellulose or cellobiose. The Gram-stain reaction was negative, but the aminopeptidase test (Cerny, 1976) and non-staining (KOH) method (Buck, 1982) showed it was typical of a Gram type positive organism. Thin sections also showed the Gram positive structure of the wall (Figure 3.2). Scanning electron microscopy showed that thin rod cells with endospores were bound to cellulose (Figure 3.3). Cells attached to cellulose particles early in growth and remained attached until the particles were hydrolysed. Small colonies on Gelrite plates were observed after 4 days incubation at 65°C when the culture was surface spread on Gelrite plates with cellobiose as substrate (Figure 3.4). The colonies were transparent circular, colourless, irregular umbonate and less than 1 mm in diameter. The surface of colonies was undulate, shiny and smooth. No growth was observed on the same medium with agar as the gelling agent, despite repeated attempts and varying the quality and concentration of agar used.

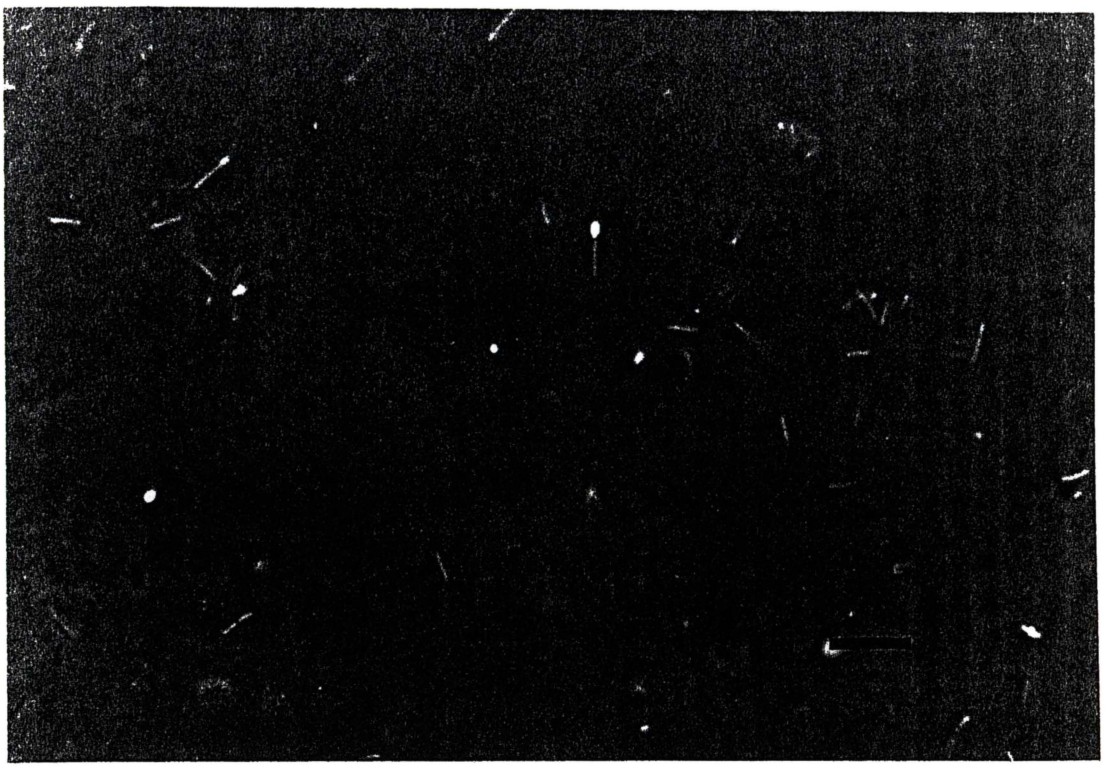


Figure 3.1 Phase-contrast photomicrograph of sporulating cells from exponential growth phase of strain COMP.A2. The strain COMP.A2 was grown on basal medium with 0.4% cellobiose (w/v) as carbon source at 65°C. Bar=10 μm.

Figure 3.2 Thin section photomicrograph showing endospore of strain COMP.A2 cells. Bar=0.2 μm.

Figure 3.3 Scanning electron photomicrograph illustrating the attachment of strain COMP.A2 cells to crystalline cellulose. Cells are thin rods with terminal endspores.

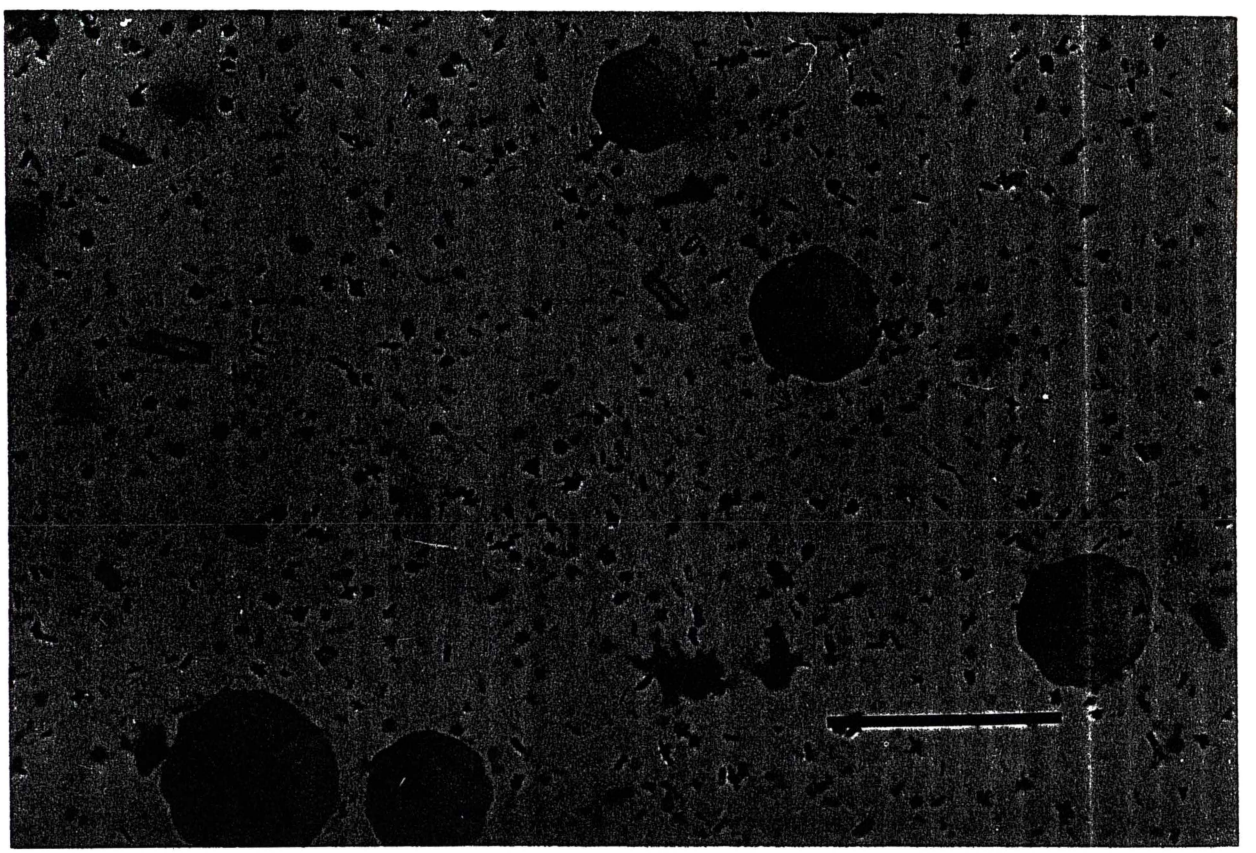


Figure 3.4 Photomicrograph of colony of strain COMP.A2 on Gelrite plate with cellobiose as substrate. Bar= 0.5 mm.

3.3.3 Physiological and nutritional properties

3.3.3.1 Effects of pH and temperature on growth

The optimum growth temperature for strain COMP.A2 was 65°C with a doubling time of 2.29 hours at log phase (Figure 3.5). No growth was observed at 55°C or 75°C even after extended incubation and there is a sharp curtailment of growth between 70°C and 75°C. The generation time at 60°C and 70°C was 3.47 and 3.72 hours at log phase, respectively. Strain COMP.A2 did not grow at pH 5.5 or pH 8.5 (Figure 3.6). The optimum pH for growth was between 6.5 and 7.5.

3.3.3.2 Substrates utilization

The utilization of substrates as sole carbon and energy source was tested by supplementation of the basal medium at pH 7.0. The results are shown in Table 3.1. Strain COMP.A2 grew well on xylan, but showed no growth on xylose. Although strain COMP.A2 grew well on Avicel, growth was not observed on Whatman filter paper (or a slurry of Whatman filter paper). The cells produced a yellow pigment when grown on cellobiose medium. Growth did not require the presence of the vitamin supplement or yeast extract.

Table 3.1 Carbon sources utilized by strain COMP.A2.

Acetate[a]	+	Arabinose	+
Pyruvate	+	Glucose	++
Glutamate[a]	+	Mannitol	w
Cellobiose	++	Maltose	++
Amorphous cellulose	++	Starch	+
CMC	w	Avicel	++
Sigmacell 20	++	Sigmacell 50	++
Dry wood pulp	+	Xylan	++

++ =good growth, + = growth, w = weak growth. a: Tests were carried out by Dr Hudson. Other substrates that were not utilized included: lactose, lactate, galactose, sorbitol, sucrose, xylose, fructose, raffinose, ribose, melezitose, rhamnose, mannose, citrate, newspaper (40 mesh), cotton (dewaxed), gum locust bean, pectin, ground *Pinus radiata* (60 mesh), inulin, mannan, steam exploded poplar. Substrates were added to the basal medium at a concentration of 0.4 (w/v) or 0.5% (w/v). Dry wood pulp, newspaper, ground *Pinus radiata*, and steam-exploded poplar were prepared in this lab.

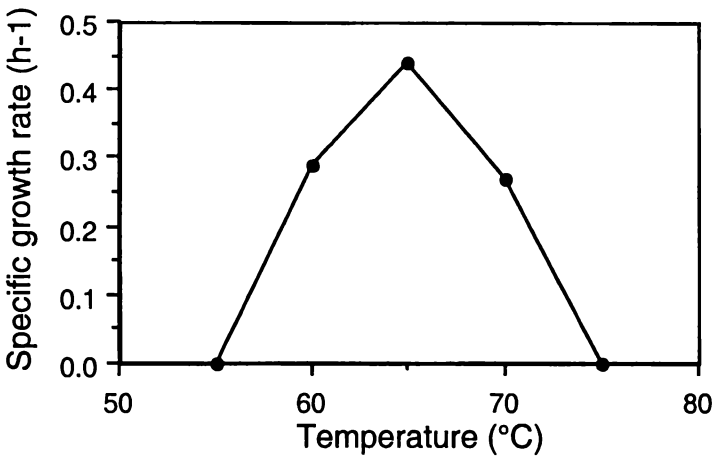


Figure 3.5 Growth rate of strain COMP.A2 at different temperatures. Incubation was carried out in basal medium containing cellobiose as substrate at pH 7.0.

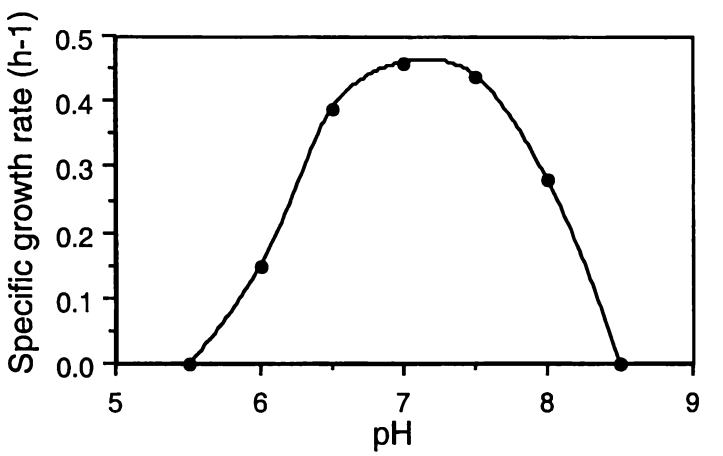


Figure 3.6 Growth rate of strain COMP.A2 at different pH values. Incubation was carried out in basal medium containing cellobiose as substrate at 65°C. Basal medium (50 ml) with MOPS or where MOPS was replaced by EPPS and MES at 50 mM was adjusted to the various pH values at 65°C. The range of pH used in the experiment is as follows: MES buffer (pH 5.5, 6.0, 6.5), MOPS buffer (pH 6.5, 7.0, 7.5) and EPPS buffer (pH 7.5, 8.0, 8.5).

3.3.3.3 Growth on insoluble substrates

Growth of COMP.A2.1 and COMP.A2 on microcrystalline cellulose Sigmacell 20 is shown in Figure 3.7 and 3.8. It appears that the mixed-culture COMP.A2.1 grew and degraded cellulose faster than the cellulolytic bacterium strain COMP.A2 alone. Similar results have been observed using amorphous cellulose as substrate. Reducing sugars were only accumulated when growth reached late log phase for COMP.A2.1 (Figure 3.7). In contrast, the growth of strain COMP.A2 correlated with the accumulation of reducing sugars (Figure 3.8). Growth of COMP.A2 on amorphous cellulose is shown in Figure 3.9. Almost all the amorphous cellulose (90%) was degraded by strain COMP.A2 at the end of growth.

3.3.3.4 Growth and end product formation

Growth of the strain COMP.A2 on cellobiose and the formation of end products are shown in Figure 3.10. Cell growth reflected the utilization of cellobiose and accumulation of glucose. Acetate and lactate were detected when cell growth reached late log phase. Table 3.2 shows the formation of end products from COMP.A2 grown on basal medium using amorphous cellulose as the substrate. As amorphous cellulose was degraded, glucose, acetate, and lactate were accumulated. Acetate and lactate were metabolized at the late stage of growth.

Table 3.2 End products formation from strain COMP.A2 grown in basal medium containing amorphous cellulose as substrate.

Incubation time (hrs)	glucose (mM)	lactate (mM)	acetate (mM)
12	2.67	0.0	0.0
13.5	3.11	0.0	0.0
15	4.0	0.0	0.0
16.5	5.49	0.0	0.0
19	8.27	5.47	0.0
21.75	9.73	1.23	0.55
24	14.47	0.85	0.77
37	8.15	0.47	0.0

3.3.3.5 Utilization of cellobiose and glucose

Growth and substrate utilisation by strain COMP.A2 is shown in Figure 3.11. In Figure 3.11 A and B, the flask was inoculated from an inoculum grown on basal medium with 20 mM glucose and an inoculum grown on basal medium with 10 mM cellobiose, respectively. A long lag phase in Figure 3.11 A was observed in comparison with that in Figure 3.11 B. The results show that cells preferred cellobiose to glucose as the substrate. Glucose was accumulated in the medium as cells grew and cellobiose was consumed. The accumulation of glucose (about 24 mM) and preference for cellobiose may be the result of released β-glucosidase activity in the culture medium and/or the presence of cellobiose phosphorylase.

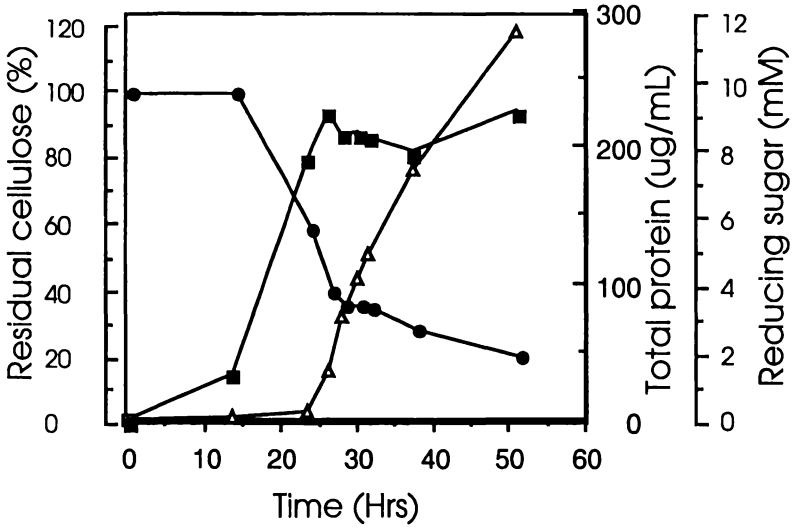


Figure 3.7 Growth of the co-culture COMP.A2.1 in basal medium containing Sigmacell 20 as substrate. Incubation was at 70°C with shaking. Symbols: reducing sugars (Δ), total protein (■), residual cellulose (●).

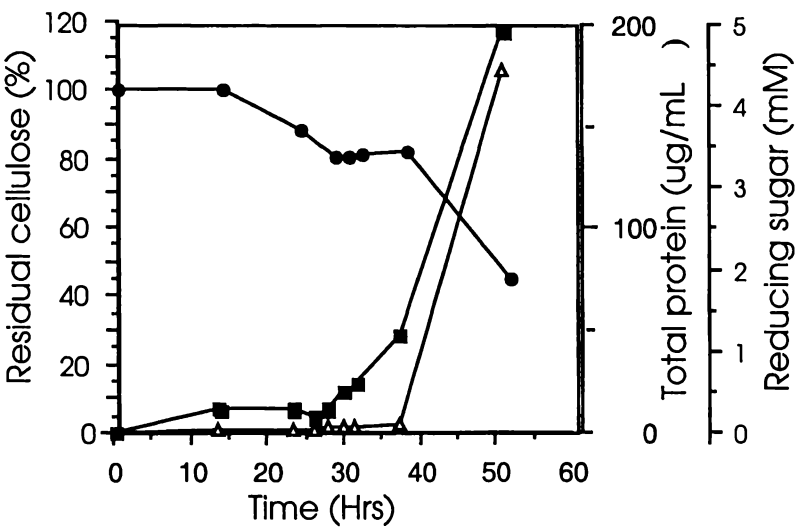


Figure 3.8 Growth of strain COMP.A2 in basal medium containing Sigmacell 20 as substrate. Incubation was at 70°C with shaking. Symbols: reducing sugars (Δ), total protein (■), residual cellulose (●).

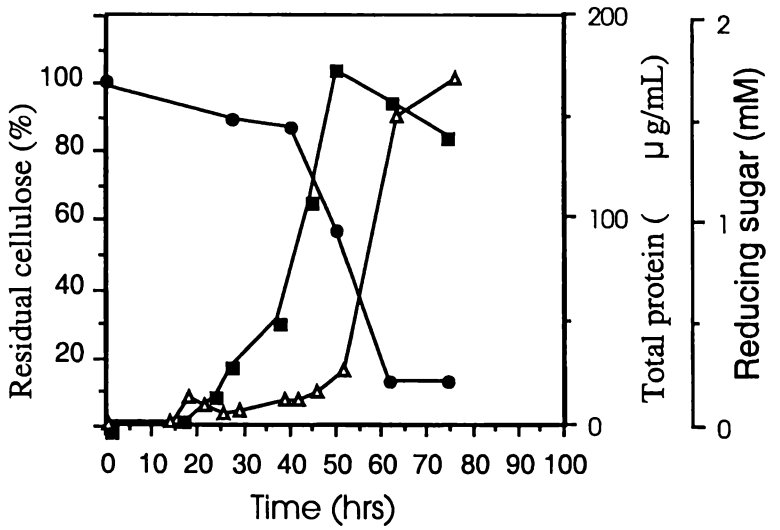


Figure 3.9 Growth of strain COMP.A2 in basal medium containing amorphous cellulose as substrate. Incubation was at 70°C with shaking. Symbols: reducing sugars (Δ), total protein (■), residual cellulose (●).

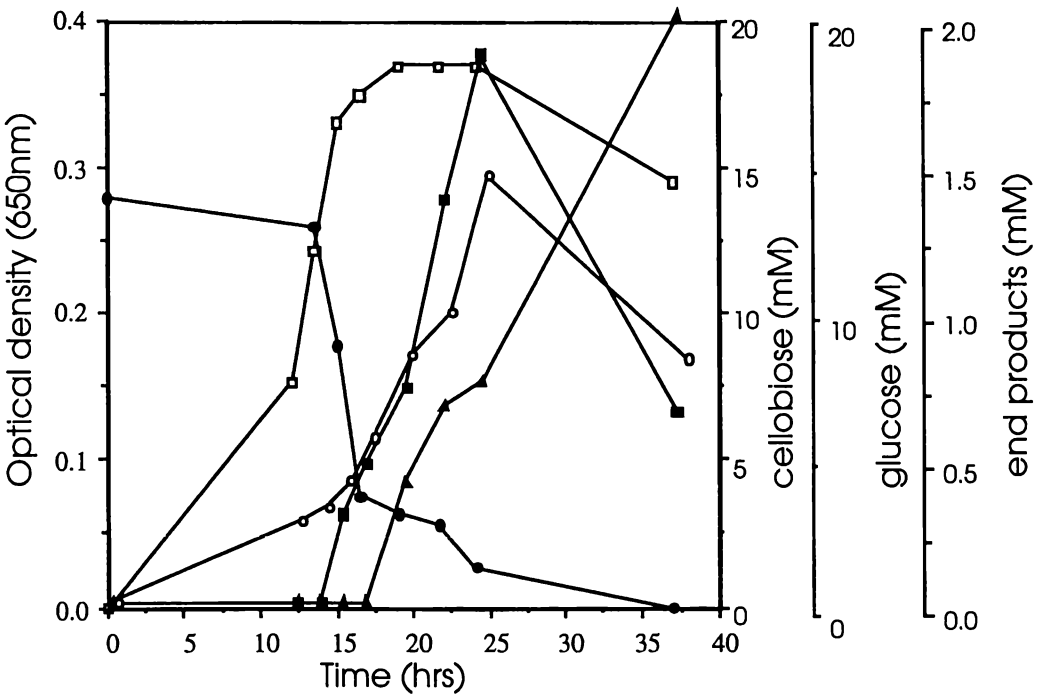


Figure 3.10 Growth time course of strain COMP.A2 in basal medium containing cellobiose as substrate. Symbols: OD at 650 nm (□), cellobiose (●), glucose (○), acetate (▲), lactate (■).

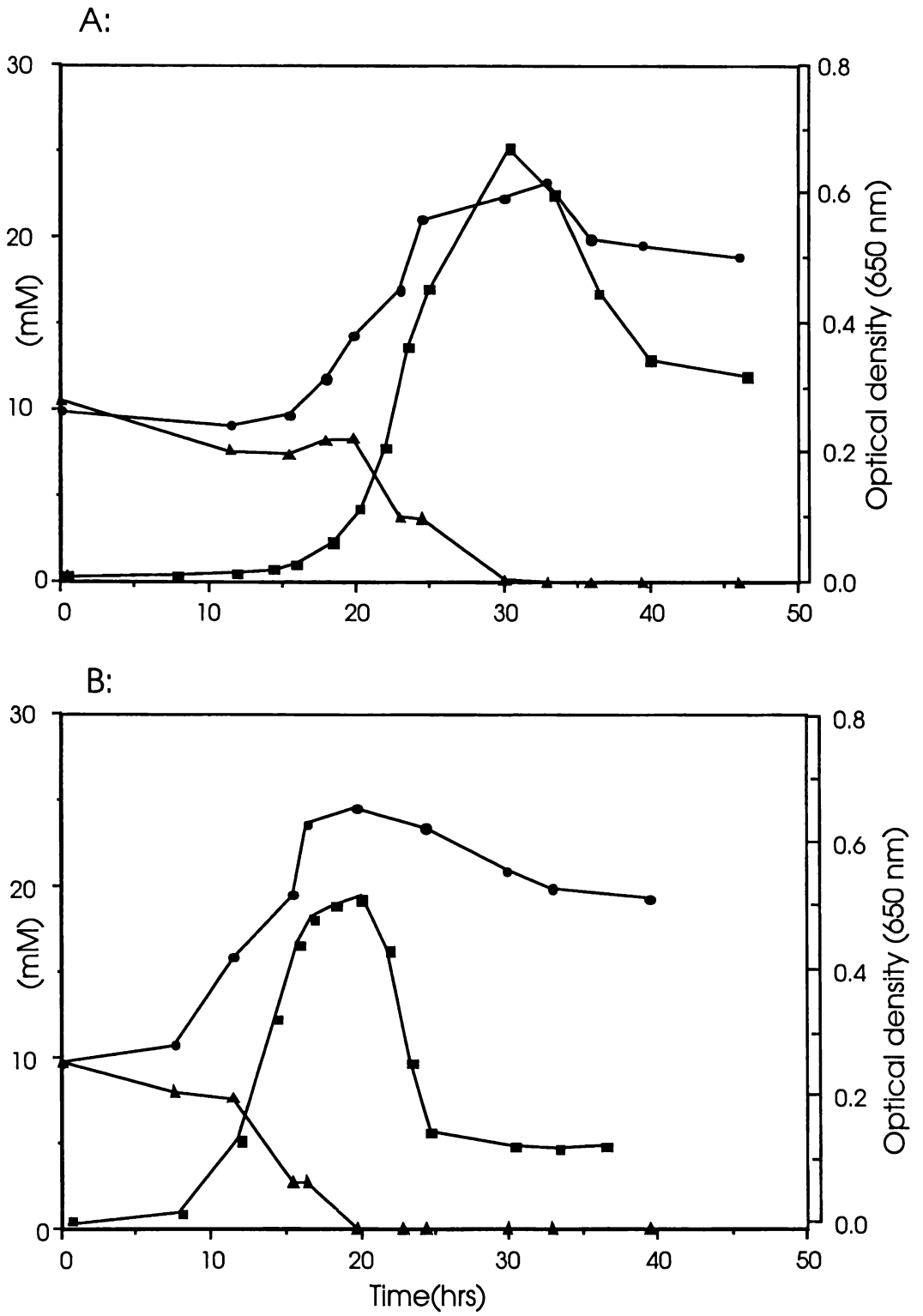


Figure 3.11 A and B. A: Growth curve of strain COMP.A2 in basal medium containing a mixture of 10 mM cellobiose and 10 mM glucose, inoculated from an inoculum grown on basal medium with 20 mM glucose. B: Growth curve of the strain COMP.A2 in basal medium containing a mixture of 10 mM cellobiose and 10 mM glucose, inoculated from an inoculum grown on basal medium with 10 mM cellobiose. Symbols: optical density at 650 nm (■), cellobiose (▲), glucose (●).

3.3.4 Sensitivity to inhibitors

Strain COMP.A2 was tested for its sensitivity to antibiotics, NaCl, agar and agarose. The results in Table 3.3 and 3.4 show that cells of COMP.A2 were sensitive to most antibiotics tested and resistant to novobiocin at a concentration of 5 μg/ml. The growth of strain COMP.A2 was inhibited by NaCl at a concentration of 2% (w/v) or NaN_3 at a concentration of 0.01% (w/v) (Table 3.4). Cells grew in the presence of 0.12% agar or 0.05% agarose, but growth was inhibited at 0.25% agar or 0.12% agarose.

Table 3.3 Antibiotic sensitivity of strain COMP.A2.

	Concentration (μg/mL)				
	1.25	2.5	5.0	10	20
Streptomycin	-	-	-	-	-
Penicillin-G	-	-	-	-	-
Novobiocin	+	+	+	-	-
Kanamycin	+	-	-	-	-
Ampicillin	-	-	-	-	-
Polymixin B	-	-	-	-	-
Neomycin	-	-	-	-	-

+ =Growth, - = No Growth. Growth was examined after stationary incubation at 65°C for two days.

Table 3.4 Agar, agarose, and NaN_3 sensitivity of strain COMP.A2

	Concentration (w/v %)						
	0.002	0.005	0.01	0.05	0.12	0.25	0.5
agar				+	+	-	-
agarose				+	-	-	-
NaN_3	+	+	-	-	-	-	-

+ =Growth, - = No Growth. Growth was examined after one day shaking incubation at 70°C.

3.3.5 Composition of cell lipids

The phospholipid and cell residue fatty acids in COMP.A2 contained 98-100 % saturated branched or straight chain fatty acids (Table 3.5). The main branched chain and straight chain fatty acids were iso $C_{16:0}$ and $C_{16:0}$, respectively. No cyclic fatty acids were found in cells. A number of related but unidentified high molecular weight components were present in the phospholipid fatty acid fraction.

3.3.6 DNA base composition

The purity of DNA extracted from COMP.A2 was estimated from its ratio of absorbance at 280:260. The ratio was 1.86 and this was regarded as acceptable for G+C mol% determination. The DNA melting temperature was determined twice, and the T_m values obtained were 90.3 and 90.7°C, respectively. Figure 3.12 shows the DNA melting curve of COMP.A2. The T_m of *E.coli* strain B (Sigma) was determined under the same conditions. The guanine-plus-cytosine content of DNA was calculated from the equation (Mandel and Marmur, 1973) as follows.

$$G+C\ \% = 2.44\ [T_m - 81.5 - 16.6\log(Na^+)]$$

$$= 2.44\ (90.5 - 81.5 - 16.6\log 0.195/2) = 62.4\%$$

So the G + C content of DNA was 62.4 mol% for strain COMP.A2.

3.3.7 Phylogenetic analysis

The full 16S rDNA sequence of strain COMP.A2 was determined and aligned to the existing database for the members of genus *Bacillus* and *Alicyclobacillus*. A phylogenetic dendrogram and 16S rDNA similarity values are presented in Figure 3.13 and Table 3.6. The results show that strain COMP.A2 is separated from the main *Bacillus* group and is closest to the phylogenetic lineage leading to *B. schlegelii*, *B. tusciae* and the new genus of *Alicylobacillus*. 16S rDNA similarity values of COMP.A2 with *Bacillus* species and *Alicylobacillus* species range from 85.3 to 88.2.

3.3.8 Comparison with *Bacillus schlegelii*

Both *B. schlegelii* and *B. tusciae* are thermophilic, facultatively chemolithoautotrophic hydrogen-oxidising bacteria. Strain COMP.A2 is phylogenetically close to *B. schlegelii* and *B. tusciae*. Autotrophic medium (AM 1) with $O_2/H_2/CO_2$ was made up to test whether strain COMP.A2 can use hydrogen as its energy source to fix CO_2. *B. schlegelii* grew well on AM 1 medium with $O_2/H_2/CO_2$ after 2 days incubation at 65°C. However, growth was not observed for strain COMP.A2 on this medium even after

incubation at 65°C for one week. This suggests strain COMP.A2 is not hydrogen-oxidizing and can not grow autotrophically.

Growth was not observed for both *B. schlegelii* and strain COMP.A2 on AM 2 medium in flasks or serum bottles after one week incubation at 65°C. No growth was observed for *B. schlegelii* on basal medium containing 0.4 % (w/v) glucose or 0.4 % (w/v) Avicel.

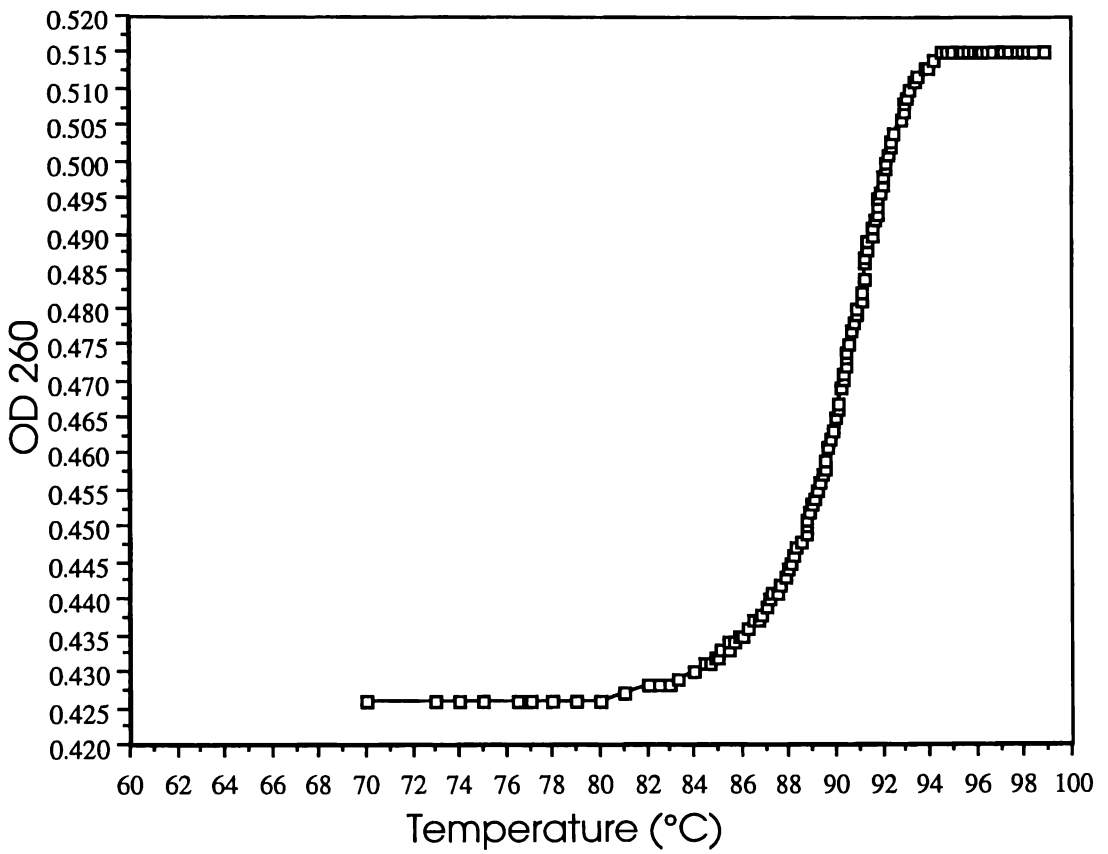


Figure 3.12 DNA melting curve of strain COMP.A2.

Table 3.5 Phospholipid and cell residue fatty acid composition of strain COMP.A2.

Fatty acids	Percentage composition (%)	
	Phospholipid fatty acids	Cell residue fatty acids
branched chain		
i14:0	0.6	1.1
i15:0	0.2	0.2
a15:0	1.3	1.5
i16:0	58.7	61.9
i17:0	0.8	0.7
a17:0	5.8	4.9
i18:0	2.6	2.3
i19:0	-	-
sum	70.0	72.6
straight chain		
14:0	0.6	0.7
15:0	2.4	2.4
16:0	24.6	22.2
17:0	1.5	1.5
18:0	0.7	0.6
sum	29.8	27.4
other		
18:1	-	-
16:0 ß-OH	0.2	-
total sum	100	100

i = iso, a = anteiso, ß-OH = beta hydroxy fatty acid.

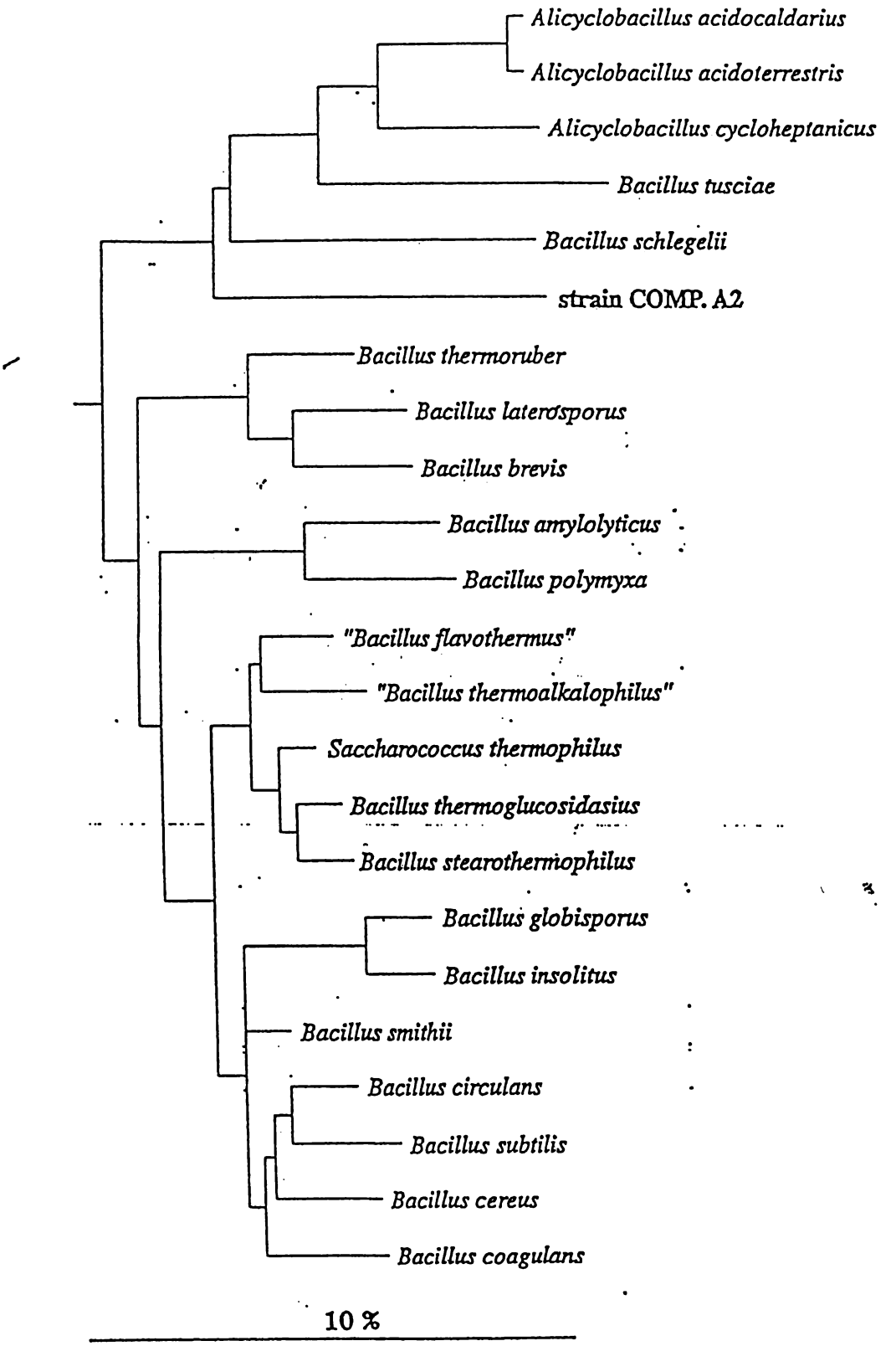


Figure 3.13 Phylogenetic dendrogram based on 16S rDNA sequence data indicating the position of strain COMP.A2 within the members of the genus *Bacillus* and *Alicyclobacillus*. The scale bar represents 10 substitutions per 100 nucleotides.

Table 3.6 16S rDNA sequence similarity values for strain COMP.A2, *Alicyclobacillus* species, and other *Bacillus* species.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
1. strain COMP.A2																								
2. *B. schlegelii*	88.2																							
3. *B. tusciae*	87.0	88.9																						
4. *A. cycloheptanicus*	87.0	87.6	90.9																					
5. *A. acidoterrestris*	87.8	88.4	89.9	93.7																				
6. *A. acidocaldarius*	87.9	88.7	90.1	93.9	99.3																			
7. *B. thermoruber*	87.0	88.1	86.4	87.9	87.5	87.9																		
8. *B. laterosporus*	85.3	86.4	84.9	86.1	85.6	86.0	94.5																	
9. *B. brevis*	86.1	86.8	85.1	86.5	86.5	86.9	95.0	95.3																
10. *B. amylolyticus*	87.0	85.4	84.0	86.4	86.5	86.6	90.8	90.8	90.0															
11. *B. polymyxa*	87.7	85.7	84.3	86.0	85.4	85.7	90.5	90.6	89.9	94.2														
12. "*B. flavothermus*"	87.5	89.0	87.2	88.7	89.2	89.2	92.1	91.0	90.6	90.1	90.1													
13. "*B. thermoalkalophilus*"	86.7	87.9	86.5	87.6	88.6	88.8	91.5	90.9	90.4	89.7	89.9	96.5												
14. *Sac. thermophilus*	87.8	88.4	87.5	88.2	89.0	89.2	93.0	91.1	91.0	91.6	91.2	96.6	96.3											
15. *B. thermoglucosidasius*	87.9	88.8	87.3	88.0	89.2	89.4	92.6	90.5	90.5	90.8	89.8	96.6	96.5	98.1										
16. *B. stearothermophilus*	87.8	88.9	88.0	87.9	89.0	89.2	92.3	91.1	90.3	90.3	89.3	96.4	96.0	97.8	98.0									
17. *B. globisporus*	85.9	86.0	84.6	85.7	86.4	86.5	90.4	90.1	90.3	90.3	91.0	93.7	93.1	93.8	92.8	92.2								
18. *B. insolitus*	85.3	86.1	84.4	85.5	86.1	86.2	90.4	90.2	89.7	91.1	91.4	93.2	92.7	93.4	92.5	91.9	97.0							
19. *B. smithii*	87.7	88.4	86.8	87.8	88.6	88.7	92.3	91.2	91.1	91.7	90.9	95.8	94.7	95.8	95.2	94.3	94.3	94.1						
20. *B. circulans*	86.9	87.5	86.1	87.0	87.1	87.5	92.2	91.3	90.8	91.9	91.7	94.8	94.5	95.5	94.5	93.8	94.3	95.3	96.4					
21. *B. subtilis*	86.8	87.0	85.5	86.6	86.6	86.9	90.4	89.8	89.8	91.6	90.4	94.1	93.7	94.9	94.2	93.1	93.5	93.7	95.0	96.3				
22. *B. cereus*	86.2	87.2	86.0	87.3	86.7	87.1	90.8	91.9	90.2	90.6	90.2	94.8	94.2	95.0	94.3	93.8	93.7	94.7	95.0	96.0	95.4			
23. *B. coagulans*	86.5	87.6	86.2	87.3	87.5	87.8	91.3	91.0	90.6	90.6	90.8	94.1	94.3	94.3	93.8	93.0	93.3	93.4	95.3	95.3	95.2	95.3		
24. *C. butyricum*	81.7	81.6	82.3	83.8	82.8	82.8	85.6	85.9	87.0	84.2	83.3	84.7	84.6	84.6	84.6	84.2	83.9	85.0	84.6	85.6	85.3	86.4	85.0	

Table 3.7 Comparison of some characteristics of strain COMP.A2 and related species.

Organism	Source	Mol% of G+C	pHopt	Topt(°C)	Alicyclic fatty acids	Hydrogen autotrophy	Cellulose hydrolysed
COMP.A2		62.4	6.5-7.5	65	—	—	+
B.stearothermophilus	ATCC 12016	54.2	6.8	60-65	—	—	—
B.tusciae	DSM2912	57	4.2-4.8	55	—	+	—
B.schlegelii	DSM2000	66-68	6.0-7.0	70	—	+	—
Alicyclobacillus cycloheptanicus	DSM4006	55.6	3.5-4.5	48	+	—	—

G+C content was determined by T_m measurement.

Data from different sources. *B.stearothermophilus* from Sharp et al.(1980), *B.tusciae* from Bonjour and Aragno (1984), *B.schlegelii* from Schenk and Aragno (1979), *A. cycloheptanicus* from Wisotzkey et al (1992), COMP. A2 from this study.

3.4 DISCUSSION

The genus *Bacillus* consists of a large and heterogeneous collection of aerobic, rod-shaped, endospore-forming bacteria which vary phenotypically and genomically (Sneath, 1984). The mol% of G+C of DNA in the genus *Bacillus* ranges from 32% to 68%. The strain COMP.A2 is related to the genus *Bacillus* on the basis of it being a Gram-positive, aerobic, endospore-forming rod.

The application of 16S rRNA (rDNA) sequence analysis and homology comparisons had an important influence on classification of the genus *Bacillus*. This is thought to be the most appropriate method for determining broad taxonomic relationships (Fox et al, 1980). Ash et al (1991) reported that there were at least three main clusters in the genus *Bacillus* based on 16S rRNA cataloguing studies. Three thermoacidophilic *Bacillus* species *B. acidocaldarius*, *B. acidoterrestris*, and *B. cycloheptanicus*, previously grouped with the genus *Bacillus*, have now been reclassified in a new genus *Alicyclobacillus*. While the case for reclassification was based on 16S rRNA sequence analyses it was also supported by good phenotypic markers such as the presence of unique alicyclic fatty acid as the major membranous lipid component, and growth at low pH (Wisotzkey et al, 1992). The 16S rRNA sequence analysis (Figure 3.13) revealed that strain COMP.A2 is distinct from other members of genus *Bacillus* and aligns closer to *B. schlegelii*, *B. tusciae* and the genus *Alicylobacillus*. A comparison of selected characteristics of COMP.A2 with related species is shown in Table 3.7. A distinguishing feature that separates *B. tusciae* and *B. schlegelii* from strain COMP.A2 and *Alicylobacillus* species is the ability to grow autotrophically using hydrogen and carbon dioxide. Both *B. tusciae* and *B. schlegelii* are facultatively chemolithoautotrophic hydrogen-oxidizing bacteria (Bonjour and Aragno, 1984; Schenk and Aragno, 1979). Strain COMP.A2 was unable to grow autotrophically in the presence of hydrogen and carbon dioxide or thiosulphate. *B. schlegelii* did not show autotrophic growth on thiosulphate. However Hudson et al. (1988) reported that *B. schlegelii* was capable of autotrophic growth using thiosulphate. The reason for these different results is not clear although the same medium and incubation condition was used. The lack of alicyclic fatty acid in the cell membrane and growth at neutral pH indicates that COMP.A2 is not a member of the genus *Alicyclobacillus*.

A feature that is unique to strain COMP.A2 is its ability to grow at high temperature on a defined medium with crystalline cellulose as sole carbon and energy source. No other thermophilic member of the genus *Bacillus* or *Alicyclobacillus* has this property. The ability to utilise crystalline cellulose is considered to be the characteristic of truly cellulolytic microorganisms (Johnson et al., 1982) and is a property only found in a few

Bacillus species (Jorgensen and Hansen, 1990; Kim, 1995; Han et al., 1995) and not reported in the genus *Alicyclobacillus*.

Taken together these four features, 16S rRNA sequence, obligate heterotrophy, absence of alicyclic fatty acids in the membrane and capability to use crystalline cellulose as substrate at high temeprature, clearly show that strain COMP.A2 is a novel isolate.

Another unusual feature of strain COMP.A2 is that it did not grow on agar based media and was inhibited by low concentrations of agar or agarose. This property has not been observed in other *Bacillus* and *Alicyclobacillus* species. It is likely that the inhibition by agar is due to the agarose which is one of the main components in agar since the inhibition by agar reflected inhibition found with agarose concentration present in agar. It has been reported that the thermophilic obligately hydrogen-oxidizing bacterium *Hydrogenobacter thermophilus* was inhibited by agar (Kristjansson et al., 1985, Kawasumi et al., 1984). The optimum growth temperature and pH of *H. thermophilus* are similar to those of strain COMP.A2. But cells of *Hydrogenobacter thermophilus* are non-spore-forming, gram-negative rods. Another unique feature is the extreme sensitivity of strain COMP.A2 to most antibiotics tested and being only resistant to novobiocin. Penicillin G sensitivity has also been observed in *B. schlegelii* (Schenk and Aragno, 1979). Recently, some autotrophic, sulfur- and hydrogen-oxidizing thermophilic bacteria isolated from hot compost were shown to have a high DNA:DNA homology with *Hydrogenobacter* strains (Beffa et al, 1996a). The isolated bacteria were sensitive to penicillin G and showed no inhibition by agar.

Strain COMP.A2 had a narrow growth temperature range (60 to 70°C) in comparison with other members of the genus *Bacillus* and *Alicyclobacillus*. The temperature range for growth is 45 to 70°C for *Alicyclobacillus acidocaldarius* (Darland and Brock, 1971), and 40 to 53°C for *A. cycloheptanicus* (Deinhard et al, 1987b). *B. schlegelii* can grow up to 75°C (Aragno, 1978). No growth occurs at 65°C for *B. tusciae* (Bonjour and Aragno, 1984).

Strain COMP.A2 is able to use xylan as substrate, but is unable to use xylose as substrate. This has been found in *C. thermocellum* which is capable of degrading xylan, but is incapable of metabolizing xylose (Wiegel et al., 1985; Slapack et al., 1987; Bender et al., 1985). CMC is a poor growth substrate for COMP.A2. Similarly it has been reported that CMC was a poor inducer of CMCase formation in *B. subtilis* AU-1 (Chan and Au, 1987) and *B. subtilis* DLG was unable to use CMC either as a growth substrate or as an inducer for CMCase (Robson and Chambliss, 1984).

The cells of COMP.A2 adhere to cellulosic substrates during the degradation process as was shown in scanning electron microscopy and phase contrast microscopy (Fig. 3.1 and Fig. 3.3). A close association between cells and cellulose is not unusual in cellulolytic bacteria and has been observed in *C. thermocellum*, *R. albus*, and *F. succinogenes* (Bayer et al., 1983; Morris and Cole, 1987; Gaudet and Gaillard, 1987).

It was shown that the mixed-culture COMP.A2.1 grew and degraded cellulose faster than the cellulolytic bacterium strain COMP.A2 alone. This has been found in many cellulolytic systems for example *Sporocytophaga* sp. and a noncellulolytic bacterium *Flavobacterium* sp. (Von Hofsten et al, 1971), *C. thermocellum* and *C. thermohydrosulfuricum* (Ng et al, 1981), *Bacteroides succinogenes* and *Treponema bryantii* (Stanton and Canale-Parola, 1980), *C. thermocellum* and *Thermoanaerobium brockii* (Lamed and Zeikus, 1980), *C. thermocellum* and *Spirochaeta caldavia* (Pohlschroeder et al., 1994). They all showed that the co-culture degraded cellulose faster than the cellulolytic bacterium alone. The usual explanation that is given is that removal of cellobiose and glucose by the non-cellulolytic bacteria reduces catabolite repression of cellulase and accelerates cellulose hydrolysis.

Glucose, acetate, and lactate were the end products when strain COMP.A2 grew on cellulose or cellobiose. No cellobiose was detected. When grown on glucose and cellobiose simultaneously, strain COMP.A2 showed a preference for cellobiose. It appears that cell growth stopped when all cellobiose was metabolized. Glucose was accumulated in the medium, which is surprising since glucose is a substrate which supports growth. One possibility is that it will take longer time for cells to adapt to growth on glucose. The accumulation of glucose was still evident when an inoculum pre-grown on glucose was used on the cellobiose/glucose mixed medium, the only difference being a longer lag phase compared to an inoculum pre-grown on cellobiose. Extracelllular glucose accumulation was considered to be the result of released β-glucosidase activity in the culture medium (Khan and Patel, 1991). The accumulation of glucose and preference for cellobiose may also be explained by the presence of cellobiose phosphorylase in the cells of strain COMP.A2. This will need to be proven by further investigation. The preference of cellobiose for growth has been reported in several mesophilic bacteria, e.g. *Cellulomonas fermentans* (Bagnara et al., 1987), thermophilic bacterium *Clostridium thermocellum.* (Ng and Zeikus, 1982), and *Thermomonospora curvata* (Bernier and Stutzenberger, 1987). Cook et al. (1993) showed that glucose and xylose were simultaneously utilized by the thermophilic glycolytic anaerobe *Clostridium thermohydrosulfuricum.*

3.5 CONCLUSIONS

Both phenotypic and phylogenetic characters have been used in the characterization of strain COMP.A2. Strain COMP.A2 is considered to be related to the genus *Bacillus* based on it a being Gram-positive, aerobic, endospore-forming rod. Phylogenetic studies of the 16S rRNA sequence analysis showed that the strain COMP.A2 forms a cluster with *B. schlegelii*, *B. tusciae* and the new genus of *Alicylobacillus*. The 16S rRNA similarity values of COMP.A2 with *Bacillus* species and *Alicylobacillus* species range from 85.3 to 88.2.

Strain COMP.A2 was capable of utilizing a wide range of substrates such as glucose, cellobiose and cellulose as the sole carbon and energy source in a chemically defined medium. The ability to grow on Avicel suggests that strain COMP.A2 produced cellulases capable of degrading crystalline cellulose. The end products of strain COMP.A2 grown on cellulose and cellobiose were glucose, acetate, and lactate. When grown on glucose and cellobiose simultaneously, strain COMP.A2 showed a preference for the cellobiose and accumulation of glucose. Further study needs to be done for understanding the mechanism of preference for the cellobiose.

The growth of strain COMP.A2 was inhibited by low concentration of agar or agarose, and is extremely sensitive to most antibiotics tested and only resistant to novobiocin. Unlike *B. schlegelii* and *B. tuscia*, strain COMP.A2 is not a facultatively chemolithoautotrophilic hydrogen-oxidizing bacterium. The growth temperature range is between 60°C to 70°C. The lack of alicylic fatty acid in the cell membrane and neutral growth pH suggests that strain COMP.A2 is not a member of the genus *Alicyclobacillus*. Strain COMP.A2 is proposed to be a new species of a new genus based on its physiological and phylogenetic characters. Ideally at least 10 strains are needed for description of new species as suggested by Sneath (Truper and Schleif, 1992). However in reality, new species and genera have been described on the basis of a few strains or even only one strain such as an extreme thermophile *Thermomicrobium roseum* (Jackson et al., 1973) and a thermophilic sulfate-reducing bacterium *Thermodesulforhabdus norvegicus* (Beeder et al., 1995).

CHAPTER FOUR

PURIFICATION AND CHARACTERIZATION OF CELLULASES FROM STRAIN COMP.A2

4.1 INTRODUCTION

The enzymatic hydrolysis of crystalline cellulose, as determined through studies on fungal cellulolytic enzymes, requires the synergistic action of at least three cellulase components: endo-1,4-ß-glucanase, exo-1,4-ß-glucanase, and ß-glucosidase (Wood, 1989). Bacterial cellulase systems however display a characteristic difference from fungal cellulases. All cellulolytic bacteria produce a variety of endoglucanases, but exoglucanases have been found only in a few bacteria (Creuzet and Frixon, 1983; Nakamura and Kitamura, 1988; Yablonsky et al., 1988, Wang et al., 1994; and Zverlov et al., 1998a).

The understanding of the mechanism of cellulose degradation by cellulases requires the purification and characterization of the individual components of the cellulase system. The isolation and characterization of cellulases has been reported from some cellulolytic bacteria. They include endoglucanases from *Clostridium thermocellum* (Petre et al, 1981; Ng and Zeikus, 1981; Romaniec et al., 1992), *Clostridium stercorarium* (Bronnenmeier and Staudenbauer, 1988a), *Bacillus subtilis* (Au and Chan, 1987), *Bacillus circulans* (Kim, 1995), *Thermomonospora fusca* (Calza et al, 1985), *Rhodothermus marinus* (Hreggvidsson et al. 1996), and exoglucanases from *C. stercorarium* (Creuzet et al., 1983; Bronnenmeier et al., 1991), *C. thermocellum* (Wang et al., 1994), *Cellulomonas uda* (Nakamura and Kitamura, 1988), and *Ruminococcus albus* (Ohmiya and Shimizu, 1988a).

The strain COMP.A2 is an endospore-forming, thermophilic, cellulolytic bacterium which produces cellulases to hydrolyze crystalline cellulose. Three types of cellulases (endoglucanase, exoglucanase, and β-glucosidase) have been detected in the extracellular supernatant of strain COMP.A2. The purification of cellulase components from the strain COMP.A2 was carried out and described in this chapter. One endoglucanase was finally purified to homogeneity. The purified enzyme showed an exo-model of action on CMC which is unusual among most other endoglucanases.

4.2 MATERIALS AND METHODS

4.2.1 Chemicals

Where possible analytical grade reagents were used, except that on some occasions reagent grade salts were used for making media. 4-methylumbelliferyl-cellobiose (MUC), *p*-nitrophenyl-cellobioside (pNPC), *p*-nitrophenyl-lactone (pNPL), microcrystalline cellulose (Sigmacell 20 and 50), carboxymethyl cellulose (CMC, low and medium viscosity), lichenan, laminarin, xylan (oat spelt and larch wood) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). PM 10 ultrafiltration membranes were from Amicon Corporation (Danvers, MA, USA).

4.2.2 Enzyme assays

All enzyme assays were carried out at pH 7.0 and 70°C unless noted otherwise. Substrates were prepared in 50 mM MOPS (Sigma, USA) buffer and adjusted to pH 7.55 at room temperature which corresponds to pH 7.0 at 70°C.

4.2.2.1 Activity on microcrystalline cellulose

Activity on microcrystalline cellulose was measured by incubating the enzyme sample with a slurry of Avicel (Asahi Chemical Industry Company Ltd, Japan) and determining the reducing sugars released using the PAHBAH method. The detailed PAHBAH method was described in section 3.2.7.1.

Aliquots (0.9 ml) of 2% or 4% (w/v) suspension of Avicel in 1.5 ml Eppendorf tubes were preheated at 70°C. An enzyme sample of 0.1 ml was added to two of the triplicate tubes, mixed and incubated at 70°C for 30 minutes (t=30). All assays were stopped by placing the Eppendorf tubes in ice-cold water. An enzyme sample of 0.1 ml was added to the remaining tube (t=0) as control. All tubes were mixed and centrifuged for 5 minutes at maximum speed in a benchtop centrifuge (Microfuge E, Beckman, USA). Supernatant, 0.5 ml, from each tube was removed and reducing sugars were assayed by PAHBAH method. Unless otherwise stated, activity on Avicel (Avicelase) is expressed as μmol (glucose equivalent) min^{-1} ml^{-1} or mg^{-1} protein. Avicel suspensions were prepared immediately before use or stored at 4°C for two days.

4.2.2.2 Activity on carboxymethylcellulose (CMC)

Activity on carboxymethylcellulose (CMC) was determined by measuring reducing sugar released from CMC. A low viscosity CMC solution (1% w/v), 0.4 ml, in 50 mM MOPS buffer at pH 7 in Eppendorf tubes was preheated at 70°C. An enzyme sample of 0.1 ml was added to two of the triplicate tubes, mixed well and incubated at 70°C for 15 minutes (t=15). All assays were stopped by the addition of 1.0 ml PAHBAH reagent. An enzyme sample of 0.1 ml was added to the remaining tube (t=0). PAHBAH assay was carried out as described in section 3.2.7.1. Unless otherwise stated, activity on CMC is expressed as μmol (glucose equivalents) $min^{-1}mg^{-1}$ protein or ml^{-1}. CMC solutions were made freshly or stored at 4°C for two days.

Activity on CMC was also determined by a viscosimetric assay. A volume of 0.8 ml of a low viscosity CMC solution (0.5% w/v in 50 mM MOPS buffer at pH 7.0) was pipetted into 1.5 ml Eppendorf tubes and incubated with 20 μl volumes of purified CMCase (0.22 μg enzyme) at 70°C. Samples were withdrawn at specified times, then chilled to stop the reaction. The relative viscosity of the incubation mixture was determined at room temperature by comparing the flow time in a 1.0 ml tuberculin syringe fitted with a blunt-ended 26-gauge needle based on the method of Fauth et al. (1991). The specific viscosity (nsp) was given by the formula $nsp = (T-T_O) / T_O$ where T_O is the flow time of water and T is the flow time of CMC solution. After determining the viscosity, the reducing sugar in samples was determined to establish the relationship between the viscosity and reducing sugar production during incubation.

4.2.2.3 ß-Glucosidase

The substrate *p*-nitrophenyl-ß-D-glucoside (pNPG), was used to measure ß-glucosidase activity. The *p*-nitrophenol released from pNPG was measured colorimetrically.

pNPG, 0.4 ml at a concentration of 2 mM, in 50 mM MOPS buffer at pH 7.0 was preheated in 1.5 ml Eppendorf tubes at 70°C. An enzyme sample of 0.1 ml was added to two of triplicate tubes and incubated at 70°C for 15 minutes (t=15). All assays were stopped by the addition of 1.0 ml of 1.0 M Na_2CO_3, then 0.1 ml of enzyme sample was added to the remaining tube (t=0). The absorbance of solution was read at 400 nm.

A standard curve was prepared from *p*-nitrophenyl (*p*-NP, Merck). *p*-Nitrophenyl solution, 0.5 ml at concentrations from 0.0 to 0.313 mM *p*-NP in 50 mM MOPS buffer at pH 7.0, was added to 1.0 ml 1.0 M Na_2CO_3. The absorbance of each solution was read at 400 nm. An extinction coefficient (ε_{400}) of 18.7×10^3 $lmol^{-1}cm^{-1}$ was

obtained and used to calculate activities. The activity on pNPG is expressed as μmol (*p*-NP) min^{-1} ml^{-1} or mg^{-1} protein.

4.2.2.4 Activity on other substrates

Other substrates were chosen for study of the substrate specificity of the purified CMCase. They included 0.25% (w/v) xylan (oat spelt and larch wood), 1% (w/v) laminarin, 1% (w/v) lichenan, 2 mM *p*-nitrophenyl-cellobioside (pNPC), 2 mM *p*-nitrophenyl-lactoside (pNPL), and 0.5 mM 4-methylumbelliferyl-cellobiose (MUC). All substrates were prepared in 50 mM MOPS buffer, pH 7.55 at room temperature. 400 μl of the substrate preparations were preheated in 1.5 ml Eppendorf tubes at 70°C and pH 7.0. An enzyme sample of 50 μl was added to two Eppendorf tubes and incubated at 70°C for 10 or 15 minutes. The reaction was stopped by the addition of 1.0 ml PAHBAH reagent to Eppendorf tubes containing xylan, laminarin, and lichenan as substrates, 1.0 ml of 1.0 M Na_2CO_3 to Eppendorf tubes containing pNPC or pNPL, and 1.0 ml glycine buffer (pH=10.4) to Eppendorf tubes containing MUC. The appropriate control was run at the same time. The PAHBAH assay method was as described in section 3.2.7.1. The *p*-nitrophenol released from pNPC or pNPL was measured colorimetrically as described in section 4.2.2.3. The GOD-perid method, described in section 5.2.2, was used for determining glucose release from pNPC or pNPL reaction mixtures. MU released from MUC was measured at 365 nm. A standard curve was prepared from 4-MU. The standard solution was made up from 0.025 to 1.6 mM 4-MU in Milli Q water. Each standard solution (0.1 ml) was mixed with 0.4 ml of 50 mM MOPS buffer and 1 ml of 0.5 M glycine/NaOH buffer (pH 10.4) was added. The solution absorbance was read at 365 nm. An extinction coefficient (ε_{365}) of 12.3 x $10^3 lmol^{-1}cm^{-1}$ was obtained and used to calculate activities. The activity on xylan is expressed as μmol (xylose equivalent) min^{-1} mg^{-1} protein. The activity on laminarin and lichenan is expressed as μmol (glucose equivalent) min^{-1} mg^{-1} protein. The activity on pNPC or pNPL is expressed as μmol (*p*-NP) or glucose min^{-1} mg^{-1} protein. The activity on MUC is expressed as the μmol (MU) $min^{-1}mg^{-1}$ protein.

4.2.2.5 Proteinase activity determination

Proteinase activity was determined by the azocasein assay method (Peek et al. 1992). Strain COMP.A2 was grown in 50 ml of basal medium containing 0.4% Sigmacell 20 or 0.4% cellobiose. Incubation was carried out at 70°C for two and one day respectively. One ml of 0.2% (w/v) azocasein in 50 mM HEPES buffer, pH 7.0 (70°C) containing 5 mM $CaCl_2$ was pippetted into 1.5 ml Eppendorf tubes and preheated for 10 min at 70°C. 70 μl of the COMP.A2 culture grown as described was added and

incubated at 70°C for 10 min. The reaction was stopped by the addition of 500 µl of 15% (w/v) trichloroacetic acid. The tubes were cooled for 10 min at room temperature, then centrifuged at maximum speed in a benchtop microfuge for 5 min. The absorbance of the supernatant was read at 420 nm. The activity of enzyme was defined as the change in absorbance at 420 nm at 70°C per hour.

4.2.3 Protein determination

The Folin-phenol method was used for protein determination in trial experiments of enzyme purification. The detailed method is described in section 3.2.7.2. The dye-binding method of Bradford (1976) was used for protein determination during the purification and characterization of the enzymes. This method is based on the change in the absorbance spectrum of Coomassie Blue on binding to protein. The maximum absorbance of dye which is 470 nm in the acidic assay reagent solution shifts to 595 nm after the dye binds to protein.

Coomassie Brilliant Blue G-250, 100 mg, was added to 50 ml 95% ethanol and mixed with 100 ml 85% (w/v) phosphoric acid. The solution was stirred thoroughly using a magnetic stirrer and filtered through a glass fibre GF/C (Whatman, England). This stock solution was stable at room temperature. The assay reagent was made by the addition of 20 ml stock solution to 80 ml R.O. water and was stable for two weeks. Protein samples were assayed in duplicate by adding 0.2 ml of sample to 1.0 ml of the assay reagent in 1.5 ml Eppendorf tubes. After 15 minutes at room temperature, the absorbance was read at 595 nm.

Bovine serum albumin (BSA, Sigma) was used as standard and was dissolved in R.O. water in the range of 0.0 to 1.25 mg/ml and stored at -20°C.

4.2.4 Effect of carbon sources on cellulase production

The basal culture medium (100 ml in 250 ml conical flasks) was supplemented with one of following carbon sources: 0.5 g xylan, 0.5 g Sigmacell 20, 10 g wet amorphous cellulose (dry weight 0.57 g). Additionally a filter-sterilized stock solution of cellobiose was added to 100 ml of autoclaved basal medium in 250 ml flasks to a final concentration of 0.4% (w/v). The flasks were inoculated with COMP.A2 (0.2% inoculum), which had been grown on basal medium containing 0.4% cellobiose, and incubated at 70°C with shaking at 120 rpm. Samples were withdrawn from the flasks and assayed for ß-glucosidase and CMCase activity. Growth of cells was determined

by the estimation of total protein in the culture medium. The method of total protein determination is described in section 3.2.7.2.

pNPG at a concentration of 2 mM was used for the ß-glucosidase assays. CMCase activity was assayed with cultures which had been diafiltered over PM 10 ultrafiltration membranes (25 mm in diameter) to reduce the background reducing sugar level. The sample (1.0 ml) plus 5 ml of 50 mM MOPS buffer at pH 7.0 was diafiltered using a PM 10 membrane and washed three times with 50 mM MOPS buffer at pH 7.0.

4.2.5 Purification of cellulase

4.2.5.1 Eluting bound cellulases with water

Attempts were made to wash off any CMCase absorbed to the pellet of centrifuged cells and residual cellulose using chilled water. The basal medium (800 ml) containing 0.3% (w/v) Sigmacell as substrate was inoculated with COMP.A2 and incubated for three days at 70°C with shaking at 120 rpm. The culture was centrifuged at 13,000 × g for 15 min. The pellet, containing cells and residual cellulose, was washed with 100 ml of chilled Milli Q water at 4°C for 15 min with shaking at 100 rpm. The washing was repeated three times with centrifugation after each washing. The washing supernatants and resuspended cell pellet were then assayed for CMCase activity.

4.2.5.2 Small scale trial of CMCase purification

The basal medium was prepared with Sigmacell 20 added as the carbon source at a final concentration of 0.3% (w/v). Three flasks were prepared and each 2 litre flask contained 800 ml of medium. After autoclaving, flasks were inoculated with strain COMP.A2 and incubated at 70°C in an orbital incubator at 120 rpm. Growth was monitored by microscopic examination and CMCase activity assay. The flasks were harvested when the growth reached late log phase. The culture broth in the flasks was combined and centrifuged at 10000 × g for 15 min at 4°C (Beckman, model J2-21M, USA). The supernatant was concentrated by ultrafiltration using a PM 10 membrane (10, 000 molecular weight cut off, Amicon) at 4°C and the concentrated supernatant (200 ml) was used as a crude preparation of CMCase for small scale trial purification.

Various chromatography techniques were evaluated for CMCase purification. These included DEAE-Sepharose, size-exclusion chromatography (Superdex 200), dye-ligand method, Phenyl-Sepharose chromatography, ion exchange chromatography (FPLC

Mono Q, Pharmacia, Uppsala, Sweden), and chromatofocusing (FPLC Mono-P, Pharmacia).

In the DEAE-Sepharose (Pharmacia) chromatography trial, the column (50 × 105 mm) was equilibrated using 50 mM MOPS buffer at pH 8.0. The sample of a crude preparation of CMCase (100 ml) was adjusted to pH 8.0 and applied to the column. After washing the column with 50 mM MOPS buffer, the column was eluted with a linear gradient of 0.0 to 1.0 M NaCl in 50 mM MOPS buffer. The fractions were collected and assayed for CMCase activity. The result showed that the resolution was poor for CMCase purification.

In the dye-ligand chromatography trial, the dye-ligand column screening kit was obtained from Professor Scopes laboratory at La Trobe University. One column from each of five dye-ligand groups and all the columns in group five were chosen to screen their binding ability for the CMCase enzyme (Scopes, 1993). The columns were equilibrated using a buffer containing 10 mM KOH and 5 mM MgCl (pH 6.0, adjusted with MES). Five ml of concentrated supernatant was loaded onto the columns and eluted with 6 ml of the same buffer at pH 6.5, followed by 1.0 M NaCl at pH 7.0. The fractions were assayed for CMCase activity and protein concentration. The results showed that CMCase enzymes and most of proteins were unable to bind to the columns.

In the size-exclusion chromatography trial, a Superdex 200 (20 × 600 mm) column (Pharmacia) and Fractogel HW-55F (TSK) column (16 × 820 mm) were used. The column was equilibrated using 50 mM MOPS buffer at pH 7.0. The sample was loaded to the column and eluted with the same MOPS buffer. The poor resolution and low recovery made this method unsuitable for the cellulase purification.

Phenyl-Sepharose chromatography, ion-exchange chromatography (FPLC Mono Q), and chromatofocusing (FPLC Mono P) were chosen for cellulases purification after further chromatography trial experiments.

4.2.5.3 Large scale purification of CMCase

a: Growth of organisms and crude enzyme preparation

The basal medium was prepared and Sigmacell 20 was added as the carbon source at a final concentration of 0.3% (w/v). Twenty flasks were prepared, each 2 litre flask contained 1.0 litre of medium. After autoclaving, flasks were inoculated with strain COMP.A2 (0.2% inoculum) and incubated at 70°C in an orbital incubator at 120 rpm.

Growth was monitored by microscopic examination and CMCase activity assays. The flasks were taken out when the growth reached late log phase. The culture broth in all the flasks was combined and harvested using an Amicon hollow fibre cartridge (H1MP01-43, 0.1 μm cutoff), connected to an Amicon DC10LA pump unit. Ammonium sulphate and NaN_3 were added to the Hollow Fibre permeate at a final concentration of 1.0 M and 0.04% (w/v), respectively, and stored at 4°C. The cell pellet and residual cellulose were stored at -20°C.

b: Hydrophobic interaction chromatography (Phenyl-Sepharose)

Phenyl-Sepharose, Fast Flow, low substitution (Pharmacia) was packed into a column (26 mm × 175 mm) to make a 70 ml bed volume. The column was washed consecutively with 20 ml of 1 M NaCl, 6 M urea, and 0.5 M NaOH. It was then rinsed with 10 bed volumes of Milli Q water. The column was equilibrated with 1 M $(NH_4)_2SO_4$ in 20 mM MOPS buffer at pH 7.0. Nineteen litres of culture supernatant in 1.0 M $(NH_4)_2SO_4$ was applied to the column in two runs. The column was washed with 1.0 M $(NH_4)_2SO_4$ and bound protein was eluted stepwise with 0.5 M, 0.4 M, 0.25 M $(NH_4)_2SO_4$, and then by 20 mM MOPS buffer at pH 7.0, Milli Q water, 25% (v/v) and 50% (v/v) ethylene glycol. Fractions were collected and assayed for enzyme activity.

c: Anion-exchange chromatography (FPLC Mono Q)

The active fractions from the Phenyl-Sepharose column were pooled and a total of 879 ml was concentrated by ultrafiltration using a PM 10 membrane at 4°C. The concentrate was diafiltered with 20 mM MOPS buffer at pH 7.0 using a PM 10 membrane. The final volume of 38.5 ml was adjusted to pH 8.0 and applied to a Pharmacia FPLC Mono Q (10/10) column, which was pre-equilibrated with 20 mM Tris-HCL buffer (pH 8.0). The bound proteins were eluted with a gradient of 0.0 to 1.0 M NaCl in 20 mM Tris-HCl buffer, at a flow rate of 1.0 ml per minute. The fractions were collected and assayed for enzyme activity. The active fractions were pooled and the ionic strength was reduced by diafiltration using a PM 10 membrane.

d: Chromatofocusing (FPLC Mono P)

The active fractions, in 25 mM Bis-Tris buffer (pH 6.3) from Mono Q chromatography, were applied to a FPLC Mono P column (HR 5/20, Pharmacia), previously equilibrated with 25 mM Bis-Tris buffer (pH 6.3). The protein was eluted with Pharmacia

Polybuffer 74 diluted 10-fold with Milli Q water, at pH 4. The fractions were collected and assayed for CMCase activity.

4.2.6 Characterization of CMCase

4.2.6.1. Molecular weight determination

Molecular weight determination was carried out by running the CMCase protein on SDS-PAGE using a PhastSystem (Pharmacia, Uppsala, Sweden). A PhastGel Gradient 10-15 and PhastGel SDS Buffer Strips were used. The sample buffer contains 20 mM Tris/HCl (pH 8.0), 2 mM EDTA, 5% SDS, 10% ß-mercaptoethanol and 0.02% bromophenol blue. The Low Molecular Weight Standards kit (Pharmacia) was used as molecular markers. The enzyme samples and the molecular weight standards were mixed with an equal volume of double strength sample buffer and heated at 100°C for 6 minutes. After cooling at room temperature, samples were centrifuged at maximum speed in a benchtop centrifuge for 5 min and approximately 1.0 µl of the samples and molecular weight standard were loaded onto the gel. The gel was run according to the method of PhastSystem Separation Technique File No.110 and stained by the silver stain method described in Appendix 2.

The molecular weight of purified CMCase A was also estimated by size exclusion HPLC using a BioSep-SEC-S3000 column (Bio-Rad, Richmond, U.S.A.). Twenty microlitres of concentrated CMCase (0.0022 mg enzyme) and Gel Filtration Molecular Weight Standards (Boehringer Mannheim Biochemia, Germany) were applied to the BioSep-SEC-S3000 column which was previously equilibrated with 25 mM MOPS buffer (pH 7.0). Gel filtration molecular weight standards were ß-galactosidase (465,000), Ig G (150,000), Fab fragment IgG (50,000), Myoglobin from skceletal muscle (17,000). The mobile phase consisted of 25 mM MOPS buffer (pH 7.0) at a flow rate of 1.0 ml per min. The molecular weight of CMCase was estimated from a plot of the log molecular weights of the standard proteins against elution volume.

4.2.6.2 Native-PAGE and activity stain

PhastGel Gradient 10-15 gels and PhastGel Native Buffer Strips were used for native-PAGE in the PhastSystem. The enzyme samples were loaded onto the gel and run under the conditions specified in the PhastSystem Separation Technique File No.120 and then silver stained.

The detection of cellulase activity on the gels was mainly based on the method of Coughlan (1988). An overlay gel was made by dissolving 2 g agar and 0.6 g CMC (low viscosity) in 100 ml MOPS buffer (pH 7.0). The solution was boiled and poured onto Gel Bond film (Bio-Rad, USA) which was warmed on a gel-casting plate (Bio-Rad, USA) at 65°C. After running the native-PAGE gel, the overlay gel was laid on the native-PAGE gel and incubated at 60°C for 30 min. It was then stained by submerging the overlay gel in 20 ml of 0.1 % (w/v) Congo Red for 15 min at ambient temperature. The overlay gel was destained by immersing in a solution of 1.0 M NaCl until the hydrolysis zones became visible.

4.2.6.3 Isoelectric point determination

Isoelectric focusing was carried out using the PhastSystem and PhastGel IEF 3-9 gel. The enzyme samples and standards were applied in the middle position and run according to the method of PhastSystem Separation Technique File No. 100. The Isoelectric Focusing Calibration kit (Pharmacia) was used as the standard. The gel was silver stained (appendix 2).

4.2.6.4 Effect of temperature on activity

A low viscosity CMC solution (400 µl at the concentration of 1% w/v) in 50mM MOPS buffer was preincubated at temperatures ranging from 50°C to 95°C in triplicate 1.5 ml Eppendorf tubes. The pH value was adjusted at room temperature to correspond to pH 7.0 at each temperature. Samples of 20-fold diluted purified CMCase A (100 µl) were added and incubated for 10 min. The reaction was stopped by the addition of 1.0 ml PAHBAH reagent. PAHBAH assay was carried out as described in section 3.2.7.1.

4.2.6.5 Effect of pH on activity

Acetate, MES, MOPS, Tris, CHES and Bis-Tris-propane (Sigma, U.S.A.) solutions (200 mM) were mixed with an equal volume of 2% (w/v) CMC (low viscosity) respectively and adjusted to various pH values at 80°C with 1.0 M NaOH or 1.0 M H_2SO_4. CMCase activity was assayed as described at 4.2.2.2 at 80°C.

4.2.6.6 Thermostability

Five-fold diluted purified CMCase A (0.55 µg of enzyme), 50 µl in 50 mM MOPS buffer at pH 7.0, were incubated in 1.5 ml Eppendorf tubes at the stated temperatures in the presence and absence of BSA. A thermistor probe (Omega, USA) sealed into an

Eppendorf tube showed that the liquid within reached the required temperature within 3 min. Duplicate Eppendorf tubes were withdrawn at intervals over the time course and cooled in ice-cold water. All samples were assayed for residual CMCase activity. The CMCase activity was assayed as described at 4.2.2.2 at 80°C for 10 minutes.

4.2.6.7 Effect of chemical reagents on activity

Solutions of 10 mM of various chemical reagents were prepared in 50 mM MOPS buffer and adjusted to pH 7.0 at 70°C. The solutions, 42 µl, were mixed with 8 µl of purified CMCase A (0.088 µg of enzyme) in 1.5 ml Eppendorf tubes and preheated at 70°C for 10 minutes. 400 µl of 1% (w/v), CMC (low viscosity) solution, was added and the samples incubated for 10 minutes. Then PAHBAH assay was followed as described in section 3.2.7.1. Each compound, which was incubated without enzyme as a control, was run concurrently.

4.2.6.8 Reaction product analysis

The reaction products of CMCase A on a variety of of cellulosic substrates were determined as follows: 50 µl volumes of test substrates (low viscosity CMC, Sigamacell 20, oat spelt and birch wood xylan, inulin, maltoheptaose, maltohexaose, maltopentaose, maltotetraose, maltotriose, maltose) at a concentration of 1% (w/v) in 50 mM MOPS buffer (pH 7.0 at 70°C) were separately incubated in 1.5 ml Eppendorf tubes with 12.5 µl of CMCase A (0.14 µg enzyme) at 70°C for 14 hours.

Additionally, 200 µl of either 4% (w/v) amorphous cellulose or 1% (w/v) cellopentaose in 50 mM MOPS buffer (pH 7.0 at 70°C) was incubated in 1.5 ml Eppendorf tubes with 200 µl and 100 µl respectively of CMCase A (2.2 and 1.1 µg enzyme) at 70°C for various time periods. Each substrate was incubated without enzyme as a control and was run concurrently.

The samples in Eppendorf tubes from each of the above experiments were withdrawn after incubation and cooled in ice cold water, and then centrifuged at maximum speed (8000 rpm) in a benchtop centrifuge for 8 minutes. The supernatants were assayed by high performance liquid chromatography (HPLC) using a Bio-Rad HPX-42A column (Bio-Rad, USA) operated at 85°C. The mobile phase consisted of Milli Q water at a flow rate of 0.6 ml/min. The Milli Q water was filtered through a 0.45 µm filter and vacuum-degassed after boiling. The eluted peaks were monitored with a refractive index detector (Erma Optical Works Ltd, Japan). Data was collected by a 3000 series Chromatography Data System (Nelson Analytical Inc, USA) using Exzel XT computer.

Standards were glucose, cellobiose, maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose and maltoheptaose. Maltooligosaccharides were chosen as standards because the full range of cellulooligosaccharides was not available. However the column separates carbohydrates on the basis of molecular weight, the maltooligosaccharides will give valid retention times, as their molecular weights are equal to those of the corresponding cellulooligosaccharides. For analysis, 20 µl volumes of standard solutions or the enzyme reaction products were injected onto the column using a 50 µl glass syringe (Hamilton, USA) and a Rheodyne 7125 injection valve (Rheodyne, USA).

4.2.6.9. Synergistic effect of CMCase A and CMCase B on Avicel.

Aliquots (0.9 ml) of 1% (w/v) suspension of Avicel in 1.5 ml Eppendorf tubes were preheated at 70°C. An enzyme sample (10 µl of CMCase A or CMCase B or the mixture of 10 µl of CMCase A and 10 µl CMCase B) was added to two of the triplicate tubes, mixed and incubated at 70°C for 30 minutes (t=30). All assays were stopped by placing the Eppendorf tubes in ice-cold water. An enzyme sample of 10 µl or 20 µl was added to the remaining tube (t=0) as control. All tubes were mixed and centrifuged for 5 minutes at maximum speed in a benchtop centrifuge (Microfuge E, Beckman, USA). Supernatant, 0.4 ml, from each tube was removed and reducing sugars were assayed by the PAHBAH method as described before.

4.3 RESULTS

4.3.1 Effect of carbon sources on enzyme production

In order to determine suitable substrates for the production of cellulase, strain COMP.A2 was grown in basal medium containing different substrates. Figure 4.1 shows the time course of growth and ß-glucosidase activity. The high reducing sugar background in the cultures makes it difficulty to assay for CMCase activity. Cultures which were diafiltered over ultrafiltration membranes to reduce the background reducing sugar level were assayed for CMCase activity only (Table 4.1). Strain COMP.A2 produced CMCase and ß-glucosidase activity in the presence of cellulose, xylan, and cellobiose (Figure 4.1, Table 4.1). However the production of CMCase and ß-glucosidase activity was higher on amorphous and crystalline cellulose medium than on cellobiose and xylan medium. The production of CMCase and ß-glucosidase activity in the log phase was about 16-fold and 2 to 3-fold higher respectively with Sigmacell 20 as substrate than the production of CMCase and ß-glucosidase activity at stationary phase with cellobiose as substrate. More CMCase and ß-glucosidase will be produced as cells continue to grow to stationary phase in the basal medium with Sigmacell as substrate. Therefore, Sigmacell 20 was chosen as the substrate for production of cellulases.

Table 4.1 Effect of carbon source on production of CMCase and ß-glucosidase by strain COMP.A2.

Incubation time (hrs)	Growth substrate (w/v)	CMCase (U/ml)[a]	CMCase (U/mg)[a]	ß-glucosidase (U/ml)[b]	ß-glucosidase (U/mg)[b]
24.5	0.5% xylan	0.0048	0.027	0.13	0.74
34	0.57% amorphous cellulose	0.024	0.24	0.46	4.6
41	0.5% Sigmacell 20	0.092	1.08	0.36	4.23
19	0.4% cellobiose	0.0056	0.059	0.15	1.58

a: CMCase activity was expressed as units per ml culture broth or per mg total protein. One unit of CMCase activity is defined as the amount of enzyme required to produce one micromole of reducing sugar (glucose equivalent) min^{-1}.

b: ß-glucosidase activity was expressed as units per ml culture broth or unit per mg total protein. One unit of ß-glucosidase activity is defined as the amount of enzyme required to produce one micromole *p*-nitrophenol min^{-1}.

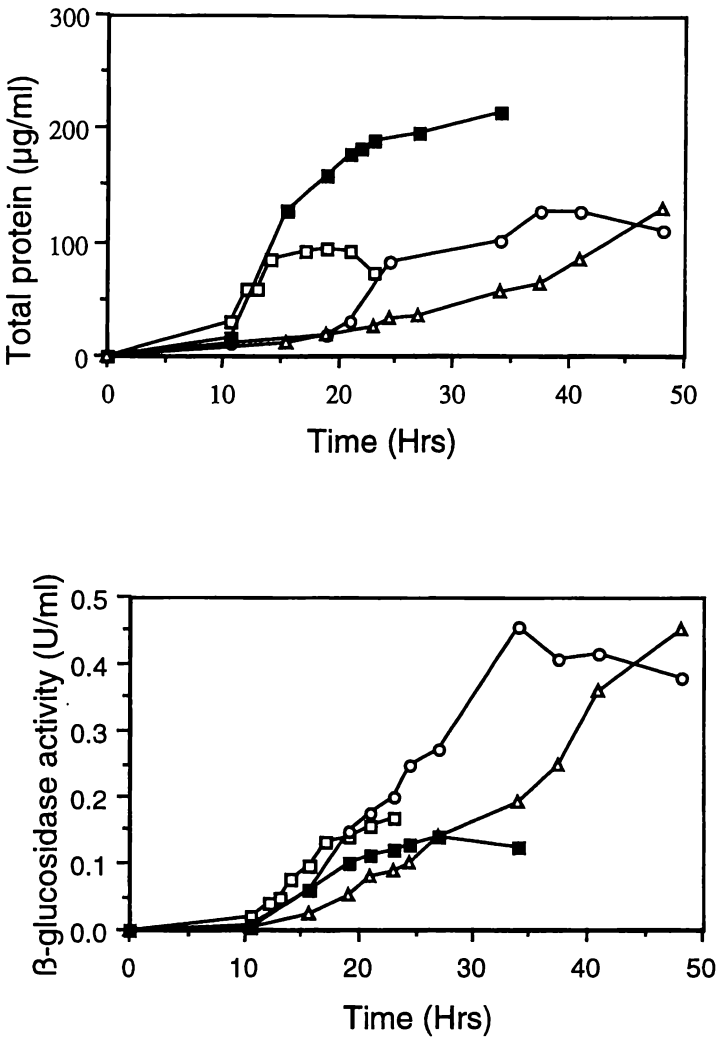


Figure 4.1 Time course of growth and ß-glucosidase production of strain COMP.A2 on basal medium containing different substrates at 70°C. Substrates include cellobiose (□), amorphous cellulose (○), xylan (■), and Sigmacell 20 (△). At the indicated time, the cultures were withdrawn from the flasks and used for determination of total cell protein and assayed for ß-glucosidase and CMCase activity.

4.3.2 Proteinase assay

The production of multiple components of cellulase is quite common among cellulolytic microorganisms. This might be caused by proteinase activity in the culture medium. Proteinase activity was assayed in culture medium of strain COMP.A2 which grew in basal medium with 0.4% Sigmacell 20 or 0.4% cellobiose. No proteinase activity was detected in both culture broth.

4.3.3 Purification of CMCase

The large scale of growth of COMP.A2 was carried out in twenty flasks with each flask containing 1.0 litre of basal medium. The culture was incubated at 70°C and harvested when growth reached late log phase. Culture supernatant was prepared for cellulases purification. The total cellulase activity (as represented by CMCase activity) in culture supernatant and whole culture broth is 434.4 and 1494.4 units, respectively. This suggests that about 29% of total CMCase activity produced by the cells was in the extracellular medium, whereas the remainder was bound to residual cellulose and/or cells-associated.

Efforts were made to recover the bound cellulase by washing CMCase enzyme absorbed to residual cellulose and cells using chilled water. The culture of strain COMP.A2, which grew on the basal medium with Sigmacell as substrate, was harvested by centrifugation. The pellet of harvested cells and residual cellulose was washed three times with distilled water and assayed for CMCase activity. The results are shown in Table 4.2. Less than 2% of the bound cellulase was successfully washed out from the residual cellulose and cell pellet. This differs from the report that *C. thermocellum* cellulase can be eluted from yellow affinity substance-cellulose using distilled water (Ljungdahl, 1989). The bound cellulases from COMP.A2 may be different from cellulases of *C. thermocellum.* Loss of the CMCase activity in the residual cellulose and cell pellet was observed after the first water wash. The reason for the CMCase activity loss is not clear.

Table 4.2 CMCase activity in residual cellulose and cell pellet and washed supernatant.

Sample	CMCase activity (U)
residual cellulose and cell pellet	51.34
first wash supernatant	1.16
residual cellulose and cell pellet (from first wash)	40.8
second wash supernatant	0.71
residual cellulose and cell pellet (from second wash)	37.7
third wash supernatant	0.73

One unit of CMCase activity is defined as the amount of enzyme required to produce one micromole of reducing sugar (glucose equivalent) min^{-1}.

4.3.3.1 Phenyl-sepharose chromatography

Nineteen litres of culture supernatant was adjusted to a salt concentration of 1.0 M $(NH_4)_2SO_4$ and 0.04% NaN_3 prior to adding to a Phenyl-Sepharose column in two runs. In the first run, 9.7 litres of culture supernatant was loaded to the column and the elution profile is shown in Figure 4.2. Twenty four per cent of the applied CMCase activity was eluted in 20 mM MOPS buffer (pH 7.0) from fraction 3 to fraction 5 (Table 4.3). In the second run, 9.3 litres of culture supernatant was applied to the Phenyl Sepharose column. The elution profile of the second run was similar to the first run. The active fractions from fraction 3 to fraction 5 in both runs (about 879 ml) were pooled and 36% of the applied CMCase activity was recovered.

4.3.3.2 Ultrafiltration and diafiltration

The pooled active fractions (879 ml) were concentrated using an Amicon Ultrafiltration cell fitted with a PM 10 membrane, then diafiltered with 20 mM MOPS buffer (pH 7.0). 54% of the applied CMCase was recovered after ultrafiltration and diafiltration. The final volume of concentrated sample was 40 ml.

4.3.3.3 Anion-exchange chromatography (Mono Q)

The concentrated sample (38.5 ml) from ultrafiltration and diafiltration was applied to a Mono Q column (10/10). The CMCase activity was eluted in three active peaks (Figure 4.3). Two of the active peaks were eluted in a linear gradient of 0.0 to 0.1 M NaCl. An additional linear gradient of 0.1 to 0.7 M NaCl eluted a significant quantity of protein containing further CMCase activity. The specific acitivity increased 37-fold in the first

active peak (Peak A, fraction 35 to 43) and 17-fold in the second active peak (Peak B, fraction 44 to 53). Table 4.4 shows the CMCase and ß-glucosidase activity in the eluted fractions. Thirty-three per cent of the CMCase activity applied to the column was eluted in Peak A, twenty-five per cent was eluted in peak B, and thirty-six per cent was eluted in peak C (fraction 70 to 95). The ß-glucosidase activity was only eluted in peak C, and represented 70% of the applied ß-glucosidase activity. The active fractions from peak C were pooled and stored in the deep freeze (-20°C) for ß-glucosidase purification.

4.3.3.4 Chromatofocusing (Mono P)

The active fractions of peak A (fraction 35 to 43) from the Mono Q column (Figure 4.3) were pooled and diafiltered in 25 mM Bis-Tris at pH 6.3. A final volume of 8 ml was applied to a Mono P column (HR 5/20). The protein was eluted with Polybuffer 74 diluted 10-fold with Milli Q water, at pH 4.0. The CMCase activity was eluted from the Mono P column (HR 5/20) when the pH reached approximately 5.4 (Figure 4.4). The active fractions (fraction 15 to 20) were pooled and showed a single protein band on SDS-PAGE (Figure 4.6). This purified CMCase (fraction 15 to 20) was designated CMCase A. A final 117-fold increase in the specific activity was achieved for CMCase A. The recovery of the original CMCase activity was about 0.77% (Table 4.5). The CMCase A preparation was stored at -80°C for later characterization. Further active fractions (fraction 21 to 24) were pooled and showed multiple protein bands on SDS-PAGE (Figure 4.6).

The active fraction (peak B, fraction 44 to 53 from Mono Q in Figure 4.3) was diafiltered in 25 mM Bis-Tris buffer at pH 6.3. The final volume of 7.7 ml was applied to the Mono P column (HR 5/20). The protein was eluted with Polybuffer 74 diluted 10-fold with MilliQ water, at pH 4.0. The CMCase activity was eluted in one active peak (Figure 4.5). The active fractions (fraction 23 to 24) were pooled and showed a major protein band and several minor bands on SDS-PAGE (Figure 4.6). This partially purified CMCase was designated CMCase B. A summary of CMCase purification is shown in Table 4.5. Avicelase was not purified to homogeneity and a summary of purification is shown in Table 4.6.

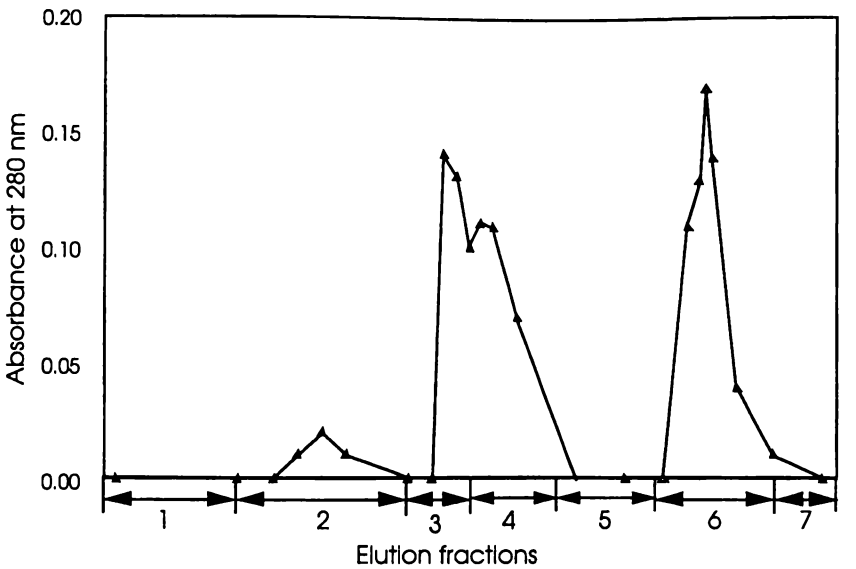


Figure 4.2. Phenyl-Sepharose chromatography of extracellular CMCase from strain COMP.A2. Elution conditions and CMCase activity of fraction are shown in Table 4.3.

Table 4.3 Elution conditions and CMCase activity of COMP.A2 eluted from a Phenyl-Sepharose column depicted in Figure 4.2.

Fraction	Elution conditions and volume	CMCase activity (Units)
1	0.5 M $(NH_4)_2SO_4$, 134 ml	0.0
2	0.25 M $(NH_4)_2SO_4$, 149 ml	6.56
3	20 mM MOPS (pH, 7.0), 80 ml	5.52
4	20 mM MOPS (pH, 7.0), 126 ml	52.2
5	20 mM MOPS (pH, 7.0), 64 ml	1.54
6	Milli Q water, 112 ml	3.47
7	Milli Q water, 44 ml	0.62

One unit of CMCase activity is defined as the amount of enzyme required to produce one micromole of reducing sugar (glucose equivalent) min^{-1} at pH 7.0 and 70°C.

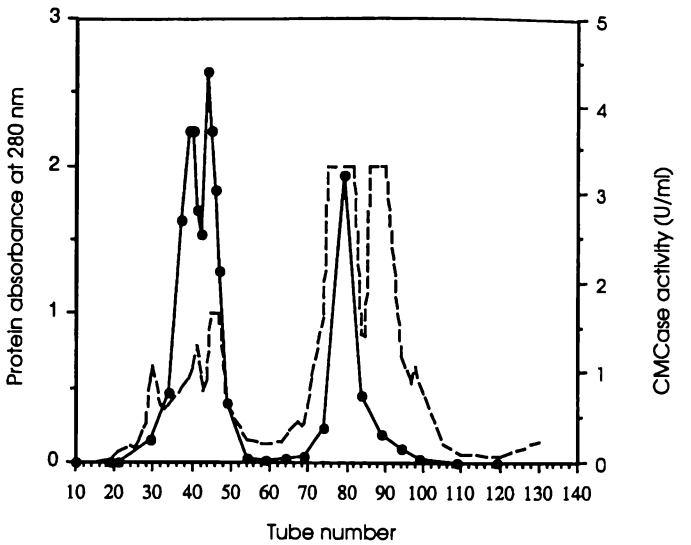


Figure 4.3. Anion exchange chromatography of CMCase active fractions (fraction 3 to 5) obtained from Phenyl-Sepharose chromatography on an FPLC Mono Q column (10/10). Fractions 1 to 58, 0-0.1 M NaCl linear gradient; fractions 59-130, 0.1-0.7 M NaCl linear gradient. Fraction size, 1 ml. (---) Absorbance at 280 nm; (•) CMCase activity.

Table 4.4 Summary of CMCase and ß-glucosidase recovery from Mono Q elution depicted in Figure 4.3

Fractions	CMCase activity (Units)	ß-glucosidase activity (Units)
25-34	4.5	0.02
35-43 (peak A)	23.76	0.0
44-53 (peak B)	17.5	0.0
60-69	0.08	0.0
70-95 (peak C)	25.5	167.5

One unit of CMCase activity is defined as the amount of enzyme required to produce one micromole of reducing sugar (glucose equivalent) min^{-1} at pH 7.0 and 70°C.

One unit of ß-glucosidase activity is defined as the amount of enzyme required to produce one micromole of *p*-nitrophenol min^{-1} at pH 7.0 and 70°C.

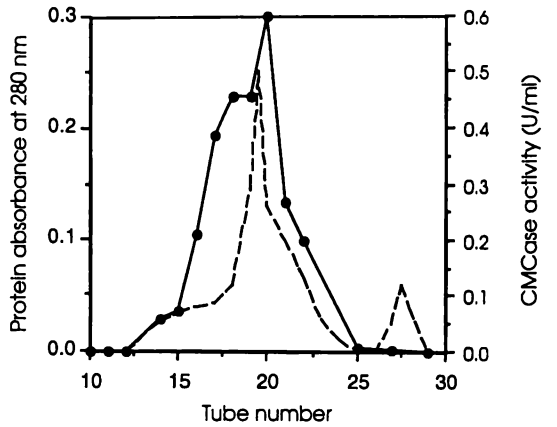


Figure 4.4. Chromatofocusing of CMCase active fractions (fraction 35 to 43 or peak A) obtained from anion exchange chromatography on a FPLC Mono P column (HR 5/20). Fraction size, 1 ml. pH=5.9 at fraction 10, pH=5.4 at fraction 13, pH=4.07 at fraction 30. (---) Absorbance at 280 nm; (•) CMCase activity.

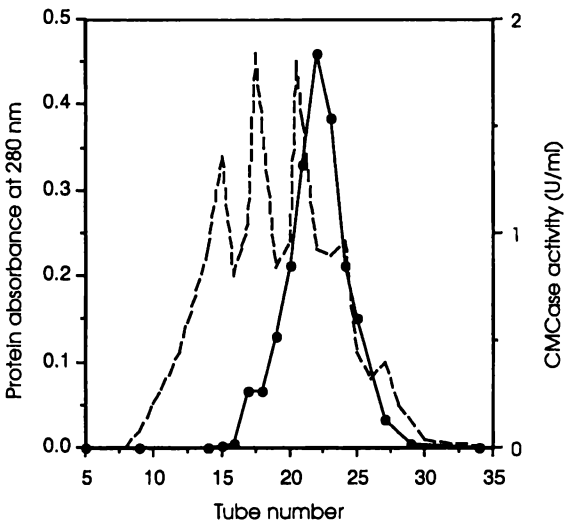


Figure 4.5. Chromatofocusing of CMCase active fractions (fraction 44 to 53 or peak B) obtained from anion exchange chromatography on a FPLC Mono P column (HR 5/20). Fraction size, 1ml. pH=5.9 at fraction 9, pH=4.07 at fraction 30. (---) Absorbance at 280 nm; (•) CMCase activity.

Table 4.5 Purification of CMCase from strain COMP.A2

Purification step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Recovery (%)	Purification factor
culture supernatant	1660	434.4	0.27	100	1
phenyl-sepharose	237	158	0.67	36	2.5
ultrafiltration	103.2	85.1	0.82	19.6	3.1
FPLC Mono Q					
Peak A (tubes 35-43)	2.43	23.8	9.8	5.5	37
Peak B (tubes 44-53)	3.9	17.5	4.5	4.0	17
Peak C (tubes 70-95)	60.3	25.5	0.42	5.9	1.6
FPLC Mono P					
CMCase A	0.11	3.33	31.7	0.77	117
CMCase B	0.20	3.42	17.1	0.79	63.3

One unit of CMCase activity is defined as the amount of enzyme required to produce one micromole of reducing sugar (glucose equivalent) per min. PAHBAH assays were conducted with 1% CMC as substrate at pH 7.0 at 70°C.

Table 4.6 Purification of Avicelase from strain COMP.A2

Purification step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Recovery (%)	Purification factor
culture supernatant	1660	237	0.14	100	1
phenyl-sepharose and ultrafiltration	103.2	28	0.27	11.8	1.93
FPLC Mono Q					
Peak A (tubes 35-43)	2.43	1.8	0.77	0.76	5.5
Peak B (tubes 44-53)	3.9	1.1	0.28	0.46	2.0
Peak C (tubes 70-95)	62.3	1.87	0.03	0.79	0.21

One unit of Avicelase activity is defined as the amount of enzyme required to produce one µmol of reducing sugar (glucose equivalent) min^{-1}.
PAHBAH assays were carried out with 1% Avicel as substrate at pH 7.0 at 70°C.

4.3.4 Characterization of CMCase A

4.3.4.1 Molecular weight determination

After running a SDS-PAGE PhastGel (gradient 10-15), the purified CMCase A electrophoresed as a single band and was estimated to be 85.1 kDa from comparison with the molecular weight standard curve (Figure 4.6 and 4.7). The major band of CMCase B was estimated to be 81.3 kDa. The CMCase A eluted as a single symmetrical peak on a BioSep-SEC-S3000 column with a retention volume corresponding to a molecular weight of approximately 174 kDa (Figure 4.8). This suggests that the native enzyme presumably is a dimer.

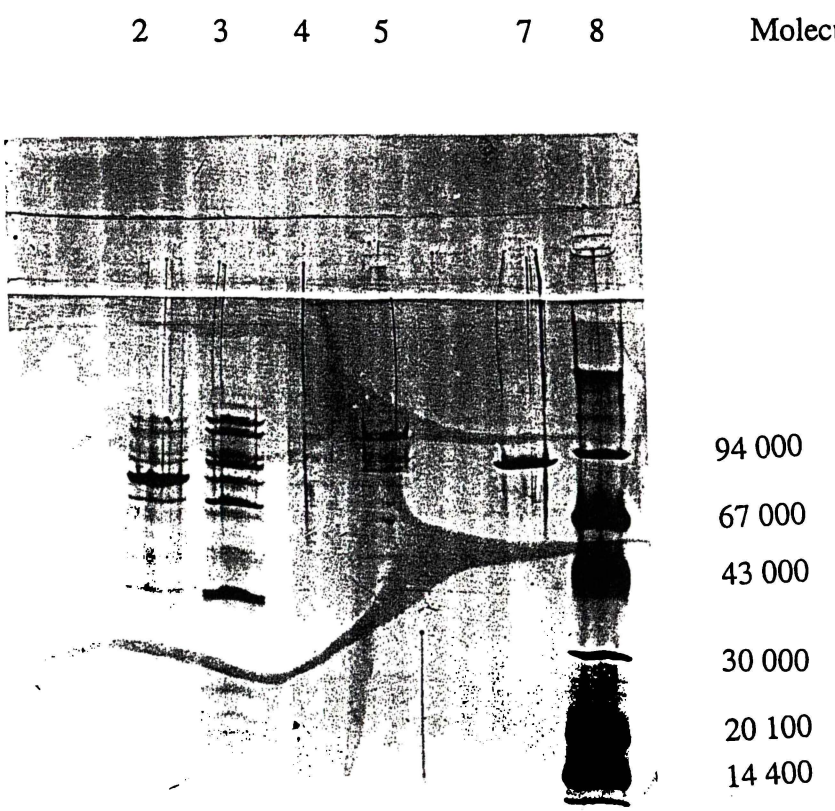
4.3.4.2 Native-PAGE and active stain

The purified CMCase A was applied to two separate lanes of a Phastgel and run as native-PAGE. The gel was divided into two halves after electrophoresis. One part was silver stained and the other was stained for CMCase activity using the overlay gel technique (Coughlan, 1988). The active band on the overlay gel aligned with a single protein band on the native-PAGE gel (Figure 4.9).

4.3.4.3 Isoelectric point

Isoelectric focusing of the purified CMCase A gave a major protein band and a minor one (Figure 4.10), with an isoelectric point estimated to be 4.12 for the major band by comparison with the standard curve (Figure 4.11). The minor protein band may represent the monomer of the enzyme and the difference in charge in the two bands may be due to the net charge changing as the dimer is formed.

Figure 4.6. SDS-PAGE of CMCase A and B of strain COMP.A2 using silver staining. Lane 8: molecular weight standards (Pharmacia): Phosphorylase B, 94,000; BSA, 67,000; ovalbumin, 43,000; carbonic anhydrase, 30,000; soybean trypsin inhibitor, 20,100; lactalbumin, 14,400.
Lane 2: CMCase B; Lane 3: fraction 21-22 in Figure 4.5; Lane 4: fraction 22-24 in Figure 4.4; Lane 5: fraction 21-22 in Figure 4.4; Lane 7: CMCase A;



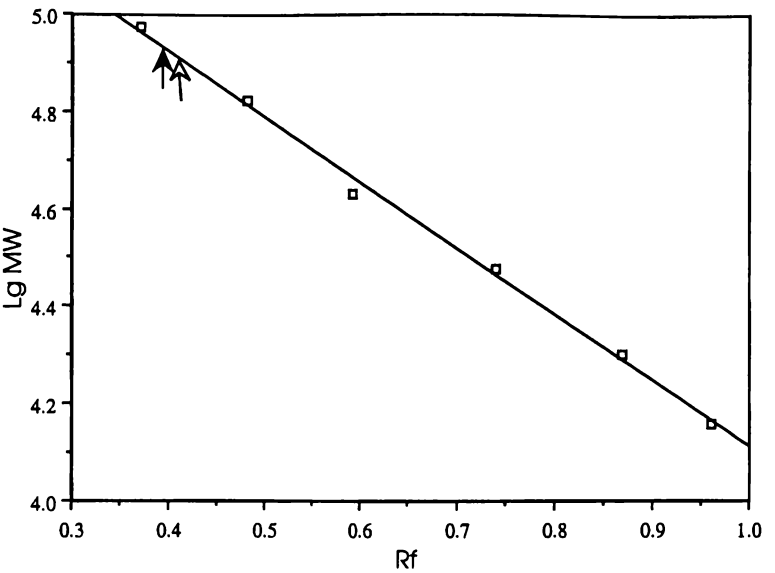


Figure 4.7. Molecular weight (MW) standard curve for SDS-PAGE of the purified CMCase A (closed arrow) and CMCase B (open arrow). Molecular weight standards (Pharmacia): lactalbumin, 14,400; soybean trypsin inhibitor, 20,100; carbonic anhydrase, 30,000; ovalbumin, 43,000; BSA, 67,000; phosphorylase B, 94,000.

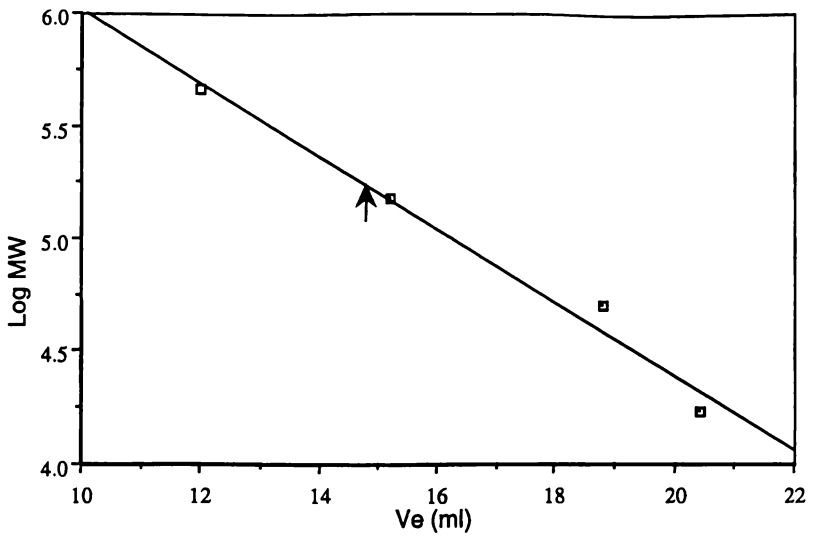
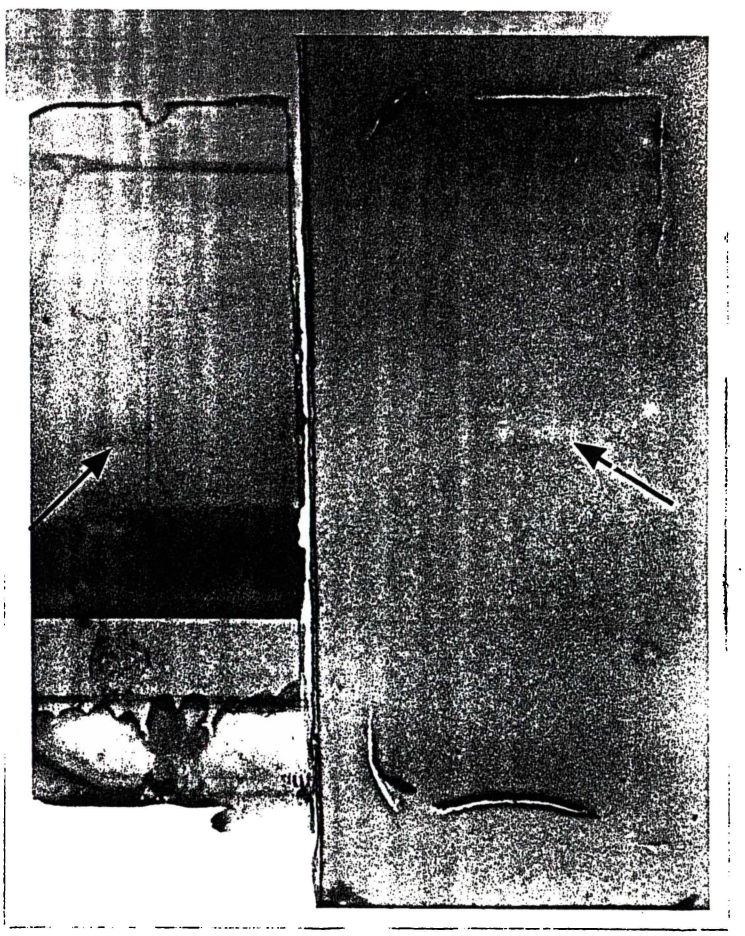


Figure 4.8. Log molecular weight (MW) vs elution volume (Ve) from size exclusion HPLC. Twenty microlitres of concentrated CMCase A (0.0022 mg enzyme) and gel filtration molecular weight standards were applied to the BioSep-SEC-S3000 column which was previously equilibrated with 25 mM MOPS buffer (pH 7.0). Gel filtration molecular weight standards were ß-galactosidase (465,000), Ig G (150,000), Fab fragment IgG (50,000), Myoglobin from skceletal muscles (17,000). Closed arrow shows the position of CMCase A.



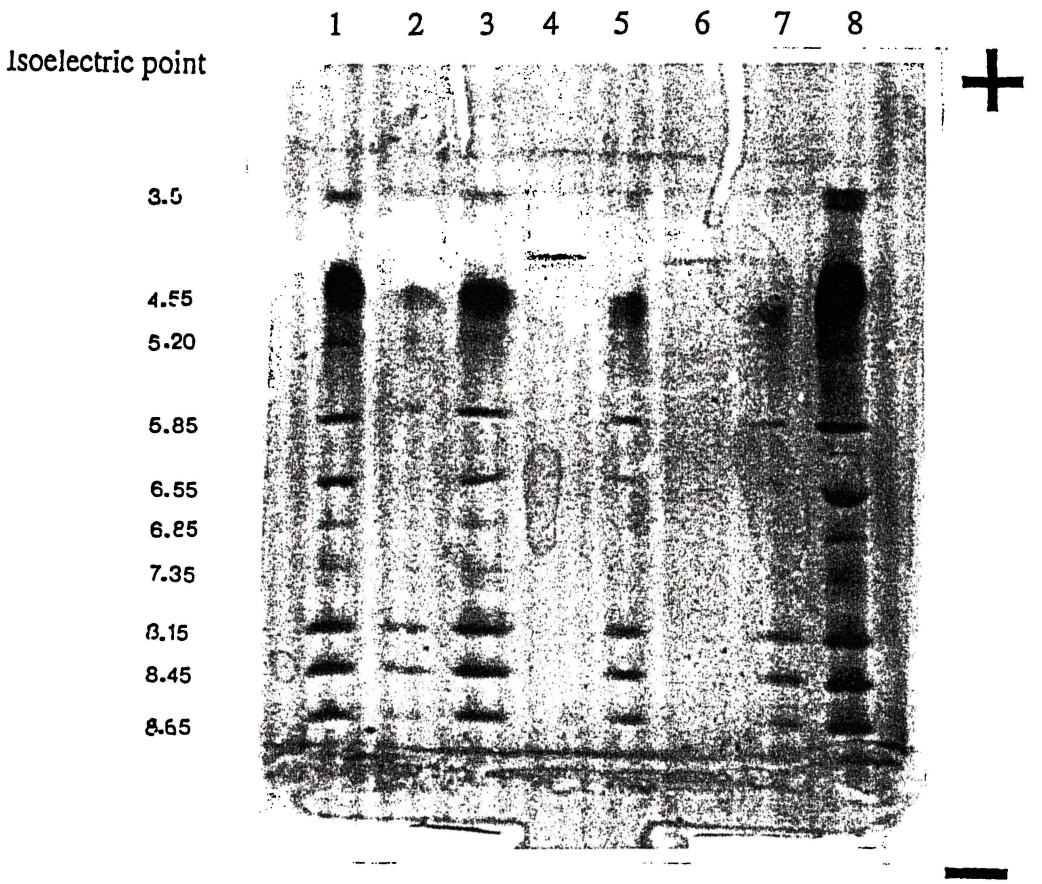
A B

Figure 4.9 A. Silver stained native-PAGE of the purified CMCase A.. B: The activity stained overlay gel containing CMC substrate. Arrows indicate protein band and active band.

Figure 4.10. Isoelectric focusing of purified CMCase A on an IEF 3-9 gel (silver stained).

Lanes 1, 2, 3, 5, 7 and 8: isoelectric focusing standards (Pharmacia): amyloglucosidase, 3.5; soybean trypsin inhibitor, 4.55; ß-lactoglobulin A, 5.20; bovine carbonic anhydrase B, 5.85; human carbonic anhydrase B, 6.55; horse myoglobin (acidic), 6.85; horse myoglobin (basic), 7.35; lentil lectin (acidic), 8.15; lentil lectin (middle), 8.45. lentil lectin (basic), 8.65.

Lanes 4 and 6: Purified CMCase A



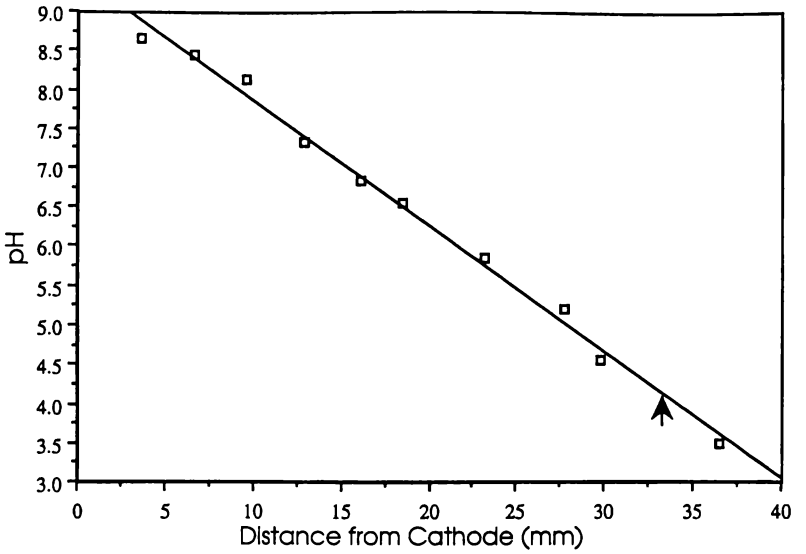


Figure 4.11. Isoelectric point standard curve for the purified CMCase A (closed arrow) in a IEF gel. IEF standard proteins (Pharmacia): amyloglucosidase, 3.5; soybean trypsin inhibitor, 4.55; ß-lactoglobulin A, 5.20; bovine carbonic anhydrase B, 5.85; human carbonic anhydrase B, 6.55; horse myoglobin (acidic), 6.85; horse myoglobin (basic), 7.35; lentil lectin (acidic), 8.15; lentil lectin (middle), 8.45. lentil lectin (basic), 8.65.

4.3.4.4 Linearity of assay

A time course assay of CMCase A activity was linear for at least 20 minutes at 70°C (Figure 4.12) and was also linear to 0.11 μg of CMCase A (Figure 4.13). The CMCase A assay for assessing activity was run for 10 or 15 minutes and the amount of enzyme added for assay did not exceed 0.11 μg.

4.3.4.5 Influence of temperature and pH on CMCase A activity

The activity of CMCase A was assayed for 10 minutes at pH 7.0 at various temperatures using 0.055 μg of purified enzyme. The activity increased up to 80°C and declined rapidly after this (Figure 4.14). The Arrhenius plot from 50°C to 80°C gave a straight line (Figure 4.15), from which an activation energy (Ea) of 46.6 kJ mol^{-1} was calculated (Smith et al, 1983).

The activity of CMCase A was assayed at 80°C for 10 minutes at various pH values using 0.055 μg of purified enzyme. The enzyme showed a pH optimum between pH 6.5 and pH 7.0 and retained 50% of maximal activity at pH 5.5 and pH 8.5 (Figure 4.16).

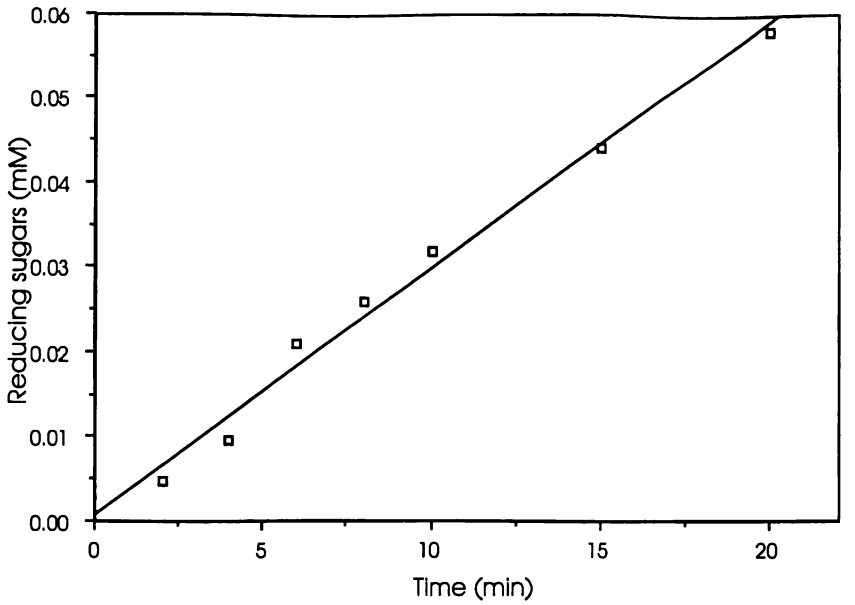


Figure 4.12. Time course of CMCase A assay. The activity of CMCase A was determined with 1% CMC (w/v) in 50 mM MOPS buffer, pH 7.0 and 70°C. 0.055 μg of enzyme was used in each assay.

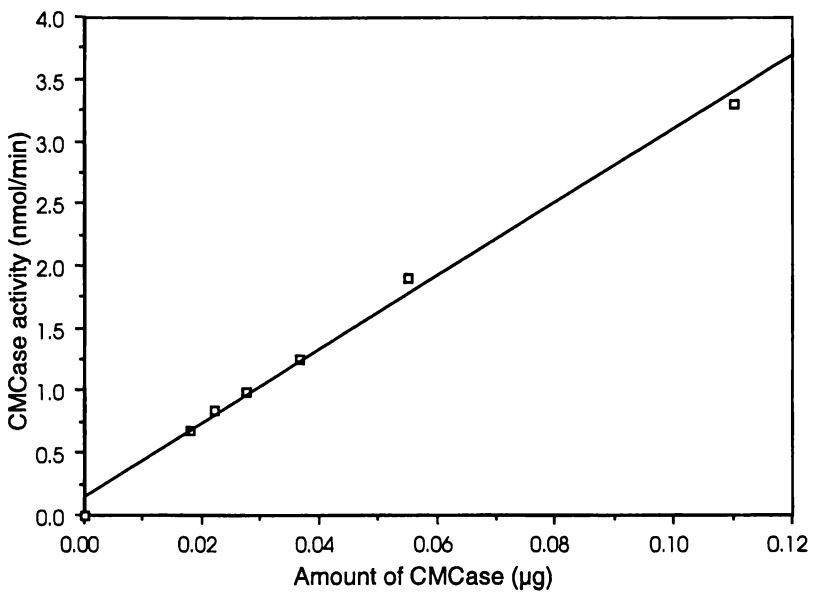


Figure 4.13. Amount of enzyme versus CMCase A activity. The activity of CMCase A was determined with 1% CMC (w/v) in 50 mM MOPS buffer at pH 7.0 and 70°C for 15 min.

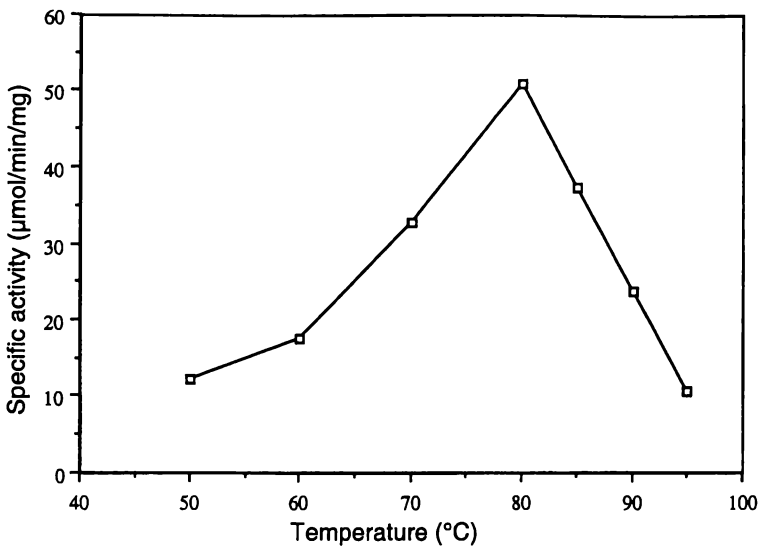


Figure 4.14. Effect of temperature on the activity of CMCase A. The temperature profile of CMCase A activity was determined with 1% (w/v) CMC via 10 min assays in 50 mM MOPS buffer, pH 7.0.

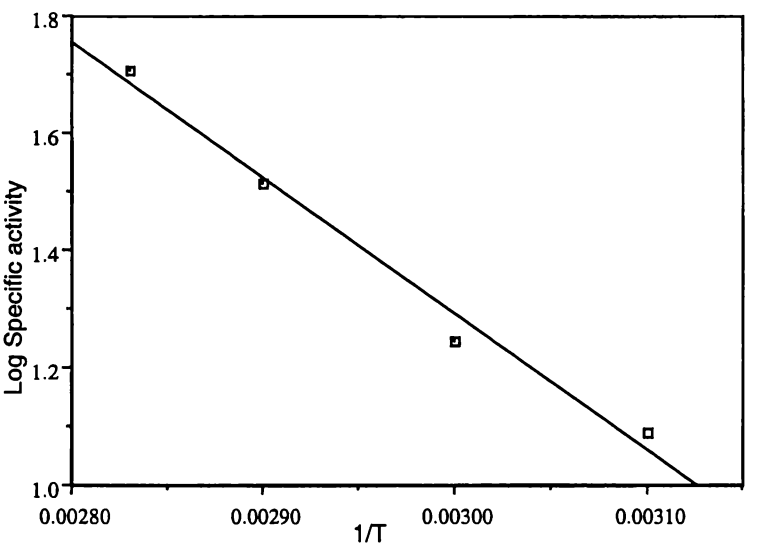


Figure 4.15. Arrhenius Plot for CMCase A from 50°C to 80°C.

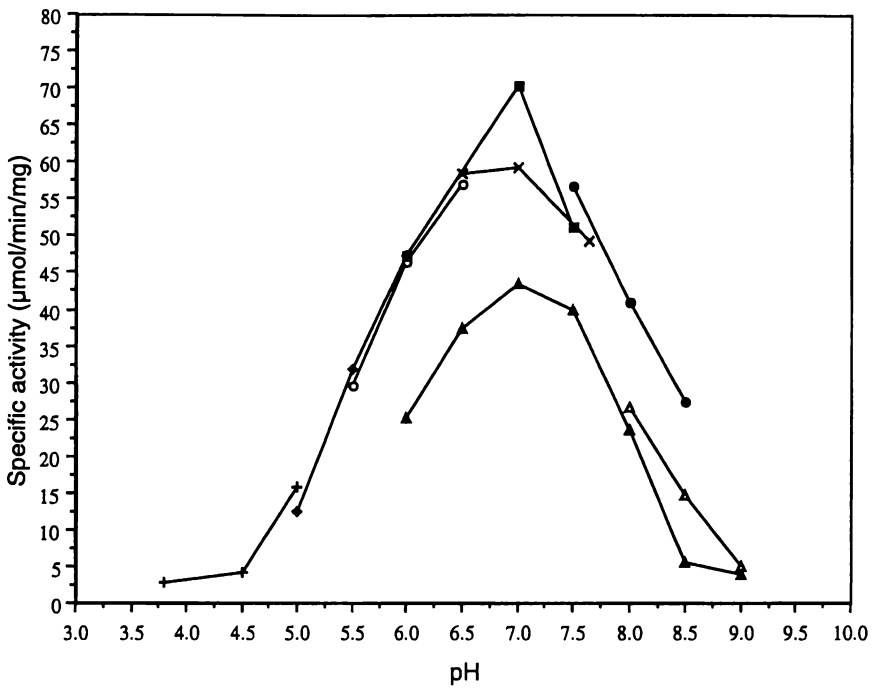


Figure 4.16. Effect of pH on CMCase A activity. Assays were done with 1% (w/v) in 0.1 M sodium acetate (+), 0.1 M sodium citrate (◆), 0.1 M MES (±), 0.1 M HEPES (■), 0.1 M MOPS (×), 0.1 M Bis-Tris propane (▲), 0.1 M CHES (Δ), 0.1 M Tris (●) at 80°C for 10 minutes.

4.3.4.6 Thermal stability of CMCase A

The thermal stability of CMCase A was determined at 70°C, 80°C, and 85°C. CMCase A maintained activity for one hour at 70°C and retained 83% of its original activity after three hours incubation at 70°C. The CMCase A activity increased slightly for 20 minutes at 80°C in the presence of BSA (Figure 4.17). A plot of time of incubation at elevated temperature versus the log of residual activity is shown in Figure 4.18. The half-life of the CMCase A in the presence and absence of BSA at 80°C and 85°C is given in Table 4.7. The results show that the half-life of enzyme doubled with the addition of BSA at 80°C and 85°C. This suggests that the thermal stability of enzyme is affected by the protein concentration. Sorbitol has been reported to significantly increase the thermostability for MUCase (Ruttersmith, 1991). The effect of sorbitol on thermostability of CMCase A has been tested. However, the high reducing sugar background of the sorbitol solution made it difficult to assay the enzyme activity by the PAHBAH method. An assay method which did not rely on reducing sugar determination would be required to test the effect of sorbitol on thermostability of CMCase A.

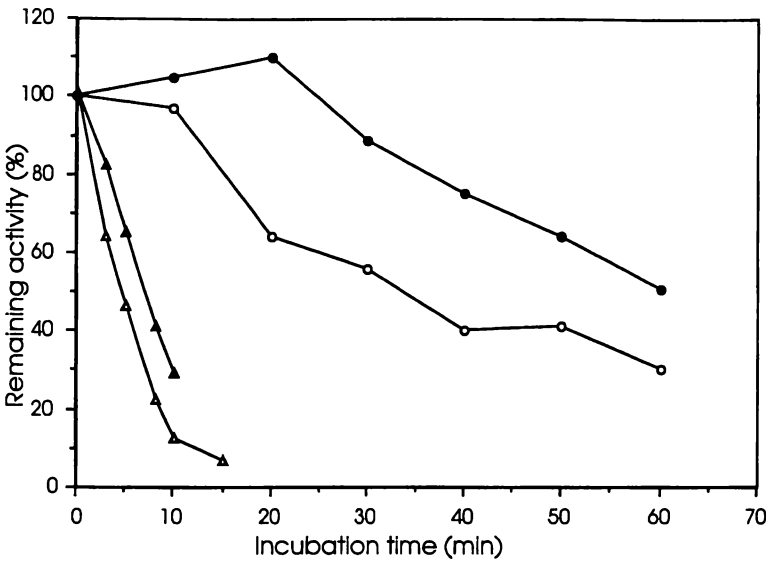


Figure 4.17. Thermal stability of CMCase A of strain COMP.A2. (●), addition of BSA (0.8 mg/ml) at 80°C; (○), no BSA addition at 80°C; (▲), addition of BSA (0.8 mg/ml) at 85°C; (Δ), no BSA addition at 85°C.

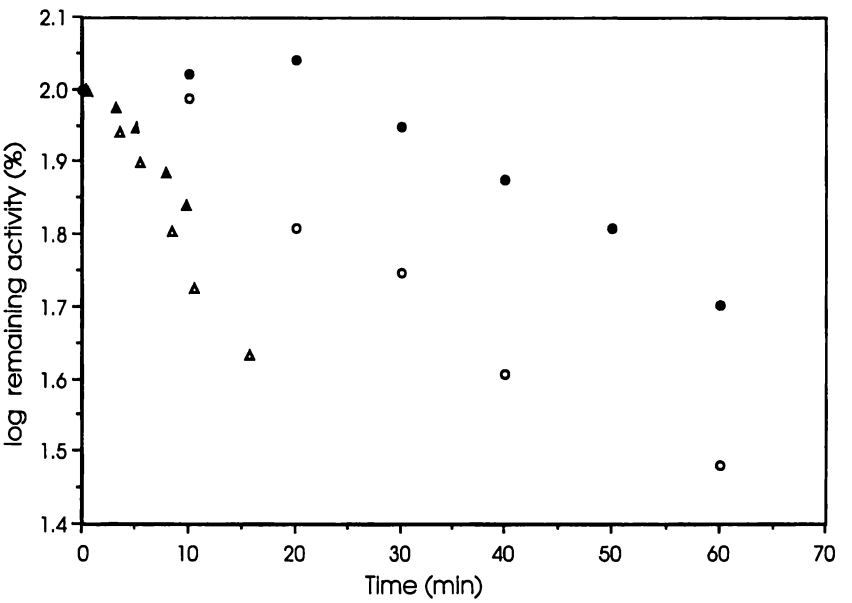


Figure 4.18. Log of residual CMCase A activity from strain COMP.A2 versus incubation time. (●), addition of BSA (0.8 mg/ml) at 80°C; (○), no BSA addition at 80°C; (▲), addition of BSA (0.8 mg/ml) at 85°C; (Δ), no BSA addition at 85°C.

Table 4.7 The half life of CMCase A from strain COMP.A2 at 80°C and 85°C

Incubation condition	$t_{1/2}$ (min)	Temperature (°C)
50 mM-MOPS,pH 7.0	32	80
50 mM-MOPS pH 7.0 +0.8 mg BSA/ml	60	80
50 mM-MOPS pH 7.0	2	85
50 mM-MOPS pH 7.0 +0.8mg BSA/ml	6	85

CMCase activity was assayed using 1% CMC (low viscosity) as the substrate.

4.3.4.7 Effect of chemical reagents on CMCase A activity

The effect of various compounds on CMCase A activity is shown in Table 4.8. Mild inhibition was shown by several divalent cations and EDTA. Enzyme activity was slightly activated in the presence of NaCl. CMCase A activity was strongly inhibited by Zn^{2+}, Hg^{2+}, and the thiol-specific inhibitor *p*-chloromercuribenzenesulphonic acid (pCMPS). The reducing agents ß-mercaptoethanol and dithiothreitol (DTT) had no stimulatory effect on CMCase A activity.

4.3.4.8 Kinetics of the CMCase A activity

Classical Michaelis-Menten kinetics were observed with CMC as the substrate (Figure 4.19a). The data from these experiments were plotted in the Lineweaver-Burk (Figure 4 19b), Hanes (Figure 4.20a) and Woolf forms (Figure 4.20b). Straight lines were obtained for all the plots. The K_m and V_{max} values were determined from these curves over the concentration range from 0.031 to 1.0% CMC (Table 4.9). The results showed that the similar K_m and V_{max} values were obtained using the different plotting methods. The K_m value for CMC of the purified CMCase A is similar to that of *Bacillus subtilis* AU-1 (Au and Chan, 1987) and *Thermoascus aurantiacus* (Tong et al., 1980). The wide range of values for different enzymes may be due to assay methods and substrates used.

Table 4.8 Effect of chemical reagents on CMCase A activity

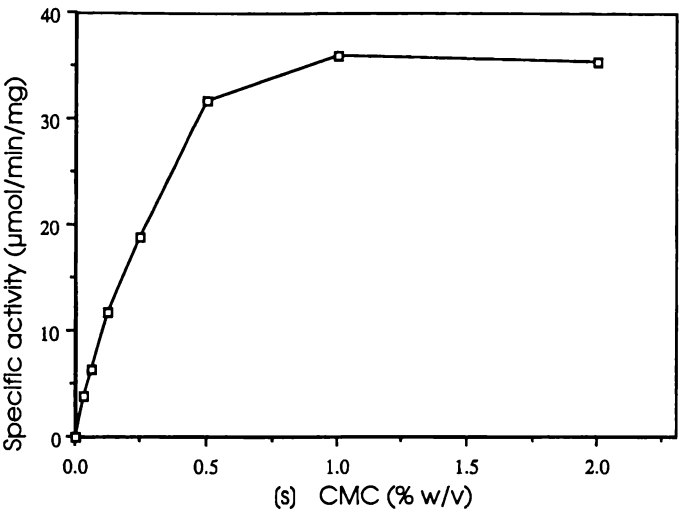
Chemical reagents	Relative activity of CMCase (%)
none	100
8.4 mM $CaCl_2$	87.3
8.4 mM $MgCl_2$	84.5
8.4 mM $SrCl_2$	93.0
8.4 mM $ZnCl_2$	12.4
8.4 mM $HgCl_2$	16.6
8.4 mM NaCl	108.4
8.4 mM EDTA	90.1
8.4 mM DTT	93
8.4 mM ß-mercaptoethanol	88.7
8.4 mM pCMPS	22
1.25% SDS	91.5
1.25% Triton X-100	94.4

100% CMCase activity is equivalent to 37.34 (units mg^{-1}). One unit of CMCase activity is defined as the amount of enzyme required to produce one micromole of reducing sugar (glucose equivalent) min^{-1}. 0.088 µg of enzyme was used in each assay.

Table 4.9 Kinetic data for the purified CMCase A

	CMC (%) concentration	K_m (CMC % w/v)	V_{max} (µmol min^{-1} mg^{-1})
Lineweaver-Burk Plot	0.031-1.0	0.34	44.7
Hanes Plot	0.031-1.0	0.40	51.50
Woolf Plot	0.031-1.0	0.42	52.67

a:



b:

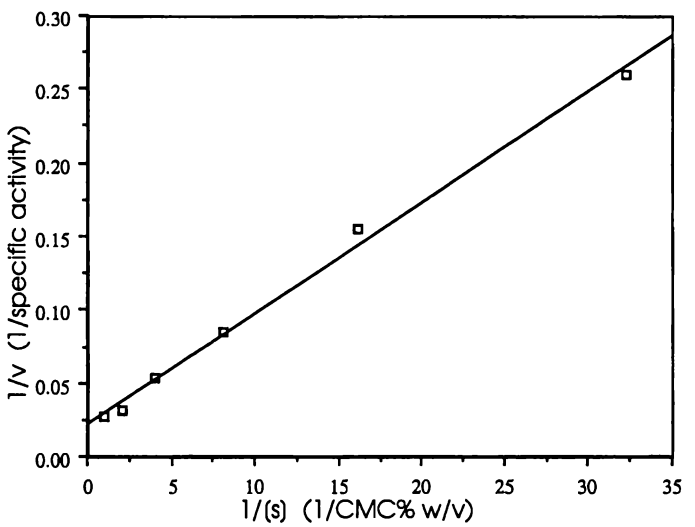
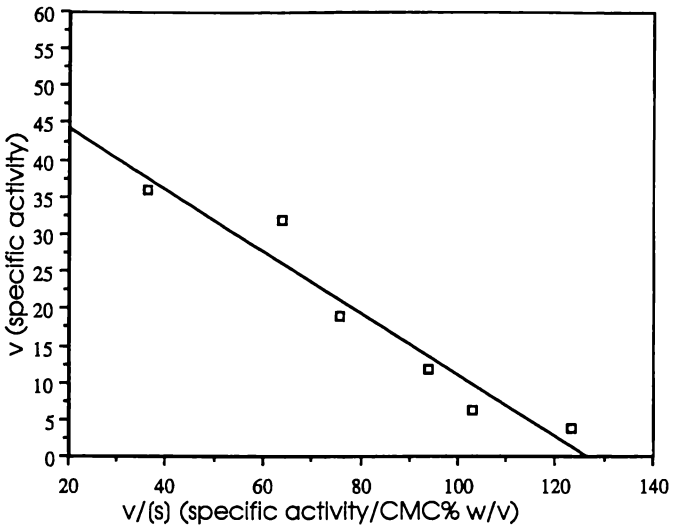


Figure 4.19 a: Michaelis-Menten plot for CMCase A. **b**: Lineweaver-Burk plot for CMCase A.

a:



b:

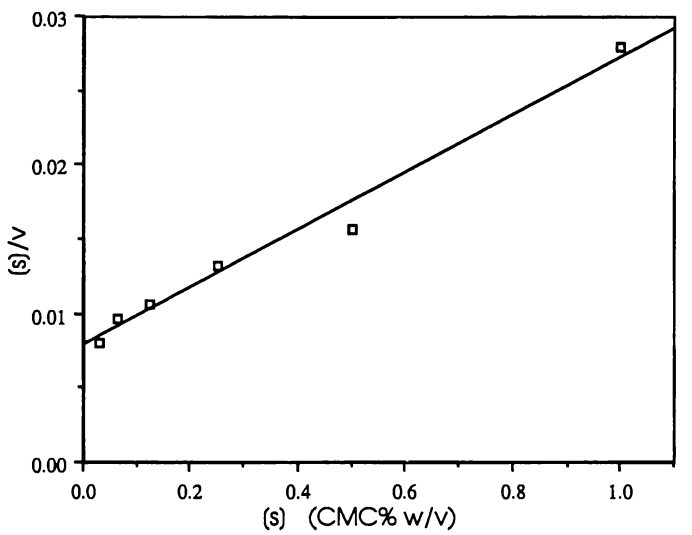


Figure 4.20 a: Woolf plot for CMCase A. **b**: Hanes plot for CMCase A.

4.3.4.9 Substrate specificity and mode of action

The purified CMCase A was tested for its ability to hydrolyze a range of compounds as potential substrates at pH 7.0 and at 70°C (Table 4.10). The enzyme showed greatest activity on CMC and had low activity on xylan and microcrystalline cellulose (Avicel). However, end products were not detected by HPLC analysis when xylan (oat spelt or larch wood) and microcrystalline cellulose (Sigmacell 20 or 50) were incubated with CMCase A at 70°C for 14 hours. *p*-Nitrophenol, methylumbelliferone, or glucose were not detected when CMCase A was incubated with the substrates MUC, pNPG, pNP-cellobiose, and pNP-lactoside at 70°C for 30 min. This suggested that the enzyme did not cleave either the aglyconic bond or the holosidic bond of MUC, pNP-cellobiose, and pNP-lactoside. The high background of reducing sugars in 1% laminarin (ß-1,3-1,6-glucan) and 1% lichenan (ß-1,3-1,4-glucan) solution made it difficult to assay the enzyme activity on laminarin and lichenan solutions using the PAHBAH method. A modified method is needed to assay enzyme activity on laminarin and lichenan. No end products were detected by HPLC when the purified CMCase A was incubated with maltooligosaccharides ranging from G2 to G7. The lack of end products from the incubation of maltooligosaccharides with the purified enzyme suggested that the enzyme did not cleave α-1,4-glucosidic bond of glucan and only degrades β-1,4-glucosidic bond.

Cellobiose was the main product of amorphous cellulose hydrolysis by CMCase A (Table 4.11). Trace amounts of cellotriose were detected when amorphous cellulose was incubated with enzyme over 30 minutes. No cellulooligosaccharides with a degree of polymerization (DP) higher than 3 were detected during the incubation.

The CMCase A enzyme hydrolyzed CMC to produce 0.45 mM cellobiose, 0.15 mM cellotriose and 0.41 mM glucose after the enzyme was incubated with CMC at 70°C for 14 hours. In order to further determine the mode of action of purified CMCase A, a viscometric assay was performed and accompanied by assay of reducing sugar production during incubation of 0.5% CMC and enzyme at 70°C. The result in Figure 4.22 showed that the decrease in viscosity of CMC solutions was slow compared to the increase in reducing sugars during the incubation.

Cellopentaose in 20 mM MOPS buffer at pH 7.0 was incubated with the purified CMCase A at 70°C for varying lengths of time. The results are shown in Table 4.12 and Figure 4.21. Cellopentaose (G5) was hydrolyzed to produce cellotetraose (G4), cellotriose (G3), cellobiose (G2), and glucose (G1). The high concentrations of cellotriose and cellobiose in the early incubation suggested that the second and/or the

third glycosidic bonds of cellopentaose were preferentially cleaved over the first and the fourth glycosidic bonds. The proportion of G3 and G1 remain constant after G5 was cleaved, and G2 increased until all the cellotetraose was degraded. This indicated that the most probable mode of action was for the enzyme to cleave the second glycosidic bond of G4 to produce G2. After 25 hours incubation, the end products were G3, G2, and G1 at a molar ratio of 1.6:2.4:1. This ratio remained unaltered during further incubation for up to 56 hours (data not shown for 56 hours). This further demonstrates that cellotriose and cellobiose are not degraded and at least four contiguous glucosyl residues were necessary for degradation by the enzyme. This may be the reason that the enzyme could not degrade MUC, pNP-cellobiose, and pNP-lactoside, whose structures are similar to that of cellotriose.

4.3.4.10 Synergistic effect of CMCase A and CMCase B on Avicel.

Synergistic effect of the enzyme activity on Avicel was tested and the results are shown in Table 4.13. The mixtures of CMCase A and CMCase B did not appear to enhance the activity on Avicel.

Table 4.10 Substrate specificity of the CMCase A from strain COMP.A2.

Substrates	Specific Activity (units/mg protein)
0.8% (w/v) CMC	30.6
3.6% (w/v) Avicel	1.7
0.25% (w/v) Xylan (oat spelt)	4.56
0.25% (w/v) Xylan (birch wood)	5.34

One unit of activity is defined as the amount of enzyme required to produce one micromole of reducing sugar (glucose equivalent for glucan substrates or xylose equivalent for xylan substrates) min^{-1} at pH 7.0 and 70°C.
Activity was not detected on MUC, *p*-NPG, *p*-NP-lactoside, and *p*-NP-cellobiose.

Table 4.11 Hydrolysis of amorphous cellulose by CMCase A at 70°C.

Incubation time (min)	Cellobiose (μmol/ml)
5	0.12
14	0.18
20	0.21
30	0.24
60	0.41
186	0.81
272	0.93

200 μl of 4% (w/v) amorphous cellulose was incubated with 200 μl of CMCase A at 70°C (2.2 μg of enzyme). Reaction products were assayed by HPLC using a Bio-Rad HPX-42A column.

Table 4.12 Time course study of hydrolysis of cellopentaose by CMCase A, assayed by HPLC.

Time (Hrs)	G5	G4	G3	G2	G
0.0	9.6	0.0	0.0	0.0	0.0
1.0	5.5	2.0	2.9	1.5	1.9
5.0	0.0	3.1	6.3	5.0	3.8
10	0.0	1.8	6.8	6.9	3.9
20	0.0	0.5	6.0	8.3	4.0
25	0.0	0.0	7.2	10.9	4.5
30	0.0	0.0	7.7	11.6	4.4

The data represents millimolar concentration of products formed.
Cellopentaose (G5), cellotetraose (G4), cellotriose (G3), cellobiose (G2), glucose (G).

Table 4.13 Avicel activity of CMCase A and CMCase B individually and combined.

Enzyme	Activity (U)
CMCase A	0.124
CMCase B	0.022
CMCase A + CMCase B	0.071

One unit of activity is defined as the amount of enzyme required to produce one micromole of reducing sugar (glucose equivalent) min^{-1} at pH 7.0 and 70°C. 10 µl of CMCase A and 10 µl CMCase B were used respectively for enzyme assay.

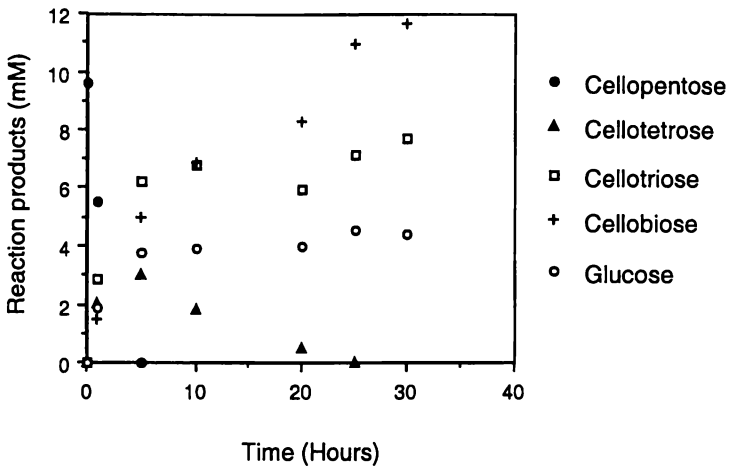


Figure 4.21. Hydrolysis of cellopentaose by CMCase A. The reaction products were analyzed by HPLC using a Bio-Rad HPX-42A column.

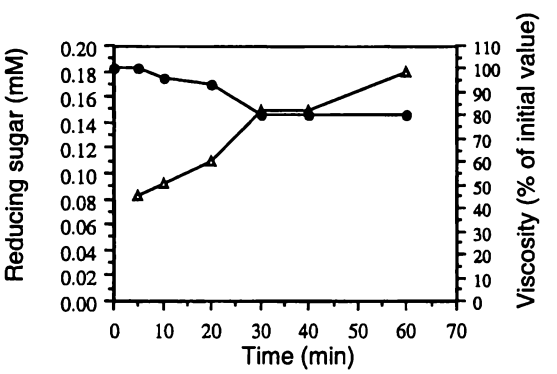


Figure 4.22. The decrease in viscosity of a CMC solution due to enzyme activity of the purified CMCase A. Viscosimetric assay was according to materials and methods. The reducing sugar was determined by PAHBAH assay. Symbol: reducing sugar (Δ), viscosity (●).

4.4 DISCUSSION

The regulation of cellulase production varies among different microorganisms, with some cellulases being synthesized constitutively and others being induced by cellulose, cellobiose and /or sophorose (Tomme et al., 1995). The results of this studies show that cellulase activity in strain COMP.A2 was induced by cellobiose, xylan, amorphous cellulose and microcrystalline cellulose. The production of CMCase activity was much higher in cellulose medium than in cellobiose and xylan medium (Table 4.1). This suggests that microcrystalline cellulose was a much stronger inducer of enzyme than other carbon sources. However this does not mean that cellulose induces directly (e.g. by binding to a receptor on the cell surface). The generally accepted mechanism for cellulase induction by cellulose is that cellulose first undergoes limited hydrolysis by cellulases constitutively produced in low amounts. The soluble hydrolysis products such as cellobiose or sophorose thus generated could then enter the cells and induce cellulase synthesis.

Almost all purified cellulases (e.g. endo- or exoglucanases) are extracellular enzymes (Paech, 1994), and culture supernatant has normally been used for cellulase purification. For the COMP.A.2 culture, approximately 29% of total CMCase activity was found in the supernatant, the remainder being adsorbed to either cells or un-degraded cellulose. It was not possible to remove this bound CMCase activity by washing with distilled water. The failure to elute the bound CMCases with water suggests that the property of the bound CMCases is different from that of the cellulosomes of *C. thermocellum* which can be eluted by distilled water or a 1% solution of triethylamine (Lamed and Bayer, 1988).

To fully understand the mechanism of action of the cellulase system of strain COMP.A2, it is essential to purify and characterize the components of cellulase system or clone and express the genes encoding for various components of the cellulase system in a suitable host. Although the gene cloning approach has become more popular nowadays as molecular biology techniques improve, the purification and characterization of the enzymes from the native host is still widely adopted. Conventional chromatography and fast protein liquid chromatography techniques (FPLC) were chosen to purify cellulases from the culture supernatant of strain COMP.A2. CMCase A was finally purified to homogeneity. CMCase B was partially purified. The CMCase A and CMCase B are only portions of the complex of cellulase enzymes produced by strain COMP.A2. It is still a challenging task to purify the other components of cellulase, especially those bound to residual cellulose and/or the cell surface which may or may not be similar to the enzymes found in the supernatant.

Multiple forms of endoglucanases have been reported in cellulolytic bacteria (Ng and Zeikus, 1981; Lamed et al., 1983b; Horikoshi et al., 1984; Au and Chan, 1987). Multiplicity of cellulases has been reported to be a consequence of multiple genes (Fukumori et al., 1989, Hazlewood et al., 1988), glycosylation (Ng and Zeikus, 1981, Calza et al., 1985; Yoshimatsu et al., 1990), and proteolytic degradation (Sami et al., 1988). Proteinase activity was not detected in culture broth of COMP.A2 which grew on Sigmacell 20 or cellobiose basal medium. This suggests that the multiplicity of cellulases did not arise from proteolytic degradation. Thus, the various components of the cellulase system from strain COMP.A2 may be encoded by different genes or the proteins were glycosylated.

A final purification factor of 117 was obtained for the CMCase A activity. This is quite high compared to that obtained for some other cellulolytic thermophiles, e.g. a purification of 14.5 was reported for an endoglucanase from *R. marinus* (Hreggvidsson et al., 1996), 6.7 for an endoglucanse from *C. thermocellum* (Fauth et al., 1991), 4.8 for a bifunctional cellulase from the extreme thermophile *'Anaerocellum thermophilum'* (Zverlov et al., 1998b). This high level of purification was obtained at the expense of a low recovery of activity. One reason for this low recovery might be synergism of CMCases, which would cause high activity figures in the early stages of purification. Subsequently, separation of the CMCases on purification would cause a decrease in total activity, without necessarily a corresponding decrease in the total amount of active protein. The ultrafiltration and diafiltration procedure also resulted in important enzyme activity losses. The loss of activity in this procedure may be due to the adsorption of the enzyme to the membrane or occurrence of the enzyme denaturation. Further losses of enzyme activity were deliberate to ensure a homogenous protein, thus some of the eluted fractions which showed activity, e.g. fractions 21 to 24 in Figure 4.4 and fractions 21 to 22 in Figure 4.5, were not collected because they exhibited multiple protein bands when run on SDS-PAGE. It needs to be pointed out that it is impossible to determine the original ratio of different CMCase activities of the culture because the assay method can not differentiate one CMCase activity from the others. Therefore, we are unable to estimate a true recovery value of the purified CMCase.

Exoglucanase (Avicelase) was not purified to homogeneity. Avicelase activity was elevated in the early steps of purification and relatively low in the later steps (Table 4.6). This suggests that the degradation of Avicel may be the result of synergistic action of multiple cellulases or Avicelase activity was lost or unstable during the purification. Since synergistic effects were not observed for the activity on Avicel using mixture of CMCase A and CMCase B, other cellulase components seem to be needed for degradation of Avicel. Most cellulolytic bacteria have been reported to

produce endoglucanases and only a few bacteria were reported to produce exoglucanase (Creuzet and Frixon, 1983; Ohmiya and Schimizu, 1988a; Nakamura and Kitamura, 1988; Yablonsky et al., 1988; Wang et al., 1994; Zverlov et al., 1998a). Whether strain COMP.A2 contains exoglucanase will remain to be clarified in a further study.

The native CMCase A purified from COMP A.2 is most probably a dimer based on the results of SDS-PAGE and size exclusion chromatography. The molecular mass of native CMCase A from COMP.A2 is larger than those of other thermostable cellulases previously reported. The endoglucanases from *C. thermocellum* (76 kDa) (Romaniec et al. 1992), *C. stercorarium* (100 kDa) (Bronnenmeier and Staudenbauer, 1988a), *Rhodothermus marinus* (49 kDa) (Hreggvidsson et al. 1996) were all reported to be monomeric. A non-thermostable endoglucanase purified from an alkalophilic *Bacillus* species has been reported to be a tetrameric enzyme with a molecular mass of 500 kDa determined by size exclusion chromatography (Yoshimatsu et al., 1990).

Multimolecular complexes with various cellulase components have been found in anaerobic cellulolytic bacteria, such as *Clostridium thermocellum* (Lamed et al., 1983a), *Clostridium* strain C7 (Cavedon et al., 1990), *Clostridium cellulovorans* (Shoseyov and Doi, 1990), *Ruminococcus albus* (Wood et al., 1982), *Bacteroides cellulosolvens* (Lamed et al., 1991). A recent report showed that *Bacillus circulans* also produced two multimolecular complexes with an extraordinary capacity to degrade crystalline cellulose (Kim, 1995). Multi-molecular complexes with cellulase activity are generally typical of anaerobic microorganisms, including bacteria and fungi that colonize anaerobic environments in the rumen, hind-gut of herbivores and sewage. So far there is no evidence to show that strain COMP.A2 produces the multimolecular complex which is responsible for cellulose hydrolysis.

With an assay time of 10 min, CMCase A displayed highest activity at 80°C and had a half life of 32 min at this temperature. This "optimum temperature", which will be dependent on the assay time period, for the CMCase A is quite high compared with that of other *Bacillus* species (Table 4.14). However, care needs to be exercised in this comparison because different assay time periods have been used by different laboratories. The thermostability of CMCase A of strain COMP.A2 is similar to endoglucanase of *Thermomonospora curvata* but lower than those from other thermophilic cellulolytic bacteria (Table 4.15). The cellulases from *Cs. saccharolytium*, *Thermotoga* sp., and *Clostridium stercorarium* showed very high thermostabilities. The most thermostable endoglucanases reported are from a thermophilic eubacterium *Rhodothermus marinus* (Hreggvidsson et al., 1996) and a hyperthermophilic archaeon *Pyrococcus furiosus* (Bauer et al., 1999). The endoglucanase from *R. marinus* and *P.*

furiosus displayed an extraordinary thermostability and had a half life of 3.5 h at 100°C and 40 h at 95°C, respectively. *R. marinus* is an obligate aerobe with optimum growth temperature at 65°C. The highly thermostable cellulase purified from *R. marinus* demonstrates that cellulases with very high thermostability are not necessarily produced only by organisms growing at very high temperatures or by anaerobic bacteria and archaea. The increase in thermostability of CMCase A from strain COMP.A2 by the addition of BSA suggests that protein concentration has an important role to play in enzyme thermostability. Similar results have been reported from cellobiosidase activity from *Bacteroides succinogenes* (Huang et al., 1988). The results are not surprising as the purified cellulase does not work alone in the natural environment. There are always other proteins and cellulases in existence. It is likely that the thermostability of CMCase A in vivo is much higher than that of enzyme in vitro.

The inhibition of CMCase A activity by Zn^{2+} and Hg^{2+} was not surprising and has been reported for many cellulases. Fauth et al. (1991) suggested that Hg^{2+} inhibition may be due to complex-formation with specific residues, e.g., thiol groups, or the result of non-specific salt formation. The inhibition of CMCase activity by Hg^{2+} was considered to be related to the tryptophan residues at the active site of the enzyme (Hurst et al., 1977; Yoshimatsu et al., 1990). It is not clear whether a similar role occurs in the CMCase A of strain COMP.A2 but the inhibitory effect of the thiol-specific inhibitor *p*-chloromercuribenzenesulphonic acid (pCMPS) on CMCase activity supports the presence of essential thiol groups in the enzyme. Similar results have been reported from other bacterial endoglucanases (Creuzet and Frixon, 1983; Romaniec et al., 1992; Au and Chan, 1987). Unlike the case with the endoglucanase from *Bacillus subtilis* (Au and Chan, 1987), the reducing agent ß-mercaptoethanol and dithiothreitol (DTT) had no stimulatory effect on CMCase A activity. The mild inhibition by several divalent cations and EDTA suggests that CMCase A is not dependent for activity on metal ions.

CMCase A showed greatest hydrolytic activity on CMC and low activity on Avicel and xylan (Table 4.10). The poor activity of this enzyme on Avicel suggests that other components of the cellulase system are necessary for degradation of crystalline cellulose. It is not clear why the end products were not detected by HPLC analysis when xylan (oat spelt or larch wood) and microcrystalline cellulose (Sigmacell 20 or 50) were incubated with CMCase A at 70°C for 14 hours. One possibility is that the cellodextrins produced by enzyme action can not be detected by HPLC due to their high molecular weight, while the relatively high sensitivity of the PAHBAH assay allowed the reducing end of cellodextrins to be detected. It is not unusual for a cellulase to show xylanase activity. Endoglucanases from *Ruminococcus albus* and cellulase I of

Thermoascus aurantiacus have been reported to display activity on xylan (Ware et al., 1989; Shepherd et al., 1981). In addition, an enzyme encoded by the *celB* gene in *Caldicellulosiruptor saccharolyticus* contains two catalytic domains with the first one showing both xylanase and cellobiohydrolase activities (Saul et al., 1989, 1990; Gibbs et al., 1992).

The activity on cellopentaose and cellotetraose by CMCase A showed that cellopentaose is the preferred substrate. This is comparable to the report that the activity of endoglucanase decreases as degree of polymerization of cellulooligosaccharides decreases (Coughlan and Mayer, 1992). The lack of activity on cellotriose by CMCase A suggests that a minimum length of polysaccharide chain is necessary for enzyme action. The minimum number of consecutive glucosyl residues for enzyme reaction varies with different endoglucanases. The endoglucanases from *C. thermocellum* (Ng and Zeikus, 1981, Fauth et al., 1991) and *B. circulans* (Kim, 1995) displayed no activity on cellotriose and four consecutive glucosyl residues were needed for enzyme reaction, while endoglucanases from *R. marinus* (Hreggvidsson et al., 1996) and *C. cellulolyticum* (Gal et al., 1997) could cleave cellotriose to cellobiose and glucose and only three consecutive glucosyl residues were necessary for enzyme reaction.

The term CMCase has commonly been regarded as synonymous with endoglucanase which randomly degrades CMC or amorphous cellulose and has no activity on Avicel (Coughlan and Mayer, 1992). The low activity on Avicel and high activity on CMC suggest that the purified CMCase A from strain COMP.A2 belongs to an endoglucanase on the basis of definition by Coughlan and Mayer (1992). However, cellobiose was the main product released from hydrolysis of amorphous cellulose or CMC and no cellulooligosaccharides with a DP higher than 3 were detected. This property, along with the observation that the decrease in viscosity of CMC solutions was slow compared to the increase in reducing sugars during the incubation, suggests that the enzyme hydrolyses amorphous cellulose or CMC in an exo-acting fashion. The definition of an exo-1,4-ß-glucanase and endo-1,4- ß-glucanase by Wood and Bhat (1988) is mainly based on the study of fungal cellulases and may not be suitable for all bacterial cellulases. Glucanases may not be totally exo- or endo- acting, but may exhibit predominately one activity, with low levels of the other activity (Tomme et al., 1996). Many cellulases have also been reported to show both endo- and exoglucanase activities (Bronnenmeier and Staudenbauer, 1990; Han et al., 1995; Tomme et al., 1996; Zverlov et al., 1998b). Recently, the endoglucanases were arranged into four groups with similar ratios of viscometric to reductometric activity based on the combined use of capillary viscometry, reducing end-group analysis (BCA measurement), and size

exclusion chromatography methods (Vlasenko et al., 1998). Group I had a higher preference for hydrolysis of the bonds near the chain end, while group IV was designated as the true endoglucanase. The purified CMCase A may belong to group I according to the arrangement of endoglucanases by Vlasenko et al. (1998) and has a preference for hydrolyzing terminal bonds of CMC and amorphous cellulose. The enzyme may act processively at the end of cellulose molecules following an initial random attack on CMC or amorphous cellulose according to the proposal of Tomme et al. (1996), in which cellulose was hydrolyzed by cellulases through a processive, or a semiprocessive, or a nonprocessive mechanism.

In conclusion, CMCase A purified from strain COMP.A2 hydrolyses CMC or amorphous cellulose in an exo-model of action following initial and random attack. This is different from the traditional definition of endoglucanases. The classification of cellulases in two exclusive groups, endoglucanases and exoglucanases, is not appropriate as suggested recently by Tomme et al. (1996). Enzymatic hydrolysis of cellulose might be more complex than we thought. The arrangement of endoglucanases into four groups and endoglucanase assay methods proposed by Vlasenko et al. (1998) may help us solve some confusion in cellulase classification. CMCase A is only one component of the cellulase system from strain COMP.A2. Other cellulase components are necessary for hydrolysis of crystalline cellulose. A search for these cellulases will be continued in further investigations.

Table 4.14 Temperature and pH optima of some purified bacterial cellulases.

Bacteria	Enzyme	pH opt.	Temp. opt.	Reference
Acetivibrio cellulolyticus (NRCC 2248)	Endoglucanase	5.0	50	Saddler and Khan, 1980, 1981
Bacillus subtilis AU-1	Endoglucanase	5.5		Au and Chan, 1987
Bacillus sp. KSM-635	Endoglucanase	9.5	40	Yoshimatsu et al., 1990
Bacillus sp. No 1139	Endoglucanase	9.0		Fukumori et al., 1985
Bacillus subtilis DLG	Endoglucanase	4.8	58	Robson and Chambliss, 1984
Bacteroides succinogenes	Cellobiosidase	6.0	45	Huang et al. 1988
Cellulomonas uda	Exocellbio-hydrolase	5.5-6.5	45-50	Nakamura and Kitamura, 1988
Clostridium josui	Endoglucanase	6.8	60	Fujino et al., 1989
Clostridium stercorarium	Endoglucanase	6.4	80	Creuzet and Frixon, 1983
Clostridium thermocellum strain LQ R1	Endoglucanase	5.2	62	Ng and Zeikus, 1981
Clostridium thermocellum strain A.T.C.C. 27405	Endoglucanase	7.0	70	Romaniec et al., 1992
Rhodothermus marinus	Endoglucanase	6-8	95	Hreggvidsson et al. 1996
Ruminococcus albus F-40	Endoglucanase	6.7	44	Ohmiya et al., 1987
Thermomonospora fusca strain YX	Endoglucanase	6.0	74	Calza et al., 1985
Thermomonospora curvata	Endoglucanase	6.0-6.5	70-73	Lin and Stutzenberger, 1995
strain COMP.A2	CMCase A	6.5-7.0	80	this study

pH opt., optimum pH; Temp. opt., optimum temperature (°C).

Table 4.15 Thermostabilities of cellulases from some bacteria and a hyperthermophilic archaeon.

Eubacteria	Enzyme	Temp	$T_{1/2}$ (min)	Reference
Clostridium thermocellum	Endoglucanase	70	300	Hon-Nami et al., 1985
Acidothermus cellulolyticus	Endoglucanase	85	60	Tucker et al., 1989
Cs. saccharolytium [a]	MUCase [b]	85	90	Ruttersmith, 1991
Cs. saccharolytium	CMCase [b]	80	378	Neal, 1987
Clostridium stercorarium	Endoglucanase	85	60	Creuzet and Frixton, 1983
Rhodothermus marinus	Endoglucanase	100	210	Hreggvidsson et al. 1996
Thermomonospora curvata	Endoglucanase	80	30	Lin and Stutzenbergen, 1995
Thermotoga sp.strain FjSS3-B.1	MUCase	108	70	Ruttersmith and Daniel, 1991
Strain COMP.A2	CMCase	80	32	this study
Archaeon *Pyrococcus furiosus*	Endoglucanase [b]	95	2400	Bauer et al., 1999

Temp, temperature (°C); $T_{1/2}$, half life of cellulase activity.

a: *Caldocellum saccharolyticum* is now designated as *Caldicellulosiruptor saccharolyticus* (*Cs. saccharolyticus*).

b: expressed in *E. coli*.

CHAPTER FIVE

PURIFICATION AND CHARACTERIZATION OF A ß-GLUCOSIDASE FROM STRAIN COMP.A2

5.1 INTRODUCTION

ß-Glucosidase (ß-D-glucoside glucohydrolase, EC 3.2.1.21) catalyses the hydrolysis of ß-glucosidic linkages between glucose and alkyl, aryl or saccharide groups (Patchett et al., 1987). The enzyme has been reported from plants, fungi, yeasts, and bacteria. The enzyme is capable of hydrolyzing cellobiose and cellodextrins to glucose and removes the inhibitory effect of cellobiose on the other components of the cellulase complex (Woodward and Wiseman, 1982).

As product inhibition and thermal inactivation of cellulases constitute two major barriers to the realization of enzymatic cellulose hydrolysis as a commercial process, thermostable ß-glucosidases provide opportunities for economically efficient breakdown of cellulose. Studies of ß-glucosidases from thermophilic bacteria, including *Clostridium thermocellum* (Ait et al., 1982), *Thermotoga* sp. strain FjSS3-B.1 (Ruttersmith and Daniel, 1993), an extremely thermophilic anaerobic strain Wai21W.2 (Patchett et al., 1987), *Thermotoga maritima* (Gabelsberger et al., 1993a,b), and *C. stercorarium* (Bronnenmeier and Staudenbauer, 1988), have shown enhanced thermostability of the enzyme relative to the ß-glucosidase from mesophilic bacteria.

Results in chapter three showed that glucose was the main end product when strain COMP.A2 grew on cellulose or cellobiose medium and produced high ß-glucosidase activity in culture on these substrates. Chapter four reported the purification and characterization of a thermostable cellulase (CMCase A). CMCase A is capable of hydrolyzing cellulose primarily to cellobiose. The active fractions (fraction 70 to 95) from Mono Q chromatography during purification of CMCases contained most of the ß-glucosidase activity (Figure 4.3). In the present chapter the purification and characterization of this ß-glucosidase is reported.

5.2 MATERIALS AND METHODS

5.2.1 Chemicals reagents

Analytical grade reagents were used. *p*-NP-substrates were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

5.2.2 Enzyme activity assays

Two substrates, cellobiose and *p*-nitrophenyl-ß-D-glucoside (pNPG), were used to assay ß-glucosidase activity. The cellobiose was hydrolyzed to glucose which was determined by use of a glucose oxidase / peroxidase kit. The *p*-nitrophenol released from pNPG was measured colorimetrically.

400 µl of a 2 mM solution of pNPG in 50 mM MOPS buffer at pH 7.0 was preincubated in Eppendorf tubes at 70°C. An enzyme sample of 0.1 ml was added to two of triplicate tubes and incubated at 70°C for 15 minutes (t=15). All assays were stopped by the addition of 1.0 ml 1.0 M Na_2CO_3. The enzyme sample of 0.1 ml was added to the remaining tube (t=0). The absorbance was read at 400 nm.

The same procedure was used in the determination of substrate specificity. 0.4 ml of *p*-NP-substrate in 50mM MOPS buffer at pH 7.0 at a concentration of 2 mM was preincubated in Eppendorf tubes at 70°C. The purified enzyme sample of 0.1 ml was added to two of triplicate tubes and incubated at 70°C for 15 minutes. The reaction was stopped by the addition of 1.0 ml 1.0 M Na_2CO_3. The purified enzyme sample (0.1 ml) was added to the remaining tube (t=0). The absorbance was read at 400 nm.

A standard curve was prepared from *p*-nitrophenol (Merck, U.S.A). *p*-Nitrophenol (0.5 ml) at the concentration of 0.0 to 0.313 mM in 50 mM MOPS buffer at pH 7.0 was added to 1.0 ml 1.0 M Na_2CO_3. The absorbance was read at 400 nm. An extinction coefficient (ε_{400}) of 5.97×10^3 $lmol^{-1}cm^{-1}$ was obtained and used to calculate activities. Unless otherwise stated, activity on each *p*-NP-substrate is expressed as µmol *p*-nitrophenol (*p*-NP) released min^{-1} ml^{-1} or mg^{-1} protein.

To measure the hydrolysis of cellobiose or other substrates (maltose, trehalose, sophorose, lactose and laminarin), 5 mM of substrates and 2% (w/v) for laminarin were made up in 50 mM MOPS buffer at 7.0. Each substrate (0.2 ml) was preheated at 70°C in Eppendorf tubes. An enzyme sample of 10 µl was added and incubated at 70°C for 15 min. The reaction was stopped by placing tubes in cold water. The supernatant (40

µl) from the tubes was mixed with 1 ml of a GOD-perid glucose test kit solution 2 (Boehringer Mannheim, Mannheim, Germany) and incubated at 37°C for 15 min. The absorbance was read at 578 nm. A standard curve was prepared from glucose in the range of 0.049 - 1.56 mM and used to calculate activities.

5.2.3 Protein determination

The dye-binding method of Bradford (1976) was used for protein determination in purification and characterization of the enzyme. The detailed method is described in section 4.2.3.

5.2.4 Location of ß-glucosidase

The basal medium (200 ml) containing 0.5% cellobiose was prepared in a one litre flask. The flask was inoculated with strain COMP.A2 and incubated at 70°C at 140 rpm. Samples were withdrawn over a time course and assayed for ß-glucosidase activity.

Culture samples (0.4 ml) were centrifuged in 1.5 ml Eppendorf tubes at maximum speed in a benchtop centrifuge to obtain cell-free supernatants and cell pellet. The cell pellet was washed and centrifuged twice with 50mM MOPS buffer at pH 7.0. The cell pellet was resuspended in the same buffer and toluene:acetone (1:1 v/v) was added to a final concentration of 10% (v/v). The tubes were mixed and allowed to stand at room temperature for 10 min. The whole cell culture, cell-free supernatants, and toluene-acetone treated cell pellet suspensions were assayed for ß-glucosidase activity.

5.2.5 Purification of ß-glucosidase

The method used for the purification of CMCases was also used to purify the ß-glucosidase. This included Phenyl-Sepharose chromatography, ion-exchange chromatography (FPLC Mono Q), and chromatofocusing (FPLC Mono P). The methods are described in section 4.2.5.2. Chromatographic steps were carried out at ambient temperature. pNPG was the substrate used for activity assays in the ß-glucosidase purification.

5.2.6 Characterization of ß-glucosidase

5.2.6.1 Molecular weight determination

The molecular weight determination and the assessment of purity were carried out by running samples on SDS-PAGE using a PhastSystem (Pharmacia). The method is described in detail in section 4.2.6.1.

The molecular weight of the purified ß-glucosidase was also estimated by analytical high performance size exclusion liquid chromatography using a BioSep-SEC-S3000 column (Bio-Rad, U.S.A.). Twenty microlitre concentrated ß-glucosidase (0.2 mg/ml) and Gel Filtration Molecular Weight Standards (Boehringer Mannheim Biochemia, Germany) were applied to the BioSep-SEC-S3000 column which had been previously equilibrated with 25 mM MOPS buffer (pH 7.0). The method was described in section 4.2.6.1.

5.2.6.2 Native-PAGE and activity stain

PhastGel Gradient 10-15 gels and PhastGel Native Buffer Strips were used for native-PAGE in the PhastSystem. The enzyme samples were loaded onto the gel and run under the conditions specified in the PhastSystem Separation Technique File No.120 and then silver stained.

The detection of ß-glucosidase activity on the gels was mainly based on the method of Coughlan (1988). An overlay gel was made by dissolving 2 g agar in 100 ml of 2 mM pNPG with MOPS as buffer (pH 7.0). The solution was boiled and poured onto Gel Bond film (Bio-Rad, USA) which was warmed on a gel-casting plate (Bio-Rad, USA) at 65°C. After running the native-PAGE gel, the overlay gel was laid on the native-PAGE gel and incubated at 60°C for 10 or 15 min until a yellow band appears.

5.2.6.3 Isoelectric point determination

Isoelectric focusing was carried out using the method described in section 4.2.6.3.

5.2.6.4 Effect of temperature on ß-glucosidase activity

Four hundred µl of a 2 mM solution of pNPG in 50 mM MOPS buffer at pH 7.0 at various temperatures was preincubated at temperatures ranging from 50°C to 85°C in

triplicate 1.5 ml Eppendorf tubes. Samples of 100-fold diluted purified ß-glucosidase (100 µl) were added to two tubes and assayed enzyme activity as described at 4.2.2.3.

5.2.6.5 Effect of pH on ß-glucosidase activity

Solutions of 200 mM acetate, citrate, MOPS, HEPES, CHES, Tris and Bis-Tris-Propane were mixed with an equal volume of 4 mM pNPG and adjusted to various pH values at 70°C with 1.0 M NaOH or 1.0 M H_2SO_4. The resultant 2 mM pNPG (400 µl) solutions were preincubated in triplicate 1.5 ml Eppendorf tubes at 70°C. Samples of 100-fold diluted purified ß-glucosidase (100 µl) were added to two tubes and assayed enzyme activity as described at 4.2.2.3.

5.2.6.6 Thermostability

Twenty five-fold diluted purified ß-glucosidase (50 µl) in 50 mM MOPS buffer at pH 7.0 was incubated in 1.5 ml capped Eppendorf tubes at temperatures of 70°C and 80°C in the presence and absence of BSA, betaine, or trehalose. Duplicate Eppendorf tubes were withdrawn at intervals over a time course and cooled in ice-cold water. All samples were assayed for residual ß-glucosidase activity as described at 4.2.2.3. A thermistor probe (Omega, USA) sealed into Eppendorf tubes showed that the liquid within reached the required temperature within 3 minutes.

5.2.6.7 Effects of chemical reagents on ß-glucosidase activity

Various chemical reagents were prepared in 50 mM MOPS buffer and adjusted pH to 7.0 at 70°C. Ten microlitres of 10-fold diluted ß-glucosidase was mixed with 40 µl of test solutions in Eppendorf tubes and preincubated at 70°C for 5 minutes. Four hundred microlitres of 2 mM pNPG was added and assayed enzyme activity for 10 minutes as described at 4.2.2.3.

5.3 RESULTS

5.3.1 Location of ß-glucosidase enzyme

A growth curve of strain COMP.A2 on basal medium containing cellobiose as substrate is shown in Figure 5.1. ß-Glucosidase activity was assayed and the results are shown in Table 5.1. In the early log phase, 90% of the ß-glucosidase activity was cell-associated. This gradually declined with culture age and in stationary phase of growth.

Approximately 62% of the ß-glucosidase activity was cell-associated when cell lysis occurred.

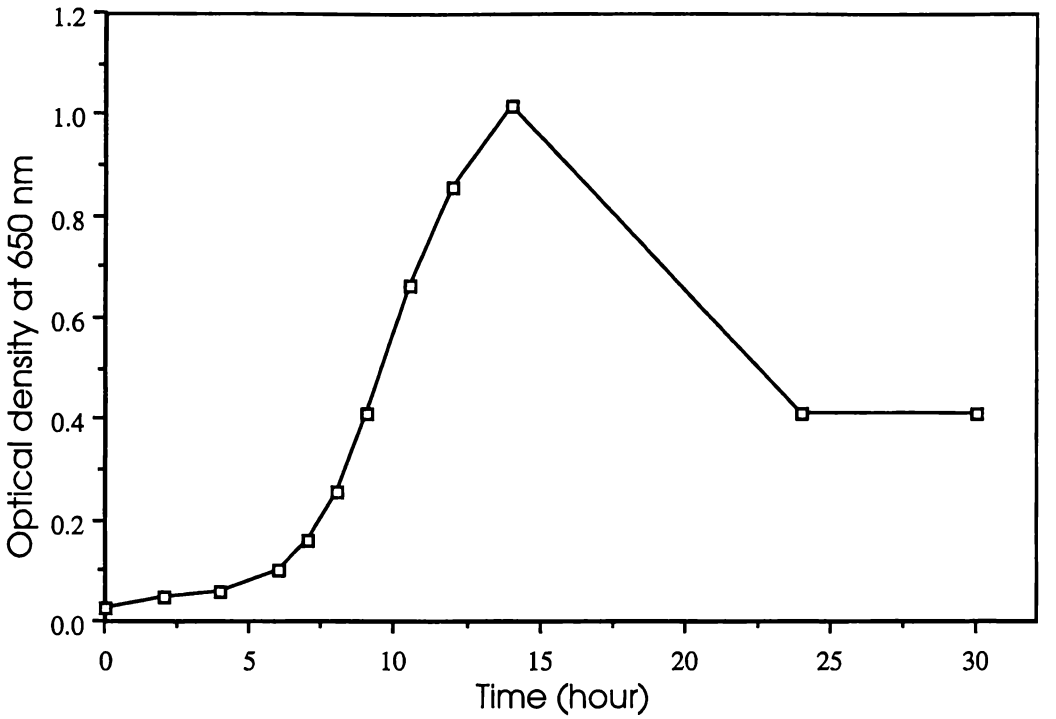


Figure 5.1. Growth curve of strain COMP.A2 on basal medium with cellobiose as carbon source and incubated at 70°C.

Table 5.1 The location of ß-glucosidase activity

Incubation time (hrs)	Sample	ß-glucosidase activity
7.0	cell culture	0.033
	culture supernatant	0.0048
	toluene-acetone treated cells	0.048
10.5	cell culture	0.108
	culture supernatant	0.014
	toluene-acetone treated cells	0.075
24	cell culture	0.16
	culture supernatant	0.079
	toluene-acetone treated cells	0.21

ß-Glucosidase activity is expressed as μmol *p*-NP released min^{-1} ml^{-1}.

5.3.2 Purification of ß-glucosidase

The active fractions (fraction 70 to 95) in Figure 4.3 stored in the freezer (-20°C) were thawed and used as the material for the ß-glucosidase purification. The ß-glucosidase purification sequence was the same as that for the CMCase purification from the Phenyl-Sepharose column to the Mono Q column (Figure 4.2 and Figure 4.3). The purification sequence for these steps is described here again for completeness.

Nineteen litres of culture supernatant, which was adjusted to a salt concentration of 1.0 M $(NH_4)_2SO_4$ and 0.04% NaN_3, was applied to the Phenyl-Sepharose column in two runs. The elution profile from the first run is shown in Figure 5.2 and Table 5.2. The elution profile of the second run was similar to the first run. Twenty-one per cent of applied ß-glucosidase activity was recovered after the two Phenyl-Sepharose column runs. The active fractions (fraction 3 to 5) from Phenyl-Sepharose were pooled and concentrated by ultrafiltration using a PM 10 membrane (10,000 molecular weight cut off, Amicon). The concentrated sample was diafiltered with 20 mM MOPS buffer at pH 7.0 using a PM 10 membrane. The concentrated sample in 20 mM Tris-HCl buffer at pH 8.0 was applied to the Mono Q column and eluted with a gradient of 0.0 to 1.0 M NaCl (Figure 5.3). The ß-glucosidase activity was eluted in fractions 70 to 95 (Figure 5.3). The active fractions were pooled and concentrated by ultrafiltration, and then diafiltered with 20 mM MOPS buffer at pH 7.0 using a PM 10 membrane at 4°C. The final sample (16.5 ml) in 20 mM Tris-HCl buffer at pH 8.0 was applied to the Mono Q column (10/10, Pharmacia) which had been pre-equilibrated in 20 mM Tris-HCl buffer (pH 8.0). The bound proteins were eluted with a linear gradient of 0.0 to 1.0 M NaCl in 20 mM Tris-HCl buffer at a flow rate 1.0 ml per minute. A significant amount of protein containing no ß-glucosidase activity was eluted in the early stage of the gradient, at NaCl concentrations from 0.15 to 0.26 M. The ß-glucosidase activity was eluted in the concentration range from 0.27 to 0.31 M NaCl. The active fractions were pooled and concentrated by ultrafiltration and diafiltered with 20 mM MOPS buffer at pH 7.0 at 4°C using a PM 10 membrane. The final enzyme solution (11.3 ml) in 20 mM Tris-HCl at pH 8.0 was reapplied to the Mono Q column (10/10). The protein was eluted in a gradient of 0.0 to 1.0 M NaCl. The ß-glucosidase was eluted in one active peak in the concentration range from 0.27 to 0.29 M NaCl (Figure 5.4). The active fractions (fraction 18 to 22) were pooled and concentrated by ultrafiltration and diafiltered with 25 mM Bis-Tris buffer at pH 7.0 using a PM 10 membrane. The concentrated sample (4.2 ml) was loaded to a FPLC Mono P column (HR 5/20, Pharmacia) which had been previously equilibrated in 25 mM Bis-Tris Buffer at pH 7.0. The protein was eluted using Pharmacia Polybuffer 74 diluted ten-fold with Milli-Q water, at pH 4.0, at a flow rate of 0.5 ml per min (Figure 5.5). The ß-glucosidase

activity was eluted from the Mono P column when the pH reached approximately 4.6 (fraction 24). The active fractions (fraction 25 to 26) showed one major protein band and two minor protein bands on a SDS-PAGE gel. These fractions contained 50% of the activity applied to the Mono P column; this activity was lost when they were reapplied to the Mono P column for further purification. The combined fractions (fraction 27 to 33) showed a single protein band after running SDS-PAGE (Figure 5.6) and were used as the purified ß-glucosidase for further characterization.

A summary of the ß-glucosidase purification is shown in Table 5.3. About 80-fold purification was achieved; however, the yield was low. The final recovery would have been higher if the ß-glucosidase activity in fractions 25 to 26 had not been lost (Figure 5.5). The loss of 79% of the ß-glucosidase activity in the first stage of the purification may be due in part to the fact that the decision to purify the ß-glucosidase activity was made after CMCases had been purified, and when the Phenyl-Sepharose column was run, only the fractions showing CMCase activity and which also contained the ß-glucosidase activity subsequently purified were collected. The fractions showing low CMCase activity and possibly containing further ß-glucosidase activity were not collected. Therefore, it is not known how much ß-glucosidase was discarded in the Phenyl-Sepharose chromatography. Recoveries would be greater if more ß-glucosidase had been collected from the Phenyl-Sepharose column.

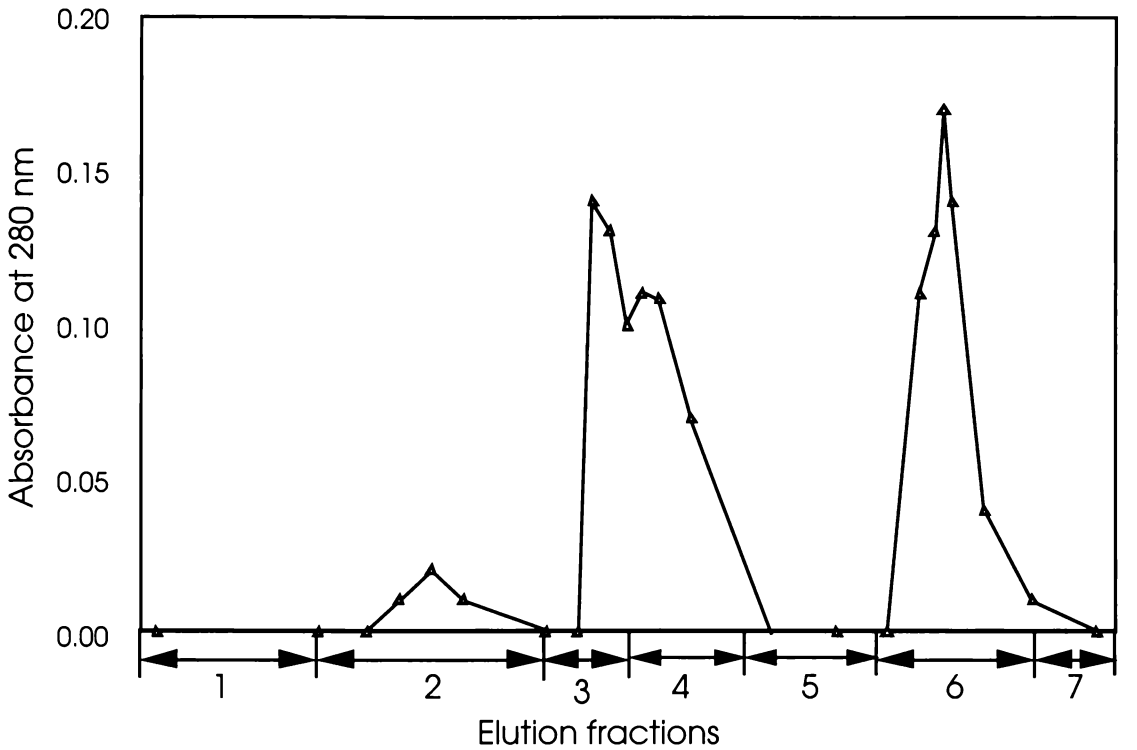


Figure 5.2 Phenyl-Sepharose chromatography of extracellular ß-glucosidase from strain COMP.A2. Elution conditions and ß-glucosidase activities of fractions are shown in Table 5.2.

Table 5.2 Elution conditions and ß-glucosidase activity of COMP.A2 eluted from a Phenyl-Sepharose column depicted in Figure 5.2.

Fraction No	Elution conditions and volume	ß-glucosidase activity (units)
1	0.5 M $(NH_4)_2SO_4$, 134 ml	0.0
2	0.25 M $(NH_4)_2SO_4$, 149 ml	1.38
3	20 mM MOPS (pH, 7.0), 80 ml	0.456
4	20 mM MOPS (pH, 7.0), 126 ml	151.2
5	20 mM MOPS (pH, 7.0), 64 ml	5.63
6	MilliQ water, 112 ml	4.03
7	MilliQ water, 44 ml	0.53

One unit of ß-glucosidase activity is defined as the amount of enzyme required to produce one micromole *p*-nitrophenol min^{-1}.

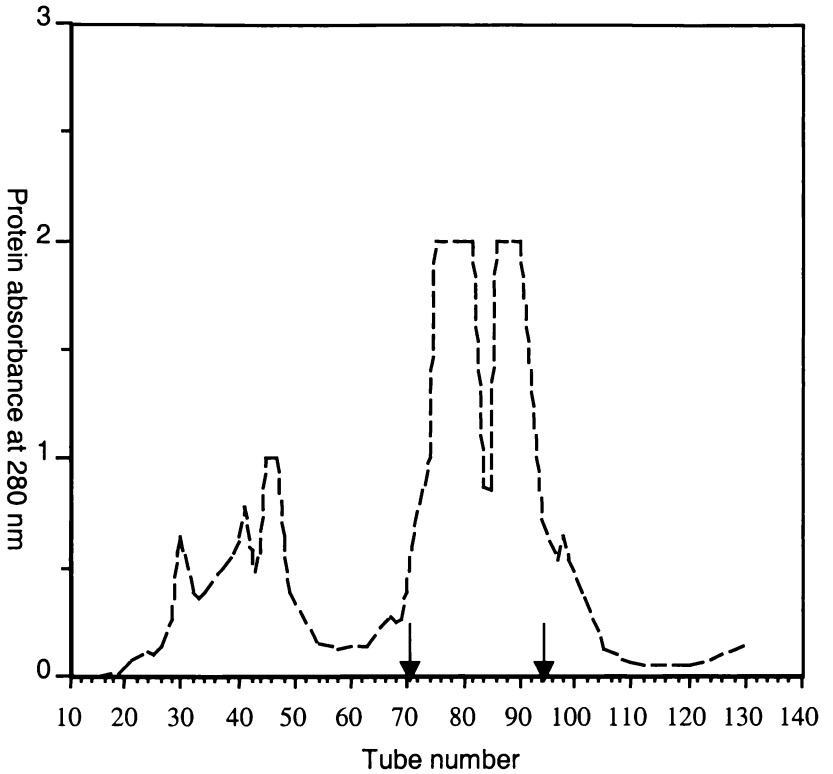


Figure 5.3 Anion exchange chromatography of ß-glucosidase activity fractions (fraction 3 to 5) obtained from Phenyl-Sepharose chromatography on an FPLC Mono Q column (10/10). Fraction 1 to 58, 0-0.1 M NaCl linear gradient; fraction 59-130, 0.1-0.7 M NaCl linear gradient. Fraction size, 1 ml. (---) absorbance at 280 nm. The ß-glucosidase activity (167.5 Units) is eluted from fraction 70 (closed arrow) and finished at fraction 95 (closed arrow).

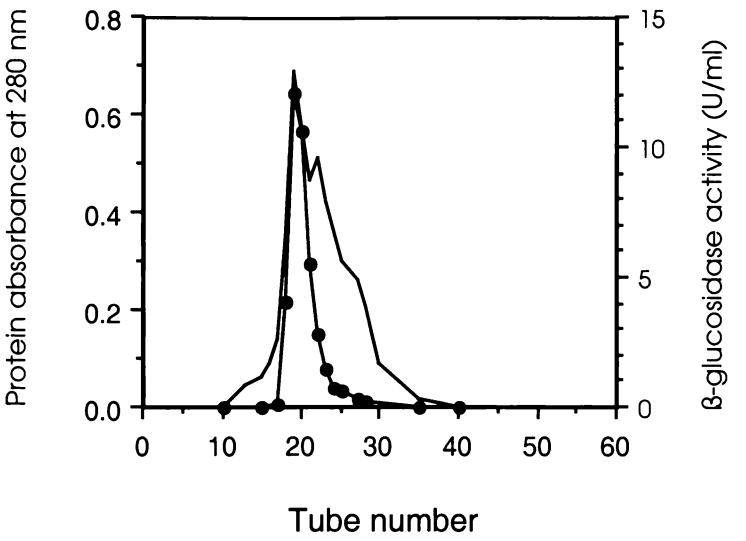


Figure 5.4 Anion exchange chromatography of ß-glucosidase activity fractions (fraction 70 to 95) obtained from a Mono Q column in Figure 5.3 on an FPLC Mono Q (10/10). fraction 1-5, 0-0.25 M NaCl linear gradient; fraction 5-60, 0.25-0.35 M NaCl linear gradient. Fraction size, 1.0 ml. (—) absorbance at 280 nm; (●) ß-glucosidase activity.

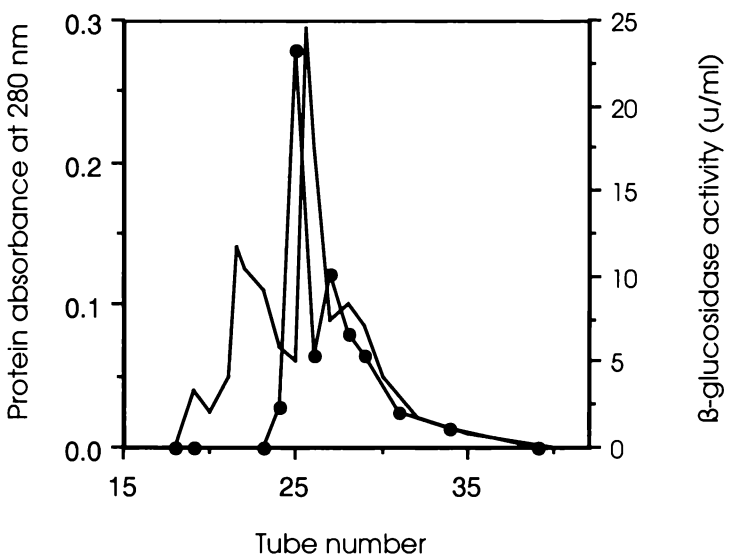


Figure 5.5 Chromatofocusing of ß-glucosidase activity fractions (fraction 18 to 22) obtained a Mono Q column in Figure 5.4 on a FPLC Mono P column. Fraction size, 1 ml. (—) absorbance at 280 nm; (●) ß-glucosidase activit

Table 5.3 Purification of ß-Glucosidase from strain COMP.A2

Purification step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Recovery (%)	Purification factor
culture supernatant	1657	2932	1.77	100	1
Phenyl-Sepharose	237	603	2.54	20.6	1.43
Diafiltration 1	103.2	250.3	2.42	8.5	1.37
FPLC Mono Q 1	62.7	174	2.78	5.9	1.57
Diafiltration 2	21.6	103.13	4.8	3.5	2.7
FPLC Mono Q 2	4.63	55.5	12.0	1.89	6.7
FPLC Mono Q 3	2.65	46.0	17.4	1.56	9.83
FPLC Mono P	0.14	19.0	136	0.65	76.8

One unit of ß-glucosidase activity is defined as the amount of enzyme required to produce one micromole *p*-nitrophenol min^{-1}. Assays were carried out using 2mM pNPG as substrate at pH 7.0 at 70°C.

5.3.3 Characterization of ß-glucosidase

5.3.3.1 Molecular weight

The purified ß-glucosidase (fraction 27 to 33 in Figure 5.5) showed a major protein band and two very faint protein bands on a Phast Gel (Gradient 10-15) after SDS-PAGE was run (Figure 5.6). The molecular weight of the major protein band was estimated to be 52.5 kDa from the molecular weight standard curve (Figure 5.7). In gel filtration on a BioSep-SEC-S3000 column, the enzyme eluted as a symmetrical peak with a retention volume corresponding to a molecular weight of about 537 kDa.

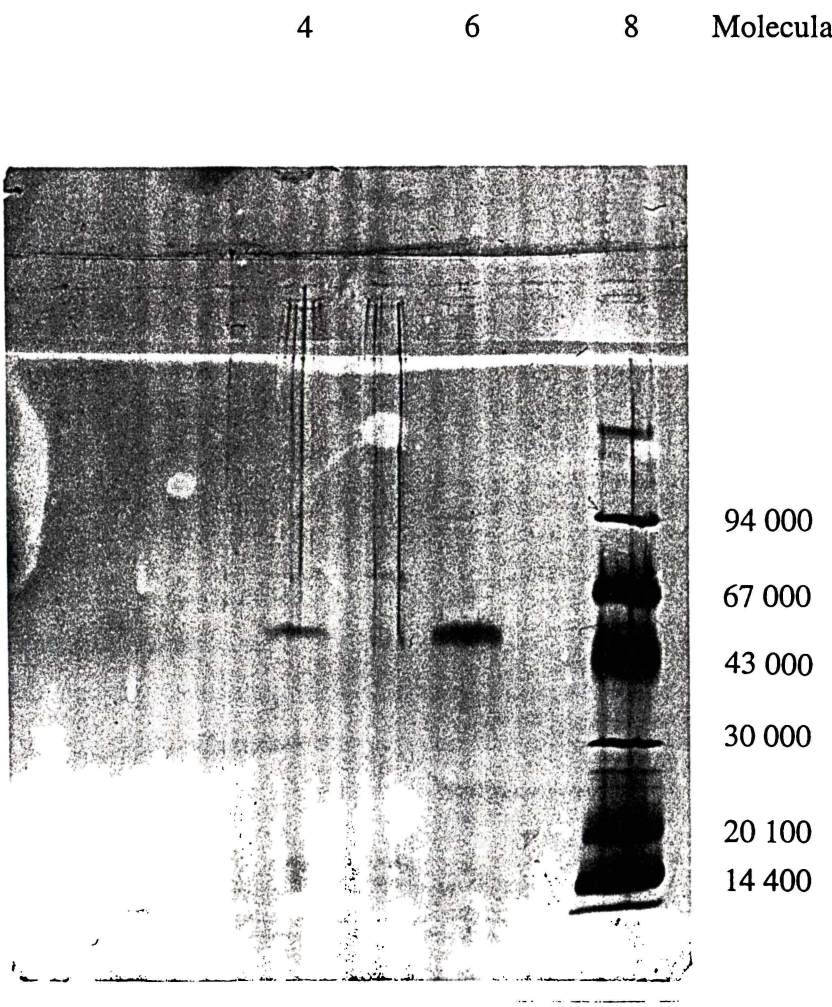
5.3.3.2 Native-PAGE and active stain

The purified ß-glucosidase was applied to two separate lanes of a Native PAGE Phastgel and run as described in section 5.2.6.2. After running, the gel was divided into two halves. One part was silver stained and the other was stained for ß-glucosidase activity using the overlay gel technique as described in section 5.2.6.2. A single yellow activity band on an overlay gel aligned with a single protein band on the native-PAGE gel. The result is not shown here due to the rapid diffusion of the yellow activity band on the overlay gel and difficulty encountered in photographing this image.

5.3.3.3 Isoelectric point

Isoelectric focussing of the purified ß-glucosidase gave a single protein band (Figure 5.8). The isoelectric point of this protein band was estimated to be 4.43 from the isoelectric focussing standards curve (Figure 5.9). The isoelectric point of ß-glucosidase from strain COMP.A2 is higher than those from other thermophilic bacteria reported by Patchett et al. (1987), Bronnenmeier and Staudenbauer (1988b), and Ruttersmith et al. (1993).

Figure 5.6 SDS-PAGE of ß-glucosidase from COMP.A2 using silver staining.
Lane 8: molecular weight standards (Pharmacia): Phosphorylase B, 94 000; BSA, 67 000; ovalbumin, 43 000; carbonic anhydrase, 30 000; soybean trypsin inhibitor, 20 100; lactalbumin, 14 400.
Lane 4 and 6: purified ß-glucosidase. Protein concentration is two-fold higher in lane 6 than lane 4.



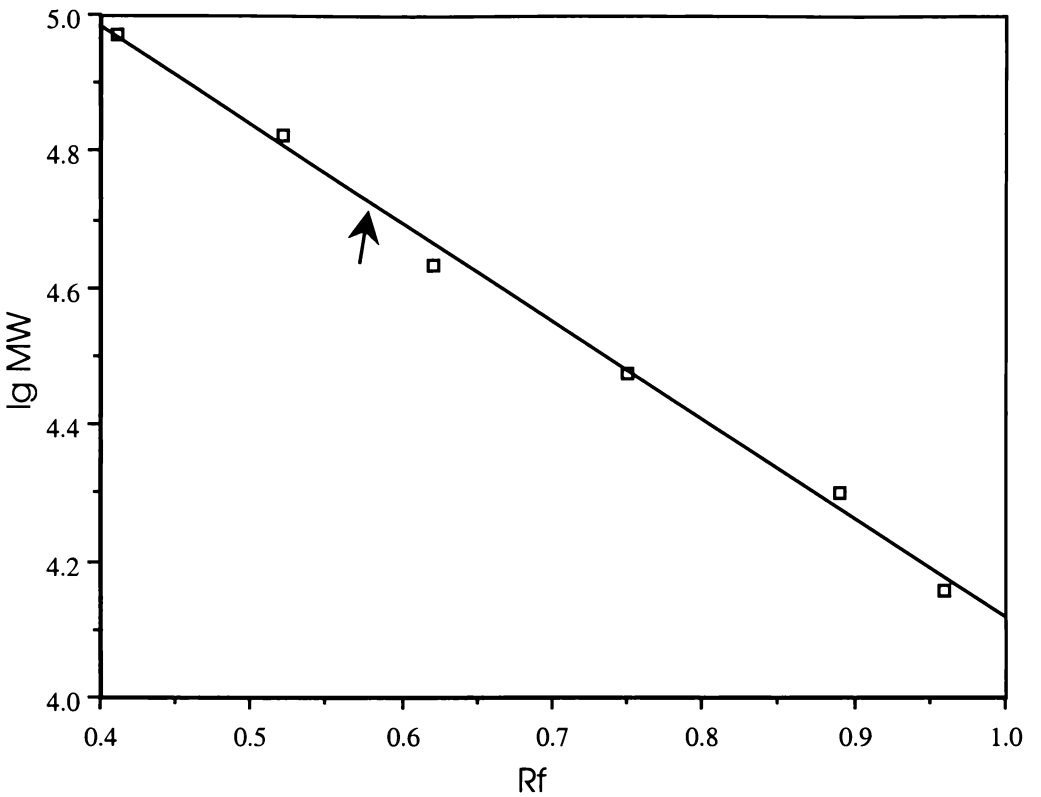
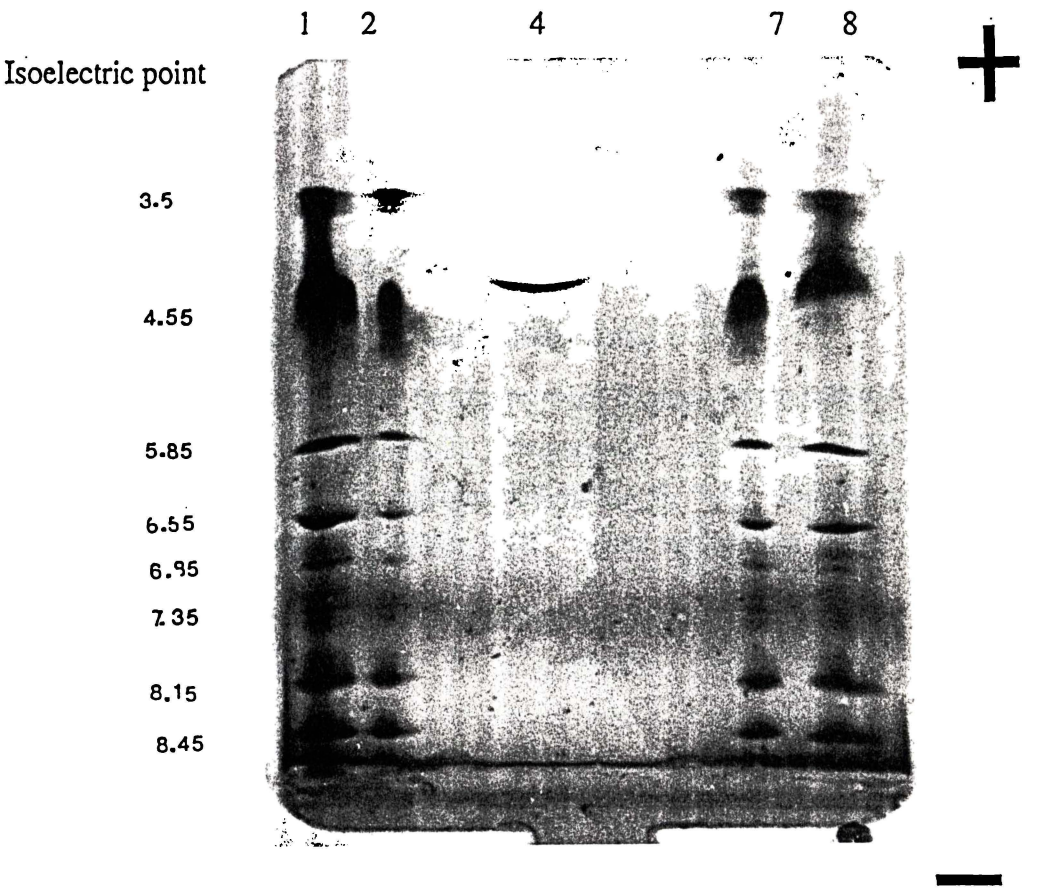


Figure 5.7 Molecular weight standard curve for SDS-PAGE of the purified ß-glucosidase (closed arrow).

Molecular weight standards (Pharmacia): Phosphorylase B, 94 000; BSA, 67 000; ovalbumin, 43 000; carbonic anhydrase, 30 000; soybean trypsin inhibitor, 20 100; lactalbumin, 14 400.

Figure 5.8 Isoelectric focusing of the purified ß-glucosidase on IEF 3-9 gel using silver staining. Lanes 1, 2, 7, and 8: isoelectric focussing standards (Pharmacia): amyloglucosidase, 3.5; soybean trypsin inhibitor, 4.55; bovine carbonic anhydrase B, 5.85; human carbonic anhydrase B, 6.55; horse myoglobin (acidic), 6.85; horse myoglobin (basic), 7.35; lentil lectin (acidic), 8.15; lentil lectin, 8.45.

Lane 4: the purified ß-glucosidase.



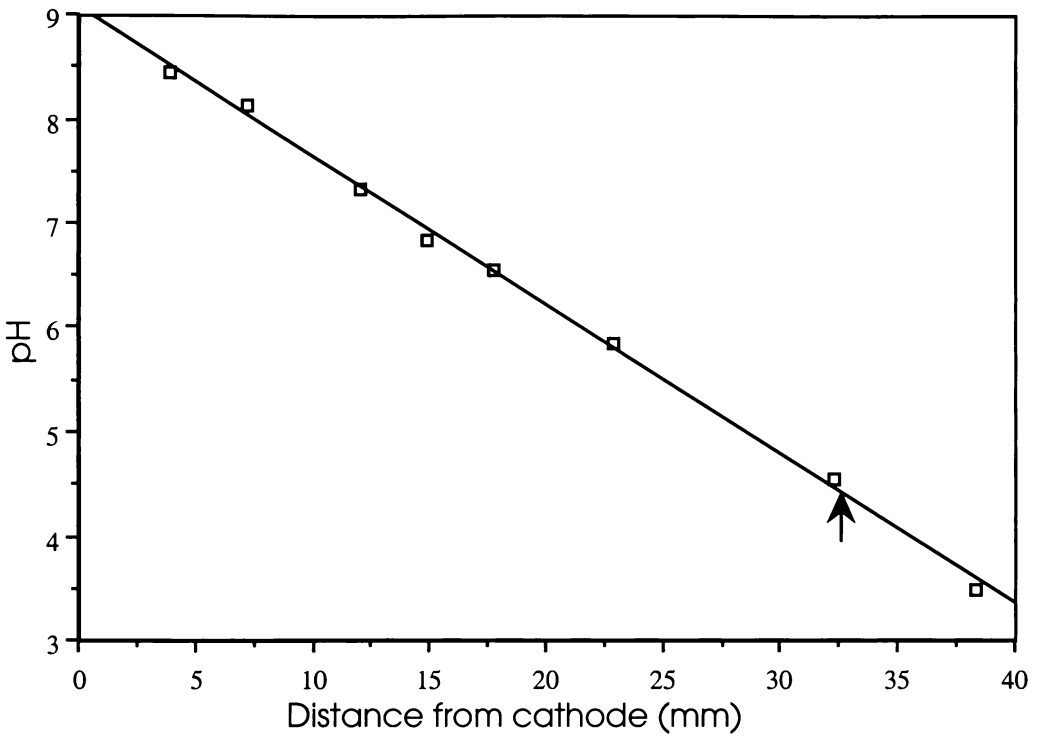


Figure 5.9 Isoelectric point standard curve for the purified ß-glucosidase (closed arrow) in a IEF gel. Isoelectric focussing standards (Pharmacia): amyloglucosidase, 3.5; soybean trypsin inhibitor, 4.55; bovine carbonic anhydrase B, 5.85; human carbonic anhydrase B, 6.55; horse myoglobin (acidic), 6.85; horse myoglobin (basic), 7.35; lentil lectin, 8.15; lentil lectin, 8.45.

5.3.3.4 Linearity of assay

A time course assay of the ß-glucosidase was linear for at least 20 minutes at 70°C (Figure 5.10). The assay time was usually 15 minutes in all assays during the ß-glucosidase purification and characterization. The ß-glucosidase activity was linear to 40 ng ß-glucosidase (Figure 5.11).

5.3.3.5 Effects of temperature and pH on ß-glucosidase activity

The purified ß-glucosidase was assayed for 15 min at various temperatures using 0.02 µg of enzyme protein. The result is shown in Figure 5.12. The enzyme showed maximum activity at 75°C, and retained 86% of its maximum activity at 80°C. Activity declined rapidly above 80°C. The Arrhenius plot was linear in the range 50°C to 75°C with pNPG as a substrate (Figure 5.13). An activation energy (Ea) of 35.4 kJ mol^{-1} was calculated from the slope (Smith et al., 1983). This value is lower than Ea values reported for ß-glucosidase of the extremely thermophilic anaerobic bacterium Wai21W.2 (Patchett et al., 1987) and *C. thermocellum* (Ait et al., 1982).

The activity of the ß-glucosidase was assayed for 15 minutes at various pH values at 70°C with 0.02 µg enzyme protein. The ß-glucosidase had a pH optimum between pH 5.5 and pH 6.5 and retained at least 50% of its maximum activity between pH 4.6 and pH 8.0 (Figure 5.14). The pH optima of bacterial ß-glucosidases are usually in the range 5.5-7.0. The pH optimum of ß-glucosidases from strain COMP.A2 was comparable to that of ß-glucosidases from *C. thermocellum* (Ait et al., 1982) and *Caldicellulosiruptor saccharolyticus* (Love and Streiff, 1987). Figure 5.14 shows that acetate and MOPS buffers display little effect on enzyme activity. Bis-Tris-propane buffers showed some inhibitory effects. The enzyme activity was strongly inhibited by Tris buffer at a concentration of 100 mM. The inhibition by Tris buffer has been reported in other ß-glucosidases, and was possibly due to changes in conformation or charge distribution (Ait et al., 1982; Patchett et al., 1987).

5.3.3.6 Thermal stability of ß-glucosidase

The thermal stability of the purified ß-glucosidase activity was determined at temperatures of 70°C and 80°C at pH 7.0 using 2 mM pNPG as the substrate. Results are shown in Figure 5.15. The plot of time versus the log of residual activity at 80°C is also shown in Figure 5.16. The estimated half-lives of ß-glucosidase in the presence and absence of additives at 70°C and 80°C are given in Table 5.4. Trehalose greatly increased the thermostability of ß-glucosidase. The half-life of ß-glucosidase was 24

minutes at 80°C and 11.75 hours at 70°C in the presence of trehalose, compared to less than 5 minutes at 80°C and 2.5 hours at 70°C in the absence of trehalose. The addition of BSA only showed small improvement in thermostability of ß-glucosidase from strain COMP.A2.

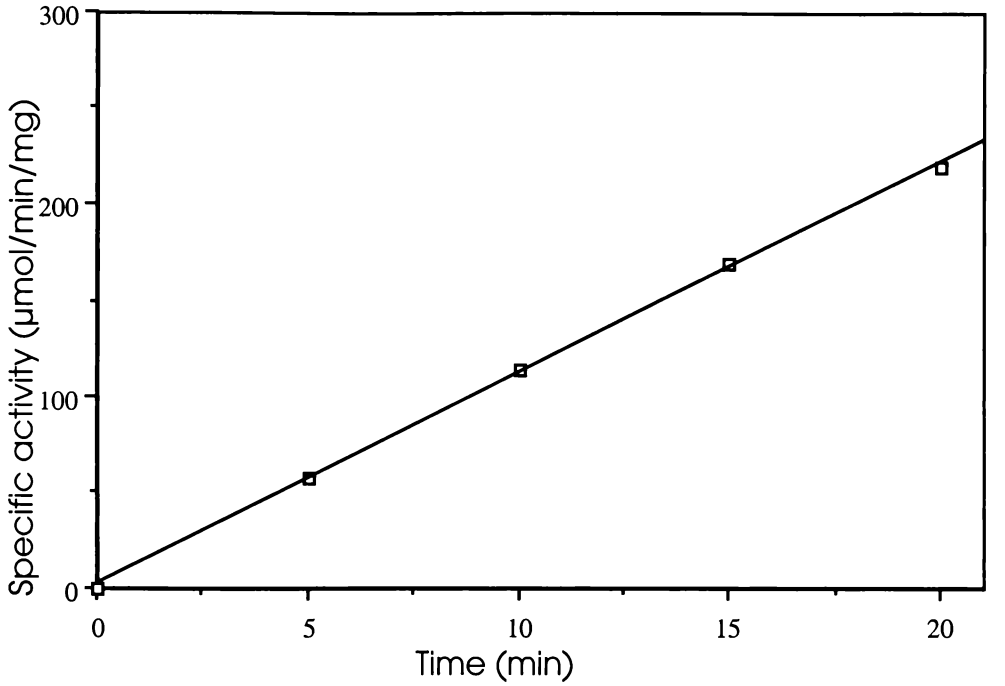


Figure 5.10 Time-course of ß-glucosidase assay. The activity of ß-glucosidase was determined with 2 mM pNPG in 50 mM MOPS buffer, pH 7.0 and 70°C. 25 ng of ß-glucosidase was used in each assay.

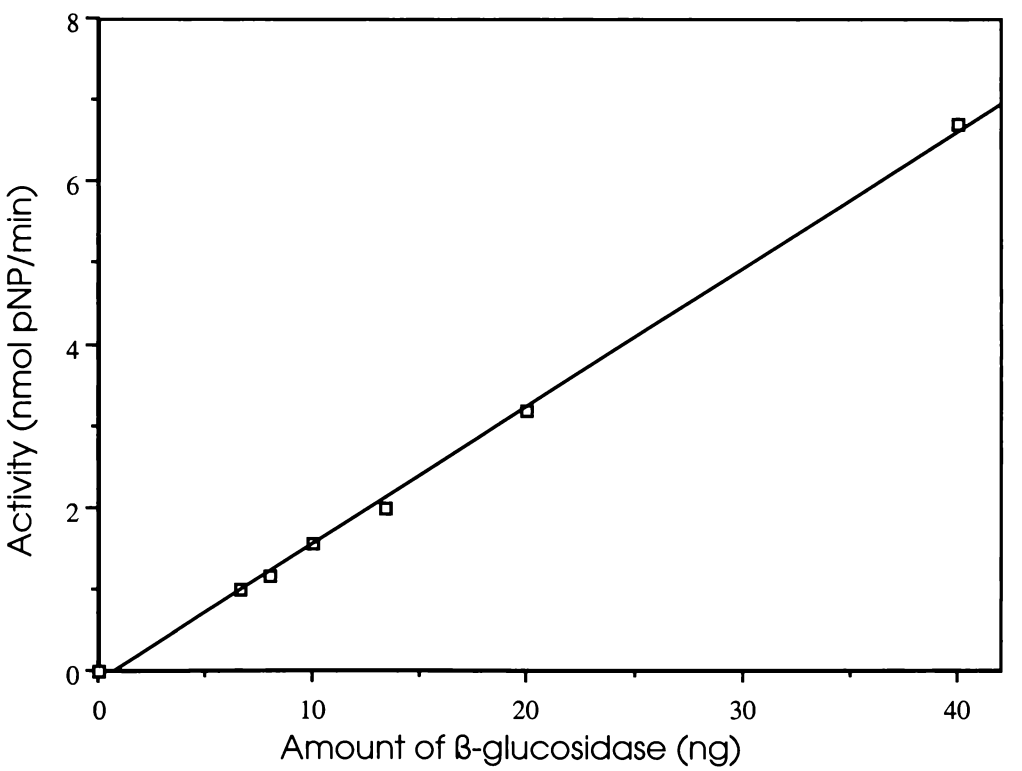


Figure 5.11 The ß-glucosidase activity versus the amount of ß-glucosidase enzyme. The activity was determined with 2 mM pNPG in 50 mM MOPS buffer, pH 7.0 and 70°C for 15 min.

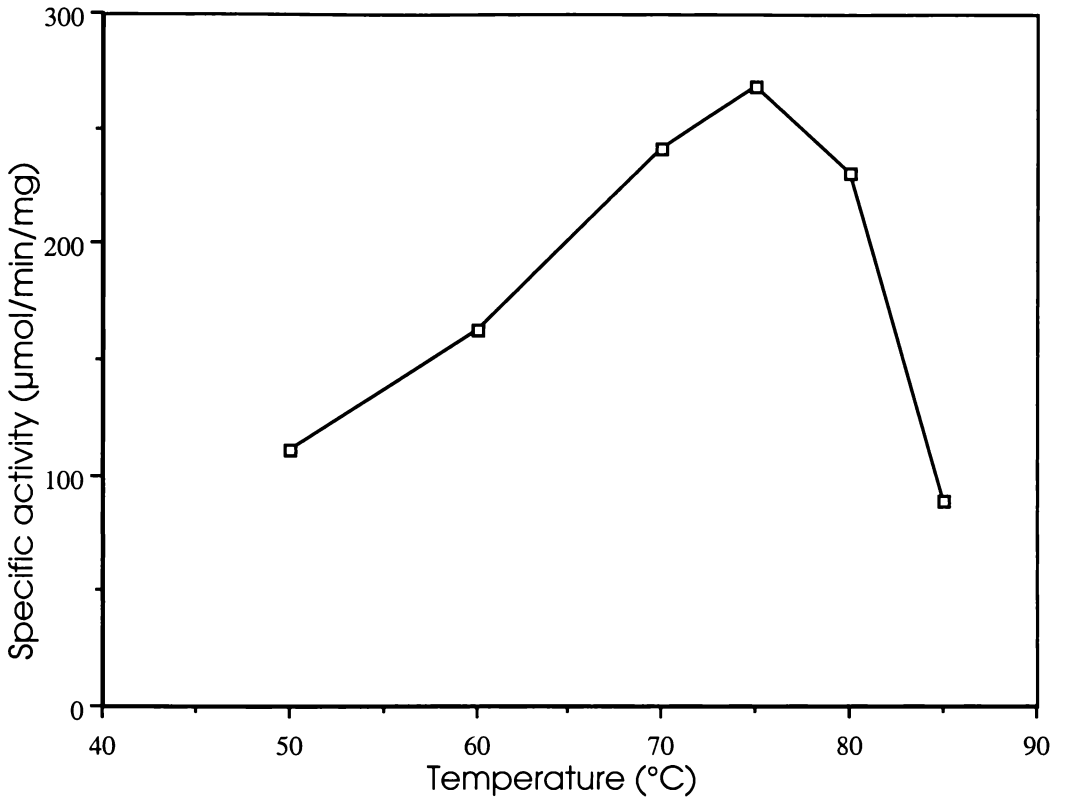


Figure 5.12 Effect of temperature on activity. The temperature profile of ß-glucosidase activity was determined with 2 mM pNPG with a 15 min assays in 50 mM MOPS buffer, pH 7.0.

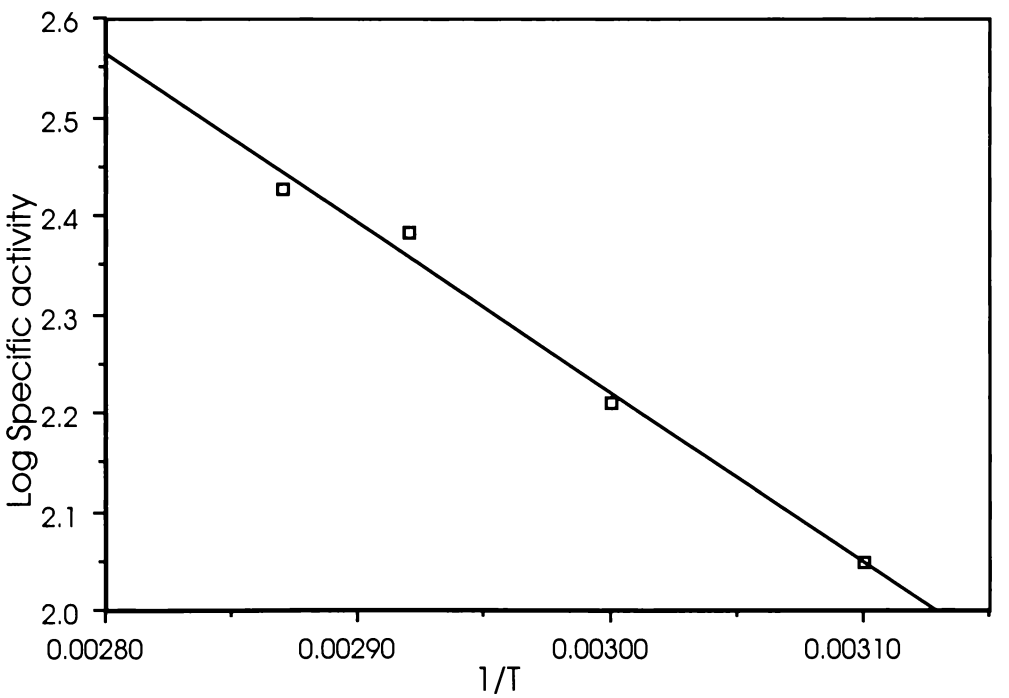


Figure 5.13 Arrhenius plot for ß-glucosidase from 50°C to 75°C.

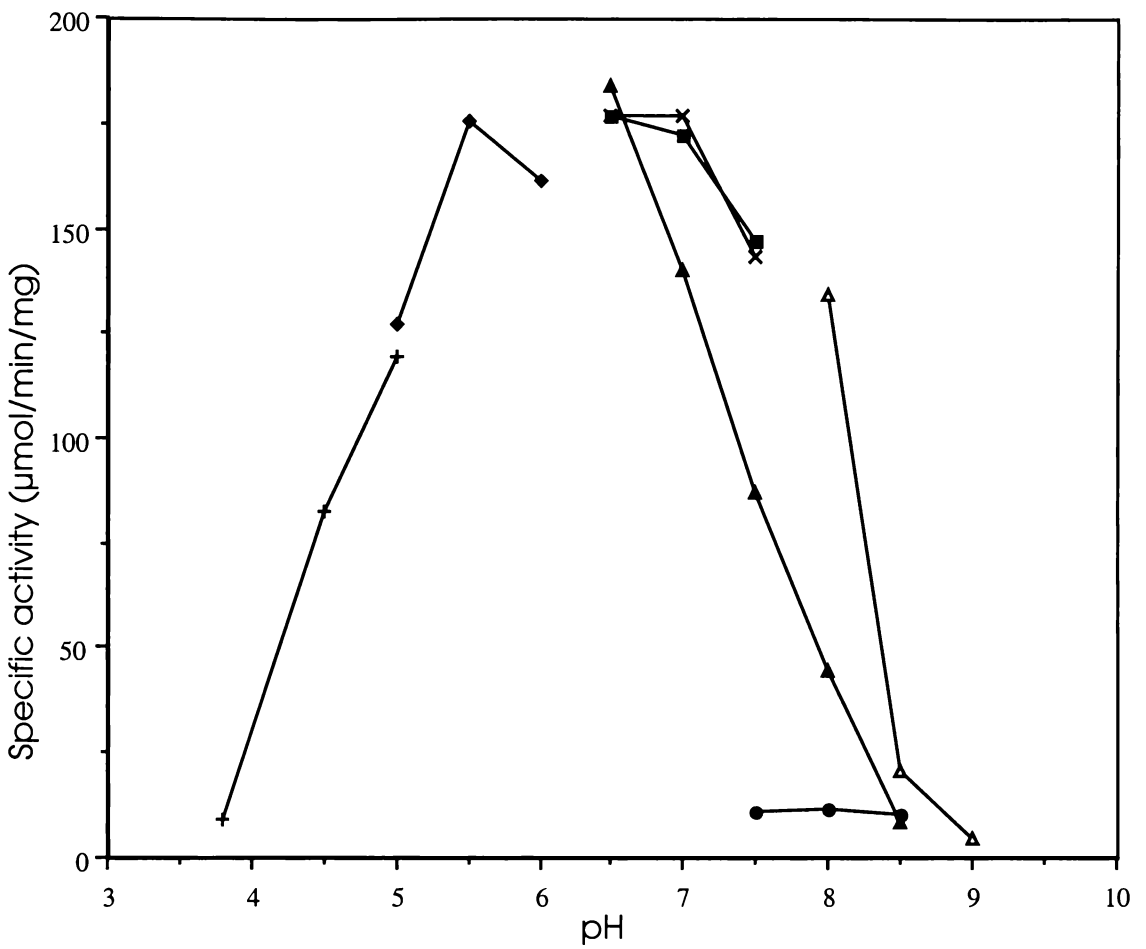
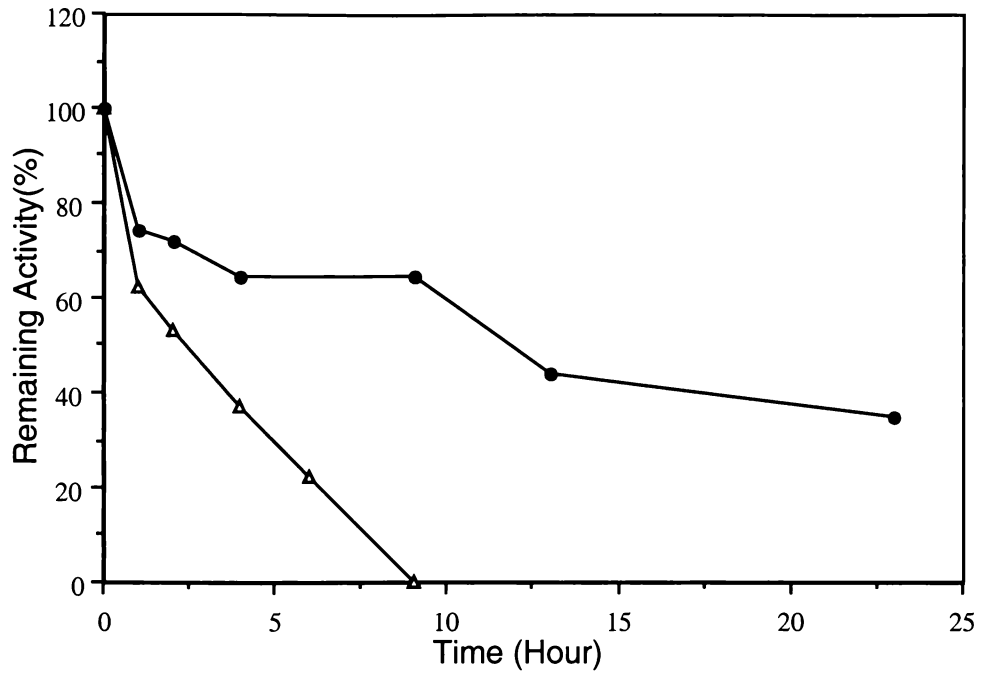


Figure 5.14 pH-activity profile for ß-glucosidase of strain COMP.A2. Assays were determined with 2 mM pNPG in 0.1 M sodium acetate (+), 0.1 M sodium citrate (◆), 0.1 M HEPES (■), 0.1 M MOPS (×), 0.1 M Bis-Tris-propane (▲), 0.1 M CHES (Δ), 0.1 M Tris (●) at 70°C.

a:



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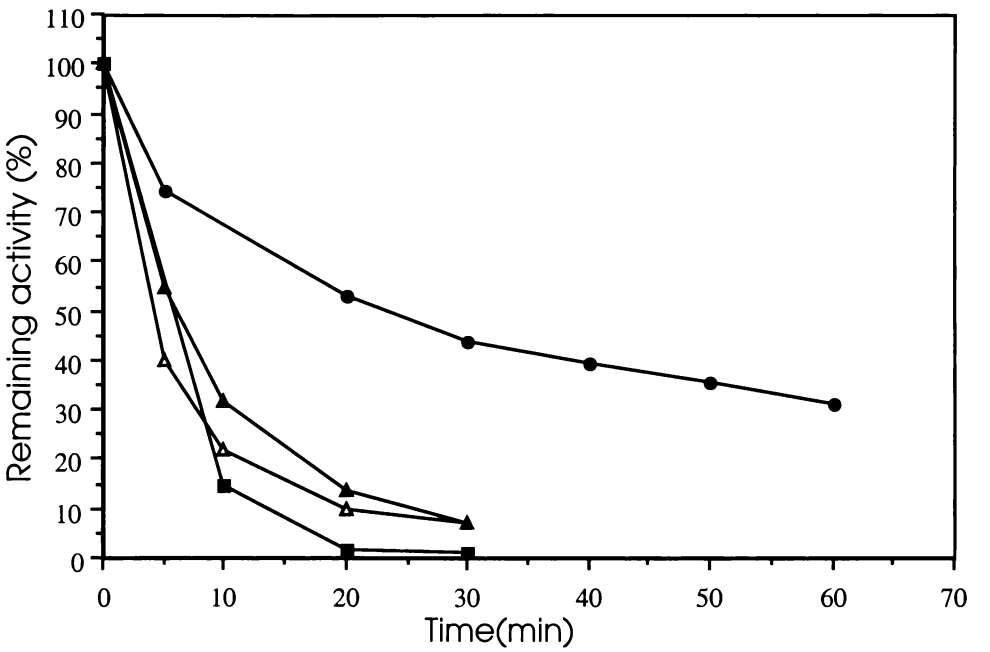


Figure 5.15 a: Thermal stability of ß-glucosidase of strain COMP.A2 at 70°C. (●), addition of 12% trehalose; (Δ), no additive. **b**: thermal stability of ß-glucosidase at 80°C. (●), addition of 12% trehalose; (▲), addition of BSA (0.8 mg/ml); (■), addition of 12% betaine; (Δ), no additive.

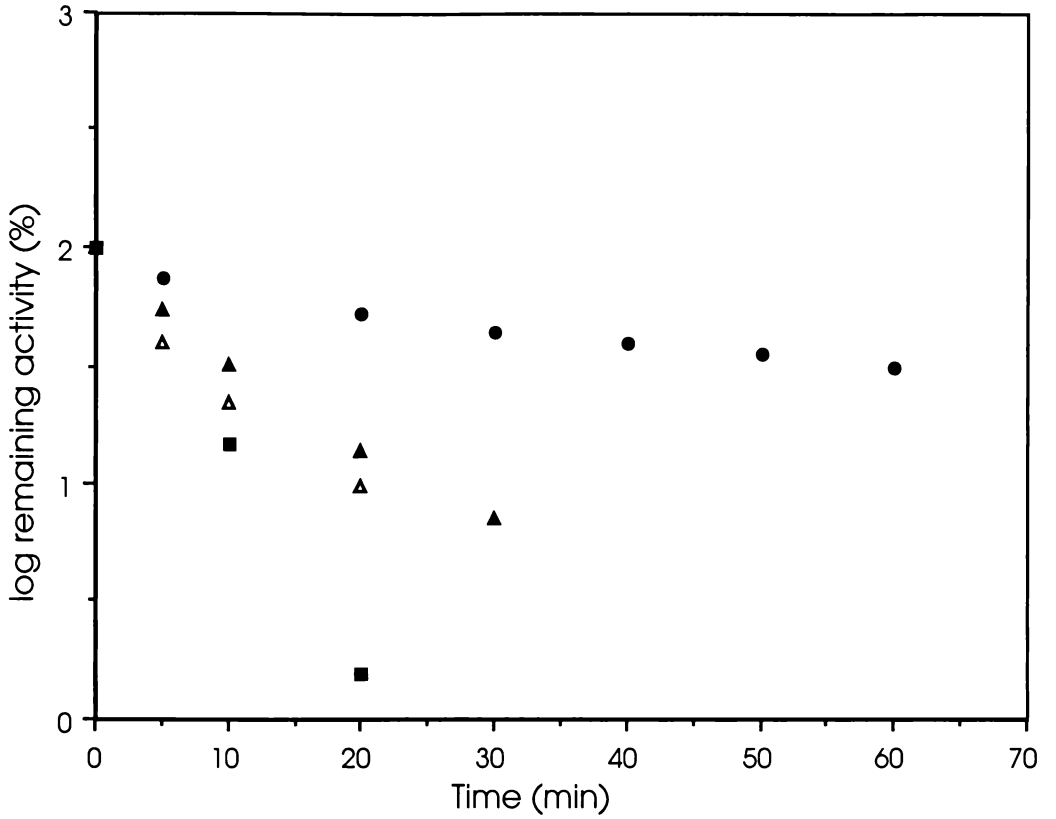


Figure 5.16 Log of residual ß-glucosidase activity of strain COMP.A2 at 80°C versus time. (●), addition of 12% trehalose; (▲), addition of BSA (0.8 mg/ml); (■), addition of 12% betaine; (Δ), no additive.

Table 5.4 Thermostability of the ß-glucosidase from strain COMP.A2.

Incubation conditions	t 1/2	Temperature (°C)
50 mM-MOPS,pH 7.0	2.5 hrs	70
50 mM-MOPS,pH 7.0		
+12% trehalose	11.75 hrs	70
50 mM-MOPS,pH 7.0	<5 min	80
50 mM-MOPS pH 7.0		
+12% betaine	<5 min	80
+0.8 mg BSA/ml	6 min	80
+12% trehalose	24 min	80

The ß-glucosidase activity was assayed using 2 mM pNPG as substrate. 40 ng of enzyme was used in each assay.

5.3.3.7 Effect of chemical reagents on ß-glucosidase activity

The effects of various chemical reagents on ß-glucosidase activity is shown in Table 5.5. It appears that metal ions were not required for the enzyme activity as EDTA and Ca^{2+} did not change activity very much. The ß-glucosidase activity was completely inhibited by Cd^{2+}, Hg^{2+}, Zn^{2+} and Ag^{+} at a concentration of 8 mM, or 2 mM *p*-chloromercuriphenylsulphonic acid (pCMPS). The enzyme activity was strongly inhibited by SDS and Cu^{2+}. The reducing agent dithiothreitol (DTT) and ß-mercaptoethanol activated the enzyme activity slightly, whereas the thiol-specific inhibitor pCMPS strongly inhibited the activity. The non-ionic detergent Triton X-100 slightly stimulated the enzyme activity.

ß-Glucosidase activity was assayed in the presence and absence of D-glucono-1,5-lactone (Sigma, St. Louis, USA) using cellobiose or pNPG as substrates at 70°C and pH 7.0. The activity was inhibited 95% in the presence of 25 mM gluconolactone with 2 mM pNPG as substrate. Inhibition of 94% and 88% was observed with 12.5 and 25 mM cellobiose using 25 mM gluconolactone. The inhibition decreased by a factor of two as cellobiose concentration doubled. This indicated that the purified ß-glucosidase was competitively inhibited by gluconolactone.

5.3.3.8 Kinetic characterisation of the ß-glucosidase

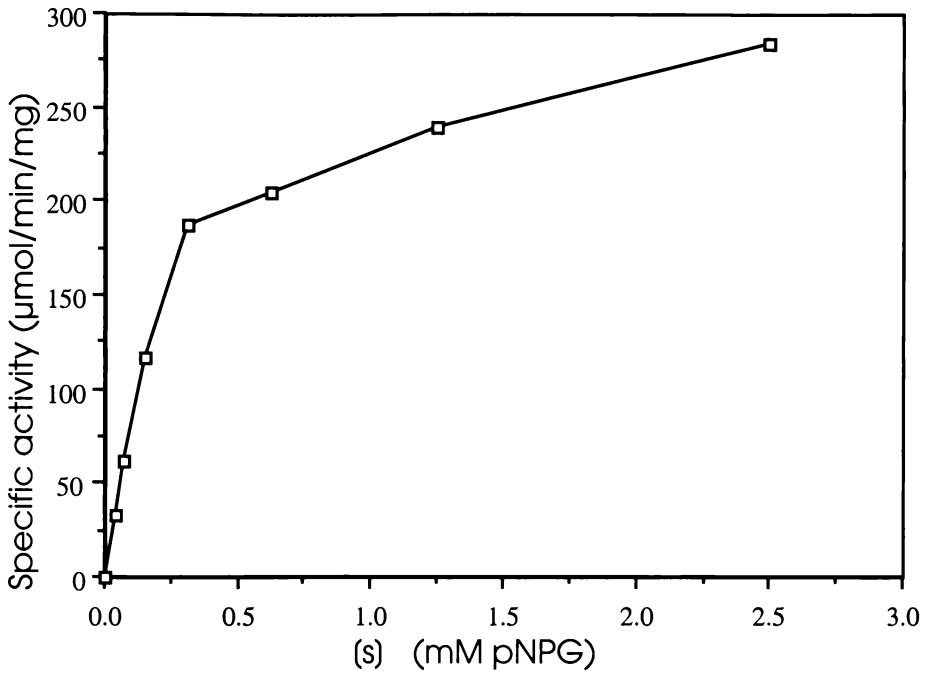
Michaelis-Menten kinetics were observed using pNPG, cellobiose and sophorose as substrates, and the data plotted in the Lineweaver-Burk, Hanes and Woolf forms (Figure 5.17 to Figure 5.22). Linearity was observed for Lineweaver-Burk, Hanes and Woolf plots. Similar K_m and V_{max} values were obtained for each of the substrates from these plots (Table 5.6).

Table 5.5 Effect of chemical reagents on activity of ß-glucosidase.

Chemical reagents	Relative activity of ß-glucosidase (%)
none	100
8 mM $CaCl_2$	79.3
8 mM $SrCl_2$	95.2
8 mM NaCl	98.2
8 mM $CoCl_2$	74.3
8 mM $NiCl_2$	56.0
8 mM $CuCl_2$	6.28
8 mM $ZnCl_2$	2.0
8 mM $CdCl_2$	0.0
8 mM $HgCl_2$	0.0
8 mM AgCl	0.0
8 mM EDTA	73.9
8% ethanol	102.9
8% glycerol	118.6
2.0% Triton-x-100	110.1
2.0% SDS	3.7
8 mM DTT	111.6
8 mM ß-mercaptethanol	109.4
2 mM pCMPS	0.0

100% ß-glucosidase activity is equivalent to 233.55 µmol *p*-NP min^{-1} mg^{-1}. Activity was assayed at 70°C and pH 7.0.

a:



b:

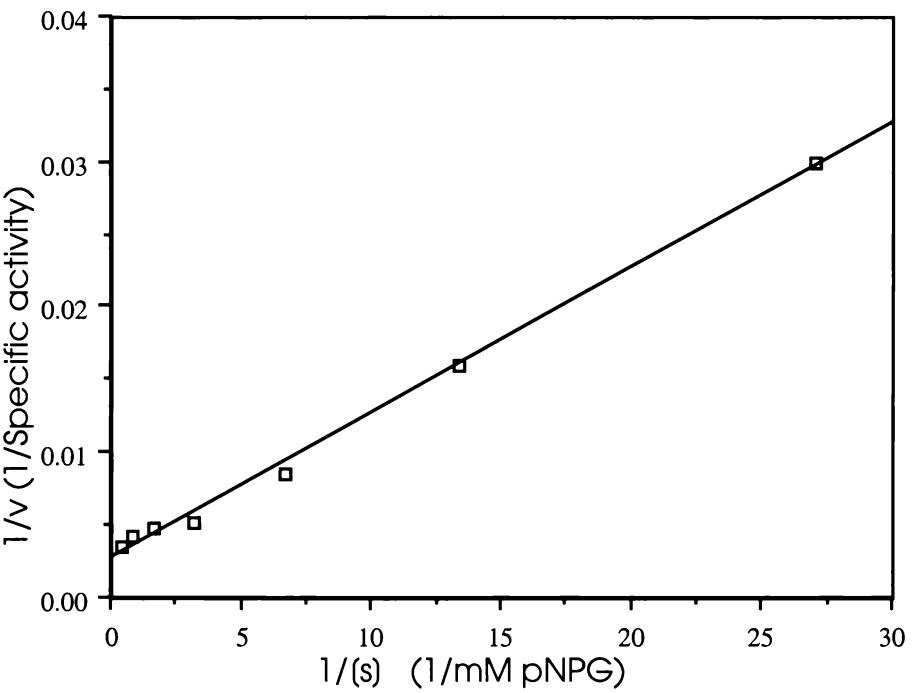
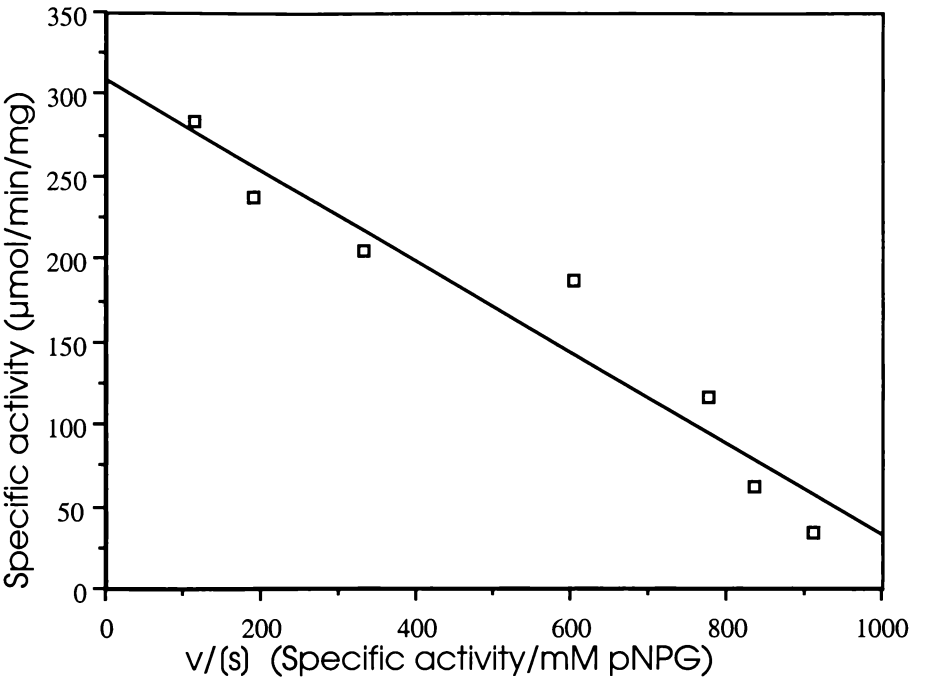


Figure 5.17 a: Michaelis-Menten plot of ß-glucosidase activity with pNPG as substrate at 70°C. **b**: Lineweaver-Burk plot of ß-glucosidase activity with pNPG as substrate at 70°C.

a:



b:

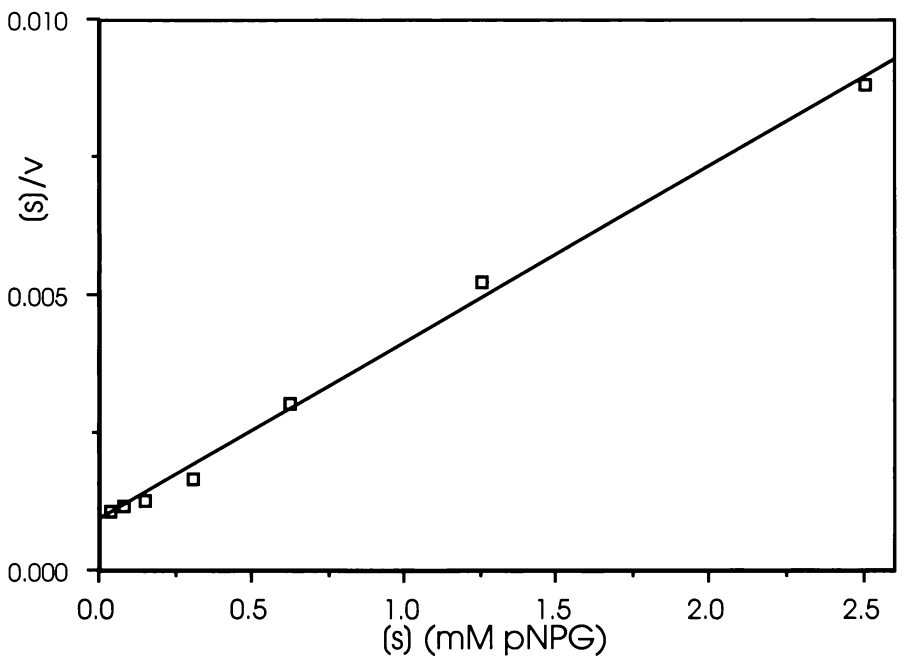
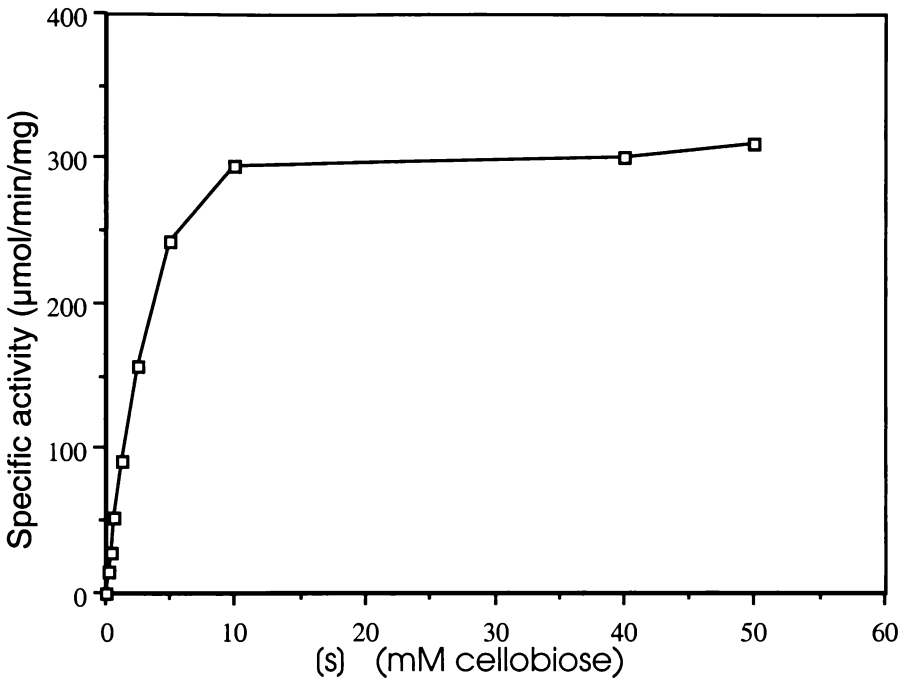


Figure 5.18 a: Woolf plot of ß-glucosidase activity with pNPG as substrate at 70°C. **b**: Hanes plot of ß-glucosidase activity with pNPG as substrate at 70°C.

a:



b:

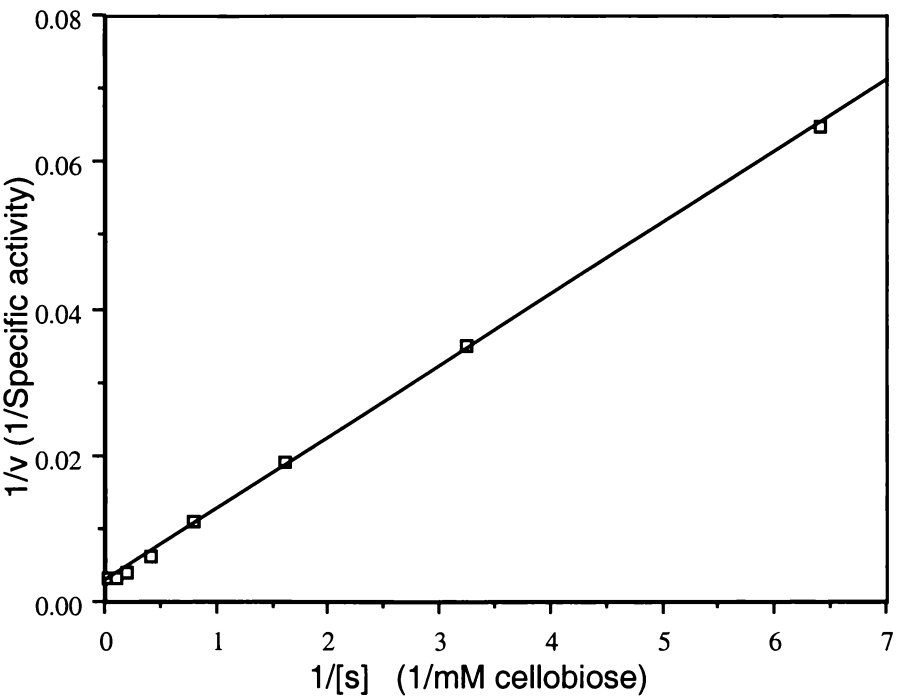
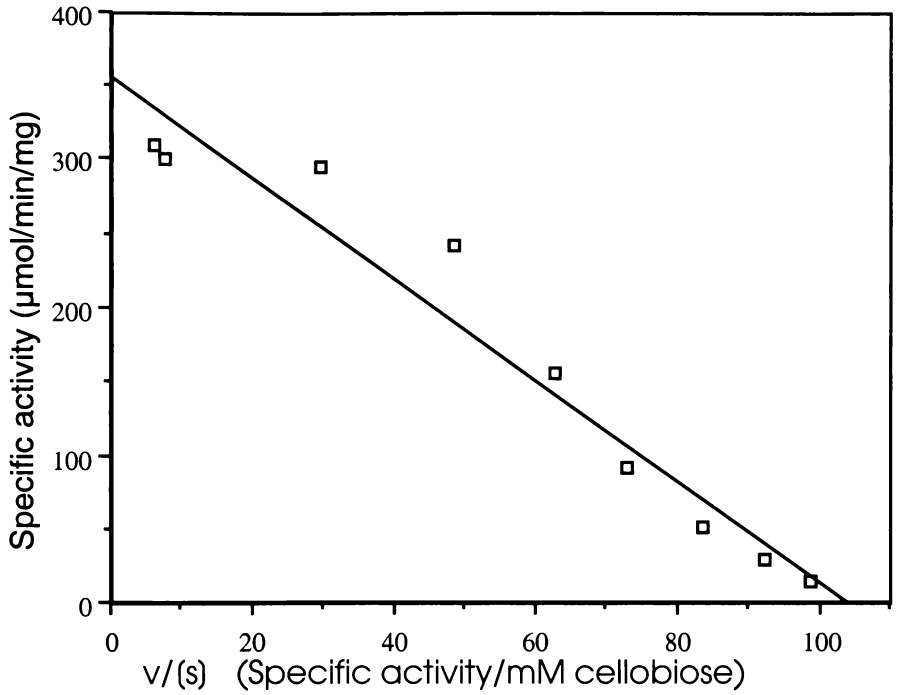


Figure 5.19 a: Michaelis-Menten plot of ß-glucosidase activity with cellobiose as substrate at 70°C. **b**: Lineweaver-Burk plot of ß-glucosidase activity with cellobiose as substrate at 70°C.

a:



b:

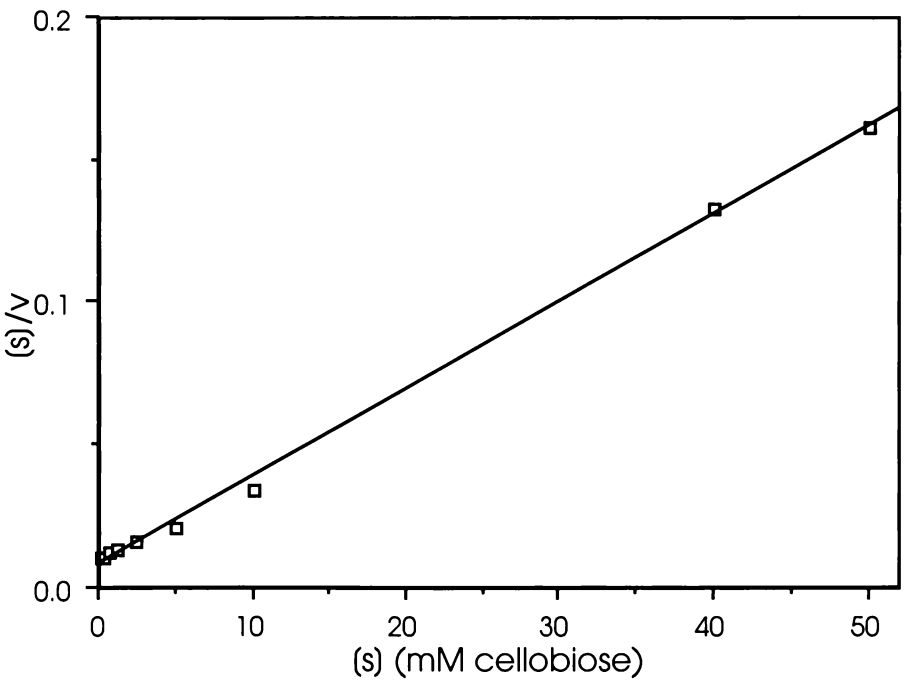
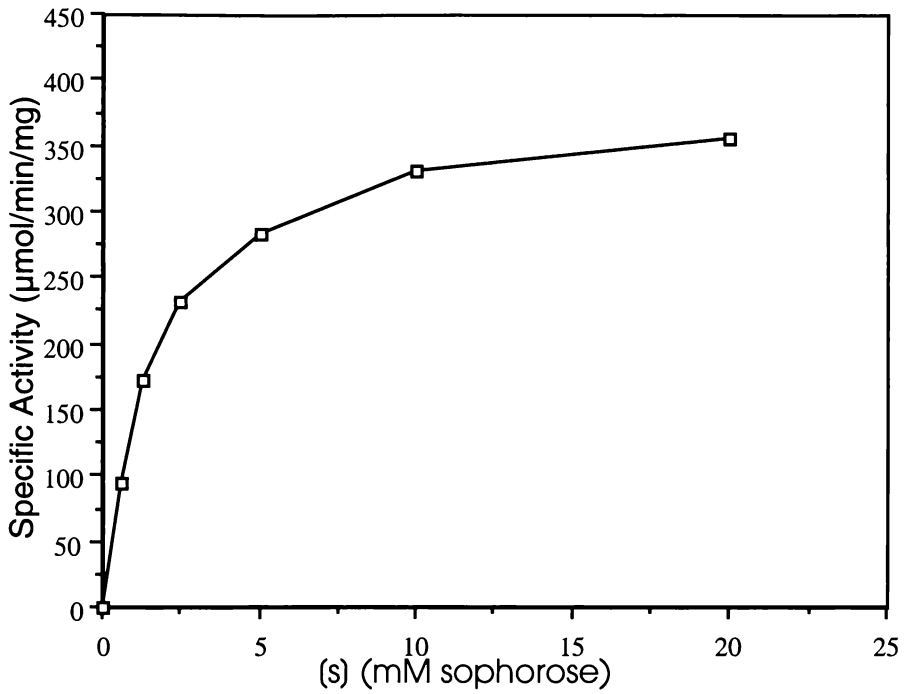


Figure 5.20 a: Woolf plot of ß-glucosidase activity with cellobiose as substrate at 70°C.
b: Hanes plot of ß-glucosidase activity with cellobiose as substrate at 70°C.

a:



b:

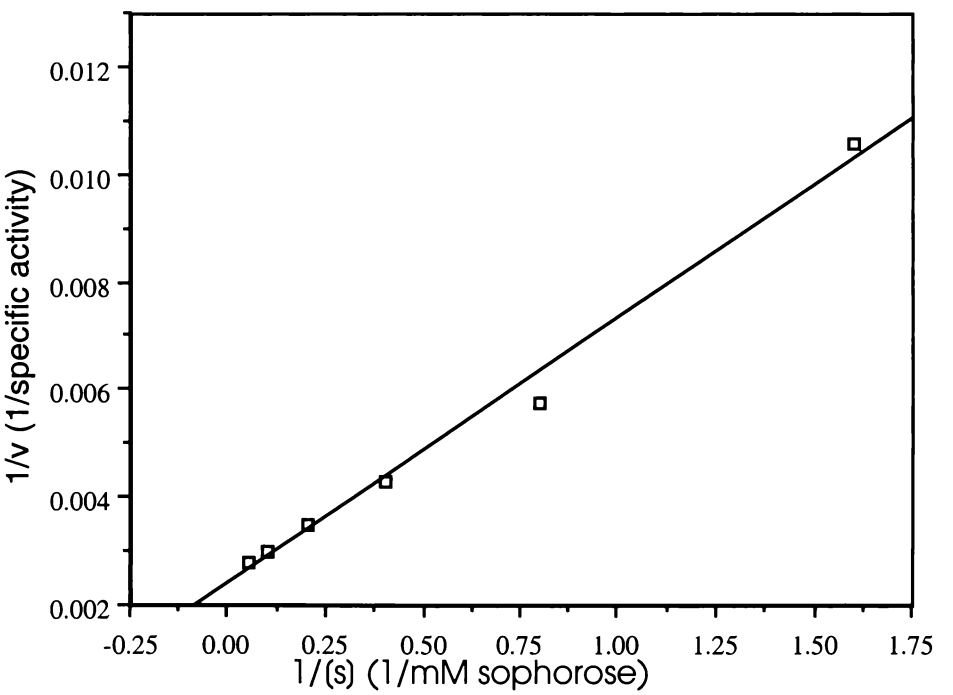
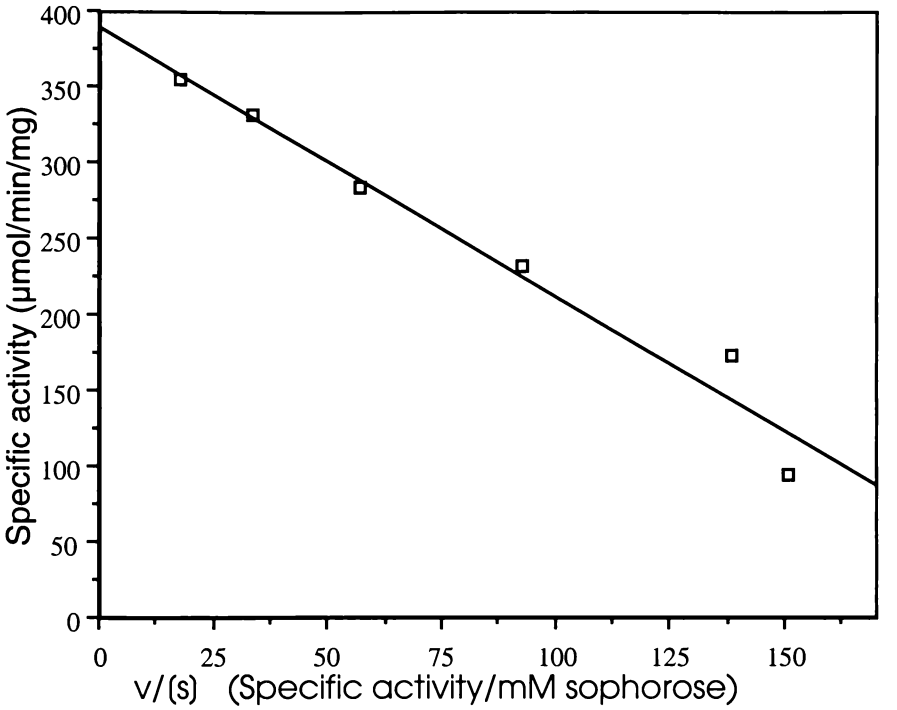


Figure 5.21 a: Michaelis-Menten plot of ß-glucosidase activity with sophorose as substrate at 70°C. **b**: Lineweaver-Burk plot of ß-glucosidase activity with sophorose as substrate at 70°C.

a:



b:

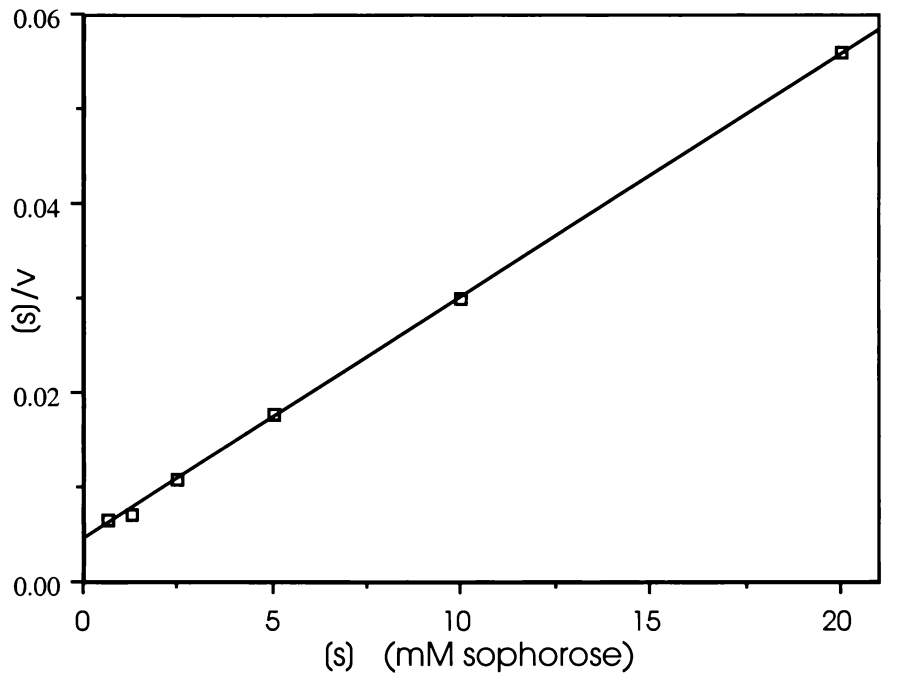


Figure 5.22 a: Woolf plot of ß-glucosidase activity with sophorose as substrate at 70°C.
b: Hanes plot of ß-glucosidase activity with sophorose as substrate at 70°C.

Table 5.6. Kinetic data for the ß-glucosidase.

Substrates	Lineweaver-Burk Plot	Hanes Plot	Woolf Plot
pNPG (range 0.037-2.5 mM)			
K_m (mM)	0.356	0.283	0.276
V_{max} (µmol min^{-1} mg^{-1})	355.9	311.3	309.22
cellobiose (range 0.156-50 mM)			
K_m (mM)	3.44	2.7	3.42
V_{max} (µmol min^{-1} mg^{-1})	352.49	326.3	356.27
sophorose (range 0.625-20 mM)			
K_m (mM)	2.05	1.74	1.77
V_{max} (µmol min^{-1} mg^{-1})	414.3	388.8	389.76

5.3.3.9 Substrate specificity

The relative activities of the purified ß-glucosidase on various substrates are shown in Table 5.7. The enzyme has significant activity on cellobiose and pNPG. The K_m values (Table 5.6) indicate that the enzyme has a higher affinity for the artificial substrate pNPG than sophorose and cellobiose. The purified ß-glucosidase was capable of hydrolyzing the ß-linked glucose dimers, sophorose (ß-1,2), cellobiose (ß-1,4), and laminarin (ß-1,3). Using the V_{max}/K_m values in Table 5.6 to determine the preferred substrate, the enzyme prefers sophorose (ß-1,2) to cellobiose (ß-1,4). The absence of activity on gentiobiose suggests that the enzyme is unable to cleave ß-1,6-linkages. The lack of activity on maltose and trehalose indicated that the enzyme was unable to hydrolyze α-linked glucosides. Low activity on lactose (galactose-ß-1,4-glucose) was observed, suggesting that the enzyme had the ability to hydrolyze glucose at the reducing end of the substrate. The activity on D-type fucoside, but not on L-type fucoside, showed that the enzyme required a D-type configuration for activity. The enzyme had no activity on CMC or xylan.

Table 5.7. Substrate specificity of ß-glucosidase.

Substrates	ß-glucosidase activity (%)	Amount of enzyme in assay (ng/ml)
1.6mM *p* NP-ß-D glucopyranoside	100	40
1.6mM *p* NP-ß-D-fucopyranoside	86.4	40
1.6mM *p* NP-ß-L-fucopyranoside	0.0	40
1.6mM *p* NP-ß-D-cellobioside	7.0	200
1.6mM *p* NP-ß-L-arabinopyranoside	4.3	400
1.6mM *p* NP-α-D-glucopyranoside	0.69	400
1.6mM *p* NP-ß-D-maltoside	0.0	400
1.6mM *p* NP-ß-D-mannopyranoside	0.09	400
1.6mM *o* NP-ß-D-galactopyranoside	18.6	40
1.6mM *o* NP-ß-D-xylopyranoside	0.31	400
4.76mM cellobiose (ß-1,4)	100	950
4.76mM sophorose (ß-1,2)	110	950
4.76mM lactose	5.5	950
1.9% laminarin (ß-1,3)	1.42	950
4.76mM gentibiose (ß-1,6)	0.0	950
4.76mM trehalose (α-1,1)	0.0	950
4.76mM maltose (α-1,4)	0.0	950
4.76mM sucrose	0.0	950
0.25% xylan	0.0	950
1.9% CMC	0.0	950

Relative rates were determined using artificial substrates at a concentration of 1.6 mM and natural substrate at a concentration of 4.76 mM except for laminarin (1.9%), xylan (0.25% filtered oat spelts xylan) and 1.9% CMC. 100% pNPGase activity is equivalent to 255.2 µmol *p*-NP min^{-1} mg^{-1}. 100% cellobiase activity is equivalent to 214 µmol glucose min^{-1} mg^{-1}.

5.4 DISCUSSION

The ß-glucosidase of strain COMP.A2 is a cell-associated enzyme (Table 5.1). More ß-glucosidase activity from strain COMP.A2 was released into medium as culture density increased following growth. Many ß-glucosidases have been reported to be cell-associated (Patchett et al., 1987; Heupel et al., 1993; Ruttersmith and Daniel, 1993). The ß-glucosidase of *C. stercorarium* and *C. thermocellum* was found to be mainly located in the periplasmic space (Bronnenmeier and Staudenbauer, 1988b; Ait et al., 1979). The localization of ß-glucosidases at the surface of cells could help to hydrolyze cellobiose which may inhibit cellulase activity.

A single band of the purified ß-glucosidase on an isoelectric focusing gel and one activity band on the overlay gel are good indication of homogeneity of the purified enzymes. However a major protein band and two very faint protein bands on a SDS-PAGE gel suggest that the purified enzyme may still contain minor non-enzymatic protein. The effect of minor non-enzymatic protein on enzyme characterization should be small because only small amount of the purified enzyme (80 or 100 fold dilution) was used in characterization.

The molecular weight of the purified ß-glucosidase was 52.5 kDa and 537 kDa respectively as determined by SDS-PAGE and gel filtration. These results suggest that the native form of the ß-glucosidase from strain COMP.A2 presumably represents the decemer forms of the enzyme. This is quite unusual as most ß-glucosidases from thermophilic bacteria have been reported to be monomers (Patchett et al., 1987; Bronnenmeier and Staudenbauer, 1988b; Ait et al., 1979; Takase and Horikoshi, 1989). There are only a few reports of oligomeric forms of ß-glucosidases which include a dimer from a hyperthermophilic bacterium *Thermotoga maritime* (Gabelsberger et al., 1993) and tetramers from an extreme thermophile *Sulfolobus solfataricus* (Nucci et al., 1993) and a hyperthermophile *Pyrococcus furiosus* (Pisani et al., 1990). Caution must be taken for molecular weight determination by gel filtration because the molecular weight may be overestimated due to the aggregation of ß-glucosidases on gel filtration (Gabelsberger et al., 1993a, b). The addition of detergents such as Triton X-100 or SDS to the purified enzyme sample and eluted solution need to be carried out in future experiments to find out whether the aggregation of enzyme molecules has a role to play to in overestimation of molecular weight on gel filtration. Price and Stevens (1993) have suggested that higher molecular weights obtained with gel filtration could be due to anomalous behaviour of glycoproteins which will often cause a smaller gel-filtration volume although the glycoprotein nature of the purified ß-glucosidase is not known. This might also be a possible explanation. The molecular weight of the native ß-

glucosidase from strain COMP.A2 should be regarded as inconclusive until further experient is carried out, and it remains possible that the native enzyme is a monomer or a dimer like most other ß-glucosidases of thermophilic bacteria. Other techniques such as glycerol gradient centrifugation and mass spectrophotometer could be employed to determine the molecular weight of the native ß-glucosidase.

The ß-glucosidase purified from strain COMP.A2 exhibited a half-life of 2.5 hours at 70°C. This is higher than that of ß-glucosidase from *C. thermocellum*, but lower than the ß-glucosidases from *Thermotoga* sp. FjSS3-B.1, *Caldicellulosiruptor saccharolyticus* and *Pyrococcus furiosus* (Table 5.8). The ß-glucosidase from the hyperthermophilic archaeon *P. furiosus* is the most thermostable ß-glucosidase reported so far, with a half-life of 85 h at 100°C and retention of 80 to 90% of the initial activity at 100°C after 30 hours (Kengen et al., 1993; Voorhorst et al., 1995). The moderate thermostability of ß-glucosidases from strain COMP.A2 reflects its growth environment and optimum temperature which is lower than that of *Thermotoga* sp., *P. furiosus* and *Cs. saccharolyticus*.

The thermostability of ß-glucosidases varies with conditions of enzyme incubation and additives added. ß-glucosidases displayed greater thermostability at pH values above the optimum pH for activity (Tong et al.,1980; Patchett et al., 1987). Addition of dithiothreitol and divalent cations increased the thermostability of ß-glucosidases from *C. stercorarium* significantly (Bronnenmeier and Staudenbauer, 1988b). Trehalose is a cryoprotectant. Dramatic thermostability increase was observed for strain COMP.A2 ß-glucosidases by adding trehalose to the purified enzyme. Trehalose may work as a substrate analog to protect the active-site amino acid residues of ß-glucosidase that would otherwise be susceptible to inactivating reactions. The stabilization of ß-glucosidase by substrate (cellobiose) or substrate analogs has been reported by Patchett et al. (1987), Plant et al. (1988), and Ruttersmith and Daniel (1993). BSA gave some improvement in thermostability of the ß-glucosidase but no effect was observed for betaine (Table 5.4). This is different from the ß-glucosidase of *Thermotoga* sp. strain FjSS3-B.1 (Ruttersmith and Daniel, 1993) for which the thermostability was significantly increased by the addition of BSA and betaine.

The half-life of CMCase purified from strain COMP.A2 was 32 min at 80°C (section 4.3.5.6), while the half-life is less than 5 min at 80°C for the ß-glucosidase. This is consistent with the report that ß-glucosidase is the least stable component of cellulase systems (Hagerdal et al., 1980; Patchett et al., 1987).

The ß-glucosidase activity was completely inhibited by Cd^{2+}, Hg^{2+}, Zn^{2+}, Ag^{+} and 2 mM p-chloromercuriphenylsulphonic acid (pCMPS). The inhibition by pCMPS and enhancement of enzyme activity by dithiothreitol and ß-mercaptoethanol suggest the presence of essential thiol groups in the enzyme molecule. Similar results have been reported for the ß-glucosidases from other bacteria (Ait et al., 1982; Patchett et al., 1987; Ohmiya and Shimizu, 1988b). Heavy metal ion inhibition could also be due to its effect on specific residues, e.g., thiol groups. The activation of the enzyme caused by glycerol may be attributed to ß-glucosyl transferase activity. Some ß-glucosidases prefer glycerol and alcohols rather than water as acceptors for the ß-glycosyl moiety from the enzyme-ß-glucosyl complex during catalysis (Umezurike, 1978, 1981). The enzyme activity was strongly inhibited by Tris buffer (100 mM) at pH 7.5 to 8.5 and at 70°C. The inhibition by Tris has been observed from ß-glucosidase of *C. thermocellum* and was explained as being due to changes in the conformation or the charge distribution of the enzyme molecules (Ait et al., 1982).

The enzyme has significant activity on both cellobiose and pNPG (Table 5.7). The activity on both aryl-ß-glucosides and cellobiose, are commonly found in cellulolytic microbes (Sharrock, 1985). The Km values show that the enzyme has a higher affinity for the artificial substrate pNPG than sophorose and cellobiose. This is not unusual and has been reported for ß-glucosidases from other cellulolytic thermophilic bacteria (Ait et al., 1982; Patchett et al., 1987; Bronnenmeir and Staudenbauer, 1988b). The lack of activity on maltose, trehalose and *p*NP-α-D-glucopyranoside suggests that the purified enzyme only degrades ß-linked glucosides. The purified ß-glucosidase was capable of hydrolyzing the ß-linked glucose dimers, sophorose (ß-1,2), cellobiose (ß-1,4), and laminarin (ß-1,3) but not gentibiose (ß-1,6). The preference of sophorose (ß-1,2) to cellobiose (ß-1,4) is comparable to the report that ß-1,2-linkages are the most susceptible hydrolysis (Ait et al., 1982). Some bacterial ß-glucosidases were able to hydrolyze all four of the ß-linked glucose dimers, sophorose, cellobiose, laminarin, and gentiobiose at different rates (Plant et al., 1988; Ruttersmith and Daniel, 1993).

According to the classification proposed by Bauer et al. (1996), family 1 glycosyl hydrolases are all exo-acting and ß-specific enzymes and show a wide range of substrate specificities. The ß-glucosidases from many thermophiles have been placed in the family 1 glycosyl hydrolases group. The broad substrate range of the COMP.A2 ß-glucosidase and its specificity for a ß-linked glucosides supports placing this enzyme with the family 1 glycosyl hydrolases. The determination of amino acid sequence of the ß-glucosidase from COMP.A2 will be required to finally confirm this.

D-glucono-1,5-lactone is an inhibitor of ß-glucosidase, and not an inhibitor of exoglucanases and endoglucanases (Deshpande et al., 1988). The purified ß-glucosidase was competitively inhibited by gluconolactone and had no activity on CMC and xylan. Therefore, the enzyme is not a 1,4-ß-D-glucanglucohydrolase (also called exoglucohydrolase, EC 3.2.1.74) or endoglucanase.

Table 5.8 Thermostabilities of ß-glucosidases from some thermophilic bacteria and one archaeon.

Bacterium	T 1/2 (min.)	Temp. (°C)	Reference
Clostridium thermocellum	63	68.5	Ait et al., 1982
Caldicellulosiruptor saccharolyticus	105 [a]	85	Plant et al., 1988
Caldicellulosiruptor saccharolyticus	840 [b]	80	Love and Streiff, 1987
Hyperthermophilic archaeon *Pyrococcus furiosus*	5100	100	Kengen et al., 1993
Thermophilic anaerobic bacterium Wai21W.2	47	75	Patchett et al., 1987
Thermotoga sp FjSS3-B.1	47	80	Ruttersmith et al., 1993
Thermomonospora curvata	10	75	Stutzenberger, 1972
strain COMP.A2	150	70	this study

T 1/2, half life of ß-glucosidase; Temp, temperature.

a and b, expressed in *E. coli.*

CHAPTER SIX

GENERAL DISCUSSION AND CONCLUSION

The taxonomic position of strain COMP.A2, which was isolated from an artificial compost, has been established based on its phenotypic and phylogenetic characteristics. The cells are Gram-positive, aerobic endospore-forming rods and are considered to be related to the genus *Bacillus*. The high G+C mol% of the DNA and 16S rRNA sequence analysis suggest that strain COMP.A2 is distinct from most members of the genus *Bacillus* and is more closely related to *B. schlegelii*, *B. tusciae* and the genus *Alicylobacillus*. However strain COMP.A2 differs from all these organisms in their major physiological characteristics. Both *B. schlegelii* and *B. tusciae* are facultatively chemolithoautotrophic hydrogen-oxidizing bacteria (Bonjour and Aragno, 1984; Schenk and Aragno, 1979), and members of the genus *Alicylobacillus* grow at pH values between 2 and 6, and contain alicyclic fatty acids in the cell membrane. None of *B. schlegelii*, *B. tusciae* or *Alicylobacillus* species is capable of utilizing cellulose as growth substrate. In contrast, strain COMP.A2 is an extremely thermophilic, cellulolytic bacterium and is unable to grow chemolithoautotrophically in the presence of hydrogen and CO_2. Alicyclic fatty acids are not found in the cell membrane of strain COMP.A2, and the optimum pH for growth is 6.5 to 7.5. Thus the phenotypic and phylogenetic markers for this isolate are quite distinct and unique.

One unusual feature for strain COMP.A2 is the lack of growth on agar-based media. This is generally unusual and has not been reported for the other members of the genera *Bacillus* and *Alicyclobacillus* as far as we know. Agarose, one of the main components in agar, was found to inhibit the growth of strain COMP.A2 at concentrations which are similar to the concentrations present in agar at which inhibition by agar was recorded. This might indicate that agarose alone is the inhibitory compound present in agar. However the inhibition mechanism by agarose is not clear. The inhibition by agar has been observed in the thermophilic hydrogen-oxidizing bacterium *Hydrogenobacter thermophilus* (Kristjansson et al., 1985, Kawasumi et al., 1984). The cell morphology of strain COMP.A2 is quite different from that of *Hydrogenobacter thermophilus* which is a non-spore-forming, Gram-negative rod.

Another important feature of strain COMP.A2 is its ability to utilize microcrystalline cellulose as the sole carbon and energy source for growth at 70°C. Accumulation of glucose in the culture medium suggests that strain COMP.A2 produced all necessary enzymes to degrade crystalline cellulose to glucose. Most *Bacillus* species reported so far are unable to grow on crystalline cellulose. Those that do are all mesophiles and the

enzymes lack thermostability (Jorgensen and Hansen, 1990; Kim, 1995; Han et al., 1995). Usually, the isolation and characterization of one strain is not enough for proposing a new genus. However strain COMP.A2 is quite different from other members of the genera *Bacillus* and *Alicyclobacillus* according to its physiological and phylogenetic characteristics. Furthermore, there are precedents for describing a new genus on the basis of a single strain, for example, the extreme thermophile *Thermomicrobium roseum* (Jackson et al., 1973) and a thermophilic sulfate-reducing bacterium *Thermodesulforhabdus norvegicus* (Beeder et al., 1995). Strain COMP.A2 is tentatively proposed as a new species of a new genus, namely *Caldibacillus cellulovorans* gen. nov., sp. nov and described as follows.

Description of *Caldibacillus* gen. nov. *Caldibacillus* (Caldi.ba.cil.lus. L. adj. *caldus* hot; M.L. masc. n. *bacillus*, small rod; M.L.masc. n. *Caldibacillus*, small, heat-loving rod). Cells are long thin rods producing terminal oval endospores which caused the sporangium to swell. Gram stain reaction is negative, but the aminopeptidase test is typical of a Gram type positive organism. Strict aerobic organism is able to grow on sugars and polysaccharides. The DNA base composition is 62.4 mol% G+C. The type and only species is *Caldibacillus cellulovorans*.

Description of *Caldibacillus cellulovorans* sp. nov. *Caldibacillus cellulovorans* (cel lu lo vor ans. M.L.neut. n. *cellulosum* cellulose; L. partic. adj. *vorans*, eating; M.L.partic. adj. *cellulovorans*, eating cellulose). Cells are long thin rods (0.6 μm by 3-10 μm), producing terminal oval endospores. The colonies are transparent, circular, colourless, irregular, umbonate and less than 1 mm in diameter on Gelrite based medium with cellobiose as carbon source. The surface of colonies is undulate, shiny and smooth. No growth was observed at 55°C or 75°C. The optimum temperature for growth is 65°C. The pH range for growth is between pH 5.5 and 8.5. The optimum pH for growth is between pH 6.5 and 7.5. Growth occurs on the following substrates: glucose, maltose, cellobiose, arabinose, acetate, glutamate, pyruvate, starch, amorphous cellulose, Avicel, Sigmacell 20, Sigmacell 50, Sigmacell 100, xylan, wood pulp. Weak growth was detected on carboxymethylcellulose, proline, and mannitol. Growth does not occur on xylose, sorbitol, lactose, mannose, cotton, sucrose, fructose, raffinose, galactose, lactate, citrate, locust bean gum, pectin, inulin. No growth was observed in the presence of 2.0% sodium chloride or 0.01% sodium azide. Growth was inhibited by streptomycin, ampicillin, polymixin B, neomycin, and penicillin G at 1.25 μg/ml, kanamycin at 2.5 μg/ml, novobiocin at 10 μg/ml as well as 0.25% agar and 0.12% agarose. Small amounts of acetate and lactate were found as end products of aerobic growth on cellobiose or amorphous cellulose. High levels of saturated branched and unbranched fatty acids (98-100%) were found in the lipid membrane. The major phospholipid fatty

acids are iso $C_{16:0}$. No cyclic fatty acids were detected. The G+C content of the DNA is 62.4 mol%. The strain COMP.A2 was isolated from an artificial compost. The type strain of *Caldobacillus cellulovorans* is the strain COMP.A2 and is deposited in the DSM at access number 11694.

Strain COMP.A2 and a contaminating saccharolytic spore-forming *Bacillus* strain form a very stable co-culture. The isolation of strain COMP.A2 from the co-culture is not successful despite numerous attempts to separate the partners. Stable co-cultures among bacteria are not unusual and have been reported by Von Hofsten et al. (1971) and Ljungdahl and Eriksson (1985). The co-culture was superior in degrading cellulose than strain COMP.A2 alone. This may reflect the manner in which cellulose is degraded in nature eg composts where cellulose is not hydrolysed by a single species of microorganisms. The cellulolytic microorganisms, which are regarded as the primary microorganisms, exist in symbiotic heterologous association with non cellulolytic secondary microorganisms (Ljungdahl and Eriksson, 1985). Cellulose is hydrolysed by the primary microorganisms to a mix of cellodextrins, cellobiose and glucose, some of which they use for cell growth and maintenance. Some of the sugars produced by the primary microorganisms are utilised by the secondary microorganisms. Removal of the excess of sugars by the secondary microorganisms, in turn, facilitates cellulose hydrolysis by the primary microorganisms. The symbiotic relationship between the primary and secondary microorganisms helps to promote cellulose degradation.

The diversity of microorganisms in compost varies with the temperature change and oxygen and nutrient availability (Blanc et al., 1999). The study by Blanc et al (1997) suggests that there is a taxonomically and metabolically diverse population of heterotrophic thermophilic spore-forming bacteria in thermogenic compost. High numbers of autotrophic bacteria (Beffa et al., 1996a) as well as large numbers of thermophilic heterotrophic bacteria related to *Thermus* strains (Beffa et al., 1996b) and *Bacillus* spp. (Blanc et al., 1997) have been isolated and characterized from hot compost. A molecular clone approach has also proven that *Thermus thermophilus* strains and thermophilic *Bacillus* spp. are the dominant bacterial populations in thermogenic composts. The actinomycetes including *Thermomonospora* species and *Microbispora bispora* were reported to be the major cellulolytic microorganisms during the thermophilic composting phase (Stutzenberger et al., 1991). It appears that strain COMP.A2 is the only thermophilic cellulolytic *Bacillus* species reported so far from compost. The lack of growth on agar based medium may be a reason for failure to isolate thermophilic cellulolytic *Bacillus* species from compost before. Strain COMP.A2 and other heterotrophic bacteria in compost form a strong symbiotic relationship. This makes difficult to segregate cellulolytic microorganisms from their

partners in compost. Ecosystems in compost are complex systems in which microorganisms occur in heterogeneous communities. It is believed that the cultured microorganisms represent only a minor fraction of the existing diversity of microorganisms in natural environments (Head et al., 1998). Many microorganisms are difficult to isolate and cultivate under conditions routinely used in the laboratory. It may lead to an incorrect interpretation of microbial ecosystem in compost mainly based on traditional microbiological methods such as isolation and cultivation of pure cultures on selective media. A molecular approach, which involves DNA extraction followed by PCR amplification and subsequent cloning of 16S rRNA genes, and the fluorescence *in-situ*-hybridisation techniques have great potential to help us to understand the microbial diversity and interactions with the microbial community of compost in the future.

It is necessary to purify and characterize the individual components of cellulase systems in order to understand the relative importance and mechanism of action of each enzyme in the hydrolysis of cellulose. In this study, one cellulase component CMCase A and one ß-glucosidase were purified to homogeneity from culture supernatant of strain COMP.A2. Another cellulase component, CMCase B was only partially purified. An attempt to purify Avicelase (exoglucanase) was not successful although high activity on Avicel was observed in the culture supernatant. One possibility for the lack of purification is that Avicelase activity was the result of synergism amongst other cellulase components, and as these components are separated then Avicelase activity will dissipate. However, no synergistic effect was observed for the activity on Avicel by mixtures of CMCase A and CMCase B. This might suggest that other cellulase components from strain COMP.A2 are needed for the degradation of Avicel.

CMCase A purified from strain COMP.A2 displayed many similar biochemical characteristics with other endoglucanases. One important difference from most other endoglucanases is that CMC or amorphous cellulose is hydrolyzed by CMCase A in an exo-model of action. Endoglucanase is traditionally defined as the enzyme which randomly degrades CMC or amorphous cellulose. This definition may not be true for all endoglucanases. The classification of endoglucanase into four groups recently by Vlasenko et al. (1998) suggests that the mode of action varies among endoglucanases. It also supports the view of Tomme et al. (1996) that the classification of cellulases in two exclusive groups, endoglucanases and exoglucanases, is not always appropriate. Enzymatic hydrolysis of cellulose might be more complex than we thought. Glucanases may not be totally exo- or endo- acting, but may exhibit predominately one activity, with low levels of the other activity (Tomme et al., 1996).

Thermostability of CMCase A and ß-glucosidase from strain COMP.A2 is moderate when compared with that of other cellulases and ß-glucosidase from *Cs. saccharolyticus* (Neal, 1987; Love and Streiff, 1987), *Thermotoga* species (Ruttersmith and Daniel, 1991; Ruttersmith et al., 1993), and *Pyrococcus furiosus* (Bauer et al., 1999; Kengen et al., 1993). This may reflect the growth environment and maximum growth temperature of strain COMP.A2. Although an aerobic thermophilic bacterium *R. marinus* has been reported to produce an extraordinary thermostable cellulase (Hreggvidsson et al., 1996). Most highly thermostable cellulases reported so far come from anaerobic extreme thermophiles (Sunna et al., 1997). For comparative enzymology and an understanding of thermostability, the ideal is to study a similar enzyme function in the same genus in closely related species growing over a wide temperature range. Sequence and structure information on the cellulases might provide good comparative models against less thermostable enzymes.

The thermostability of CMCase A and ß-glucosidase activity from strain COMP.A2 was improved by the addition of BSA and trehalose, respectively. Two-fold increase for the half-life of CMCase A at 80 and 85°C was observed by the addition of BSA. Addition of trehalose lead to about five-fold improvement of the half-life of the ß-glucosidase at 80°C and 70°C. These results support that CMCase A and ß-glucosidase together with other cellulases and hemicellulases are more stable under the environment conditions in situ due to the presence of substrates and substrate analogs as well as high protein concentration.

Product inhibition and thermal inactivation of cellulase constitute two major barriers to the realization of enzymatic cellulose hydrolysis as a commercial process. The thermostable CMCase and ß-glucosidase from strain COMP.A2 promise to be advantageous in cellulose degradation. Although we have purified one CMCases and one ß-glucosidase, the enzymes purified in the present study represent only a portion of the cellulases in strain COMP.A2. A search for other cellulase components and their roles in degradation of crystalline cellulose will be continued. Sometimes the isolation of cellulase components is quite difficult and time-consuming. The preferred approach is to clone and express the genes encoding cellulases in a suitable host such as *E. coli* and *B. subtilis*. This has been proven to be quite successful in the study of cellulase systems of *C. thermocellum* and *Cs. saccharolyticus*. The same approach should be adopted in any future study of cellulase systems of strain COMP.A2. Finally, it should be pointed out that cellulase systems from strain COMP.A2 only form part of a spectrum of polysaccharide hydrolases produced by microorganisms in the compost. It will be a challenge to understand how the plant biomass is degraded cooperatively by strain COMP.A2 and other microorganisms in the compost.

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Appendix 1:

a: Wolfe's Modified Mineral Elixer:

Nitrilotriacetric acid	1.5	g
$MgSO_4 . 7H_2O$	3.0	g
$MnSO_4 . H_2O$	500	mg
NaCl	1.0	g
$FeSO_4 . 7H_2O$	100.0	mg
$Co(NO_3)_2 . 7H_2O$	100.0	mg
$CaCl_2$ (anhydrous)	100.0	mg
$ZnSO_4 . 7H_2O$	100.0	mg
$CuSO_4 . 5H_2O$	10.0	mg
$AlK(SO_4)_2$ (anhydrous)	10.0	mg
Boric acid	10.0	mg
$Na_2MoO_4 . 2H_2O$	10.0	mg
Na_2SeO_3 (anhydrous)	1.0	mg
Distilled water	1.0	L

Suspend the nitrilotriacetric acid in 500 ml water. Dissolve it by titrating with 2-3 N KOH until the pH is stabilized at 6.5. Add the rest of the ingredients and dissolve in the order they are listed. Finally, adjust the volume to 1.0 L.

b: Trace element solution of Badzoing (Badzoing et al., 1978)

Nitrilotriacetric acid	12.8	g
$CuCl_2$	20	mg
$MnCl_2 . 4H_2O$	100	mg
$FeCl_2 . 4H_2O$	300	mg
$ZnCl_2$	100.0	mg
$CoCl_2 . 6H_2O$	170.0	mg
$Na_2MoO_4 . 2H_2O$	10.0	mg
H_3BO_3	10.0	mg
distilled water	1.0	L

Appendix 2. Silver staining method

Step	Solution	Time (min)	Temp (°C)
1	30% (w/v) ethanol, 10% (w/v) acetic acid	4	50
2	30% (w/v) ethanol, 10% (w/v) acetic acid	4	50
3	0.5% (w/v) glutaraldehyde, 0.1% (w/v) sodium thiosulphate in 30% (w/v) ethanol, 0.4 M sodium acetate, pH 6.0	8	40
4	Milli Q water	3	50
5	Milli Q water	3	50
6	Milli Q water	3	50
7	Milli Q water	4	50
8	Milli Q water	4	50
9	0.1% (w/v) silver nitrate + 50 µl formaldehyde/200 ml	10	40
10	2.5% (w/v) disodium carbonate + 200 µl formaldehyde/500 ml	0.4	30
11	2.5% (w/v) disodium carbonate + 200 µl formaldehyde/500 ml	6	30
12	5% (v/v) acetic acid	2	50
13	Milli Q water	3	50
14	Approx. 20% (w/w) glycerol	3	50
15	MilliQ water	0.1	40