CHAPTER 1

1.0 INTRODUCTION

Cellulose is unarguably the most abundant natural organic substance on earth and is the predominant component throughout the plant kingdom, therefore it could be manipulated for the benefit of mankind. This renewable source of energy could be tapped for use with the help of research activity from engineers, biochemists, biologists and agriculturists. Thus for many years dating back to 1910, when the first plant constructed by Ewen and co-workers was designed to produce ethanol from sawmill dust, several improvements have been developed to make use of this natural cellulolytic material (Ramamurthy *et al.,* 1992).

For many years, researchers have been engaged in developing or improving technological methods and equipment for hydrolysing wood and other cellulose-containing substances for various industrial purposes (Sakata *et al.,* 1985; Tewari *et al.,* 1987; Tewari *et al.,* 1988; Malek *et al.,* 1988; Julian *et al.,* 1990, Saxena *et al.,* 1992; Schafener & Toledo, 1991; Shiang *et al.,* 1991; Chen & Wayman, 1992; Teunissen, 1992; Wayman *et al.,* 1992; Doran & Ingram, 1993; Chaudhuri & Sahai, 1993). The potential technology of harnessing energy from cellulose as a renewable resource has resulted in the search for cellulase enzymes that can completely degrade cellulose to glucose, already utilizable form. Biomass degradation of the highly crystalline cellulose, both efficiently and cheaply is still a challenging research for the current cost sensitive markets.

Attempts have been performed in using mixed microbial cultures to improve on the hydrolysis of cellulose. These attempts included co-culturing of microorganisms to complement each other on cellulases enzyme production. These mixed cultures were meant to improve on the production of biomass, and cellulolytic enzymes for cellulose digestion (Manonmani & Sreekantiah, 1987; Soundar & Chandra, 1987; Pavlostathis *et al.,* 1990; Bailey & Viikari, 1993; Gutierrez-Correa & Tengerdy, 1997). A lot of research on cellulose hydrolyzing microorganisms has involved fungi, although for the past two decades effort has been directed towards the study of bacterial cellulases from the biochemical to molecular level. Organisms that can produce a complete set of cellulase complex enzyme systems are capable of an efficient hydrolysis of cellulose to glucose and cellobiose.

Dunning and Lathrop (1945) designed a system, which could hydrolyze agricultural wastes such as corncobs, cottonseed hulls, flax shaves at hulls and sugarcane bagasse to produce sugars that could be fermented to ethanol. The method of hydrolysing cellulose involved the use of concentrated hydrochloric acid. The cellulose products yielded by this method had other toxic

materials as a result of chemical complexity of the natural cellulose. The disposal of this waste material after glucose extraction to date would pose problems of pollution to the already strained environment.

Bioconversion of abundant cellulosic biomass to man-manufactured products which include single cell protein (SCP), ethanol, methanol, butenediol, acetic acid, sugars, biogas, chemical feedstock and liquid fuels by employing microbial fermentation has necessitated the research for efficient cellulases which can hydrolyse cellulose *in vitro* (Leisola & Linko, 1976, Manonmani & Sreenkantiah, 1987, Julian *et al.,* 1990; Dolan & Ingram, 1993; Bhat & Bhat, 1997; Tsao *et al.,* 1999; Ho *et al.,* 1999; Gong *et al.,* 1999; Sun & Cheng, 2002). In the late 19th century, attempts were made to use acid or alkali pretreatment of cellulose followed by enzymatic hydrolysis to improve on the glucose yield (Millet & Baker, 1975; Mandels *et al.,* 1976; Tewari *et al.,* 1987; Meyer *et al.,* 1992; Tenborg *et al.,* 1998; Taherzadeh *et al.,* 1998; Lee *et al.,* 1999). However there is need from an environmental viewpoint to substitute fossil fuels with environmentally friendly renewable sources of energy. This need has motivated scientists to look for treatment of solid or solubilised organic cellulose wastes into bioconvertable fuels and other products (Rogers *et al.,* 1972; Wayman *et al.,* 1992; Saxena *et al.,* 1992; Taherzadeh *et al.,* 1998). The production of energy from renewable organic wastes like cellulose materials is affected by the total potential energy that can be tapped from these sources which in turn is a measure of the efficiency of converting the raw material source to the energy carrier of interest. Other parameters that can affect and hinder production of fuels are the cost of producing, harvesting and converting the organic sources (Miller & Churchill, 1986; Horikoshi, 1999). There is no doubt that cellulose is a good raw material for renewable source of energy, considering its availability and abundancy in nature. Cellulose as energy source can be of higher efficiency of conversion to fuels than current sources because there is room for technical improvements and higher rate of processing.

Major natural sources of cellulolytic enzymes include bacteria, fungi and plants (Gilkes *et al*., 1991; Pearson *et al.,* 1991; Horikoshi, 1999). Production of cellulases by bacteria and fungi has attracted interests by scientists. Scientists are studying on organisms that can produce cellulases that are thermostable, have temperature optima, broader pH optima and have application potential (Ito, 1997; Bhat & Bhat, 1997; Ito *et al.,* 1998). Organisms isolated from natural environments such as hot springs, alkaline lakes and sea waters can produce enzymes with these properties. The screening of microorganisms that produce products of potential industrial applications has attracted the attention of many scientists in the developing and developed world. This quest for cellulases with potential industrial applications motivated this work on cellulase producing microorganisms from Zimbabwean hot springs. There are a number of advantages for isolating enzymes from thermophilic microbes.

The advantages are:

a) vast majority of microorganisms to select a producer strain,

b) enzymes from these organisms have potential economic value,

c) selective growth of certain microbes at high temperatures because of low oxygen solubility,

d) their enzymes can be stable in extreme conditions under which the microorganism thrive,

e) fermentation techniques using microbes have high yields,

f) recovery of fermentation products downstream is easy and

g) microorganisms can be genetically manipulated to improve protein production and quality (Miller & Churchill, 1986; Mosier *et al.,* 1999).

As organisms grow, they don't produce enzymes that are independent of each other. The plethora of enzymes produced tend to interact and hence the process of proteolytic posttranslational modifications of other proteins turning these **prepro** proteins into functional proteins (Creighton, 1993). Most microbes produce protease enzymes that have a number of functions and the regulation of protease production is complex. Microbial proteases are produced as many gene products (Pero & Sloma, 1993) or as a control response to environmental stimuli. The proteolytic cleavage of polypeptide chains after synthesis occur in certain classes of proteins, primarily for those proteins destined for extracellular excretion in addition to the removal of signal peptides. The pro-enzyme will have an extracellular function and under goes the proteolytic processing modification outside the cell system (Creighton, 1993). Sometimes the proteolytic interaction of extracellular proteins can be detrimental resulting in malfunctioning or degradation (inactivation) of the modified protein. Some bacterial proteases have been shown to undergo auto proteolysis when they reach the extracellular region of the cell. The extracellular conditions are sometimes suitable for induction of autocatalysis e.g. *Bacillus* subtilisin protease (Ikemura & Inouye, 1988). Another process of interest is the production of proteases that have no clear function and whose production have been postulated to be for protein turnover or for sporulation This proteolytic enzyme production is triggered by depletion in either carbon or nitrogen nutrient sources (Pero & Sloma, 1993). It has been observed that, protease synthesis begins at the end of logarithmic growth in a number of microorganisms. The onset of protease production can affect extracellular parameters, which include enzyme levels. Therefore it's paramount that the regulation, production and level of one microbial parameter cannot be independently ascertained. In the present study both endoglucanase and protease productions were studied. It appeared that the protease enzyme affected endoglucanase levels during cultivation.

Naturally, microbes produce extracellular enzymes at very low levels. The production of cellulase enzymes by microorganisms is quite low compared to the market demand, therefore there is need to improve on yield levels. Some fermentation techniques maintain optimum conditions to maximize product levels. A target enzyme of interest can be cloned and then its regulation, synthesis and production at molecular level enhanced. Although fungal cellulase enzyme studies have been extensive (Harmova *et al.,* 1986; Ali &Akhand, 1992; Yazdi *et al.,* 1990; Meyer *et al.,* 1992; Schimdhatter & Canevascini, 1992) bacterial cellulases particularly that of *Bacillus* sp. has recently received much attention at molecular level (Nakamura *et al.,* 1987, Blanco *et al.,* 1988; Horikoshi, 1999; Takami & Horikoshi 2000) with the aim of improving the production output and characteristic or qualities of the enzyme. This involves the study of cellulase enzymes' physiological aspects from the natural producer strain and cloned sources. Cellulase regulation and synthesis from various wild and cloned sources like eukaryotes and prokaryotes are ongoing research activities (Baird *et al.,* 1990; Ozaki *et al.,* 1990 and 1991; Shima *et al.,* 1991; Park *et al.,* 1991; Hansen-Sonne *et al.,* 1993; Lindhl *et al.,* 1994; Miyatake *&* Imada 1987; Dei *et al.,* 2000; Hakamada *et al.,* 2001; Wicher *et al.,* 2001).

The endoglucanase enzyme was produced and other important enzymes investigated when *B. subtilis* CHZ1 was cultured on a cheap source of medium extracted from industrial food wastes. Conditions were optimized for the production of the endoglucanase enzyme in batch experiments. Production of the endoglucanase was affected by the concomitant proteolytic activity and this prompted the study of the protease enzyme and then considers its potential application.

This project covers the microbial physiology of an endoglucanase production by a *Bacillus* strain. The other work done was on the production of other hydrolytic enzymes using cheap source of raw materials for medium preparation. As a result of the high activity expressed by the protease enzyme, it was purified and studied. A molecular study of the endoglucanase after cloning into *E. coli* was also carried out.

CHAPTER 2

2 LITERATURE REVIEW

2.1 Natural occurrence of cellulose

Cellulose has become the pivotal point in commercial and industrial applications. Cellulose has found applications in food, clothing, paper and wood industries. It is also being used as a natural fertiliser. However, it poses an environmental pollution problem.

Plant and microbial polysaccharides fall into a number of well-defined important structural entities giving rise to cell components and food reserve groups (starch). Naturally, cellulose occurs in abundance in wood as lignified cellulose, fungi, tunicates and bacteria (Colvin, 1980). The cellulose from various raw materials can be processed into papers, fibres and textiles. Agricultural wastes like stalks, stems and husks of cereal grains have cellulose as the predominant component and can be recycled as organic fertilisers. Plant polysaccharides are classified based on structure rather than function. These plant polysaccharides have notable features of certain types of sugar residues as main polysaccharide backbone component. Other different sugar units can be attached to the main chain. Sometimes hydroxyl groups are substituted with O-acetyl, O-methyl derivatives or carboxylic acids of methyl esters. The differences in main sugar components give rise to their different biological functions (Aspinall, 1980; Bacic, 1988; Morohoshi, 1991).

2.2 NATURAL CELLULOSE PHYSIOCHEMICAL PROPERTIES

Natural cellulosic materials comprise predominantly of two distinct sections, namely microfibrillar and matrix components. These two sections have different sugar units and differ in their residues bond linkages.

2.2.1 The Microfibrillar component of cellulose

The microfibrillar component constitutes the predominant material of natural cellulose. The microfibril component is made up of long, thin and microfibril like structures of β(1,4) linked glucose units. The linear homopolymer of D-glucopyranosyl residues per native cellulose molecule vary from 15 to 15 000 conferring a varying degree of polymerisation (Morohoshi, 1991). The existence of an elementary fibril nature of cellulose has been supported by electron microscope studies and X-ray diffraction crystallography patterns (Gardener & Blackwell, 1974; Aspinall, 1980; O'Sullivan, 1997). However, this has not been conclusively resolved because of the possibility of artifacts and misinterpretation of visual images and electron scattering. Cellulose chains occur naturally in paracrystalline lattice as a result of the intra-molecular and inter-molecular hydrogen bonds. The hydrogen bonds arise between intra-chain and inter-chain hydroxyl groups of microfibril cellulose chains (figure 1). This array of cellulose structure confers a certain order of inflexibility hence the cellulose's considerable tensile strength. The linear polyglycosans of cellulose are extended in a microfibril, forming more or less ordered regions (crystallites) that are intervened with amorphous regions of varying degrees of disorderliness. This phase of cellulose is distinguished by a certain degree of crystallinity and homogenous chemical

composition. Cellulose can occur in a number of different forms. Designations of cellulose by Millet and Baker (1975) were after reacting cellulose with 20 % (w/v) sodium hydroxide, which macerate the cellulose polymer. The products that were obtained from such treatment gave rise to two forms of cellulose designated cellulose I and II. However, initial studies postulated the existence of possible parallel and antiparallel configurations of the cellulose chains thereby giving rise to different forms of cellulose designated as cellulose I, cellulose II, cellulose III and cellulose IV. Gardner and Blackwell (1974) resolved that cellulose microfibril have same polarity and are parallel orientated.

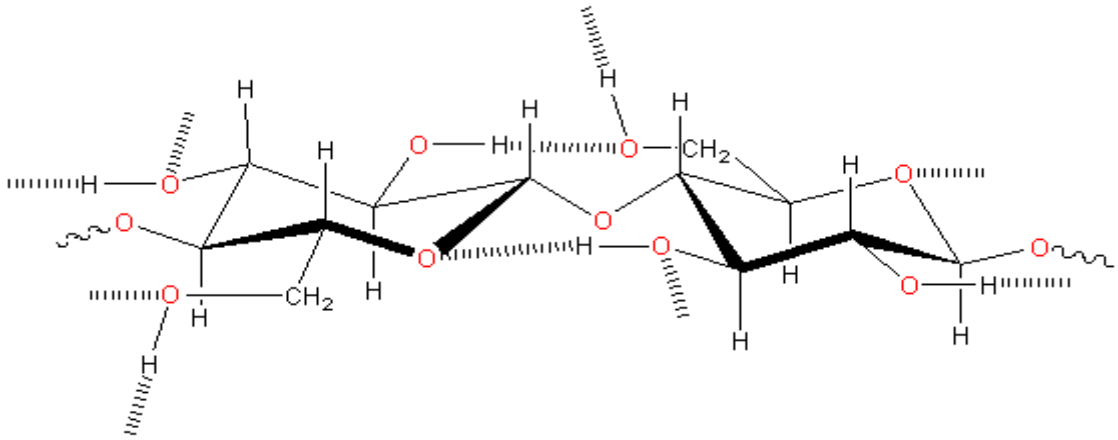


Figure 1. Conformation of β(1,4)-glucan linkages in microfibril component showing sticking out hydroxyl groups involved in intramolecular hydrogen bonding between adjacent glucose residues. (adapted from Aspinall, 1980)

Currently, native cellulose is known as cellulose I and other forms such as cellulose II, cellulose III and cellulose IV are obtained after subjecting the native cellulose to mechanical or thermal treatment (Langan *et al.,* 1999).

2.2.2 The Matrix component of cellulose

The matrix component of native cellulose is made up of different parts that are identified after solubilization, separation and analysis of these components from the microfibrillar component. The matrix components were grouped into hemicellulose, lignin, pectin, protein and phenolic compounds. The compositions of these materials are extensively complex and vary in different species, cells, and stage of cell development. The elements of matrix phase are heterogeneous polymers that vary in bond linkages and numbers of the sugar residues. The matrix component further complicates cellulose's natural composition by having variations in proportions of its heteropolymer units (Table 1).

Cellulose is made up of β(1,4) linked glucose sugar units only and hemicellulose is a heteropolymer made up of several sugar units with several bond links. Hemicellulose can easily be hydrolysed by acids to their monomer components that are glucose, mannose, galactose, xylose, arabinose, rhamnose, glucuronic acid, methyl glucuronic acid and galacturonic acid (Dekker & Richards, 1976). The variation in the sugar compositions gives rise to softwood or hardwood hemicellulose. The major difference between these two hemicellulose forms lies on the concentration of xylan. Hemicellulose of hardwood type has a higher content of xylan.
The lignins are polymers of phenylpropanoid residues that are almost exclusively derived from p-coumaryl, (p-hydroxyphenyl) coniferyl (guaicyl) and sinapyl (syringyl) alcohols. Lignin and hemicellulose are linked together by ester, ether and glycosidic chemical bonds.

Table 1. Microfibril and matrix heteropolymers constituents in nature.

Component	Constituents	Glucan(s) types
a) microfibril	cellulose	β(1,4) glucan
b) matrix	i) pectins	rhamnogalacturonan, homogalacturonan arabinan, galactan, arabinogalactan I, rhamnogalacturonan II
	ii) hemicelluloses	xylan, glucomannan, mannan, xyloglucan galactomannan, glucuronomannan, callose β(1,3) glucan, β(1,3)-β1,4) glucan, arabinogalactan, galactoglucomannan
	iii) proteins	extensin, arabinogalacta protein, others including enzymes
	iv) phenolics	lignin (coniferyl and sinapyl), ferulic acid and others (coumaric acid and traullic acid)

(adapted from Aspinall, 1980 and Morohoshi, 1991)

These linkages are quite strong and are extremely resistant to chemical and enzymatic hydrolysis. The other naturally occurring cellulose extractives found include terpenoids and steroids, fats and waxes and inorganic components like carbonates, silicates, oxalates and phosphates (Kai, 1991;

Morohoshi, 1991). Studies on hemicellulose involved its extraction and purification, then followed by physical and chemical analysis.

Plant polysaccharide nomenclature is based on the sugar involved in the main backbone chain. The other bonds that have also been identified in hemicellulose components are α(1,4); α(1,3); α(1,5); β(1,3); β(1,5); β(1,6) or a combination of these bonds giving rise to β(1,3-1,4); β(1,3-1,5) or α(1,3-1,5) branch linkages (Aspinall, 1980). Major residues found in the matrix component of plants are α-L-rhaminose, α-L-fucose, α-L-arabinose, α-D-galacturonic acid, β-D-mannose, β-D-galactose, β-D-glucose, β-D-apiose, β-L-aceric acid and ketodeoxyoctulosonic acid.

Subjecting the native cellulose to treatments for analysis can introduce chemical modifications; hence the resultant cellulose product will differ from the natural form. Therefore we can summarise and conclude that natural cellulose comprises of microfibril of β(1,4) glycosides linked polysaccharides, reinforced, or impregnated with plasticing matrix resin of heteropolymers. This description of natural cellulose presents a variable and ill-defined concentration and chemical composition.

2.3 USES OF CELLULOSE AND CELLULOSE DERIVATIVES

Besides the biotechnological by-products from cellulose produced by microbial fermentations discussed in chapter one, cellulose is being manipulated for other uses. Some commercial products currently produced from cellulose and cellulose derivatives are being used for plastics, textiles, packaging, films, lacquers, explosives and pharmaceuticals. For instance, food and pharmaceutical industries require polysaccharides and cellulose as stabilisers or thickening agents to improve on the viscosity of their food, pastes, ointments and creams. These thickening agents vary in monosaccharides composition and properties (Scaman, 2000).

Also paint and oil industries use cellulose derivatives that have high degree of polymerisation for protecting their colloidal products, as emulsifiers or pigments suspending and dispersing agents (Tothill & Seal, 1993). The chemically modified cellulose or cellulose derivatives that are used in paint and oil industries include hydroxyethyl cellulose (HEC), ethylhydroxyethyl cellulose (EHEC), hydroxypropylmethyl cellulose (HPMC) and carboxymethyl cellulose (CMC). The clothing industry is no exception in the use of microfibril cellulose for making cotton thread and fibres.

Cellulose derivatives have found much use by researchers in studying microbial cellulases (Leisola & Linko, 1976; Mandels *et al.,* 1976; Hotten *et al.,* 1983; Amanda *et al.,* 1997). After realising the complexity of cellulose and the enzymes involved in the digestion of cellulose,

modified cellulose was synthesised for cellulase enzymatic assaying. Commercially available cellulose derivatives used for cellulase assays have little resemblance to natural cellulose considering that some of these cellulose forms lack the cellulose matrix component. Otherwise, the substrates used to screen for cellulolytic enzymes should at least be closely related to the target substrates like agricultural wastes. The accessibility and extent of cellulose derivative hydrolysis becomes much important when a microorganism produces a single cellulase enzyme component. Currently marketed cellulosic products that are substrates for cellulase enzymes include filter paper, Avicel, Sigmacel, Solfa floc, cellulose azure, sodium salts of CMC and HEC. Similar cellulose derivatives have hydrophilic characters and high porosity resulting in their use in chromatography work by scientists.

Recent studies have shown that cellulose derivatives like cellulose acetate, cellulose-propionate and cellulose-acetate-butyrate can be used as membrane supports that can covalently immobilise enzymes. Such immobilised enzymes have shown better storage stabilities, and could give more enzymatic activity. This has been shown to greatly improve the crystallinity of the enzyme molecules (Murtinho *et al.,* 1998).

2.4 METHODS OF HYDROLYSING CELLULOSE

There are various methods of hydrolysing cellulose. These are physical, chemical and enzymatic methods. Physical or chemical methods of hydrolysing cellulose are sometimes incorporated as preliminary pretreatment stages to make cellulose more accessible to enzymatic hydrolysis (Tewari *et al.,* 1987; Meyer *et al.,* 1992). The physical and chemical pretreatment methods alter the fine structure and disrupt or open up lignin-carbohydrate association and weaken the crystalline complex of cellulose. By-products from natural cellulose inhibit microbial hydrolysis or fermentation of cellulose. Strategies have been developed to minimise production of cellulosic hydrolysates that inhibit fermentation of cellulose (Taherzadeh *et al.,* 1998; Tengborg *et al.,* 1998).

2.4.1 Physical and mechanical methods of hydrolysing cellulose

2.4.1.1 Grinding method

Grinding method is usually incorporated as an initial stage to ease cellulose hydrolysis by cellulase enzymes. After the grinding pretreatment, the bioavailability of the cellulose substrate for both enzymes and microorganisms is significantly increased. Vibrator balling, roll and hammer

milling can produce fine sand dust from wood. Grinding of wood generates fine particles that can be easily hydrolysed by cellulase enzymes, fungi and bacteria. Studies on compression milling or pressing and drying of cellulose showed an increase in moisture content retention by the cellulose thus improving the properties of the cellulose for enzymatic and bacterial hydrolysis (Ryu & Mandels, 1980; Haggkvist & Odberg, 1998).

2.4.1.2 Irradiation methods

Illumination of wood or straw by gamma rays or high velocity electrons affects the crystalline nature of cellulose. This can be facilitated by first subjecting the cellulose to photosensitizers like sodium nitrite which induce photodegradation (Rogers *et al.,* 1972). The cellulose becomes water permeable and easy to hydrolyse by enzymatic method.

2.4.1.3 Use of high and low temperatures

Temperatures of 200°C in solvents like kerosene, dry air, nitrogen or oxygen enhances hydrolysis of cellulose but this requires specialised equipment. The degree of polymerisation of cellulose enhance its strength, can be greatly reduced if the temperature is rapidly dropped to –75°C. Repeated freezing and thawing process can further augment the hydrolysis of cellulose with this method. This process results in reducing the compactness of cellulose because its microfibril intra- and intermolecular hydrogen bonds are weakened (Millet & Baker, 1975; McMillan, 1994).

2.4.2 Chemical methods of hydrolysing cellulose

2.4.2.1 Alkali and acid hydrolysis

Sodium hydroxide is the most widely used alkali agent. Sulphuric, hydrochloric and phosphoric acids have esterification reactions that facilitate hydrolysis of the matrix and microfibril components of cellulose. Both the acids and alkalis macerate the cellulolytic plasticing elements (matrix components) to a decrystallised cellulose (Mandels *et al.,* 1976; McMillan, 1994). It was observed that alkali treated straw that was fed to ruminants showed an improved nutritional value to the host ruminants. Sugarcane bagasse digestibility by *Cellulomonas* bacteria was shown to increase by about 40 % after sodium hydroxide pretreatment (Han & Callihan, 1974), 80 % with *Trichoderma viridie*, 90 % with a mixed culture of *Aspergillus* and *Trichoderma* strains (Manonmani & Sreekantiah, 1987). Alkali treatment improved the dissolution of hemicelluloses

part of natural cellulosic material resulting in the conversion of crystalline cellulose form I to cellulose form II that is readily hydrolysed by *Trichoderma reseei* cellulases (Rahkamo *et al.,* 1998).

2.4.2.2 Organosolv hydrolysis

This involves the pretreatment of cellulose with an aqueous solvent in the presence of a catalyst. The aqueous phase and the catalyst perform the hydrolysis of carbohydrate as well as lignin-lignin associations. Widely applied solvents are alcohols, glycerol, dioxane, phenol and ethylene glycol in the presence of catalysts like $FeCl_3$ or $Al(SO_4)_3$ (Holtzapple & Humphrey, 1984). Hydrogen peroxide and ozone can be used to enhance biodegradation by solubilising lignin and hemicellulose thereby exposing the microfibril chains of cellulose. Some of the solvents mentioned here can transform cellulose from cellulose I to other forms that are more susceptible to enzymatic hydrolysis (Holtzapple & Humphrey, 1984).

2.4.2.3 Ammonia treatment of cellulose

This method of hydrolysing cellulose involves the use of either aqueous or gaseous ammonia at 130°C. The ammonia hydrolysis method has been employed to ligninocellulosic materials fed on ruminants, and there was improved digestibility by the animals. The ammonia was realised to improve the swelling of wood. The ruminants gut flora will then produce enzymes that in turn will digest the cellulose that the ruminants feed on (Holtzapple *et al.,* 1991; McMillan, 1994). A scanning electron microscope examination of ammonia treated cellulose samples indicated an increase in pore size and porosity. Ammonia pretreatment method is more effective with agricultural residues like corn straws than with woody materials (Dunning & Lathrop, 1945; Mandels *et al.,* 1976; McMillan, 1994).

2.4.2.4 Delignification pretreatment

This process of cellulose hydrolysis can be achieved with the use of agents that cause pulping, such as the treatment of cellulose with sodium chlorite. In the presence of acetic acid, lignin solubilization is enhanced when pulping agents like sodium chlorite are used. Another pulping agent that can be used is ammonium bisulphate. Most of the processes are selective in removing lignin. Pulping agents like ammonium bisulphate allow the usage of the end product after the delignification pretreatment (Royer & Nakas, 1987).

2.4.2.5 Steaming of cellulose

Steam treatment of aspen chips fed to sheep resulted in a remarkable live weight gain. Steam treatment reduces degree of polymerisation of cellulose. Steam method is the mostly used method for pretreatment of ligninocellulosic materials. In this method, chipped material is treated with high pressure saturated with steam and then pressure is swiftly reduced. The reduction in pressure causes the material to undergo an explosion decomposition (McMillan, 1994). Steaming natural cellulose material also causes partial hydrolysis through deacetylation of its pectin and hemicellulose components. The hydrolysis products from pectin and hemicellulose cause an acidic cellulosic mixture. The acidic environment thereby generated is conducive to hydrolysis of the cellulose by acidic cellulase enzymes (Millet & Baker, 1975).

2.4.3 Enzymatic hydrolysis of cellulose

Chemical and physical agents used in pretreatment of cellulose are capable of breaking down bonds of the matrix component of natural cellulose thereby reducing its crystalline regions such that there is little resistance to enzymatic attack. Due to the fact that the structure of natural cellulosic material has multiple bonds, a single cellulase enzyme is not capable of total hydrolysis owing to the complexity of the structure. Besides cellulases, other enzymes like endo- and exo-β-1,4-xylanases, hemicellulases, and lichenases, β(1,3)(1,4) glucanases and pectinases pectin methyl esterases, polygalacturonases, and poly-α-1,4-D-galacturonide lyase are required for a complete hydrolysis of native cellulose. For an efficient hydrolysis of cellulose a system of enzyme entities collectively called cellulases are required.

The cellulases enzyme system is a complex of three different enzymes. This further complicates the assays of cellulase activity. The cellulase enzymes breakdown cellulose to glucose, cellobiose and different length sized oligosaccharides. The following is a schematic summary of the cellulase enzymes involved in cellulose hydrolysis (Kluepfel, 1988; Gilkes, 1991; Schmidhalter & Canevascini, 1993; Ito, 1997).

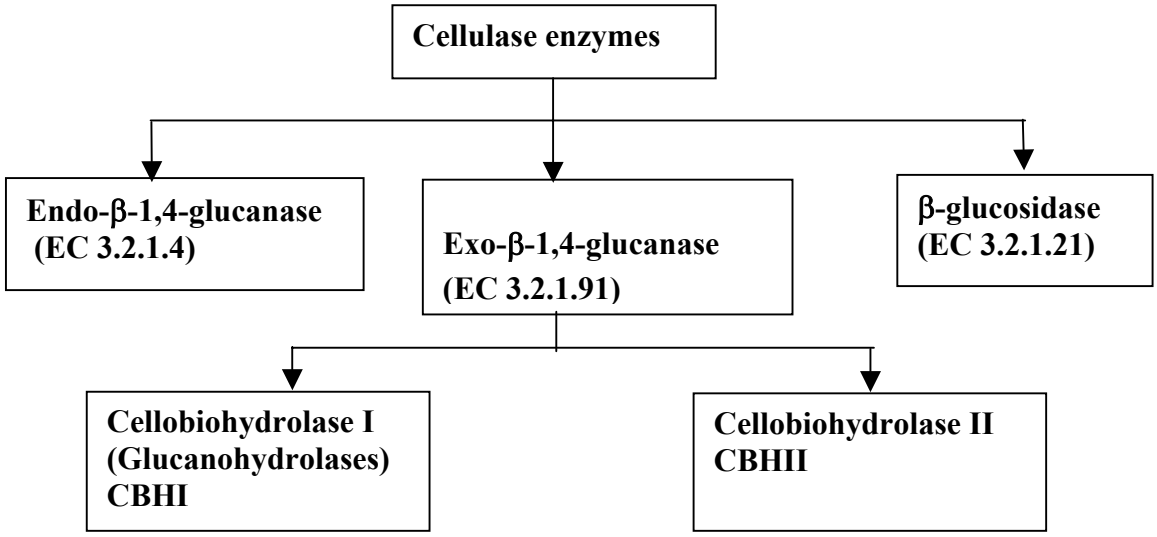


Figure 2. Cellulase enzymes involved in cellulose hydrolysis (Kluepfel, 1988).

The cellulase enzyme complex comprises of three different enzyme activities that act synergistically (i.e. endo-β-1,4-glucanase (EC 3.2.1.4), exo-β-1,4-glucanase (3.2.1.91), and β-glucosidase (3.2.1.21)) to efficiently decrystallise and hydrolyse native cellulose. These cellulase enzymes have different substrate specificities. Endo-β-1,4-glucanase activity is involved in random hydrolysis of internal β-1,4 glycosidic bonds of amorphous cellulose microfibril chains producing glucose, cellobiose and different sized celloligosaccharides. The exo-β-1,4-glucanase or 1,4-β-glucan cellobiohydrolase has two types of cellobiohydrolase activity namely CBHI and CBHII (Schmidhalter & Canevascini, 1993). Exo-β-1,4-glucanases that include cellobiohydrolase preferentially liberate cellobiose (glucose dimers) from either end of cellulose chain and glucanohydrolases liberate glucose monomers. The exo-β-1,4-glucanase hydrolyses microfibril cellulose chains from the non-reducing ends (reduces the crystallinity of cellulose) and gives more sites for endo-β-1,4-glucanase enzyme. The cellobiose disaccharides are then split into glucose moieties by the activity of β–glucosidase or β–D-glucoside glucohydrolase. This enzyme is sometimes regarded as non-cellulolytic enzyme because it is not specific to β-1,4 linkages although it participates in the complete hydrolysis of cellulose. This enzymatic mode of cellulose hydrolysis was however postulated based on fungal cellulases and can be different with bacterial cellulases because most bacterial cellulases do not have all enzyme activities as illustrated in figure 2 (Person *et al.,* 1991; Coutinho *et al.,* 1993). Some bacterial cellulases have been shown to possess endoglucanase activity only or both endoglucanase and exoglucanase activity (Béguin, 1990).

All cellulases have modular structures with one catalytic domain (CD) linked to one or several non-catalytic modules called cellulose binding domains (CBD). These two domains are structurally and functionally independent (figure 3). The catalytic domain is responsible for cellulose hydrolysis of β-1,4-glycosidic bonds while CBD mediates the attachment of cellulases to cellulose. The non-catalytic module is involved in protein-carbohydrate (ligand binding) and protein-protein (cellulosomes) interaction.

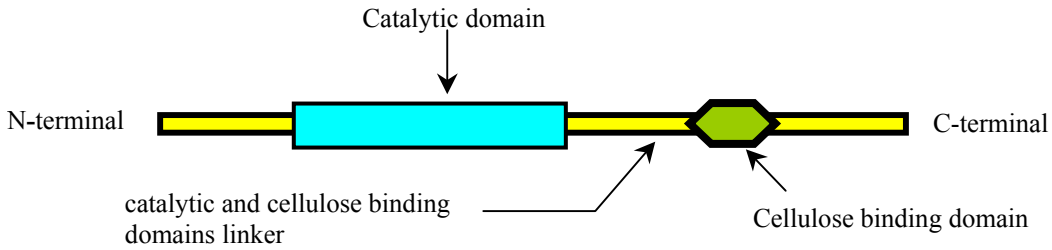


Figure 3: Architectural primary sequence organisation of cellulase domains of glycosyl hydrolase family-5A.

CBD domains are ubiquitous and occur in glycosidic hydrolase enzymes. CBD domains show a wide spectrum of substrate binding properties that include cellulose, xylan, chitin, and cellulose derivatives. There are differences within CBD domains from different sources regarding affinities to substrates. All cellulase enzymes are structurally either all α-helix domains or a combination of α/β barrels or all β-sheet domains. Endoglucanase family-5A glycosyl hydrolases, the class of the *Bacillus subtilis* CHZ1 endoglucanase under investigation, have $(\alpha/\beta)_8$ barrel folds and have two highly conserved Glu active site amino acid residues (figure 4). Enzymatic hydrolysis of cellulose microfibrillar structures by bacterial endo-β-1,4-glucanase enzyme is generally thought to be an acid/base catalysis mechanism promoted by Asp and/or Glu residues. The formation of an oxocarbonium ion is facilitated and stabilised by negatively charged adjacent Asp or Glu or His residues in the active site cleft (Henrissat *et al.,* 1989; Gilke*s et al.,* 1991; Kawaminami *et al.,* 1998; Gebler *et al.,* 1992). There are 8 invariant amino acid sequences that are highly conserved on the active sites of bacterial endo-β-1,4-glucanases. These are 2 tryptophan, lysine, serine, 2 tyrosine, glutamate and histidine residues, see figure 4A.

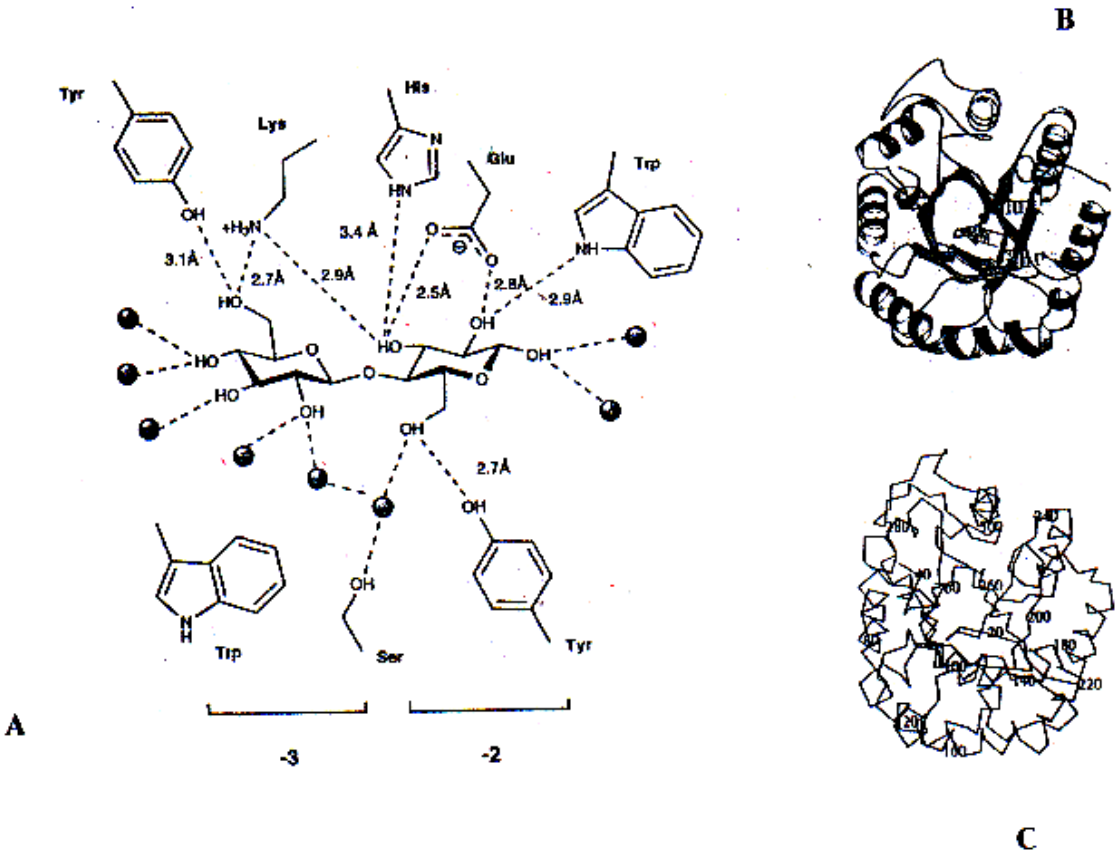


Figure 4: (**A**) Schematic representation of an endoglucanase enzyme interacting with cellobiose moiety of cellulose microfibril, (**B**) ribbon diagram of the enzyme showing the catalytic acid/base and nucleophile Glu residues positions and (**C**) its stereo C_{α}-trace. (adapted from Davies *et al.,* 1998)

The mode of stereochemical hydrolysis of β-glycosidic bonds of cellulose by cellulases proceeds by either a double-displacement catalytic mechanism (retention of configuration) or by a single-displacement catalytic mechanism (inversion) (figure 5). Inverting enzymes provide protonic assistance (from an Asp residue) to a glycosidic hydroxyl group and then a catalytic base deprotonates a water molecule for nucleophilic substitution at the anionic centre of the leaving glycoside group. The inversion hydrolysis mechanism involves formation of a covalent glycosyl-enzyme intermediate. This reaction proceeds with the aid of an enzymatic nucleophile and a catalytic base/acid residue that facilitates formation of oxocarbonium ion-like transition states (Gilkes *et al.,* 1991; Varrot *et al.,* 2000; Schülein, 2000).

The cellulase enzymes are now grouped into 13 families (5-9, 12, 45 and 48) based on their CD and CBD amino acid sequence similarities and hydrophobic clusters (Henrissat & Bairoch, 1993; Linder & Teeri, 1997; Schülein, 2000). The families are also grouped according to either retention or inversion mechanism of cellulose hydrolysis. Cellulose hydrolysis mechanisms were obtained

from NMR spectrophotometry analysis (Kawaminami *et al.,* 1998; Gilkes *et al.,* 1991; Schülein, 2000; Varrot *et al.,* 2000).

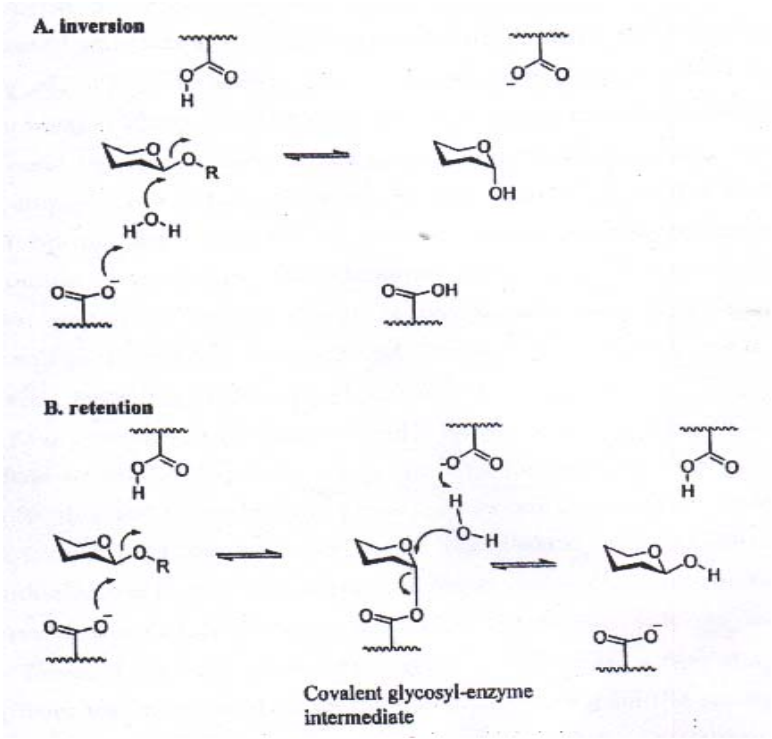


Figure 5. The proposed mechanisms of cellulose hydrolysis by glycoside hydrolases.

(adapted from Schülein, 2000)

2.5 THERMOPHILIC BACTERIA AND THEIR ENVIRONMENTS

Thermophilic biotopes have temperatures ranging from 40°C to 350°C, and these can be solar heated soils, decaying vegetation, industrial waste, self-heating soils and ore tailing soils (Bomio *et al.,* 1989; Ratledge, 1988; Hansen-Sonne *et al.,* 1993; Alfredsson *et al.,* 1988; Kristjansson *et al.,* 2000). A lot of naturally high temperature biotopes of geothermal origin occur widely around the earth's surface and these can be thermal springs, hot pools, geysers or steam emerging fumaroles and their pH varies from 0.1 to 12.0 (Krulwich & Guffanti, 1989; Martins *et al.,* 2001). Springs are a form of subterranean waters reaching the earth surface. Springs vary in volume output, temperature and chemical composition hence the need to determine their microflora that can exploit these environments. In these springs cellulase-producing microorganisms have been screened and obtained (Gilkes *et al.,* 1991; Priest, 1993; Ito, 1997; Zeikus *et al.,* 1998; Vieille & Zeikus, 2001).

Many springs have distinctive pH, temperature and or salty mineral water taste or smell such as iron or sulphur, as is the case with the Chimanimani Hot Spring's water of Zimbabwe that has high sulphur and fluoride ions concentration. These ions or compounds associated with the spring water are essential for bacterial metabolic pathways. There are several oxidation forms of sulphur that can act as both electron donors and acceptors, depending on the environmental conditions. The most widely used forms of sulphur by prokaryotes are sulphates, sulphite, sulphide and thiosulphate.

Thermal spring's flora has a wide spectrum of temperature tolerant microorganisms ranging from thermotolerant to thermophilic depending on the spring's temperature. Basing on optimum growth temperature, microbes can be classified as psychrophiles (these grow below 20°C), mesophiles (grow from 20°C up to 40°C) and thermophiles (grow from 40°C to 80°C) (Madigan *et al.,* 1997). Of late, the term hyperthermophiles is used to refer to microbes that are high temperature survivors at 100°C and above, and their enzymes are referred to as extremozymes (Kristjansson & Hreggvidsson, 1995; Kristjansson *et al.,*, 2000). Some of these extremozymes can function at high salt concentration and are known as halozymes. Other extremozymes can catalyse reactions at extreme conditions of pressure, acidity and highly alkaline conditions. Those extremozymes that function at extreme alkaline conditions are known as alkalozymes. Some thermotolerant microorganisms can grow at a temperature range of 30°C to 60°C. Availability of certain biotic and abiotic factors are subjected to alterations and these factors define distribution of spring's pH, oxygen tension, light, temperature, pressure and water chemical composition, hence define microflora which can survive in these springs. Subterranean and spring waters are poor nutrient environments, therefore are colonised by only a few types of microflora. The research on new strains or mutants that can be suitable cellulolytic sources of cellulases continues. The strains should be fast growing microbes, hypercellulolytic and be able to produce thermostable cellulase enzyme systems. This motivated the research on cellulase producing microorganisms from Zimbabwean hot springs with the hope of screening and isolating microbes producing interesting cellulase.

2.6 MODE OF THERMOPHILIC ADAPTATIONS

Recently studies have been focussed on enzymology, physiology and molecular genetics of thermophilic microorganisms to elucidate their mechanisms of adaptation to extreme conditions of high or low temperatures and extreme pH values. The adaptation by these microbes to extreme conditions has allowed their enzymes and fine biochemical products to be exploited for industrial, commercial and pharmaceutical applications.

2.6.1 Morphology and physiology of thermophiles

Since spring waters are short of nutrients, the microflora of these niches have few microbes that can exploit and survive. The bacterial shapes have evolved in such a way that they can cope with these environments i.e. bacterial cells are either small, cocci, or rods to ease transport of materials across their cell membranes. A noticeable feature of hot springs ecosystems is that they do not have much multicellular organisms.

Thermophilic microbes have evolved quite extensively so that they are able to occupy these ecological niches. They have shown adaptations through modifications of their physiological systems, enzymes and cell membranes lipid structures to sustain these extreme habitat conditions (de Mendoza *et al.,* 1993; Horani & Priest, 1994). The detailed literature referenced in this report is from *Bacillus* genus because the microorganism used for this study is of this family.

2.6.2 Nature of cell membrane fatty acids and lipids of thermophiles

Thermophiles and extremophiles membrane structures should adapt to environmental changes that occur in order to maintain their cellular integrity hence the range of cell membrane lipids from simple to complex long chain diglycerol tetraethers observed in these microorganisms. The nature of fatty acids of various thermophilic microbes is that they are temperature dependent. This biochemical adaptation and transition is necessary for thermophiles to survive in their habitats. Thermophilic microbe adaptation has been observed to be as a result of the rigid-to-fluid phase transition of biomembranes due to the structural, quantitative and qualitative nature of their cell membrane lipids and fatty acids. Microbes grown at temperatures below 20°C were observed to have relatively high amounts of unsaturated fatty acids compared to the same microbes grown at 40°C and above. An increase in temperature resulted in a shift in the fatty acid synthesis of saturated type of fatty acids (Kaneda, 1991; Priest, 1993; Horan & Priest, 1994; Markossian *et al.,* 2000; Yumoto *et al.,* 2000).

Alkalophilic *Bacillus* species have branched, and long chain fatty acids that include pentadecanoic acid (iso-$C_{15:0}$ and anteiso-$C_{15:0}$), heptadecanoic acid (iso-$C_{17:0}$, anteiso-$C_{17:0}$, iso-$C_{17:1}$) and hexadecanoic acid (iso-$C_{18:0}$) as major membrane fatty acid constituents. Table 2 summarises the nature of lipids found in thermophilic microorganisms. The lipid-compositions shift when there are changes in pH or temperature by thermophilic microbes had been observed to occur in *Bacillus* microorganisms (Kaneda, 1991 & 1997; Sturr *et al.,* 1994; Aono *et al.,* 1995; Nicolaus *et al.,* 1995; Markossian *et al.,* 2000; Yumoto *et al.,* 2000). An increase in temperature results in an increase in the amount of high melting point iso- fatty acids and a decrease of the low melting point anteiso- fatty acids particularly iso-$C_{17:0}$. Extreme thermophiles have evolutionary evolved

differently and their cell membranes contain appreciably higher quantities of high melting point iso-branched fatty acids and have tetraether lipids (see section **2.6.3**) than moderate thermophiles. The cell envelope of thermophilic bacteria is generally a composition of cytoplasmic lipid membrane peptidoglycan wall and a proteinaceous surface layer. Additional functional surface inclusions like capsules, slimes, fimbriae and flagella may be present. The cell wall in many other gram-positive bacteria is composed of peptidoglycan and one or more anionic polymers. The peptidoglycan layer is mainly techoic acids that are ribitol phosphate polymers including glycerol techoic acids, lipotechoic and teichuronic acids (Lennarz, 1970).

Table 2. Cell membrane fatty acids and lipid components of moderate and extremophile microbes.

Moderate thermophiles	Extremophiles
Iso-C_{15}	Iso-$C_{20:0}$
Iso-$C_{14:0}$	n-$C_{20:0}$
n-$C_{14:0}$	Iso-C_{30}
Iso-$C_{15:0}$	Iso-C_{25}
Anteiso-$C_{15:0}$	squalene
Iso-$C_{16:0}$	n-iso$C_{8:0}$
n-$C_{16:1}$	anteiso-$C_{19:0}$
Iso-$C_{17:0}$	Iso-C_{19}
Anteiso-$C_{17:0}$	hopanoids
Iso-$C_{17:1}$	
Anteiso-$C_{17:1}$	
$C_{18:0}$	
$C_{18:1}$	
n-$C_{18:1}$	

(adapted from Priest, 1993)

The synthesis of phospholipids and glycolipids is a membrane bound pathway in *Bacillus* genus. The major lipids are phosphatidylethanolamine, phosphatidylglycerol, lysine esters of phosphatidylglycerol and a small amount of cardiolipin (diphosphatidylglycerol) (Rothman & Kennedy, 1977). In *B. subtilis* strains diglucosyldiglyceride, monoglucosylglyceride and neutral 1,2-diglycerides are the major lipid components (Bishop *et al.,* 1975).

2.6.3 Extremophiles with novel lipid adaptations

The membrane lipid structure and biosynthetic pathways are quite different from thermophiles producing different lipids. Their glycerolipids lack the usual ester linkages between glycerol

backbone and fatty acyl moiety. Instead of ester bonds there are ether linkages. Ether linkages are quite stable and can withstand high temperatures and pH. A notable feature with extremophiles lipids is the presence of the isoprenoids diethers and tetraethers. The tetraether and diether molecules have phytanyl side chains that are covalently bonded to the glycerol-like backbones or substituted four-carbon or branched nine-carbon polyol structures called tetriol and nonitol (Madigan *et al.,* 1997; Priest, 1993). The tetraether molecules have phytanyl side chains that are covalently linked to form biphytanyl chains (figure 6). These complex lipids allow formation of monolipid layers instead of bilayers.

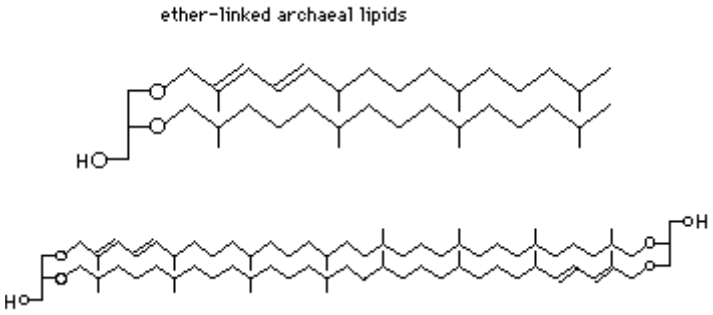


Figure 6. Illustration of the different phytanyl and biphytanyl hydrocarbon lipid structures of archaea microorganisms showing the ether linkages (adapted from Madigan *et al.,* 1997)

Monolayer structures are stable to heat compared to the bilayers that can peel off. Tetraethers exist in several species of extreme thermophilic archaea, which include extreme halophiles, methanogens, thermoacidophiles and hyperthermophiles (Kates, 1993; Langworthy, 1978; Nishihara *et al.,* 2000). The tetriol and nonitol lipid backbone compositions differ in different thermophilic bacterial and fungal species. Cell membrane lipids of thermophilic microorganisms have free hydroxyl groups that are sometimes phosphorylated, glycosylated or sulphated. Extreme thermophiles are known to have lipid structures of compounds like squalene, hexaisoprenoids, (C-30), pentaisoprenoids, (C-25) and tetraisoprenoids, (C-20). Archaea microorganisms have sterols like structures called hopanoids and are assumed to play the same role as sterols in eukaryotic cells.

2.6.4 Thermophilic enzyme adaptation mechanisms

Naturally enzymes originating from thermophiles have to be robust to function at these extreme conditions of either pH or temperature. The primary, secondary, tertiary and sometimes quartenary structure of thermophilic proteins are similar to mesophilic proteins and perform the same function although they are more stable to denaturing agents. Thermophilic proteins have the same temperature-activity profile as is observed with mesophilic proteins. The enhanced molecular

mechanism to stabilise thermophilic proteins has been explained by numerous stabilising ways (Zeikus *et al.,* 1998; Vieille & Zeikus, 2001). An explanation to that is the presence of substituted critical amino acid residues capable of forming stable hydrophobic interactions, hydrogen or anionic bonds thus producing protein conformation that has an interior that is densely packed (Yutami *et al.,* 1978; Zeikus *et al.,* 1998). Another stabilising way has been observed which is a result of the protein structure's interior having a high percentage of small hydrophobic amino acids (Stellwagen & Wilgus, 1978; Vieille & Zeikus, 2001). The small amino acids allow supplementary non-covalent hydrophobicity interaction and hence stabilise the protein's conformation.

Stabilisation of thermophilic proteins is enhanced with their interaction with Ca^{2+} ions. Ca^{2+} ions stabilising effect is documented in α-amylases, polygalacturonases and proteases (Igarashi *et al.,* 1998; Strongin *et al.,* 1979). An explanation to this is a result of the increased number of salt bridges between amino acid residues and metal ions (Na^{+} or Ca^{2+}) that enhances the compactness of the densely packed hydrophobic interior of these thermophilic proteins.

The involvement of extrinsic parameters like polyamines and diamines in thermophilic microbial cells has been assumed to be responsible for nucleic acids stabilisation, protein biosynthesis and some enzyme action in *Thermus* and *Bacillus* species (Oshima, 1978). Other intracellular factors like salts, high protein concentration, coenzymes, high substrate concentration, activators and general stabilisers such as thermamine have been shown to contribute to the thermostability of thermophilic proteins (Vieille & Zeikus, 2001).

2.7 MICROBIAL CELLULASES

2.7.1 Fungal cellulases

Several commercial preparations of cellulase are of fungal origin based on *Trichoderma reesei* (*viridie*) cellulases (Tolan & Toddy, 1999; Bhat & Bhat, 1997; Pere *et al.,* 2001). A number of the studied fungi that produce cellulase enzymes include, *Streptomyces, Actinomadura, Saccharomonospora, Trichoderma, Neurospora, Penicillum, Candida, Piromyces, Aspergillus and Rhizopus* strains (Harmova *et al.,* 1986; Yazdi *et al.,* 1990; Meyer *et al.,* 1992; Ali & Akhand, 1992; Schimdhatter & Canevascini, 1992; Haltrich & Steiner, 1994; Oksanen *et al.,* 2000; Sinha *et al.,* 2000; Belghith *et al.,* 2001; Pere *et al.,* 2001).

Fungal cellulase preparations are efficient in hydrolysing crystalline cellulose to glucose as a result of secretion of complete cellulase enzyme systems (Schmidhalter & Canevascini, 1993). Production of fungal cellulase is sometimes in very high concentration in submerged cultures and

do not seem to form complexes with each other as bacterial cellulase do (Kuhad *et al.,* 1997). However, fungal cellulase production is regulated by induction and catabolite repression by cellulase degradation products (Lin & Wilson, 1987; Meyer *et al.,* 1992), although similar cellulase regulatory production mechanisms occur in some bacteria (Gilkes *et al.,* 1991). Most fungal cellulases are mesophilic and enzyme production takes longer periods hence the search from thermophilic and cellulolytic sources like bacteria. Simple sugar like glucose has been shown to cause catabolite repression of endoglucanase in *P. chrysosporium* and *T. reesei* (Kuhad *et al.,* 1997). Multiple forms of endoglucanase i.e. (EGI, EGII, EGIII, EGIV and EGV) have been purified from *T. reesei* (Kuhad *et al.,* 1997) approximating about 5 % of the total secreted proteins. In addition to five endo-β-1,4-glucanase forms of the cellulase enzyme systems produced by *T. reese*i, at least two cellobiohydrolases (CBHI and CBHII) and two β-glucosidases (BLGI and BLGII) (Kubicek & Pentilla, 1998; Kubicek *et al.,* 1991) are also produced. The multiple forms of endo-β-1,4-glucanase had M_r values of 48, 48 and 37 kDa with different pI values of 5.4, 5.7 and 4.8 respectively.

Since *Trichoderma reesei* is the most widely studied cellulolytic microorganism, it has gone beyond the development of the strain in trying to improve the enzyme production and yields with the mutant strains. This biotechnological approach was employed and realised an increase in filter paper activity from 83 to 250 FPU g^{-1} substrate (Ryu & Mandels, 1980). Although cellulolytic enzyme production in fungi is widespread as in bacteria, the enzyme components vary greatly in each category. The current model on the cellulase enzyme's synergism in hydrolysing cellulose is based on fungal cellulases.

Recent research studies have been directed towards improving both bacterial and fungal cellulases in anticipation of a superior cellulase system compared to the already established ones (Schomburg, 1994; Ito, 1997; Zeikus *et al.,* 1998; Schülein, 2000).

2.7.2 Bacterial cellulases

Cellulolytic microorganisms have been isolated from environments such as rumen, soil compost, manure, municipal solid waste, brewery sludge, sewerage sludge, wood material, hot springs, marine sediments, alkaline environments and geysers. The majority of work on cellulase producing microbes has been done with fungi, although over the past two decades a lot of effort has been on bacterial cellulases. Among the bacterial isolates explored to date are *Bacillus, Clostridium, Cellulomonas, Acetovibrio, Bacteroides, Microbiospora, Ruminococcus, Streptomyces, Thermonospora, Cytophaga, Micrococcus, Sporocytophaga and Pseudomonas* species (Mullings & Parish, 1984; Dhillion *et al.,* 1985; Au & Chan, 1986; Prasertsan & Doelle,

1986; Waldron & Eveleigh, 1986; Gilbert *et al.,* 1987; Ghanghas & Wilson, 1987; Sami *et al.,* 1988; Gilkes *et al.,* 1991; Navarro *et al.,* 1991; D'Elia & Chesbro, 1992; Paul & Varma, 1993;). Many cellulase systems in aerobic microorganisms are free enzymes. Aggregates of multienzymes structures called cellulosomes were first identified in anaerobic thermophilic and cellulolytic clostridial species (Belaich *et al.,* 1997, Doi *et al.,* 1994; Karita *et al.,* 1997) and in bacteria and fungi (Chen *et al.,* 1998; Fanutti *et al.,* 1995). Larger complexes of cellulosomes called polycellulosomes have been identified too. Cellulosomes are mostly difficult to disrupt without loss of total enzyme activity as well as individual components (Gilbert *et al.,* 1987).

Several cellulase components of these microbial sources have been purified to homogeneity in the 1980s and their chemical and physical properties determined. Isolation of cellulolytic bacteria has been narrowed to a certain framework of desired characteristics or properties. Workers are researching on bacteria that produce cellulases that have the characteristics suitable for the intended biotechnological applications. Suitable cellulolytic microbes are fast growing and hypercellulolytic.

Bacterial cellulase systems are quite ubiquitous and have comparable hydrolysing activity to those of fungal origin. Most bacterial microbes particularly bacilli have been considered non-cellulolytic because they produce only endoglucanase component of the cellulase enzyme systems (Au & Chan, 1986; Robson & Chambliss, 1986; Gilkes *et al.,* 1991, Dhillion *et al.,* 1985; Christakopoulos *et al.,* 1999; Mawadza *et al.,* 2000). Endoglucanases of bacterial origin have M_r ranging from 23 to 146 kDa and most of them have been purified by gel filtration followed by ion exchange chromatography (Gilkes *et al.,* 1991). However, bacilli M_r range from 35 to 82 kDa (Park *et al.,* 1991; Han *et al.,* 1995; Kim *et al.,* 1995; Mawadza *et al.,* 2000). The pH optima of bacterial cellulases are quite broad ranging from 4 to 7 although Horikoshi and co-workers have reported alkaline optima of 10 (Horikoshi, 1999). Endoglucanase production is growth dependent for some microorganisms but for some *Bacillus subtilis*, cellulase production begins at the onset of the stationary phase and is constitutively produced (Dhillion *et al.,* 1985; Mawadza *et al.,* 1996). The mechanism for cellulase action in bacteria may differ from those of fungi as noted by the differences of their optima of pH and temperature. A notable feature of a number of bacterial endoglucanases is that, they do not effectively hydrolyse crystalline cellulose. Until recently few workers have purified and characterised *Bacillus* endoglucanase capable of hydrolysing crystalline cellulose (Nakamura & Kitamura, 1982; Kim *et al.,* 1995; Han *et al.,* 1995; Li *et al.,* 1998; Mawadza *et al.,* 2000). Bacterial endoglucanases can be assumed to have a different mode of cellulose hydrolysis compared to the fungal type. Hence, the assay procedures might need to be modified to suit different enzyme sources

Much of the molecular cloning of cellulases from microbial sources has been embarked on for the past decade (Gilkes *et al.,* 1991; Schülein, 2000). Nevertheless, few strains are being pursued with the hope of identifying organisms that may produce cellulase with different properties. This hope has led scientists to direct their efforts into extreme conditions researching on new strains, and in mutagenesis of current cellulase enzymes.

2.8 THE GENUS *BACILLUS*

The genus *Bacillus* is a heterogeneous group of bacteria that produce a range of biotechnologically important products such as insect toxins, peptide antibiotics and fine biochemical products like enzymes. *Bacillus* facultative aerobes and anaerobes have a unifying characteristic of forming very resistant spores. Of the more than 1134 *Bacillus* strains identified to date, only 65 validly describe the species in the genus (Priest, 1993). New species are constantly being isolated and new genome structures are being unravelled (Nielsen *et al.,* 1995; Yumoto *et al.*, 2000). The genus *Bacillus* has shown species that have identical morphological, biochemical and physiological characteristics. *Bacillus* sp. produce a large number of extracellular enzymes that include proteases, amylases, cellulase, polygalacturonases, xylanases, glycosidases and other hydrolases. *Bacillus* strains can express and secrete individual gene products at very high levels if conditions are optimised. With our current understanding of genetics of expression, there is potential for exploitation of *Bacillus subtilis* for commercial purposes. *Bacillus* species are subdivided into six groups, based on phenotypic similarities, 16S rRNA and DNA-DNA relatedness data as shown in figure 7 (Ash *et al*., 1991; Priest, 1981) and the observable characteristics of each of the bacilli group are summarised in table 3.

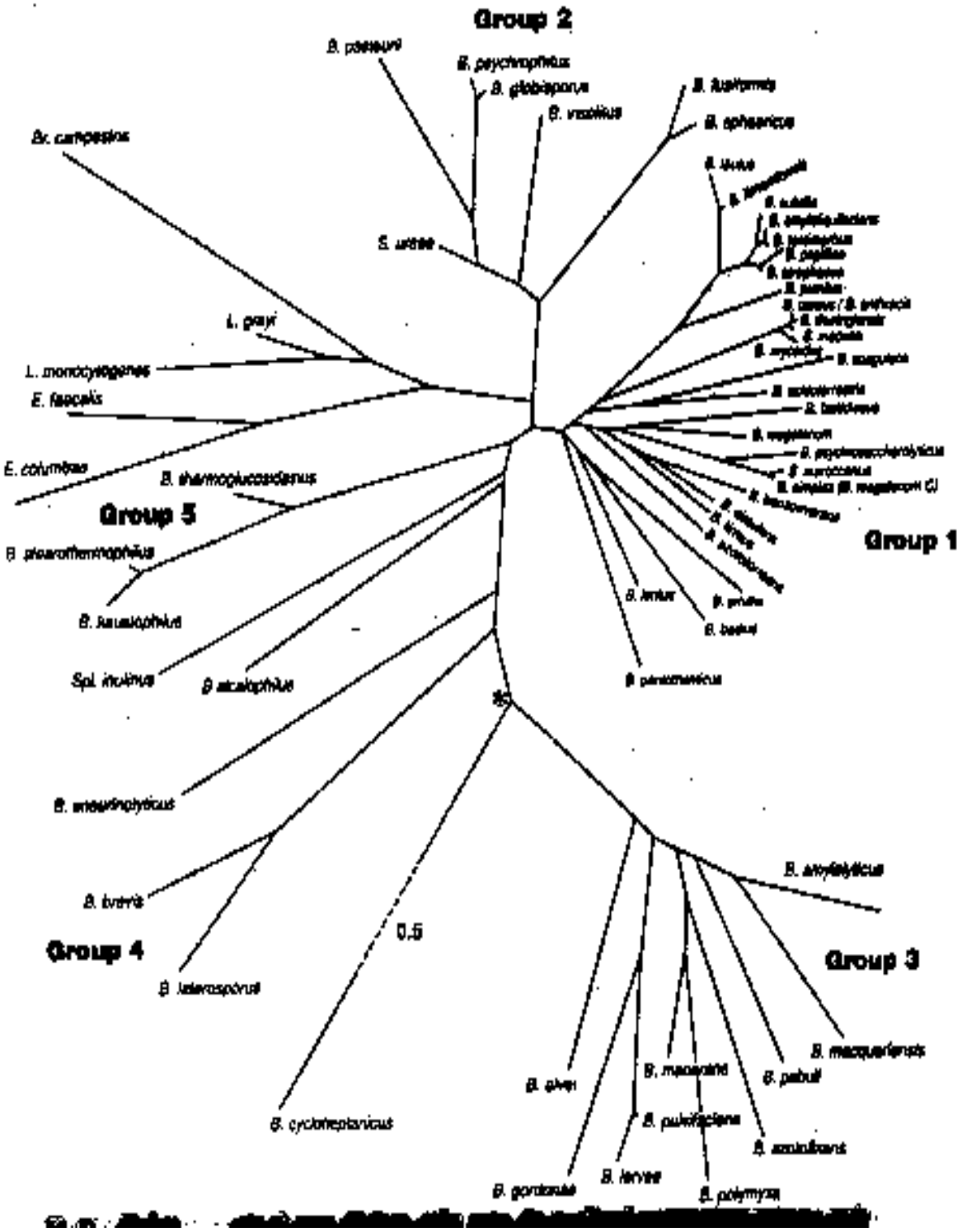


Figure 7. An overview of the general diversity and heterogeneity of *Bacillus* species illustrated by the phylogenetic tree based on 16S rRNA sequence and DNA-DNA relatedness.
(adapted from Priest, 1993)

Table 3. Classification of *Bacillus* strains into groups basing on phenetic and chemotaxanomic data in addition to phylogenetic information.

Class	Example	Group characteristics
Group 1	*Bacillus subtilis*	facultative anaerobes that grow well under anaerobiosis, require complex media, produce acids from various sugars as carbon sources, endospores are ellipsoidal swell mother cell, most species are commensals and pathogenic.
Group 2	*Bacillus sphericus*	all strains yield acids from sugars, grow weakly in absence of O_2 though *B. cereus* and *B. licherniformis* are facultative anaerobes, sporulate centrally or subterminally don't swell mother cell, all are alkalophiles, occur widely in soils, associated with ruminants gut systems.
Group 3	*Bacillus macerans*	strict anaerobes produce acids from sugars, produce ellipsoidal endospores swell the mother cell, producers of antibiotics e.g gramicidin, are involved in food spoilage.
Group 4	*Bacillus brevis*	produce spherical spores, do not make use of sugars as carbon sources for growth, and rather prefer acetate or amino acids.
Group 5	*B. stearothermophilus*	thermophilic bacilli with extensive heterogeneity, range from facultative anaerobes to strict aerobes, do not act on sugars, produce oval spores that swell the mother cell, this group has acidophilic *Bacillus* species with membranous ω–alicyclic fatty acids.

(adapted from Priest, 1993)

2.9 AN OVERVIEW OF *BACILLUS* SPP. AND APPLICATIONS OF THEIR SECONDARY PRODUCTS

Current biotechnology is attempting to address problems on nutrition, health, growth promotion, genetics, product quality, waste management monitoring and environmental bioremediation. These studies include bioconversion of agricultural wastes and human wastes to animal feed products and fuels respectively. Further biotechnological applications involve developing improved microbes for human waste treatment, agricultural, municipal and industrial organic wastes or wastewater (Bomio *et al.,* 1989; Brownell & Nakas, 1991; Selinger *et al.,* 1996; Cheung & Anderson, 1997; Morgavi *et al.,* 2000).

Methanol production, waste and wastewater treatment and sewage treatment now involve the use of thermophilic bacteria, *Bacillus* sp included. Biological control of fungal pathogens that infect plants and insects resistance, are some of the various applications of *Bacillus* sp. (Madigan *et al.,* 1997). The mutual associations of plants and *Bacillus* sp. particularly those plants that were

cultivated in low-nutrient soils had been investigated in an attempt to improve agricultural output (Kloepper *et al.,* 1989). Some inoculums of *Bacillu*s sp. are now used to improve crop production because of their nitrogen fixing metabolic capabilities. Some *Bacillus* strains are known to produce plant stimulating hormones (gibberellins) and fungal antibiotics that are useful in controlling fungal diseases in fruits, vegetables, field crops and flower crops (Handelsman *et al.,* 1990). One important agricultural application is the use of *Bacillus* strain in controlling *Phytophthera* fungal infections of wheat crops.

The study of food spoilage and infectious diseases has led to an awareness of the potential involvement of pathogenic and non-pathogenic *Bacillus* species (Gill, 1982; Hemila *et al.,* 1989). Bacilli involvement in food spoilage has necessitated a comprehensive study of the strains involved. For medical treatment, and control mechanism of an infectious bacterium, a thorough microbial study of the microbe in question has to be undertaken. The potential pathogenicity of *Bacillus* species has been demonstrated through secretion and expression of toxins after cloning toxin genes (Saris *et al.,* 1990; Ihde & Armstrong, 1973). However, *B. subtilis* does not appear to possess indigenous virulence factor genes, although it can acquire and incorporate foreign toxic genes. Food industries require a comprehensive knowledge of food poisoning microbes in order to be able to improve food-processing conditions as a way of reducing and destroying the possible spoiling bacteria.

Considering population growth worldwide and the increasing need for antibiotics and other products of medical value, there is a there is a technical need to improve on *B. subtilis* strains that have been genetically engineered to produce proinsulin (Mosbach *et al.,* 1983) and bacitracin antibiotic (Morikawa *et al.,* 1980). These processes can be scaled up in order to produce these products.

2.10 MICROBIAL ENDOGLUCANASE AND PROTEASE INTERACTION

Microorganisms produce hydrolytic enzymes that are involved in several functions. The enzymes make carbon, phosphate, nitrogen and other nutrients available for perpetuation. Among the enzymes produced include endo-β-1,4-glucanase and proteases. Endo-β-1,4-glucanase is one of the enzymes of the cellulase enzyme systems involved in hydrolysing cellulose meanwhile proteases are enzymes that have several roles in different organisms. The most important function of proteases in microorganisms is in protein turnover, including turnover of its own proteins (enzymes included), when nutrients become depleted. Bacterial proteases are also involved in proteolytic modifications or processing steps preceding protein production (Creighton, 1993). The

processes include removal of secretory signal sequences before the protein folds into a mature protein. Bacterial extracellular proteases are widely used in industry.

Endo-β-1,4-glucanase enzymes have been shown to have different sizes than the predicted product from their genes. The difference in enzyme size is accounted by post-translational modification after the extrusion from the cell. The onset of protease production is associated with the decline of endo-β-1,4-glucanase activity in the crude supernatant (Gilkes *et al.,* 1991; Au & Chan, 1986).

Microbial proteases are divided into endo- and exo-proteases. Endo-proteases are sub-classified into four categories based on their mechanism of action i.e. serine, acid, metallo and sulfhydryl proteases (Pero & Sloma, 1993; Rao *et al.,* 1998).

The serine proteases have serine residue on their active sites and are inhibited and irreversibly inactivated by small amounts of organoflourides such as diisopropylfluorophosphate (DFP) and phenylmethylsulfonyl fluoride (PMSF), which react with the serine residues. This class of proteases has been studied extensively and have pH optima around 8.0. Three types of protease substrate specifities are found in this group i.e. trypsin-like, chymotrypsin-like and elastase-like enzyme. Trypsin-like protease enzyme splits proteins at internal peptide bonds where lysine and arginine occupy on the protein substrate. Chymotrypsin-like enzyme cleaves a protein when there are adjacent bulky hydrophobic residues on a protein substrate. The third serine protease i.e. elastase-like enzyme, acts on a protein's small hydrophobic regions. The mode of hydrolysis of serine proteases is through a charged relay system (Kraut, 1977).

Metalloproteases is not a common microbial protease enzyme. Metalloproteases require divalent metal cations like Zn^{2+} for activity and are completely inhibited by chelators such as EDTA and 1,10-phenanthrone.

A third class of microbial protease enzymes is thiol or sulfhydryl proteases that have a sulfhydryl group on their active site. Their activity is stimulated by ditheiothreitol and cysteine but inhibited by oxidising agents that react with sulfhydryl groups like mercurials. Other inhibiting reagents for this group of proteases include dibromoacetone, iodoacetic acid, heavy metals, alkylating agents, Nα-p-tosyl-L-lysine chloromethyl ketone (TLCK) and N-tosyl-L-phenylalanine chloromethyl ketone (TPCK). Thiol protease enzymes have molecular weights that range from 25 to 40 000 Da and employ basically the same catalytic mechanism but because of evolutionary diversity, sizes, specifities and kinetic properties, they vary considerably. The mechanism of hydrolysis involves nucleophilic attack by cysteine resulting in formation of a tetrahedral intermediate and an acyl enzyme intermediate leading to hydrolysis (Angelides & Fink, 1979a & b).

A fourth subclass is the acid or aspartic proteases that are active in acid conditions. This class of proteases is inhibited by biazoketones. This group as well, is not well established in microorganisms. This group of proteases is quite common in animals.

Currently the common extracellular proteases obtained from *Bacillus* species are the metalloproteases and alkaline serine proteases (subtilisin like) (Tsuru *et al.,* 1966; Ward, 1983; Takami *et al.,* 1989; Pero & Sloma, 1993; Rao *et al.,* 1998). Microorganisms are known to produce proteases at very low levels with high proteolytic activity. Thermostable alkaline proteases are of interest because of their increasing commercial importance, particularly in detergent manufacturing and other related industries (Ward, 1983; Horikoshi & Akiba, 1982; Ito *et al.,* 1998; Keay *et al.,* 1972). Research on proteases from new species of microbes can broaden the potential applications of these enzymes.

2.11 APPLICATIONS OF CELLULASES AND OTHER HYDROLYTIC ENZYMES

The majority of industrial enzymes are hydrolytic in nature. Enzymes are increasingly being used for a large variety of industrial functions and consequently a lot of effort is directed into their cloning and over-expression (Schomburg, 1994; Bhat & Bhat, 1997; Ito *et al.,* 1998). Most industrially applied enzymes are used as biochemical catalysts.

Some classical examples of commercially functioning enzymes are; amylase in starch industry; bacterial protease and cellulases in detergent, beer and meat industry; glucose isomerase in making high fructose corn syrup, and rennin and chymotrypsin in cheese making industry. Different enzymes have been developed for different industrial purposes.

Cellulase enzymes are industrially applied in cleaning processes, in the production of alcohol, for predigestion of grains used in feedstocks, and in the production of coffee. An endoglucanase enzyme from *Dictyoglomus* DSM 6262 that breaks down the cotton microfibrils, has been used to soften new jeans as an alternative method to harsh stone washing and for biopolishing of cotton fabrics (Mathrani & Ahring, 1992; Schülein, *et al.,* 2000). A major research area that has attention for the past century is the biodegradation of cellulose biomass using cellulases (Sun & Cheng, 2002). This application has not been economically exploited due lack of cellulases that can hydrolyse cellulose efficiently in addition to the high cost of the enzymes.

There are several antibiotics and other important products like proinsulin that are produced by genetically engineered *Bacillus* species that can be of medical value (Morikawa *et al.,* 1980; Mosbach *et al.,* 1983). The products require further research in order to scale-up production.

The enzymes that are industrially applied in the laundry cleaning agents, dehairing, paper industry and animal manure wastes, odour treatments are cellulases, proteases, xylanases and lipases (Holtz *et al.,* 1991; Takami & Horikoshi, 2000; Horikoshi & Akiba, 1982). The overview of global enzyme market by the year 2000 is summarised in table 4. Enzyme-based detergents are value-added products on market worldwide. Of those enzymes applied in detergent industries, proteases

constitute 30 % (US dollars value) of the total global market (Srinivasan *et al.,* 1999). Although alkaline proteases have gained ground in the detergent industry, current studies have been focussed on the inclusion of amylases, lipases and cellulases in household detergents (Ito, 1997; Ito *et al.,* 1998).

Xylan usage as a renewable source of energy had no meaningful results for the commercial production of fuels. Research on xylanase-producing strains is ongoing to identify cellulases-free xylanases that can be applied in an environmentally friendly way in bleaching paper pulp without the use of toxic chlorine compounds and also without adversely affecting the quality of the paper. Amplification and cloning of these enzymes for paper application purposes is in progress in various laboratories (Paice *et al.,* 1988; Bertrand *et al.,* 1989; Linder *et al.,* 1994; Kulkarni *et al.,* 1999; Takami & Horikoshi, 2000; Luthi *et al.,* 1990; Subramaniyan & Pema, 2000).

Table 4. World market value of enzymes of biotechnological importance by year ending 2000.

Biotechnological application	Market world values (US$)
Food industries	475
Detergent additives	300
Medical diagnostics	125
Biocatalysts	15

(adapted from Srinivasan *et al.*,, 1999 and Takami & Horikoshi, 2000)

Major commercial and industrial enzyme suppliers produce enzymes that are genetically engineered to improve yields. The limitations associated with most enzymes are due to loss during isolation from natural sources, instability, and activity within narrow temperature and pH range as well as functioning in aqueous systems (Schomburg, 1994). Recombinant DNA technology can be used to improve the yield of a protein. This can be accomplished by transferring the gene encoding the protein of interest into a microorganism capable of producing it in larger quantities. Recombinant DNA technology can also allow genetic encoding of altered enzyme characteristics like stabilities to pH and temperature, substrate reactivities and specificities to suit the intended application. Table 5 summaries some of the major uses of the currently marketed enzyme. Although the discussion above is focussed on the production of a single-gene product, similar

work for modifying pathways and amino acid substitution is under way to overproduce various enzymes.

An attempt to use microbial cellulase enzyme was on covalently immobilising *Penicillum funiculosum* cellulase enzyme onto PVA using 1-ethyl-3-(3-dimethylamino propyl) carbodiimide (EDC) and then the enzyme's activity was compared with an unimmobilised one (Mishra *et al.,*, 1983). The results demonstrated that there was a 30 % increase in rate of hydrolysis of alkali treated bagasse and much retention of enzymatic activity that could be continuously used for 5 days. Hence cellulase enzymes can be immobilised to improve on product yield.

Table 5. Some of the commercial and industrial applications of enzymes from thermophilic microbes.

Enzyme	Applications
Protease	detergents additives, leather processing, cheese making.
Amylase	liquefying agents, textile industry, saccharification of starch.
Xylanase	reduction of fluid viscosity, pulp and paper bleaching, juices clarification.
Pectinase	wine and juice clarification, extraction of oils, colours or flavours, reduction of fluids viscosity.
Cellulase	detergent additives, animal feedstock preparations, increase food palatability.

2.12 CLONING OF CELLULASE ENZYMES

The intended purpose of cloning in most cases is to over-produce the cloned gene product. The key objective to this strategy in enzyme technology is to increase the specific activity of an enzyme. In an attempt to overproduce cellulase enzymes from various prokaryotic sources, workers for the past decade have embarked on cloning their respective genes (Robson & Chambliss, 1987; Kim & Pack, 1993; Min *et al.,* 1994; Meinke *et al.,* 1995; Fanutti *et al.,* 1995; Sumitomo *et al.,* 1995; Kim *et al.,* 1995; Miyatake & Imada, 1997; Sanchez-Torres *et al.,* 1996; Blanco *et al.,* 1998; Riedel & Bronnenmeier, 1998; Dai *et al.,*, 2000; Murashima *et al.,* 2002).

Several approaches have been used in cloning cellulase genes, yielding different results. Quite a number of these cellulase genes of fungal and bacterial origin were cloned by firstly performing genomic DNA extraction from the producer strain. After DNA extraction it is then partially digested using restriction enzymes. The DNA fragments generated were then cloned into several vectors that include; pJM109, pJM108, pC164, pET, pUB110, pUC18 or 19, and pGEM (Cantwell & McConnell, 1983; Cantwell *et al.,* 1985; Robson & Chambliss, 1987; Kim & Pack, 1993; Min *et al.,* 1994; Sumitomo *et al.,* 1995; Kim *et al.,* 1995; Sumitomo *et al.,* 1995; Kim *et al.,* 1995; Miyatake & Imada, 1997; Miyatake & Imada, 1997). The plasmid vectors have different characteristics. For example pUB110 is for *Bacillus* hosts, pET is a good expression vector for *E. coli* hosts or have ease cloning properties e.g. pGEM-T vectors for cloning of PCR products. The clones were then transformed into specific replicating hosts which were *E. coli* (Shima *et al.,* 1991; Sumitomo *et al.,* 1995; Kim *et al.,* 1995; Sanchez-Torres *et al.,* 1996; Miyatake & Imada, 1997; Miyatake & Imada, 1997; Akhtar *et al.,* 1997; Khanongnuch *et al.,* 1999), *Bacillus subtilis* (Lee *et al.,* 1987; Lee & Pack, 1987; Lo *et al.,* 1988; Sumitomo *et al.,* 1992; Sumitomo *et al.,* 1995; Khanongnuch *et al.,* 1999; Joseph *et al.,* 2001) or eukaryotes like fungi, plants and mice (Hinchliffe *et al.,* 1984; Cantwell *et al*., 1985; Olsen & Thomsen, 1991; Zhang *et al.,* 1997; Dai *et al.,*, 2000) for replication or expression. The produced mutants were screened for cellulase activity on a plate containing a cellulose derivative substrate, which is identified by staining the plate with a dye like Congo Red (Teather & Wood, 1982; Cantwell & McConnell, 1983; Robson & Chambliss, 1987; Her *et al.,* 1999; Srivastava *et al.,* 1999) or by confirming enzyme activity from the tissues of the mutants.

Most of the cloned cellulase genes were carried out by generating a genomic library although in a few cases the genes were not expressed (Sanchez-Torres *et al.,* 1996; Khanongnuch *et al.,* 1999). Cloning of endoglucanase enzymes from several sources by some scientists produced mutants that had different levels of expression; 6.2 fold (Chun *et al.,* 1995), 3 fold (Sharma *et al.,* 1987), 3.8 fold (Kim *et al.,* 1998). Other workers reported extracellular activity as high as 70 % (Lo *et al.,* 1988) and 50 % (Soutschek-Bauer & Staudenbauer, 1987) than their parent strains. However, lower enzyme activity from clones compared to the parent wild strain have been reported (Akhtar *et al.,* 1997; Presutti *et al.,*, 1991). The C-terminal sequence of endoglucanase gene was observed to affect transportation of the enzyme extracellularly and truncation of this signal sequence improved extracellular enzyme expression by 70 % in the *E. coli* cells (Lo *et al.,* 1988). Similar truncation by Kim and other independent workers showed that there was no effect on the basic enzyme properties (Kim *et al.,* 1995). All the current cloned endoglucanase enzymes have Mr weight values ranging from 32 to 65 kDa though most of their precursor proteins predicted from

their genes would give a Mr value of 50-55 kDa. The much difference in sizes obtained has been attributed to posttranslational processings.

Among the endoglucanase genes cloned are those of *Bacillus* species (Blanco *et al.,* 1998; Baird *et al.,* 1990; Robson & Chambliss, 1987; Kim *et al.,* 1995; Khanongnuch *et al.,* 1999; Nakamura *et al.,* 1987; Sanchez-Torres *et al.,* 1996; Lo *et al.,* 1988; Akhtar *et al.,* 1997; Ito, 1998; Horikoshi, 1999; Takami & Horikoshi, 2000; Gilkes *et al.,*, 1991; Baik & Park, 1990; Park *et al.,* 1993; Ozaki *et al.,* 1990 and 1991; Park *et al.,* 1991). The coding region of the endoglucanase gene for *Bacillus* species vary from 1.2 kb to 3.5 kb with corresponding varying open reading frames in producer strains and cloned hosts. This explains the variation in sizes of the endoglucanase enzymes. Although there is this variation, all the endoglucanase amino acid sequences contain a short hydrophilic carboxylic terminal and a long hydrophobic N-terminal end. The production of tailor made enzymes for specific applications was part of the current study.

Tobacco and potato plants have been used as bioreactors in cloning cellulase genes of *Acidothermus cellulolyticus* (Dai *et al.,*, 2000). The two transgenic plants had 0.05 % to 2.6 % cellulases of the total soluble protein harvested from the leaves. The enzyme activity was the same as the native protein produced by the wild strain. The production of cellulases in the potato has a dual-crop application because the potato tubers will still serve as food and the vines as enzyme "bioreactors".

Another cellulase cloning technique that has been used is the Polymerase chain reaction. Following the pioneering discovery of the full genome sequences of important microorganisms and gene sequences stored in the database of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov), reference sequences can now be used for cloning of cellulase enzymes using PCR technique (Sanchez-Torres *et al.,* 1996; Joseph *et al.,* 2001). The PCR technique requires knowledge of the sequence of the gene or of a functionally or structurally related protein. This information can be obtained from several databases on internet. There are database search programs such as BLAST or FAST, ENZYME, PROSITE, SCOP and Swissprot that can be used on the basis of either sequence or protein structural or enzymatic functional similarities to search for their sequences (Hegyi & Gerstein, 1999). With the help of PCR primer computer program like DNMAN, primers for amplifying the respective gene can be designed and thereafter cloned.

Gene expression can be improved by fusing the cloned gene with strong promoters (Kim & Pack, 1993; Sumitomo *et al.,* 1995; Sumitomo *et al.,* 2000). The other aspect in cloning cellulase enzymes that is equally important is the development or the use of an expression system that is capable of producing large quantities of the recombinant enzyme. Production of the product has to be at low cost and preferably from low-value processing plant.

After cloning, it maybe essential to improve the enzyme's properties by protein engineering and protein design techniques. It is unfortunate that *de novo* design of proteins is limited to small peptides or domains of a protein (Schomburg, 1994). Protein engineering has been predominantly used as a tool in the study of catalytic mechanism of enzymes. Protein engineering and protein design can improve properties like thermostability, resistance to feedback inhibition, stability to heavy metals, resistance to oxidising agents and high or low pH stability of the protein thereby suiting desired application. A simpler way of improving thermostability of a protein can be by altering the protein's conformation to assume that of a naturally similar and existing protein structure of an extremophile protein (Zeikus *et al.,* 1998; Vieille & Zeikus, 2001).

An improvement on an enzyme's properties can be accomplished by incorporating protein design techniques like site-directed mutagenesis, nested deletion mutagenesis or fusing endoglucanase gene domains from different microorganisms i.e. DNA shuffling or chimerization (Doran & Ingram 1993; Ozaki *et al.,* 1994; Wang & Jones, 1997; Ness *et al.,* 1999; Tomme *et al.,* 1994; Kim *et al.,* 1998). Table 6 summarises some of the amino acid residue substitutions or protein structure alterations that can be performed for an intended protein property. Fusion of DNA shuffling and site-directed mutagenesis techniques improved thermostability of an endoglucanase enzyme by 20˚C (Hakamada *et al.,* 2001). The mutated protein retained 50 % residual enzyme activity at 80˚C compared to the wild type that retained the same amount at 60˚C. Site-directed mutagenesis has been shown to improve various enzyme properties. It was used to improve the substrate spectrum of endoglucanase enzyme (Wang & Jones, 1997), acid-stability and non-calcium dependability in amylase enzyme activity (Zeikus *et al.,* 1998).

Table 6. Targets for proteins design and predicted mutated protein property acquired

Property	**Amino acid substitution or Protein alteration**
Thermostability	introduction of disulphide bridges, introduce Pro on β-loops, increase of internal hydrogen bonds, improvement of internal hydrophobic packing
Stability to Oxidation	conversion of Cys to Ala or Ser, conversion of Met to Gln, Val, Ile or Leu. conversion of Trp to Phe or Tyr,
pH stability	alteration of surface charge groups, replacement of internal His, Cys and Tyr, replacement of internal ion pairs.
Improved enzymatic properties	alteration of specifity, increase of turnover number, alteration of pH profile

(adapted from Schomburg, 1994 and Vieille & Zeikus, 2001)

Wang and Jones (1997) showed that an engineered fungal endoglucanase active site could recognise a substrate that had one more glycosyl unit. However, other workers cloned endoglucanase enzymes from *Bacillus macerans* and reported reduction in enzyme activity after site-directed mutagenesis (Hahn *et al.,*, 1995). Amino acid residues within the active site region of a protein can be replaced to study the involvement of the amino acid residues in catalysis (Kormos *et al.,*, 2000. Hahn *et al.,*, 1995). Other workers used site directed mutagenesis to improve endoglucanase activity by changing Tyr245 to Gly (Sakon, 1998). Other workers had shown increased endo-β-1,4-glucanase activity by non-mutagenesis methods involving deletion of a part of the carboxyl-proximal loop (Lo *et al.,* 1988; Meinke *et al.,* 1995) and by altering the promoter region or fusing with other gene sections from other microorganisms (Sumitomo *et al.,* 1994; Sumitomo *et al.,* 1995; Vieille & Zeikus, 2001).

Endoglucanase thermostability and susceptibility to proteases has been improved through glycosylation using the eukaryotic machinery of the yeast or mammalian cells (Zhang *et al.,* 1997; Olsen & Thomsen, 1991; Soole *et al.,* 1993). Glycosylation of the enzyme inhibited proteolysis by altering the residues that can be recognised by the proteolytic enzymes. These posttranslational modifications also included improvement of thermostability of endoglucanase enzymes.

Gene cloning leads to the understanding of macromolecules involved in a pathway or enzymatic catalysis allowing protein engineering and designing of new properties or specifities of proteins and enzymes. A protein's 3-D structure gives information on structure and function relationship, allowing identification of residues having potential effects on a protein's biological performance (Schomburg, 1994).

2.13 OBJECTIVES AND AIMS

To the best of our knowledge, the hot springs in Zimbabwe have not been studied in detail with respect to the microflora present and the potential biotechnological applications of the microbes. There have been recent attempts (Muronde & Zvauya, 1992; Mawadza, 1998; Mawadza & Zvauya, 1996) to study microbes from these Zimbabwean thermal sources. The current work enhances the scientific knowledge available on microorganisms from Zimbabwean hot springs at the biochemical and molecular level.

2.13.1 Main objectives

The main objective of this work was to isolate a strain that had high extracellular endoglucanase expression from Chimanimani hot springs of Zimbabwean. Furthermore, the microbial physiology

of the endoglucanase enzyme production from the strain was studied at the biochemical and molecular level with specific emphasise on the endoglucanases biotechnological applications.

2.13.2 Specific objectives

a) To isolate an endoglucanase producing strain from Zimbabwean hot spring and study the production characteristics of the enzyme under various conditions of temperature, pH carbon source and nitrogen source.
b) To formulate a cheap medium for the production of endoglucanase and other hydrolytic enzymes by the isolated strain
c) To clone the endoglucanase gene and analyse it in an attempt to overexpress enzyme production.
d) To improve on the thermostability of the endoglucanase by site directed mutagenesis and perform trials as an animal feedstock additive.

CHAPTER 3

3 MATERIALS AND METHODS

3.1 MICROBIAL SOURCE AND SCREENING

The source for an endoglucanase producing microorganism was from Chimanimani hot springs in Zimbabwe. The hot spring is located in the Eastern Highlands. The region has a savanna type of vegetation. There are two hot springs near each other at the site. At the time of sampling, the hot springs had temperature values of 44 and 45°C and pH values of 9.0 and 8.5 respectively. Water samples were collected from the hot springs in sterile vials and transported to the laboratory for enrichment and cultivated on the same day.

A 10 % (v/v) water inoculum was used to inoculate a 500 ml Erlenmeyer flask containing a working volume of 200 ml nutrient broth. The same inoculum volume was used in modified M162 medium (Degryse *et al.,* 1978) containing per litre: 1 % (w/v) carboxymethyl cellulose (CMC), 2.5 g yeast extract, 2.5 g tryptone, 10.0 g carboxymethyl cellulose, 9.1 g Na_2HPO_4, 0.9 g KaH_3PO_4 and 10.0 ml mineral solution. Stock mineral solution was prepared with 1.0 g nitriloacetic acid, 0.2 g NaOH, 0.4 g $CaSO_4.H_2O$, 2.0 g $MgCl_2.6H_2O$ and 5.0 ml ferric citrate (0.1 M). The shake flasks were incubated at 40°C with shaking at 200 rpm overnight. One millilitre of the hot spring water was plated on nutrient agar plates and incubated at the same temperature overnight. Microorganisms that grew in nutrient and CMC broth medium were

further isolated by plating on M162 agar plates with 1 % (v/v) CMC to obtain discrete colonies. The colonies were streaked and subcultured onto M162 agar medium for short-term storage. These pure isolates capable of utilising CMC as carbon source were grown in M162 broth overnight and used to prepare 50 % (v/v) glycerol culture stocks that were stored at –80°C for long term storage. Screening of cellulase producers among the isolated colonies was carried out by inoculating 5 % (v/v) of the overnight precultures into a 100 ml working volume of M162 medium containing 1 % (w/v) CMC as carbon source. Five millilitres samples were collected after 5 h and 24 h during cultivation. The samples were centrifuged, followed by assaying extracellular carboxymethyl cellulase activity from the crude supernatants as described by Ghose, (1987).
Endoglucanase activity was determined by using 0.2 ml of culture supernatant added to 1.8 ml of 1 % (w/v) hydroxyethylcellulose (Fluka product 54290, DP 4540, DS 0.9-1.0) in 0.05 M citrate-phosphate buffer at pH 6.0. The mixture was incubated at 50°C for 10 min. The reducing sugars produced by the endoglucanase activity were measured using dinitrosalicylic acid (DNS) method as described in section **3.5.5**. The enzyme blank tube was incubated with 1.8 ml of substrate only, after which 0.2 ml of the enzyme sample was then added immediately followed by pipetting DNS reagent. The reducing sugars (glucose equivalents) were used to calculate the units of enzyme expressed in nanokatal ml^{-1} where 1 kat = 1 mol s^{-1}, glucose equivalents released (Ghose, 1987).

3.2 STRAIN CHARATERISATION AND IDENTIFICATION

3.2.1 Strain morphology, physiology and biochemical studies

The microorganism that had endoglucanase activity after culturing was characterised, identified and used for further studies. Gram staining, presence of catalase and oxidase activities and reduction of nitrate were tested using standard protocols (Cowan & Steel, 1993). Spores were stained using the standard spore-staining method. The strain was further characterised for anaerobic growth, starch hydrolysis, β-galactosidase, arginine hydrolases, ornithine decarboxylase, citrate utilisation, gelatin hydrolysis. The commercial API-50CHB strips were used to test for fermentation of 49 carbohydrates and hydrolysis of esculin (BioMérieux, Marcy-I'Etoile, France). Strips were inoculated as recommended by the manufacturer and incubated at 37°C. Results were read after 12 h and 24 h. The strain was deposited with the Germany Collection of Microorganisms, Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSM), Braunschweig, Germany. The microorganism was kept on nutrient agar slopes for short-term storage at 4°C and was sub-cultured every month. The microorganism was kept in glycerol 50 % (w/v) at -80°C (for long-term storage).

Characterisation of the cellulase producer strain with the preliminary screening conditions showed that it was a *Bacillus subtilis* species and was named *Bacillus subtilis* CHZ1.

3.2.2 16S rRNA gene amplification and analysis

Genomic DNA extraction for SSU rRNA (16S rDNA) amplification was carried out as described by Rainey and co-workers (1996). The following bacterial specific primers were used to run a polymerase chain reaction (PCR) to amplify the 16S rRNA gene: F9 (9-27, *E. coli* position) 5′ GAAGAGTTTGATCCTGGCTCAG3′as forward primer and the reverse primer was R1544 (1544 - 1528, *E. coli* position) 5′CTACGGCTACCTTGTTACGA3′. The PCR mixture was made in a total reaction volume of 25 µl, containing 0.3 µl each of the forward and reverse primers (100 pmol μl^{-1} each), 3 µg of *B. subtilis* CHZ1 genomic DNA, 0.75 units of *Taq* DNA polymerase (Roche Molecular Biochemicals) and 4.0 µl of *Taq* DNA buffer. The reaction mixture was subjected to a pre-PCR denaturation at 94°C for 5 min followed by 30 cycles of heat denaturation at 94°C for 30 s, primer annealing at 55°C for 30 s and extension at 72°C for 1 min. Final extension of the amplified DNA fragments were done at 72°C for 10 min. This amplification was carried out in a model GeneAmp PCR System 9700 (PE Applied Biosystems). An amplified product of 1,500 bp was purified from a 1 % (w/v) agarose gel using the Qiagen Purification kit (Qiagen).

The purified PCR product was sequenced using the *Taq* dideoxy chain termination method using ABI Prism® BigDye™ Terminator Cycle Sequencing Ready Reaction kit v2.0 in a GeneAmp PCR System model 9700 (PE Applied Biosystems) with appropriate primers as described by the manufacturers protocol. Sequencing was performed using ABI 3100 DNA Analyser capillary electrophoresis sequencing system. After nucleotide sequencing of the full 16S rRNA gene, the sequence of the strain was used for similarity identification using BLASTN tool search in the GenBank database.

3.3 OPTIMISING GROWTH AND ENDOGLUCANASE PRODUCTION CONDITIONS FOR *B. SUBTILIS* CHZ1

Bacillus subtilis CHZ1 was cultured in M162 medium varying conditions of initial pH value, temperature and carbon source in an attempt to optimise conditions for the growth and enzyme production. Samples were collected during fermentation to determine growth and endoglucanase

production profile at these different conditions. All shake flasks cultivations performed during optimising growth and endoglucanase conditions by the strain were done in duplicate.

3.3.1 Effect of initial pH on growth and endoglucanase production

Stock solutions of 2.0 M buffers (Gerhardt *et al.,* 1994) were used to determine effect of initial pH on growth and endoglucanase production by the *Bacillus* strain. The buffers were prepared as follows: pH 4.0 (acetate), 5.0 (citrate-phosphate), 6.0 and 7.0 (phosphate), and 8.0 and 9.0 (Tris-HCl). Separately autoclaved 10 ml volumes of 2.0 M of the buffer solutions were added into a 500-ml shake flask with 190 ml of M162 medium in different flasks to introduce different initial pH values in the medium. A 5 % (v/v) overnight preculture was prepared with a 10 ml of the medium for each flask (Keay *et al.,* 1972). The shake flasks were then incubated at 45°C and shaken at 200 rpm in an Innova 4000 Applikon Shaker (New Brunswick Scientific). Samples (5.0 ml) were collected at intervals and cell density of the culture was determined on a Shimadzu UV-120-02 spectrophotometer (OD_{600nm}). Changes in pH during cultivation were monitored. After cell density determination, the samples were centrifuged using a bench centrifuge to get a cell-free supernatant and these samples were kept frozen at –20°C, until endoglucanase analysis. The endoglucanase assay is described in the enzyme assay procedure **3.1.**

3.3.2 Effect of temperature on growth and endoglucanase production

The effect of temperature on growth of the *Bacillus subtilis* CHZ1 and endoglucanase production was determined in the temperature range 30 to 80°C. A 10 ml overnight preculture of the *Bacillus* strain was prepared to inoculate 190 ml of the M162 medium with an initial pH value of 6.0 in 500 ml Erlenmeyer flask. The shake flasks were then incubated in Innova 4000 Applikon Shakers set at different temperatures. Samples (5.0 ml) were collected at 2 h intervals and cell density of the culture was determined at 600 nm on a Shimadzu UV-120-02 spectrophotometer. After cell density determination, the samples were centrifuged using a bench centrifuge to have cell-free supernatants and then the samples were kept frozen at –20°C, until endoglucanase analysis as described in the enzyme assay procedure **3.1.**

3.3.3 Effect of carbon source on the growth and endoglucanase production

The effect of carbon source on the growth and endoglucanase enzyme production by the *Bacillus* strain was determined by substituting the carboxymethyl cellulose substrate in M162 medium with the following sugars at 0.25 % (w/v) concentration: mannose, sucrose, starch, maltose, cellobiose,

glucose and galactose. The M162 working medium volumes, inoculum preparations, sample treatment and endoglucanase assays were carried out as described in **3.1**. The shake flasks were incubated in Innova Applikon Shaker at 45°C.

3.4 OPTIMISING ENDOGLUCANASE ASSAY CONDITIONS

3.4.1 Effect of temperature and pH on endoglucanase activity

A crude enzyme supernatant was used to determine effect of temperature and pH on the endoglucanase activity. The effect of temperature on endoglucanase activity was ascertained by assaying the enzyme's activity at different temperatures between 30 and 100°C. The enzyme assays were carried out with 1.0 % (w/v) hydroxyethyl cellulose (HEC, Fluka product 54290, DP 4540, DS 0.9-1.0) substrate prepared in 0.05 M sodium citrate-phosphate buffer of pH 6.0.
The effect of pH on endoglucanase activity was determined by using HEC substrate prepared in 0.05 M buffers, prepared as described by Gerhardt *et al.,* 1994 in **3.3.1** with pH values ranging from 4.0 to 11.0 at 50°C.

3.5 TOTAL REDUCING SUGARS DETERMINATION AND ENZYME ASSAYS

The following five hydrolytic enzymes were assayed in culture supernatants; amylase, endoglucanase, polygalacturonase, protease and xylanase. The free reducing sugars and that produced during enzyme assays were determined using the 3,5-dinitrosalicylic (DNS) acid reagent (Ghose, 1987). The DNS reagent was prepared by dissolving 10.6 g of 3,5-dinitrosalicylic acid and 119.8 g of NaOH in 1416 ml of distilled water followed by adding 306 g of Na-K tartrate, 7.6 ml of melted phenol and 8.3 g of Na-metabisulfate. Glucose standards of 2.5 μmol ml^{-1} to 10 μmol ml^{-1} were prepared from a stock of 2.0 mg ml^{-1}. An amount of 1.5 ml citrate-phosphate buffer (0.05 M) pH 6.0 was added into a test tube followed by 0.5 ml of the culture supernatant. DNS reagent (3.0 ml) was then added and the mixture vortexed. The tubes were placed in boiling water for 5 min and cooled under cold water. The colour formed was measured by reading absorbance at 540 nm. A linear calibration was used to convert absorbance readings to glucose concentrations.

3.5.1 Protease assay

Protease activity was determined by a modification of the method described by Kole *et al.,*, (1988) without adding $CaCl_2$. An enzyme sample (1.0 ml) was added to 1.0 ml of 0.5 % (w/v) Azocasein (Sigma product no. A 2765) in 0.2 M Tris/HCl buffer, pH 8.0. The mixture was incubated for 60

min at 50°C. The reaction was then stopped by adding 3.0 ml of 10 % (w/v) trichloroacetic acid and centrifuged at 3000 rpm for 10 min to remove a yellow precipitate. The absorbance of the supernatant was measured at 440 nm using a Shimadzu UV-120-02 spectrophotometer. One unit of enzyme activity was arbitrarily defined as the amount of enzyme that causes an increase of 0.01 O.D. units change min^{-1} at 440 nm. The rate of product formation was linear over the 60 min incubation period. The enzyme assays were done in duplicate for each sample.

3.5.2 Xylanase activity determination

Xylanase activity was assayed according to Bailey and Poutanen, (1989). A 1.8 ml substrate solution of 1 % (w/v) 4-O-methyl glucuronoxylan from oat spelts in 0.05 M citrate-phosphate buffer pH 5.3 was added to a 0.2 ml volume of culture supernatant. Then the tubes were incubated at 50°C for 5 min and the reducing sugars produced were measured by DNS method as described in section **3.5**. Enzyme activity was expressed as nanokatal ml^{-1} using xylose as standard. The reducing sugars (xylose) were used to calculate the units of enzyme in nanokatal ml^{-1}, where 1 kat = mol s^{-1} xylose equivalents released.

3.5.3 Amylase assay

The amylase activity was measured as described by Giraud and co-workers, (1991). Culture supernatants (0.1 ml) were added to 0.8 ml of 1.2 % (w/v) soluble starch substrate (Sigma) prepared in 0.05 M citrate-phosphate buffer of pH 6.0 followed by incubation at 40°C for 1 h. The reducing sugars produced were determined by the DNS method as described in **3.5**. Enzyme units were expressed as equivalent to μmoles ml^{-1} min^{-1} of glucose produced using glucose as the standard.

3.5.4 Polygalacturonase assay

Polygalacturonase (PGase) activity was determined by measuring the release of reducing groups or galacturonic acid according to Schwan and Rose, (1994). Diluted supernatant (3.0 ml) was added to 2.0 ml of 0.1 % (w/v) of pectin solution in 0.1 M citrate-phosphate buffer pH 6.0. The mixture was then incubated at 40°C for 1 h and the reducing sugars were measured by DNS method as described in section **3.5**. PGase activity was expressed as μmole min^{-1} galacturonic acid equivalent released using galacturonic acid as standard.

3.6 FORMULATION OF MEDIUM FOR HYDROLYTIC ENZYMES PRODUCTION FROM FOOD WASTES

This part of the project was an attempt to culture and produce enzymes using a cost-effective medium and considers its possible industrial applications. The media for culturing the *Bacillus* strain were prepared using waste materials from the local food industries. The components for preparing the media were sorghum malt flour, barley malt flour, defatted soya, spent yeast and opaque beer brewery wastewater. The wastewater, sorghum malt flour and barley malt flour were collected from a traditional opaque beer brewery plant. The wastewater was collected at the point of discharge into municipality treatment works. Defatted soya was obtained from an oil manufacturing company and spent yeast from a clear beer factory.

3.6.1 Food wastes medium preparation

The following amounts of the raw/waste materials were mixed in 2.64 L of opaque beer brewery wastewater; 64.4 g sorghum malt flour, 6.0 g barley malt flour, 48.0 g spent yeast, and 48.0 g defatted soya. The mixture was allowed to hydrolyse by incubating at 50°C for 5 h while shaking at 200 rpm in an Innova 4000 Incubator Shaker (New Brunswick Scientific). The hydrolysate was then centrifuged at 6 000 rpm for 10 min. The supernatant was used as the culturing medium (CWW medium). The medium was sterilised for 15 min at 250°C.

3.6.2 Shake flask batch fermentation

The shake flask experiments were carried out to determine the effect of eliminating each of the raw material components individually during CWW medium preparation. Media (180 ml) were autoclaved in 500 ml shake flask and 20 ml volume of 2.0 M sodium citrate-phosphate buffer added (pH 6.0 separately autoclaved). Then 10 ml of each sterile medium was pipetted into separate previously sterilised 50 ml Erlenmeyer flasks to prepare 12 h old inoculum precultures of the *Bacillus* strain. The precultures were grown in a shaking incubator at 200 rpm at 40°C. Samples (5.0 ml) were taken at intervals during the experiment and biomass was determined by measuring optical density (O.D.) at 600 nm on a Shimadzu UV-120-02 spectrophotometer. The remainder of the samples were centrifuged at 6 000 rpm for 10 min and the supernatants kept frozen at -20°C in 2 ml aliquots until required for enzyme analysis. The experiments were carried out in duplicate.

3.6.3 Fermentor batch cultivations

The fermentor experiments were also carried out in duplicate in 2 L Applikon fermentors with working volumes of 1 L using the CWW medium. CWW medium (900 ml) was autoclaved in the fermentor and 100 ml of 2.0 M citrate-phosphate buffer (pH 6.0 autoclaved separately) was added aseptically. A 50 ml, 12 h old preculture was used as an inoculum. The cultivation conditions were controlled at pH 6.0 ± 0.1 adding 2 M HCl and 2 M NaOH, 40°C, airflow rate of 30 L h^{-1} and stirrer speed of 600 rpm. Another set of fermentor batch experiments were carried out using the same conditions but at a cultivation temperature of 50°C. Samples were collected at intervals and treated in the same way as described for the shake flask cultivations. Fermentor batch cultivations were carried out in duplicate.

3.7 PURIFICATION OF A PROTEASE ENZYME FROM *BACILLUS SUBTILIS* CHZ1

3.7.1 Effect of nitrogen source on the protease production

Effect of nitrogen source on the production of protease was determined by replacing the tryptone and yeast extract as nitrogen source in M162 medium with the following as the only nitrogen sources: NH_4NO_3, KNO_3, NH_4Cl, $(NH_4)_2SO_4$, urea, yeast extract, peptone and tryptone. Shake flask experiments were run in 1 L Erlenmeyer flasks containing 200 ml of the modified M162 medium (Degryse *et al.,* 1978) supplemented with 0.3 % (w/v) nitrogen source. Cultivation of the *Bacillus subtilis* CHZ1 for protease production was carried out by inoculating 5 % (v/v) of an overnight preculture to make up a 200 ml working volume of the M162 medium containing 1 % (w/v) CMC as carbon source. Cultivation was stopped after 8 h. The samples were centrifuged at 4°C after reading O.D. at 600 nm, followed by assaying extracellular protease activity by a modification of the method described by Kole *et al.,*, (1988) without adding $CaCl_2$ as described in section **3.5.1**. All reagents used were of analytical grade unless otherwise stated. The experiments were done in duplicate.

3.7.2 Culturing for protease purification

The *Bacillus* strain was cultured in a 2 L Applikon fermentor containing 1 L of the modified M162 medium supplemented with 0.3 % (w/v) tryptone. The fermentation was carried out under regulated batch cultivation conditions of pH 6.0, 50°C, 600 rpm agitation speed and an airflow rate of 30 L h^{-1} for 5 hr.

3.7.3 Protease purification and SDS-PAGE analysis

Microbial cells were removed by centrifuging at 6 000 rpm for 10 min after fermentation and the culture supernatant was used for enzyme purification. Dry ammonium sulphate was slowly added to the fermentation supernatant at 4°C with stirring until 60 % (w/v) saturation. The mixture was then centrifuged at 6 000 rpm for 10 min. The protein pellet was resuspended in 0.05 M Tris/HCl buffer pH 8.0 containing 0.02 % (w/v) sodium azide and dialysed overnight against this buffer to desalt the protein at 4°C. After dialysis, 5 ml of the sample was loaded on a Sephadex G-50 chromatography column (34 x 400 mm) equilibrated with 0.05 M Tris/HCl buffer. Fractions of 5.0 ml were collected using a flow rate of 3.0 ml min^{-1}. The collected fractions were assayed for protease activity and protein content. Fractions with protease activity were pooled and freeze-dried to half volume. The freeze-dried sample was thawed and 5.0 ml was loaded on S-Sepharose fast flow cation exchanger (34 x 300 mm) equilibrated with 0.05 M Tris/HCl buffer. Fractions of 3.0 ml volumes were collected by eluting at 0.2 ml/min using a gradient mixer containing 50 ml of 0.05 M Tris/HCl pH 8.0 buffer and 0.2 M NaCl solution. The fast flow purification process was run several times for enzyme characterisation. An aliquot of the pooled protease fractions from S-Sepharose run was concentrated by freeze-drying before running it on a 10 % SDS polyacrylamide gel electrophoresis to check for purity. SDS-PAGE gels were silver stained as described by Oakley *et al.,* (1980).

3.7.4 Protein content determination

Protein concentration was determined using Bicichoninic acid (BCA) method (Sigma assay kit) and bovine serum albumin was used as the protein standard (Smith *et al.,* 1986; Walker, 1994). The reagents for the assay were:

Reagent A: 0.1 g sodium bicichoninate, 0.16 g sodium tartrate (dihydrate), 2.0 g $Na_2CO_3.H_2O$, 0.4 g NaOH, and 0.95 g NaH_2CO_3 dissolved in 100 ml distilled water (adjusted to pH 11.25 with 10 M NaOH).

Reagent B: 0.4 g $CuSO_4.5H_2O$ dissolved in 10 ml of distilled water.

The standard working reagent was then made from mixing 50 volumes of Reagent A and 1 volume Reagent B.

The standard assay was performed by mixing 0.5 ml of the sample or protein standard with 0.5 ml of the BCA standard working reagent. The mixtures were incubated at 37°C for 30 min, cooled to room temperature and then absorbance read at 562 nm.

3.7.5 Effect of temperature and pH on protease activity

An optimum temperature and pH condition for the activity of the protease was determined from the purified sample. The effect of temperature on protease activity was determined by assaying the enzyme activity at temperatures between 30 and 100°C at pH 8.0

The optimum pH for the enzyme's activity was determined by carrying out the enzyme assay in 0.5 % (w/v) azocasein substrate prepared in 0.2 M buffer solutions prepared as described by Gerhardt *et al.,* 1994 with pH values in the range 4.0 - 11.0 at 50°C. Protease activity assay is described in section **3.5.1**.

3.7.6 Effect of temperature and pH on protease stability

Protease thermostability was determined by subjecting the enzyme to temperatures between 30 and 80°C for 5 h. Volumes of 10 ml of the purified protease were incubated in incubators set at temperatures between 30 and 80°C. Then 1 ml samples were collected at 1 h intervals for 5 h and kept frozen at -20°C and later assayed for residual protease activity at 50°C and pH 8.0.

pH stability studies were carried out by diluting the enzyme samples to 50 % (v/v) with the respective buffers (pH 4.0 to 11.0 as described in **3.3.1**) and then incubating the mixtures at 30°C for 8 h. Samples were collected from these enzyme/buffer mixtures at 2 h intervals and kept frozen at -20°C until required for residual protease activity determination. The stability experiments were done in duplicate.

The effect of freezing at -20°C and thawing on protease activity was evaluated using the crude enzyme. Freezing and thawing process did not have any significant effect on the protease activity.

3.7.7 Effects of metal ions and other inhibitors on protease activity

Final concentrations of 0.5 mM and 2.5 mM salt solutions of Ca^{2+}, Mn^{2+}, Zn^{2+}, Cu^{2+}, Ni^{+}, Mg^{2+}, Fe^{2+}, Hg^{+}, Co^{2+}, and Ag^{+} were added to the purified protease enzyme to determine the effect of the metal ions on protease activity. The mixtures were incubated at 50°C for 1 h and the residual enzyme activity was assayed. Phenylmethane-sulfonyl fluoride (PMSF) being a serine protease inhibitor was included at 0.5 and 2.5 mM concentrations, to aid identification of the *Bacillus* protease. The effects of citrate, iodoacetamide and EDTA on protease activity at 0.5 and 2.5 mM concentrations were also studied. Residual protease activity was determined as described in the protease assay and expressed as a percentage of the control. The established temperature and pH optimum conditions of 50°C and 8.0 respectively were used to assay protease activity when

testing the effects of the metal ions and inhibitors on the enzyme activity. This experiment was carried out in duplicate.

3.8 AMPLIFICATION AND CLONING OF ENDO-β-1,4-GLUCANASE (*celG*) GENE OF THE *B. SUBTILIS* CHZ1

3.8.1 Bacteria strains, plasmids and culturing conditions

The *B. subtilis* CHZ1 strain was used as the source of the gene (*celG*) encoding the endoglucanase enzyme. *E. coli* DH5α cells (F^-ϕ80d*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *end*A1 *rec*A1 *hsd*R17(r_k^- m_k^+) *deo*R *thi*-1 *pho*A *sup*E44 λ^-*gyr*A96 *rel*A1) were used for transformation of the cloned plasmids. Plasmid pMOS*Blue* was used to clone a fragment of the endoglucanase gene. The pGEM-T Easy vector was used for cloning the complete *celG* gene and the resulting plasmids transformed and expressed in *E. coli* DH5α (Promega Corp., Madison, USA). *B. subtilis* CHZ1 and *E. coli* strains were cultured overnight at 37°C in Luria-Bertani (LB) broth. Only *E. coli* DH5α clones were cultured in LB broth with 100 μm ml^{-1} ampicillin. Constructed plasmids obtained with the full gene were expressed in *E. coli* DH5α cells.

3.8.2 *B. subtilis* CHZ1 genomic DNA extraction

A modified method for the extraction of genomic DNA from Gram-positive cells described by Mitterndoff and Thompson, 1993 was used. The strain was cultured in 200 ml working volume of the M162 medium supplemented with 1 % (w/v) CMC prepared in 500 ml shake flask. Fermentation with the strain was carried out overnight at 45 °C. The cells were pelleted at 4°C in a 50 ml working volume centrifuge tube. The cells were then washed once with 10 ml solution A (10 mM Tris/HCl pH 8.0, 25 % (w/v) sucrose) and resuspended in 10 ml of solution A containing lysozyme (5 mg ml^{-1}). The suspension was incubated at 37°C for 10 min and then EDTA and SDS were added to final concentrations of 0.1 M and 0.2 % (w/v) respectively. After 10 min of cell lysis, phenol-chloroform (25:24) extraction was performed twice on the cell lysate and once with chloroform solvent. The genomic DNA was precipitated with twice the volume of absolute ethanol and then washed twice with one volume of 70 % (v/v) ethanol. The DNA was pelleted using a bench centrifuge followed by drying in a vacuum dryer, resuspended in 1ml water containing RNAse (1 in 20 of 10 mg/ml) and dialysed for 24 h against sterile 4 L of Tris/EDTA buffer (10 mM Tris/HCl pH 8.0, 1 mM EDTA). The purity of the DNA was assessed from the A_{260}/A_{280} and A_{260}/A_{230} extinction ratios (Kalia *et al.,* 1999).

3.8.3 Endoglucanase (*celG*) gene fragment amplification

Primers specific for the endoglucanase gene were designed based on the conserved regions of an endoglucanase gene sequence of the *Bacillus subtilis* DLG (Robson & Chambliss, 1987). The sequences of the primers were 5′ATA**TCTAGA**GCAGCAGGGACAAAAACGCCA3′ as forward primer and 5′TAT**GGATTC**CTTAGGTTTATGCAGCGTCAC3′ as the reverse primer. The first three nucleotides on each primer are breather bases then followed by bolded *BamH*I and *Hind*III restriction sites respectively. The primers were synthesised at the University of Cape Town, (South Africa). The PCR reaction was carried out using Superthem *Taq* polymerase (Qiagen, South Africa). Amplification of the endoglucanase probe from the *B. subtilis* CHZ1 was done by adding 100 ng of genomic template DNA in 50 µl volume containing the following quantities: buffer, 2.0 µl; equimolar mixture of 25 mM dNTPs, 2.0 µl; *Taq* polymerase enzyme, 2.0 µl; Primer mix, 10.0 µl; 25 mM $MgCl_2$, 5.0 µl; and sterile distilled water, 5.0 µl. The PCR conditions were set as follows: a pre-PCR denaturation of 5 min at 94°C followed by 35 cycles of denaturation for 1 min at 94°C, annealing temperature of 55°C for 1 min, extension for 2 min at 72°C and a post-PCR extension of 2 min at 72°C. The PCR results were analysed on a 1 % (w/v) agarose gel containing 0.5 µg ml^{-1} ethidium bromide. The probe was extracted from the gel using a Sephaglas Gel Purification kit (Amersham, UK).

3.8.4 Endoglucanase gene fragment cloning and sequencing

The PCR product after amplification was digested with the restriction enzymes *Hind*III and *BamH*I to obtain sticky ends with the defined sites (Promega, South Africa). The restriction enzyme digestion reaction was performed as per the manufacturer's recommendation. The pMOS*Blue* vector was digested with the same set of restriction enzymes to generate complementary sticky ends that could be used for ligation. After digestion of the plasmid, it was dephosphorylated using alkaline phosphatase (Sambrook *et al.,*, 1989) to avoid religation and increase the chance of insertion. The digestion reaction mixtures were phenol extracted before the ligation reaction could be performed. The ligation reaction was performed overnight at 16°C with T_4 DNA ligase according to the manufacturer's instructions (Amersham, UK). The cloned plasmids were transformed by heat-shock into previously prepared competent *E. coli* DH5α cells. The transformed cells were plated on Luria-Bertani medium agar plates containing isopropyl-β-D-thiogalactopyranoside (IPTG), 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) and Ampicillin (Amp) to enable blue/white colony selection of recombinants (Sambrook *et al.,*, 1989). The desired white clones were then picked and transferred into LB broth with 100 µmoles/ml

ampicillin for an overnight culturing. The cells were then harvested and plasmids extracted using hexadecyltrimethyl ammonium bromide, Sigma: H5882, (CTAB) mini-preps method as described below.

A volume of 1.5 ml of overnight cultures was transferred to sterile eppendorf tubes. The tubes were centrifuged at 13 000 rpm for 5 min. The supernatant was decanted and cells resuspended in 300 µl of STET buffer (8 % (w/v) sucrose, 0.1 % Triton X-100, 50 mM EDTA, 50 mM Tris-HCl pH 8.0). 6 µl of fresh lysozyme enzyme (10 mg ml^{-1}) was added and kept at room temperature for 5 min. The tubes were placed in boiling water for 45 sec and centrifuged for 10 min at 13 000 rpm. The slimy pellet was removed using suction mechanism. To the remaining aqueous phase, 20 µl of 5 % (w/v) CTAB was added and mixed gently by inverting, then allowed to stand at room temperature for 5 min. The eppendorf tubes were centrifuged for 10 min at 13 000 rpm. The pellet obtained was dissolved in 300 µl of 1.2 M sodium chloride. The DNA was then precipitated by 750 µl of absolute ethanol and pelleted by spinning for 15 min at 13 000 rpm. The DNA was washed twice with 70 % (v/v) ethanol and allowed to air dry. Eventually the DNA pellet was dissolved in 50 µl of TE/RNAse (1 in 20 of 10 mg/ml RNAse stock). 10 µl of the extracted plasmids from each strain was then digested with *BamH*I and *Hind*III to check insertion of the PCR endoglucanase probe. The plasmids were sequenced at University of Cape Town, Dept of Biotechnology in South Africa. The established sequence was BLAST searched for homology from the established database sequences. The probe was intended for the subsequent work on screening of positive clones containing full endoglucanase gene by southern hybridization technique.

3.8.5 Full endoglucanase (*celG*) gene primer designing

The sequence of *B. subtilis* CHZ1 endoglucanase gene fragment was used to BLAST search for possible cellulase gene homology in GenBank databases. The obtained endoglucanase gene lengths varied in sizes but *Bacillus* sp. 79-23 gene (accession number AF045482) showed the highest homology with the cloned endoglucanase fragment (Jung *et al.,*, 1996). The endoglucanase gene sequence of *Bacillus* sp. 79-23 was used to design primers for the amplification of the full structural endoglucanase gene with the aid of a DNMAN computer programme. The primers were synthesised by Thermo Hybaid GmbH, Interactiva Division, Germany.

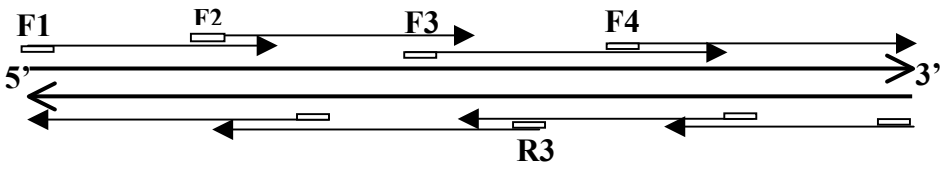
3.8.6 Endoglucanase (*celG*) gene amplification and cloning

A PCR run to amplify the full endoglucanase gene was done in a GeneAmp PCR System (model 9700; Applied Biosystems) using appropriate primers and *B. subtilis* CHZ1 genomic DNA as template. The primer sequences were: 5′ATCCGGCAAAGCAAGGCT3′ as the forward primer and 5′CGCCATATGCGCATTTGATA3′ as the reverse primer. PCR components added for amplification of the *celG* gene of *B. subtilis* CHZ1 were: 100.00 ng of DNA template; 2.50 µl of 10X STR buffer with $MgCl_2$; 0.15 µl of *Taq/Pfu* DNA polymerase; 1.00 µl of each of forward and reverse primers at 100 pmol μl^{-1} and 19.75 µl of sterilised distilled water.
The standard conditions of PCR were: a pre-PCR denaturing step of 5 min at 94°C, 30 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 50 °C, and extension for 2 min at 72°C, then a final post-PCR extension for 10 min at 72°C, using *Taq* DNA polymerase (Promega). PCR amplification was carried out in a GeneAmp PCR System 9700 (PE Applied Biosystems). The amplified product was purified after running the sample on 0.8 % (w/v) agarose gel with a Qiagen Gel purification kit (Qiagen, Germany). The purified endoglucanase gene product was cloned into pGEM®-T Easy vector (Promega) and then 5 µl of the constructed plasmids was used for transformation of 50 µl of electro-competent *E. coli* DH5α cells. Electroporation was done using Bio-Rad Gene Pulser II with the conditions; capacitance of 25 µF, resistance of 400 Ω and a voltage of 2.5 V (Miller, 1994). After transformation, 1 ml of LB broth was added and the cell suspension transferred to eppendorf tubes and the tubes incubated at 37°C for 1 h. An amount of 200 µl of the cells were plated on IPTG/X-Gal/Amp plate to screen for positive clones. White clones were then cultured overnight in LB broth with 100 µg ml^{-1} ampicillin. Plasmid mini-preps were performed on the cultures using the standard plasmid preparation protocol (Sambrook *et al.*,, 1989). The plasmids were digested with *Eco*RI and *Not*I restriction enzymes to confirm gene cloning.

3.8.7 *celG* gene sequencing and analysis

The *celG* gene was sequenced using the *Taq* dideoxy chain termination method using ABI Prism® BigDye™ Terminator Cycle Sequencing Ready Reaction kit v2.0 in a GeneAmp PCR System model 9700 (Applied Biosystems) with appropriate primers as described by the manufacturers protocol. Sequencing was performed on an ABI 3100 DNA Analyser capillary electrophoresis sequencing system. Sequencing of the obtained fragments was carried out using a 3100 Genetic Analyzer (Applied Biosystems). The first sequencing run was carried out with the primers used to

amplify the *celG* gene. The sequencing reactions produced lengths of about 700 bp from both 5′ and 3′ terminals of the DNA fragment. A second set of primers was designed from the 700 bp sequencing results to proceed sequencing from 5′→3′ and 3′→5′ inner ends of each fragment (figure 8). After sequencing the two DNA strands, gene annotation was then carried out with the aid of DNAMAN computer program. The positions of the other primers used for gene sequencing are shown in table 7. Comparison with endoglucanase gene sequences in the databases was conducted using BLASTN tool and amino acid sequence alignment was performed using CLUSTALW program (Thompson *et al.,* 1994; Holm & sander, 1994). The sequence of *celG* gene has been deposited in the GenBank database under accession number AY044252.



ent

Figure 8. Primer walking sequencing strategy of the endo-β-1,4-glucanase gene. Four primers were designed, namely **F1**, **F2**, **F3** and **F4** as forward primers and **R1**, **R2**, **R3** and **R4** as reverse sequences for the other DNA strand. The primers produced nucleotide sequences that overlap as shown in the illustration. Each subsequent primer was designed from the preceding sequence giving rise to four strands for each DNA strand. The sequences were analysed on a computer. denotes a primer (═)

3.8.8 Confirmation of *celG* orientation in pBEGc3 using T7 promoter primer

Orientation of the *celG* gene in the clone was checked by sequencing one end of the fragment with a T7 promoter primer. The PCR reaction was performed using the *Taq* dideoxy chain termination method using ABI Prism® BigDye™ Terminator Cycle Sequencing Ready Reaction kit v2.0 in a GeneAmp PCR System model 9700 (Applied Biosystems) and then followed by nucleotide sequencing on ABI 3100 DNA Analyser capillary electrophoresis sequencing system.

Table 7. Primers used for sequencing *celG* gene and the corresponding positions on the amplified fragment.

Primer sequence	Primer position
5′GGAAGATGAGAAACAAGAAGC3′	494 – 515
5′TGACGGTAATCCAAACCA3′	1222 – 1239
5′ACCAAAGATTCGACGAGGGAT3′	1814 – 1831
5′CGCCATATGCGCATTTGATA3′	1932 – 1949
5′TTATTTGAAGCTGCGGAC3′	1324 – 1344
5′TCACATCACCGTTTGGTTCG3′	771 – 791

3.8.9 Expression of *celG* gene in *E. coli* DH5α

A 190 ml LB broth medium containing 100 μg ml^{-1} ampicillin was inoculated with a 5 % (w/v) overnight preculture of *E. coli* DH5α cells transformed with pBEGc3 constructs. The same preculture size and medium without ampicillin was used to culture *B. subtilis* CHZ1 to compare endo-β-1,4-glucanase production profile. All flasks were then incubated at 37°C with shaking at 200 rpm in an incubator (Innova Applikon Shaker). Five millilitre samples were collected at intervals and cell density of the culture was monitored by absorbance at 600 nm (Ultrospec 1000-UV/Visible spectrophotometer, Pharmacia Biotech). To one of the *E. coli* broth culture that attained an OD_{600nm} = 0.7, endoglucanase expression was induced with 1.0 mM IPTG. The remaining samples after OD determination were centrifuged and supernatants kept at -20°C, until endo-β-1,4-glucanase analysis. The cell pellet was suspended in one volume of 0.1 M phosphate buffer at pH 7.0 and placed on ice. The cells were disrupted by ultrasonication using UP400S sonicator (Dr. Hielscher GmbH, Stahnsdorf, Germany) at 60 W cm^{-2}, 3x120 s. The cell debris was separated by centrifugation at 12 000 rpm for 20 min at 4°C and the samples stored at –20°C until enzyme analysis.

3.9 SITE-DIRECTED MUTAGENESIS TO INCREASE THERMOSTABILITY OF THE ENDOGLUCANASE

Considering the properties of the endoglucanase enzyme of *B. subtilis* CHZ1 and its potential biotechnological application, site-directed mutation was attempted to increase thermostability of the enzyme to use it as an animal feedstock. The processing of the cellulolytic material fed to ruminants results in attaining a high temperature that require a thermostable enzyme for a pre-digestion process. The celG enzyme was aligned using CLUSTALW tool with that of a highly thermostable *Thermotoga neopolitana* endoglucanase (Bok *et al.,* 1998). The *Thermotoga* endoglucanase shows thermostability at 106°C with half-life of 130 min at the temperature. The amino acid residue that could be substituted on celG enzyme to produce a mutated and stable enzyme was established considering the following factors. The substitution of Gly residues with Pro in the interior could reduce unfolding during temperature elevation, substitution of temperature-sensitive residues like Arg and Lys and deletion of the C-terminal end. With the aid of computer modelling using SPBDV tool, stabilisation energies were calculated to confirm disruption of the 3-D structure of the celG enzyme. The primers for site-directed mutagenesis were designed using DNMAN computer program to substitute Gly residue with Pro at position 1168-1170 of the full endoglucanase gene. The primers were, 5′GCCGCAGGAACCGCTTCTTTTAC3′ as the forward primer and 5′AAAAGAGCTTGGGATATATGTCA3′ as the reverse primer. Site-directed mutagenesis was performed using the Site-directed Mutagenesis kit (QBioegene) using the manufacturer's recommendations (figure 9). A PCR run to amplify the mutated *celG* gene was performed in a GeneAmp PCR System (model 9700, Applied Biosystems) using the mutation primers and pET clones harbouring *celG* gene as the DNA template. The PCR run components were added as follows: 0.2 ng of DNA, 0.3 µl of each of 100 pmol/µl primers, 5.0 µl of 5X reaction mixture, 1.0 µl of *Tfu* DNA polymerase and 21.9 µl of sterilised distilled water. The following diagram illustrates the inverse-PCR protocol performed. The use of celG cloned in pET would permit overexpression of the mutated enzyme.

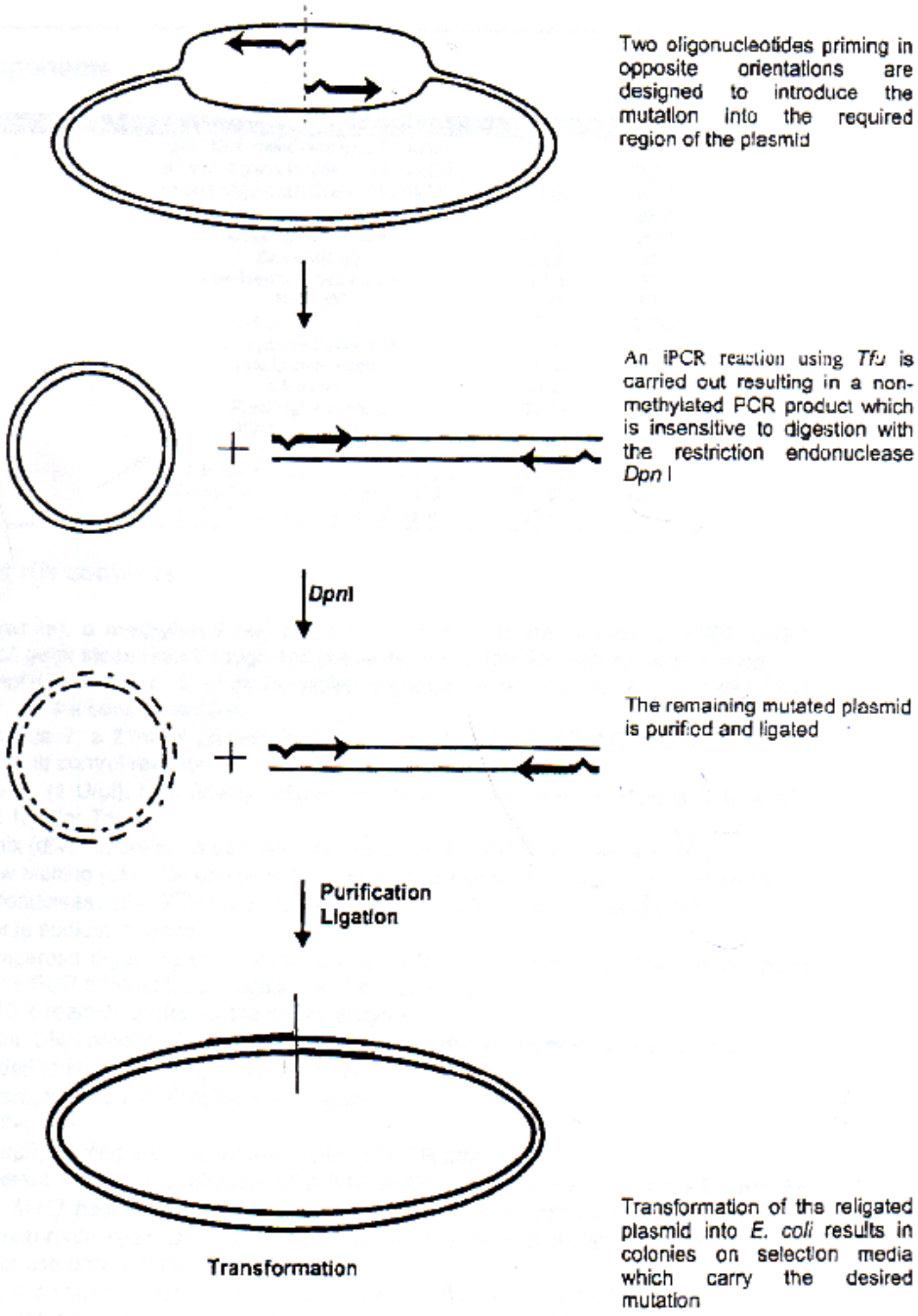


Figure 9: Overview of the site-directed mutagenesis protocol (Bioegene, 2000).

CHAPTER 4

4 RESULTS

4.1 Screening of cellulase producing microorganisms

The selection medium (M162) and nutrient broth used to isolate cellulase producer strains from Chimanimani hot spring's water were able to support growth for various microorganisms. Screening produced microbial cultures that grew at 40°C. Thirteen discrete colonies were isolated and cultured in M162 broth containing 1 % (w/v) carboxymethylcellulose to identify isolates that had extracellular cellulase expression. Their culture supernatants were then assayed for endoglucanase activity. One isolate that produced the extracellular endoglucanase activity was characterised and identified using physiological, biochemical and 16S rRNA nucleotide sequence before optimising conditions for endoglucanase production.

4.1.1 Taxonomic characterisation of strain

The same isolate reacted positive by Gram stain. Using the phase contrast microscope the strain was identified as a rod-shaped bacterium (figure 10). The morphology and general biochemical characteristics of the strain were consistent with the description of other *Bacillus subtilis* species.

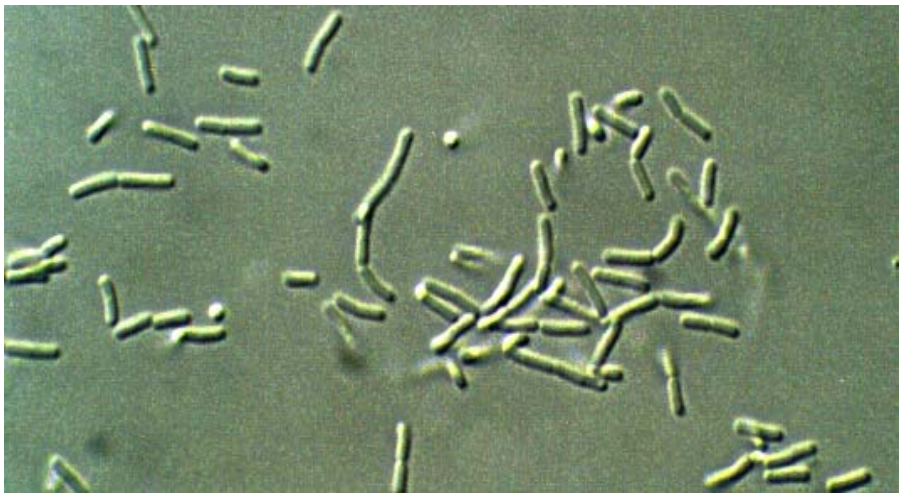


Figure 10: Phase contrast photomicrograph observation of the *B. subtilis* CHZ1 with a X1 000 magnification.

The microbial train CHZ1 varied in length from 2.0 to 5.0 m depending on the stage of cell growth and its width is 0.8 m. The bacterium is motile and forms a terminal spore that is slightly swollen. It is an obligate aerobe. Table 8 shows some of the results obtained after biochemical,

morphological and physiological studies of the microoganism using API-50 CHB identification test kit (BioMérieux, Marcy I'Etoile, France).

The cell morphology of the strain was followed during cultivation using a phase contrast microscope. Four phase cell developments were observed. These were single rods, diplobacillus, streptobacillus and spores. Single rods and diplobacilli were predominant forms in the log phase and early exponential phase, followed by slimy streptobacillus arrangement in the exponential and early stationary phases. Spores started to develop in mid stationary phase. This conforms very well with the observed cell changes in cell morphology during cultivation in the *Bacillus* genus (Priest, 1993; Nielsen *et al.,* 1995; Driks, 1999; Nicholson *et al.,* 2000).

4.1.2 16S rRNA gene DNA analysis

The amplified 16S rRNA gene gave a product of 1,500 bp fragment. The size of the amplified 16S rRNA gene conforms very well with similar genes of other *Bacillus* species. This PCR product was sequenced and analysed to further identify and classify the isolate. Using the BLASTN search tool, the sequence was 100 % homologous to other alkalophilic *B. subtilis* strains (Ash *et al.,* 1991; Priest, 1981).

Based on the identification methods by Cowan and Steel (1993), the 16S rRNA gene sequence analysis and, the physiological and biochemical identification results in table 8, the strain was identified as a *Bacillus subtilis* species and designated the name *Bacillus subtilis* CHZ1.

Table 8: Some of the biochemical and morphological characteristics of the *B. subtilis* CHZ1 from the Chimanimani hot springs, Zimbabwe.

Characteristics/Test	Result
Anaerobic growth	-
Optimal growth T°C	40
Spore formation	+
Spore shape	oval and terminal
Motility	+
Catalase reaction	+
Oxidase activity	+
Carbohydrate utilisation:	
glucose	+
mannitol	+
sorbitol	-
rhaminose	-
sucrose	+
melibiose	-
amygdaline	-
arabinose	-
fructose	+
inositol	-
starch hydrolysis	+
β-galactosidase production]	+
Arginine hydrolases	+
Ornithine decarboxylase	+
Citrate utilisation	+
H_2S	-
Urease activity	+
Tryptophan decarboxylase	-
Indole production	-
Acetoin production	+
Kohn's gelatine (gelatinase activity)	+
NO_2 production from NO_3	+
NO_2 reduction to N_2	-
Gram reaction	+

+positive reaction, - negative reaction

4.2 OPTIMISING GROWTH CONDITIONS FOR ENDOGLUCANASE ENZYME PRODUCTION

4.2.1 Effect of initial pH and temperature on growth and endoglucanase production

The optimum temperature for growing *B. subtilis* CHZ1 in M162 medium was 40°C with a doubling time of about 30 min. There was no growth at 55˚C and above (figure 11).

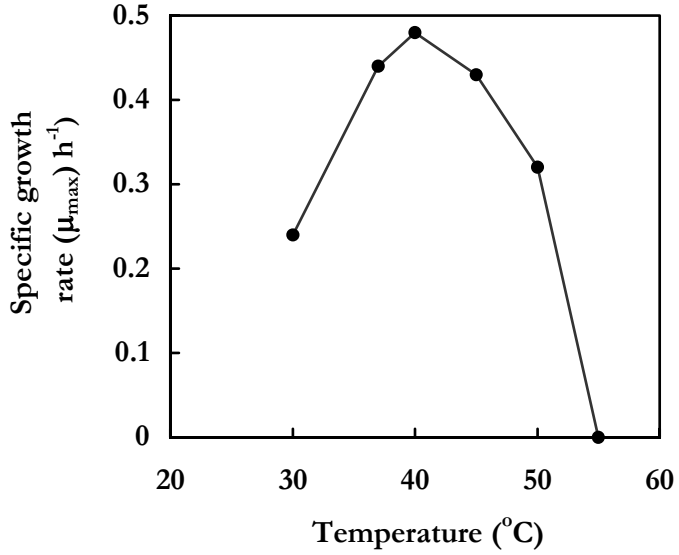


Figure 11. Effect of temperature on the growth rate of *B. subtilis* CHZ1 cultured in M162 medium with an initial pH of 6.0 (this experiment was done twice).

The CHZ1 isolate grew well in the medium with initial pH values in the neutral to alkaline region (7.0–9.0) (figure 12) although optimum growth rates were obtained in the medium with an initial pH of 8.0.

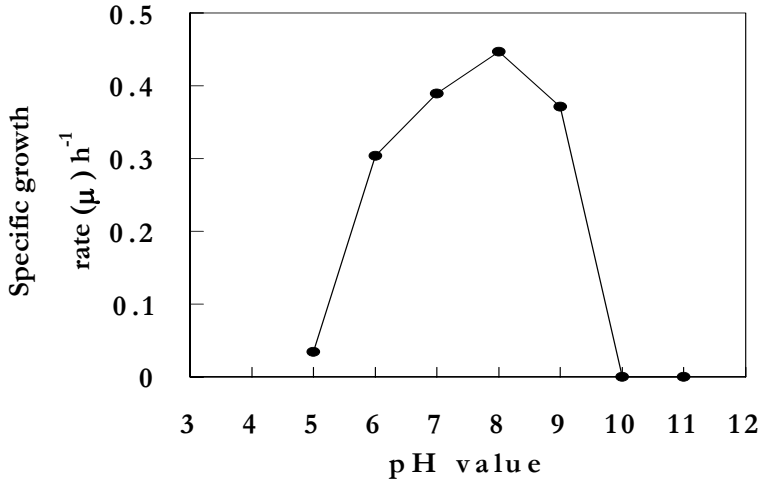


Figure 12. Effect of initial pH on the growth of *B. subtilis* CHZ1 grown in M162 medium at a temperature of 40°C (this experiment was done twice).

This pH is about the pH value of its natural habitat that is at 8.45 and a temperature of 45°C. The *Bacillus* strain produced the endoglucanase on medium with initial pH values between 5.0 and 9.0, with highest enzyme activity of about 100 nkat ml^{-1} (figure 13).

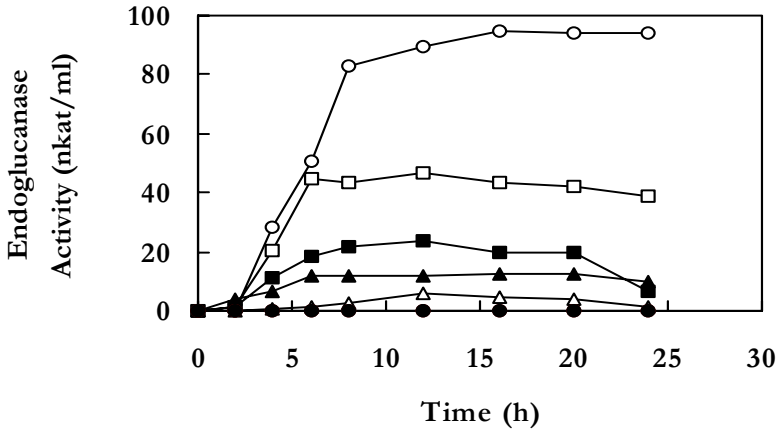


Figure 13. Endoglucanase production by *B. subtilis* CHZ1 at a cultivation temperature of 45 °C using M162 medium and various initial pH values: 5 (Δ); 6 (○); 7 (□); 8 (■); 9 (▲) and 10.0 (●). The 0.05 M buffers used were acetate (pH 4.0); citrate/phosphate (pH 5.0 - 6.0); phosphate (pH 7.0 -8.0) and Tris/HCl (pH 9.0 -10.0) (this experiment was done twice).

The production of endoglucanase enzyme by *B. subtilis* CHZ1 was optimal at 50˚C producing 120 nkat/ml of enzyme activity. However relatively high activities were obtained at 45˚C. Endoglucanase production profiles were similar at temperature values, 37 and 40˚C yielding about 50 nkat ml^{-1} after 10 h however there was a rapid drop in enzyme activity during the early stationary phase after 16 h of cultivation at 50˚C (figure 14). No Filter paper activity (FPAse) and cellobiase activity was detected in the culture supernatant.

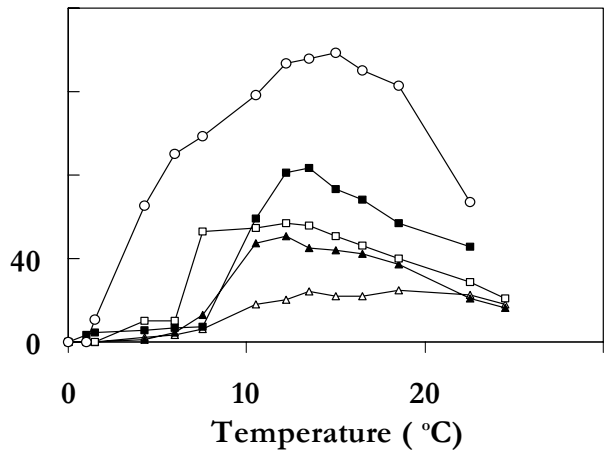


Figure 14. Endoglucanase production by the *B. subtilis* CHZ1 cultured at various temperatures and an initial pH of 6, (0.05 M citrate/phosphate buffer): 30°C (Δ); 37°C (▲); 40°C (□); 45°C (■); 50°C (○) and 55°C (●), this experiment was done twice.

4.2.2 Effect of carbon source on growth and endoglucanase production

Concentrations of 0.25 % (w/v) sugars were used to replace CMC as carbon source in M162 medium. Effects of carbon source substitution on growth and extracellular endoglucanase production by *B. subtilis* CHZ1 was determined. The strain was able to utilise all the carbon substrates. The optimal biomass and enzyme levels obtained were different with each carbon source as shown in table 9. Highest biomass was obtained with galactose within a period of 15 h. Endoglucanase was produced constitutively when the isolate was cultured using the different carbon sources although highest endoglucanase activity was with sucrose yielding 129 nkat ml^{-1} after 27 h. Mannose, maltose, starch and CMC gave high enzyme activity, while low endoglucanase activities were observed with galactose and glucose although they had high biomass levels. This *Bacillus* strain produces endoglucanase enzyme constitutively.

Table 9. Effect of different carbon sources on the endoglucanase production by the *Bacillus subtilis* CHZ1 cultivated at 40˚C and pH 6.0, this experiment was done twice.

Carbon source	Time taken to reach optimal biomass (h)	Biomass attained (A_{600nm})	Maximum enzyme activity (nkat ml^{-1})
Mannose	22	2.07	123
Sucrose	27	2.95	129
Starch	27	2.65	108
CMC	20	3.74	125
Maltose	12	3.30	123
Cellobiose	23	4.27	123
Glucose	23	3.44	97
Galactose	15	5.18	86

Enzyme production is maximal in M162 synthetic medium with an initial pH value of 6.0 and a temperature of 50˚C yielding about 120 nkat ml^{-1} after 5 h of cultivation. The optimum conditions for the endoglucanase activity produced by *B. subtilis* CHZ1 were pH 6.0 and a temperature of 70˚C.

4.3 PRODUCTION OF ENZYMES BY A *B. SUBTILIS* ON FOOD WASTES MEDIUM

4.3.1 Effect of medium composition on endoglucanase production and other hydrolytic enzymes.

Growth profiles of the *B. subtilis* CHZ1 during its culturing in shake flask and fermentors are shown in figure 14. The bacterium grew rapidly in the CWW formulated medium reaching stationary phase within 5 h in both shake flask and fermentor experiments. Microbial growth was related to the changes in free reducing sugars that correspondingly decreased with biomass formation. Reducing sugar levels fell rapidly during the exponential phase as a result of both biomass and product formation (figure 15). In the fermentor experiments, an OD of 9.0 were attained at 50°C compared to 2.0 at 40°C. Highest biomass levels were obtained with medium in which defatted soya was omitted. However, omitting sorghum malt flour resulted in low biomass. Preparation of the CWW medium without spent yeast resulted in low biomass obtained during cultivation.

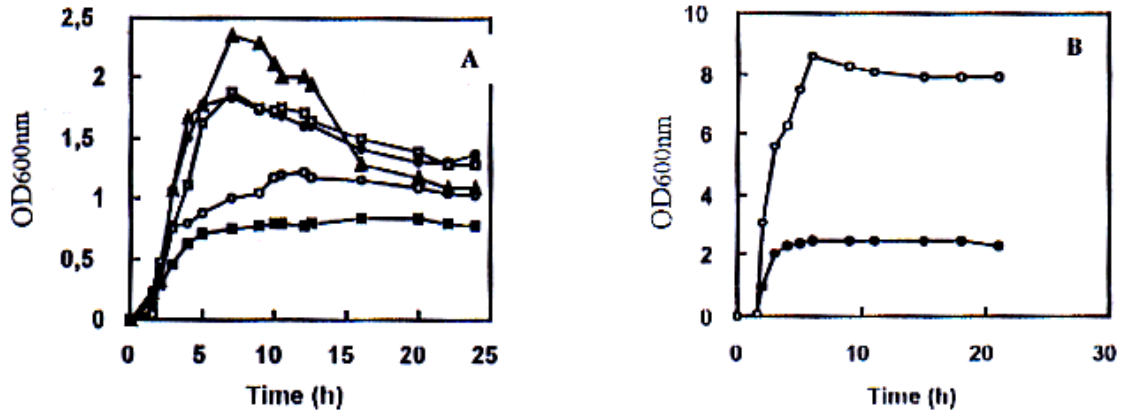


Figure 15: Graph **A** shows effect of different CWW medium components on the growth of the *B. subtilis* CHZ1 in shake flask cultivations: (▲) no defatted soya, (□) no barley malt, (■) no sorghum malt, (○) no spent yeast and (●), with all components. **B** shows fermentor cultivations using CWW medium containing all components at (○) 40°C and (●) 50°C.

The effect of the absence of each component during the medium preparation on growth and hydrolytic enzymes production by the *Bacillus subtilis* strain was investigated. The CWW medium without sorghum malt flour had low reducing sugar content hence that medium gave low biomass levels during cultivation. Shake flasks without barley malt flour and defatted soya showed similar growth profiles to the CWW medium with all components.

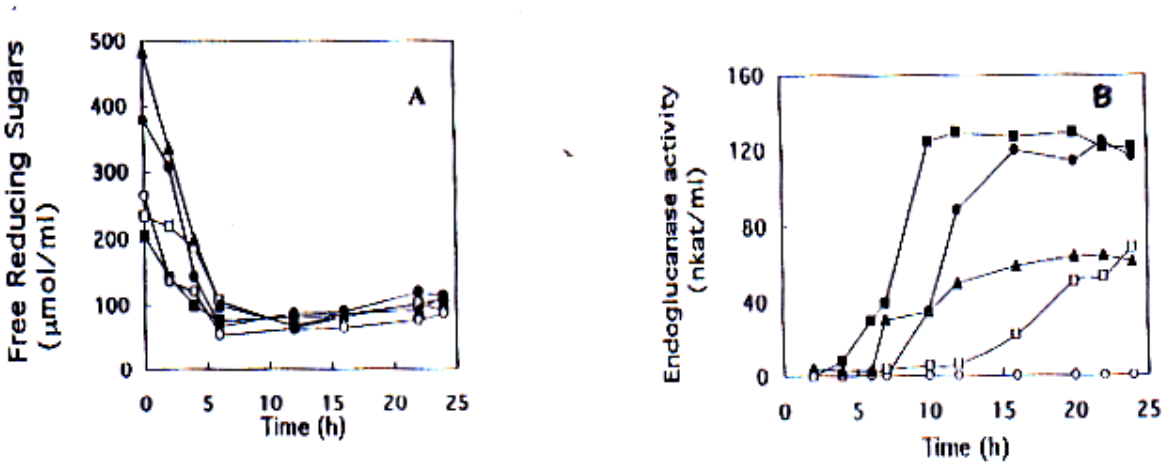


Figure 16: Cultivation of the *Bacillus* strain in CWW media containing different components; **A**: free reducing sugar profiles and **B**: endoglucanase enzyme profiles in (▲) no defatted soya, (□) no barley malt, (■) no sorghum malt, (○) no spent yeast and (●), CWW medium containing all components

There was a slight increase in free reducing sugars in both shake flasks and fermentor cultivations after 10 h (figure 16A). There was no remarkable influence on growth when defatted soya or barley malt flours were excluded.

The endoglucanase production profile by the *Bacillus* strain using different CWW media was followed. All the components except sorghum malt flour were observed to be essential for the maximum endoglucanase enzyme production (figure 15B). Much of the enzyme was produced in the exponential phase. The formulated medium with all the components and without sorghum malt gave the highest amount of endoglucanase activity. The absence of defatted soya and barley malt resulted in reduced enzyme production appreciably while no endoglucanase activity was obtained in the absence of spent yeast. Endoglucanase levels obtained and the time taken to reach the stationary phase by the *Bacillus* sp., quite comparable to those obtained using synthetic medium. Besides the production of endoglucanase by the *Bacillus* strain on these food wastes medium, other hydrolytic enzymes that included amylase, protease, xylanase and polygalacturonase were produced. These hydrolytic enzymes were assayed from the samples collected from the shake flasks and fermentor experiments. Amylase and protease production levels by the *Bacillus* strain are shown in figure 17.

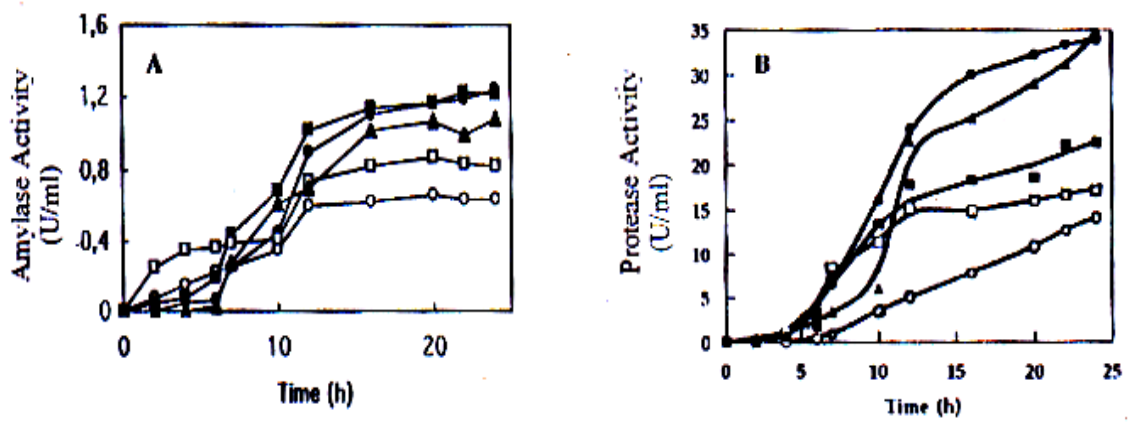


Figure 17: Enzyme production by *B. subtilis* CHZ1 in shake flask fermentations using differently prepared CWW media; **A** and **B** are amylase protease enzyme profiles respectively in (▲) no defatted soya, (□) no barley malt, (■) no sorghum malt, (○) no spent yeast and (●) CWW medium with all components.

The media containing no sorghum malt or defatted soya, and that containing all the components gave the highest amount of amylase activity. Levels of amylase enzyme production were reduced in the absence of spent yeast. The amylase enzyme was produced in the late exponential and early stationary phase while the medium with all components and that without defatted soya produced the highest amount of protease enzyme. The media without spent yeast gave the lowest amount of protease enzyme.

Effects of various components on xylanase and polygalacturonase were also determined (figure 18). CWW medium containing all preparation components resulted in maximum xylanase production.

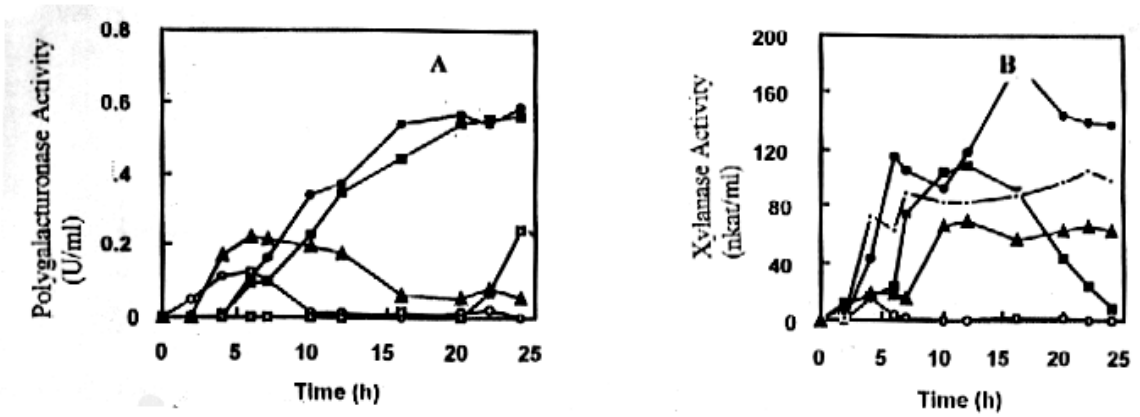


Figure 18: Enzyme production by the *B. subtilis* CHZ1 in shake flask with cultivations with differently prepared CWW media; (▲) no defatted soya, (□) no barley malt, (■) no sorghum malt, (○) no spent yeast and (●), CWW with all components

Elimination of defatted soya, barley malt and sorghum malt reduced the levels of xylanase enzyme production, while no xylanase was produced in the absence of spent yeast in the medium. The media containing all the components and also that without sorghum malt led to very high polygalacturonase levels. The absence of defatted soya and spent yeast decreased the

polygalacturonase and xylanase enzyme levels substantially. No polygalacturonase enzyme was produced without barley malt and spent yeast in the media.

4.3.2 Effect of temperature on hydrolytic enzyme production in fermentors

Production of hydrolytic enzymes was carried out in 1 L working volumes at 40°C and 50°C in fermentors using the developed CWW medium. The production of endoglucanase and amylase enzymes in fermentor cultivations is shown in figure 19. Endoglucanase was produced in the early exponential phase reaching maximum levels at 5 h while amylase was produced in the late exponential phase. For both enzymes, the lag phase was short in controlled fermentor experiments compared to the shake flask fermentations and their levels dropped rapidly at 50°C reaching negligible levels within 20 h. However, at 40°C the enzyme levels remained constant up to 24 h.

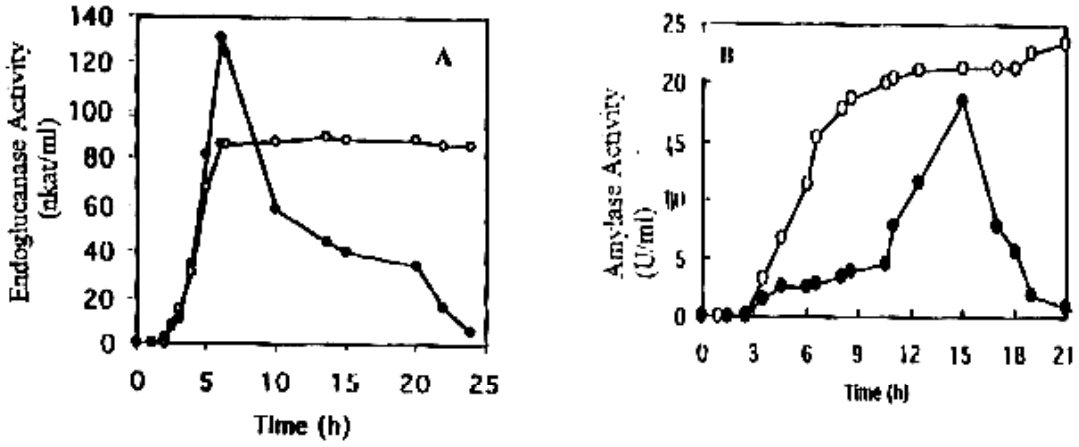


Figure 19: Enzyme production profiles of endoglucanase (**A**) and amylase (**B**) by the *Bacillus* strain in fermentor cultivations using CWW medium at (○) 40°C and (●) 50°C.

The xylanase enzyme activity during fermentor cultivations produced two peaks at both 40°C and 50°C with one peak at 5 h and the other at 12 h respectively (figure 20).

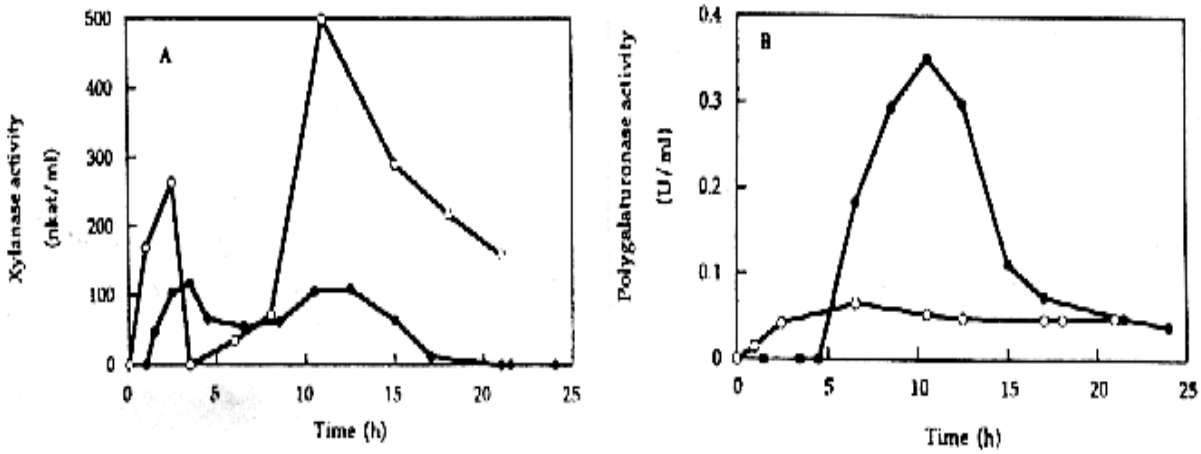


Figure 20: Xylanase and polygalacturonase production profiles by the *B. subtilis* in fermentor cultivations using CWW medium at (○) 40°C and (●) 50°C.

The enzyme levels then dropped after 12 h for both temperatures. High levels of amylase and xylanase were obtained when a cultivation of 40°C was used. The polygalacturonase was produced in the late exponential and stationary phases. The production of protease enzyme during fermentor cultivations is shown in figure 21. Higher levels of protease enzymes were obtained at 50°C compared to cultivations at 40°C. Much of the protease enzyme was produced in the late exponential and the early stationary phase under both temperature conditions.

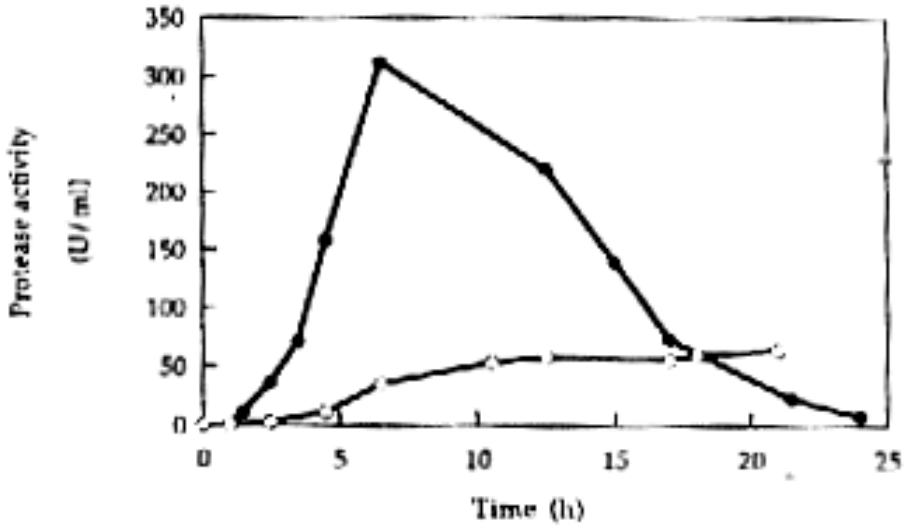


Figure 21: Protease enzyme production by *B subtilis* CHZ1 in fermentor cultivations using CWW medium at (○) 40°C and (●) 50°C.

The onset of extracellular protease expression by the *B. subtilis* CHZ1 strain coincides with an overall change in extracellular enzyme levels in both fermentor and shake flask experiments. As a result of autolysis and proteolytic activity from the protease an expected drop in enzyme was observed, rapidly so in fermentors because of very high protease activity. The formulated medium

based on food related wastes was able to support growth of the *B. subtilis* CHZ1 producing amylase, endoglucanase, protease, xylanase and polygalacturonase.

4.4 Purification of Protease produced by *B. subtilis* CHZ1

4.4.1 Effect of nitrogen source on protease production

The supplemented organic and inorganic nitrogen sources to M162 medium were added to determine the effect of the nitrogen source on the production of the alkaline protease by the *Bacillus* strain. All the nitrogen sources increased the proteolytic production except NH_4NO_3 source (table 10). In general, the *Bacillus* strain produced higher biomass and protease activity in M162 medium with organic nitrogen compared to inorganic sources. Highest protease production was achieved with Tryptone while lowest with NH_4NO_3. Peptone and yeast extract are good inducers of protease synthesis.

Table 10: Microbial growth and production of a protease by the *B. subtilis* CHZ1 using M162 medium (Degryse *et al.,* 1978) supplemented with different nitrogen sources. The assays were determined after 8 h of cultivation.

Nitrogen source	Biomass obtained A_{600nm}	Specific Activity (U/D)
KNO_3	0.475 ± 0.087	1.73 ± 0.16
NH_4NO_3	0.352 ± 0.04	0.31 ± 0.06
NH_4Cl	0.489 ± 0.022	2.31 ± 0.049
$(NH_4)_2SO_4$	0.262 ± 0.002	1.95 ± 0.39
Urea	1.028 ± 0.012	2.10 ± 1.06
Yeast extract	0.997 ± 0.022	3.00 ± 0.67
Peptone	0.794 ± 0.041	2.32 ± 0.48
Tryptone	1.952 ± 0.028	3.00 ± 0.42
Control	1.730 ± 0.033	1.19 ± 0.78

± standard deviations of means of duplicate values obtained from different assays

4.4.2 Sephadex G-50 and S-Sepharose purification run

The purification steps were carried out at 4°C, and table 11 summarises the steps used in purifying the protease enzyme from the culture supernatant. Ammonium sulphate precipitation and

subsequent dialysis of the sample had little effect on the removal of impurities. Most of the impurities were removed during the S-Sepharose fast flow column chromatography and low amounts of the protease protein were obtained hence the use of silver stain on SDS-PAGE gels to observe the protein bands.

Table 11: Purification of an alkaline protease from the *B. subtilis* CHZ1

Purification step	Total protein (mg)	Total Activity (U)	Specific Activity (U/mg Protein)	Yield (%)	Purification fold
Culture Supernatant	198	8240	42	100	1.0
$(NH_4)_2SO_4$ precipitated & dialysed	175	7700	44	93	1.1
Sephadex G-50	15	1440	96	43	2.3
S-Sepharose fast flow	2.6	3420	1315	41	31

The protease activity was found in the second peak from the S-Sepharose run (figure 22). The protease enzyme bound to the column and was eluted by the gradient eluant formed by Tris/HCl buffer and NaCl solution. Sodium chloride concentration had very little effect on the protease activity and its use as an eluant from the S-Sepharose column chromatography had no significant effect on the protease activity (data not shown).

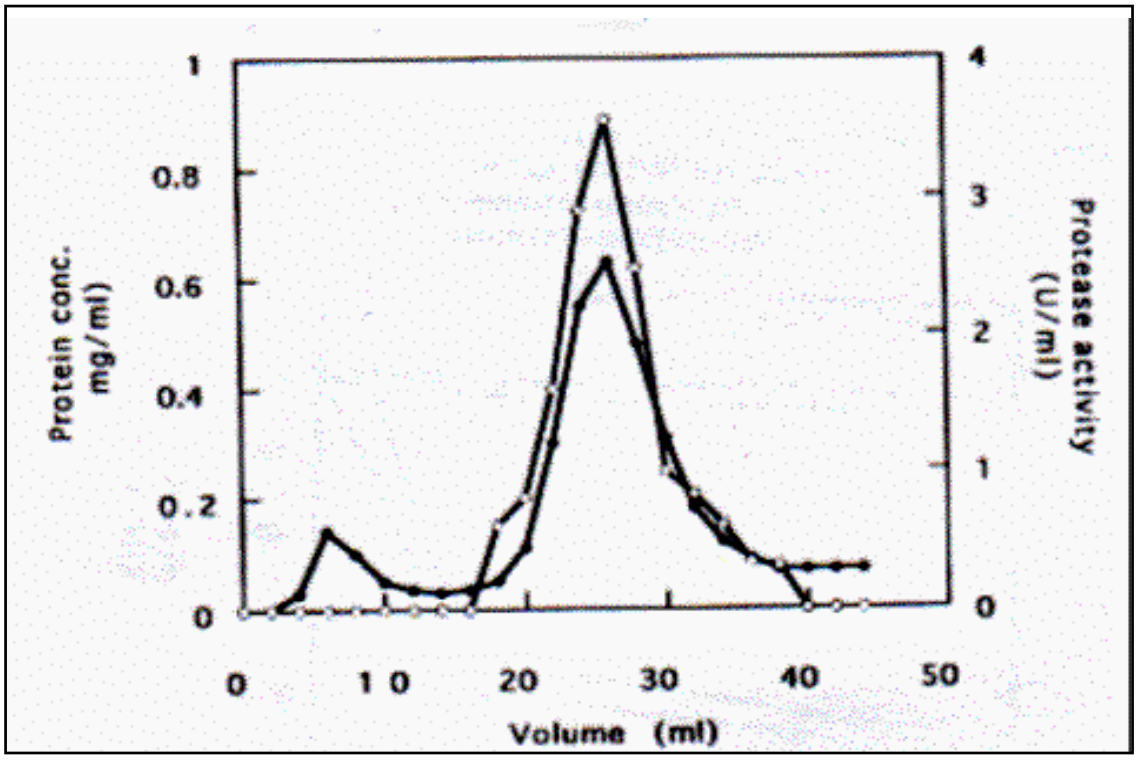


Figure 22: Purification profile of a *Bacillus* sp. Protease enzyme from M162culture supernatant on a S-Sepharose fast flow column after Sephadex G-50 gel filtration chromatography and freeze drying of the pooled fractions. Profiles show (●) Protein concentration and (○) Protease activity.

The purification of the protease was carried out at cold conditions to minimise autodigestion and proteolysis that could mislead by appearing as multiple forms of the enzyme of SDS-PAGE gel. The purified protease enzyme had a single band on an SDS-PAGE gel after silver staining indicating homogenous protein of 35 kDa (figure 23). A plot of logarithmic molecular weight of standards against band mobility was used to determine the size of the unknown. The molecular weight of the CHZ1 protease falls in the range of 25 – 35 kDa as reported for serine proteases (Priest, 1978; Keay, 1970).

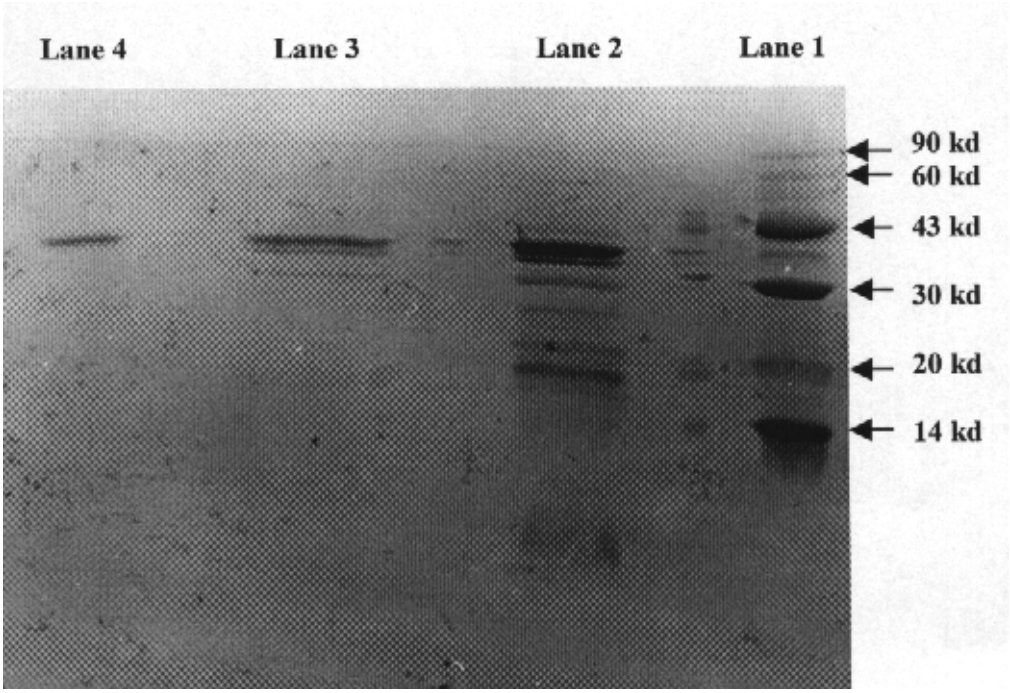


Figure 23: A 10 % (w/v) SDS-PAGE analysis of the purified protease enzyme using silver staining method. **Lane 1**: Molecular weight standards; **Lane 2**: *Bacillus subtilis* CHZ1 culture supernatant precipitated with 60 % (w/v) ammonium sulphate; **Lane 3**: Ammonium precipitate supernatant, dialysed and run on Sephadex G-50 column and **Lane 4**: S-Sepharose purified protease enzyme sample.

4.4.3 Effect of temperature and pH on the protease activity

To determine the optimum temperature of the protease enzyme, activity assays were performed at temperature values of 30˚C to 80˚C at pH 8.0 (figure 24). The optimal pH activity was determined in the range 4.0 to 12.0 at 50˚C (figure 25). The maximum activities for the protease enzyme using azocasein substrate were temperature of 60˚C and pH 8.0. The protease activity of CHZ1 strain was high in neutral to alkaline pH range of 7.0- 10.0 and the enzyme had very little activity outside this pH at 40˚C. The optimum conditions for the purified protease enzyme were the same as those for the crude product.

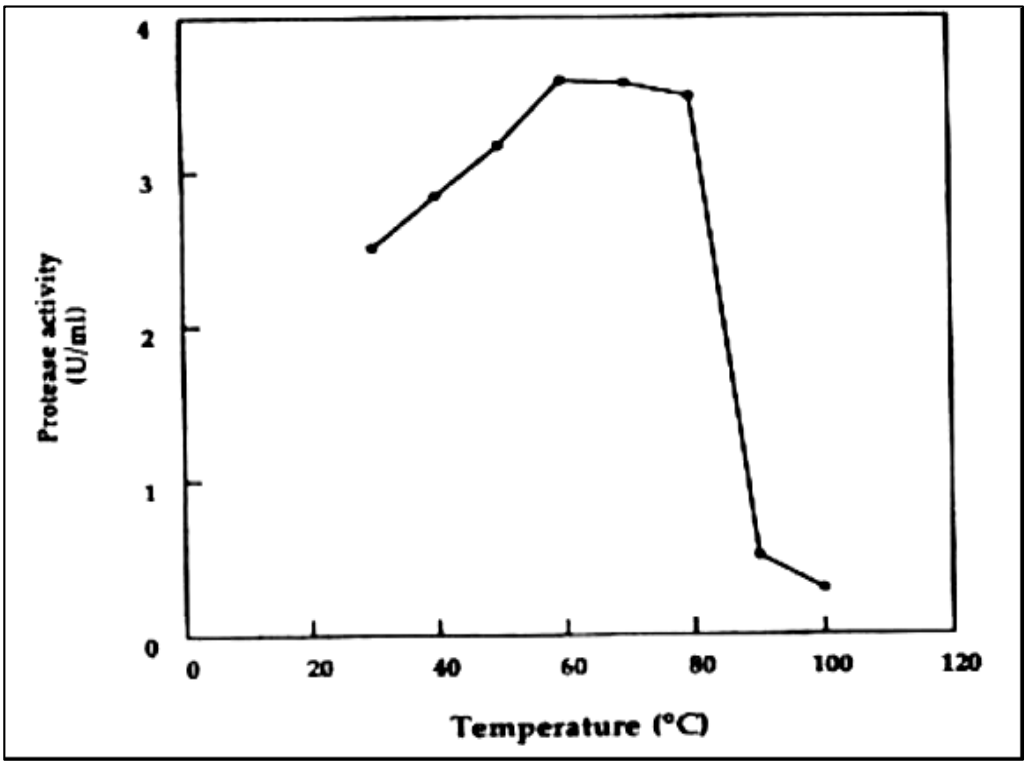


Figure 24: Effect of temperature on the purified protease enzyme activity using 0.5 % (w/v) azocasein substrate prepared in 0.2 M Tris/HCl buffer pH 8.0.

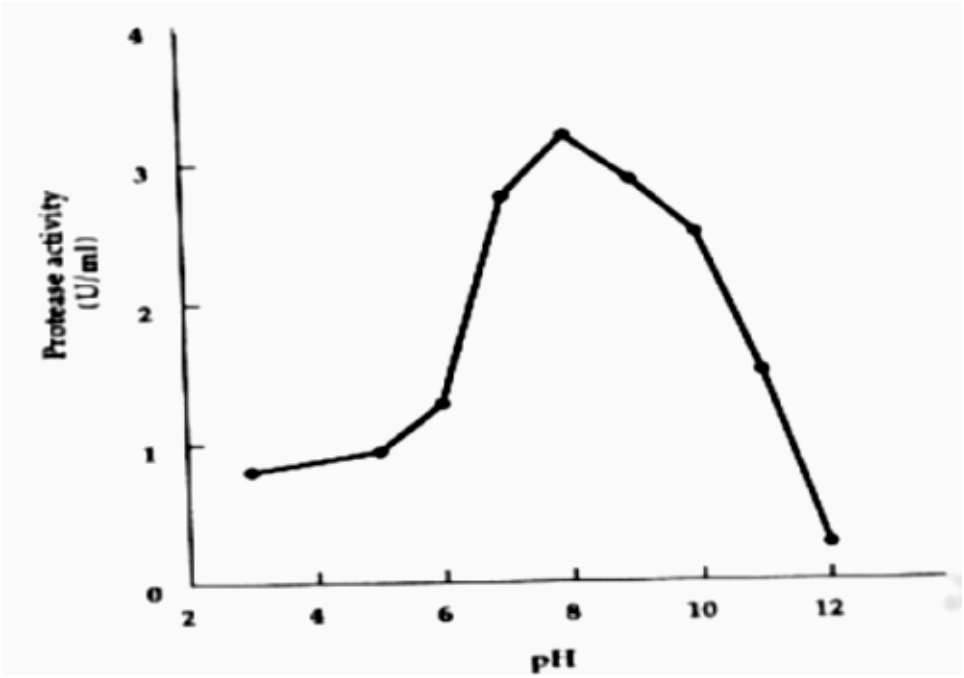


Figure 25: Effect of pH on the activity of the purified protease enzyme produced by the *Bacillus*. The buffers used were; 4.0-6.0 citrate/phosphate buffer; 7.0-8.0 phosphate buffer and 9.0-12.0 carbonate/bicarbonate buffer.

At temperatures below 50˚C the enzyme was stable, retaining about 90 % of the initial protease activity after 5½ h (figure 26).

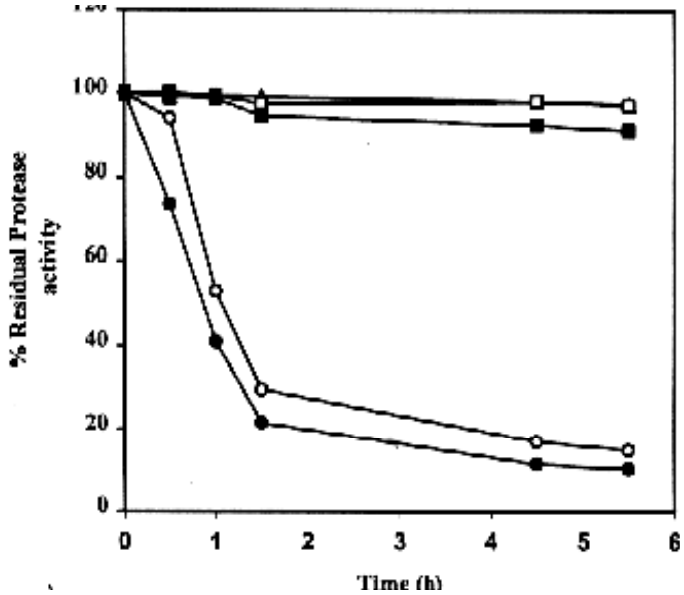


Figure 26: Thermostabilities of the purified protease enzyme produced by the *Bacillus* strain over a period of 5½ h in 0.02 M Tris/HCl buffer pH 8.0 at (△) 30˚C, (□) 40˚C, (■) 50˚C, (○) 60˚C and (●) 70˚C.

At 60˚C the enzyme retained 50 % of its activity for 1 h. The protease enzyme retained 80 % activity in buffers of pH range 7.0-9.0after 8 h by virtue of its pH activity profile it is an alkaline protease (figure 27). In buffers below 6.0 and above 10.0, the protease enzyme rapidly lost its activity within 2 h.

4.4.4 Effect of metal ions and other inhibitors on protease activity

Table 13 summarises the protease activity in the presence of different effectors such as metal ions, surfactants and other compounds. From the obtained results, it shows that a number of metal ions had no effect on the activity of the protease of *Bacillus subtilis* CHZ1. Zn^{2+}, Cu^{2+} and Ca^{2+} metal ions had no effect on the enzyme's activity meanwhile Mg^{2+}, Fe^{2+} and Mn^{2+} increased the activity by about 20 %. However, Hg^{+}, Ni^{2+}, Co^{2+}, Ag^{+}, PMSF and EDTA inhibited protease activity at both 0.5 mM and 2.5 mM concentrations. Iodoacetamide, a sulfhydryl inhibitor, reduced the enzyme's activity by 13 % at 0.5 mM and 2.5 mM concentrations. Citric acid at 0.5 mM had no effect but inhibited protease activity

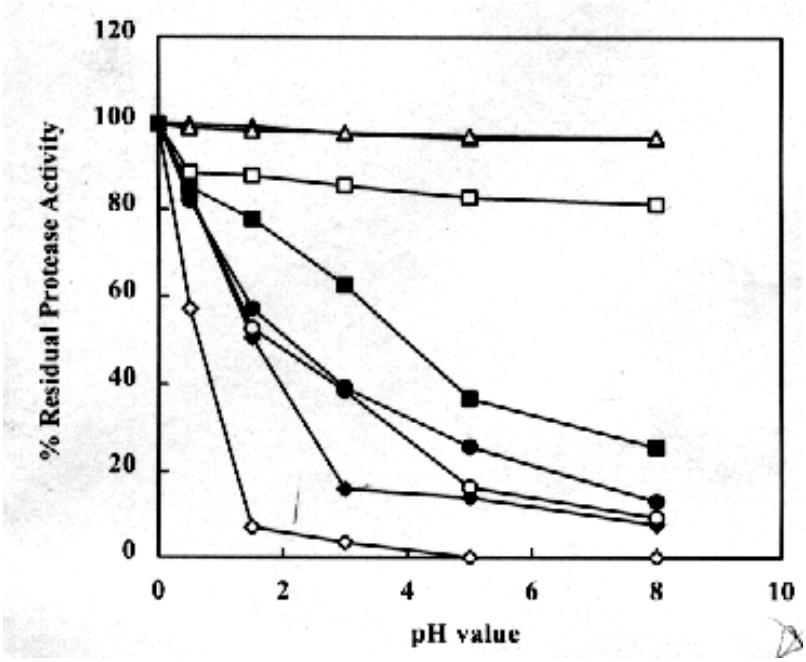


Figure 27. Influence of pH on the stability of the protease enzyme: (○) 4.0, (●) 5.0, (△) 6.0, (▲) 7.0, (□) 8.0, (■) 9.0, and (♦) 10.0 and (◊) 11.0.

by 12 % at 2.5 mM. This protease is produced by *B. subtilis* CHZ1 did not show enhanced proteolytic activity in the presence of Ca^{2+} ions unlike other *Bacillus* serine protease that have shown either enhanced activity or thermostability (Manachini *et al.,* 1988; Ward, 1983). It has been shown that a number of microbial alkaline proteases require Ca^{2+} ions to be thermostable at elevated temperatures.

Metal cofactors could be involved for the proteolytic activity of the protease because the enzyme retained 64 % of its initial activity in the presence of 2.5 mM EDTA, a chelator of metal ions. Although metal ion chelators are rarely known to affect *Bacillus* protease, the reduction in activity suggests requirement of metal ions for maintenance of the protein's integrity.

Table 12: Effect of inhibitors and metal ions on the purified protease activity from a *Bacillus subtilis* CHZ1 isolated from Chimanimani hot springs. **A** is a residual activity in 0.5 mM and **B** in 2.5mM concentration solutions.

Metal ion inhibitor	% Residual protease activity	
	A	**B**
Control	100 ± 2.5	100 ± 2.9
Cu^{2+}	100 ± 2.5	100 ± 3.6
Mg^{2+}	117 + 2.4	119 ± 2.0
Fe^{2+}	119 ± 1.8	111 ± 2.0
Ca^{2+}	100 ± 3.9	100 ± 2.1
Mn^{2+}	100 ± 1.3	120 + 3.1
Hg^{+}	91 ± 2.2	78 ± 3.0
Ni^{+}	88 + 4.2	76 ± 2.1
Co^{2+}	96 ± 2.4	88 ± 3.8
Zn^{2+}	100 + 4.2	100 ± 5.6
Ag^{+}	87 ± 1.6	76 ± 3.4
PMSF	73 ± 3.4	0
EDTA	92 ± 2.8	64 ± 2.3
Citric acid	103 + 3.8	88 ± 1.1
Iodoacetamide	87 ± 4.7	87 ± 3.6

± standard deviations of means of duplicate figures obtained from the assays

The purified extracellular thermostable protease produced by the *Bacillus subtilis* CHZ1 has characteristics consistent with those of the alkaline serine proteases.

4.5 Amplification and cloning of an endoglucanase (*celG*) gene of a *B. subtilis* CHZ1

4.5.1 Amplification and cloning of the celG gene fragment

The amino terminal sequence of an endoglucanase enzyme produced by another *Bacillus* strain from a Zimbabwean hot spring studied by Mawadza and co-workers (2001) had 100 % homology to that of *B. subtilis* DLG studied by Robson and Chambliss (1987). The endoglucanase enzyme properties from these two strains had similar properties with *B. subtilis* CHZ1. The *B. subtilis* DLG endoglucanase was therefore used as the reference for designing primers to amplify CHZ1 endoglucanase gene fragment. This was based on the assumption since *B. subtilis* CHZ1 endoglucanase properties were similar to the other *B. subtilis* genes, it was most likely that their gene structure were also similar. The endoglucanase gene from *B. subtilis* CHZ1 gene was named *celG* gene.

The primers of amplifying *celG* gene fragment were designed from the conserved regions of *Bacillus* endoglucanase genes. The gene fragment was intended for screening an endoglucanase gene from *B. subtilis* genomic library. The *celG* gene did not encode the carboxyl terminal end.

PCR amplification of this truncated endoglucanase gene yielded a fragment size of 1 200 bp (figure 28).

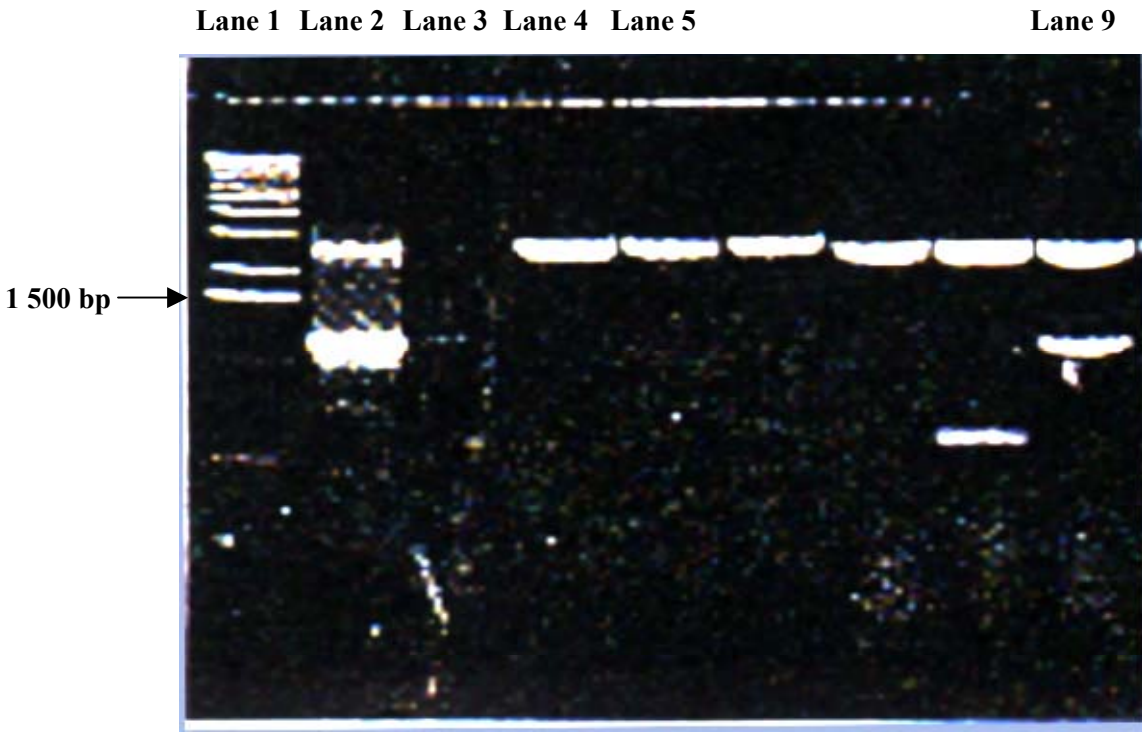


Figure 28: Various putative plasmid clones digested with *BamH*I and *Hind*III restriction enzymes and resolved on a 0.8 % (w/v) agarose gel then stained with ethidium bromide. **Lane 1**: DNA molecular weight marker; **Lane 2**: PCR amplified *celG* gene fragment; **Lane 3**: Control PCR amplification run; **Lane 4**: linearised pMOS*Blue* vector**; Lane 5** to **8** and **10**: clones used to screen for *celG* gene fragment and **Lane 9**: desired clone with the 1.2 kb *celG* fragment.

This amplified truncated gene was cloned into pMOS*Blue* vector and sequenced. The *E. coli* clones harbouring the pMOS*Blue* constructs were cultured in LB broth and checked for endoglucanase activity expression. The truncated gene yielded three quarters of the mature endoglucanase gene sequence. The clones did not show expression of endoglucanase activity. This was confirmed by assaying both extracellular and intracellular supernatants after whole cell culture sonication. Similar work has shown that such truncated gene can express an active endoglucanase enzyme. A BLASTN search with the truncated gene sequence gave greater than 98 % sequence homology with several alkaline *Bacillus* endoglucanase genes.

4.5.2 Amplification and cloning of full length *celG* gene

The lack of prior knowledge of the *B. subtilis* CHZ1 DNA sequence restricted us to use the strain that gave the highest percentage homology with the truncated endoglucanase gene for designing

primers to amplify the full-length *celG* gene. Since the *Bacillus* sp. (accession no. AF045482) had the highest homology, its gene sequence was used as a reference gene sequence. The *celG* gene amplification yielded a product of 2 600 bp as was determined on agarose gel (figure 29).

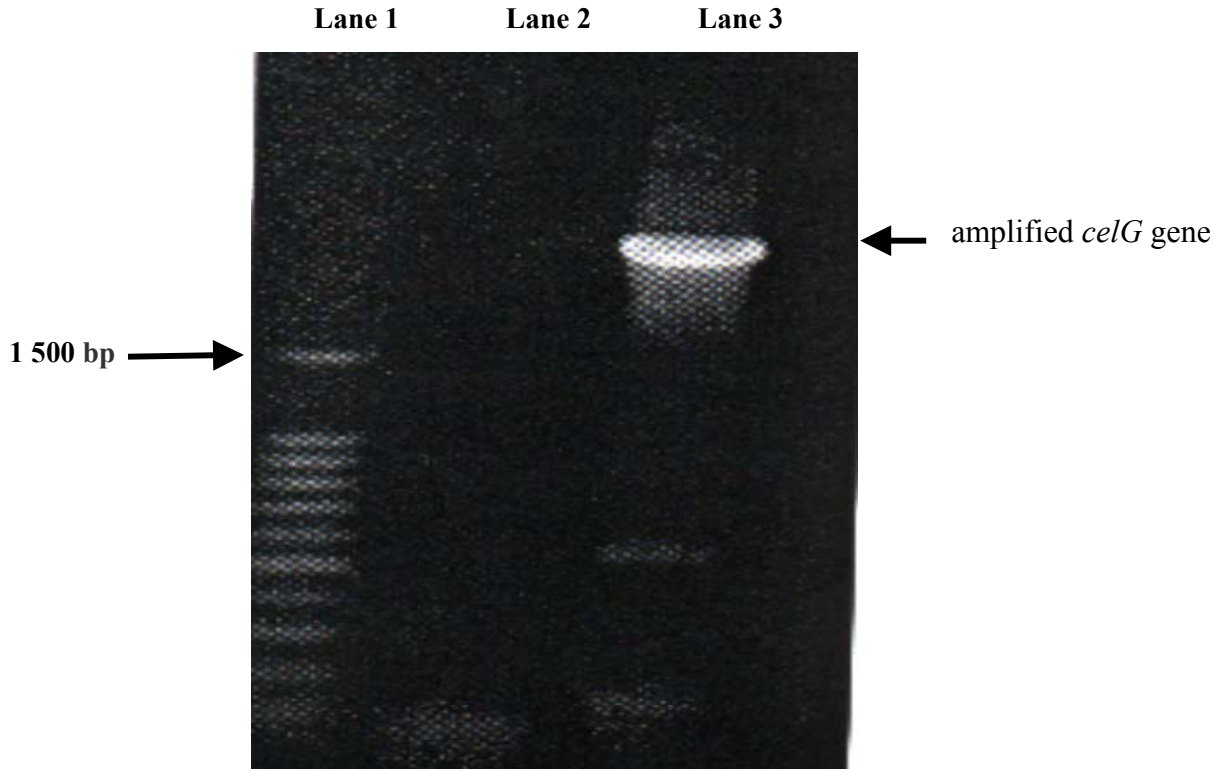


Figure 29: PCR amplification of the endoglucanase gene from the *B. subtilis* CHZ1 genomic DNA. **Lane 1**; 100 bp ladder DNA marker; **Lane 2**: Control PCR amplification run and **Lane 3**: PCR amplification run with *B. subtilis* CHZ1 genomic DNA.

The full-length *celG* gene product was cloned into pGEM-T Easy (Promega) vector to give a construct named pBEGc3. Cloning into pGEM-T Easy was made possible because of the deoxythymine (T-nucleotide) overhangs on both 3′ and 5′ ends of the vector that will allow easy ligation with DNA generated by *Taq* polymerase that will be having 5′ deoxyadenine (A-nucleotide) overhangs. A schematic representation of the amplified DNA fragment containing *celG* cloned into pGEM-T Easy multiple cloning site is shown in figure 30.

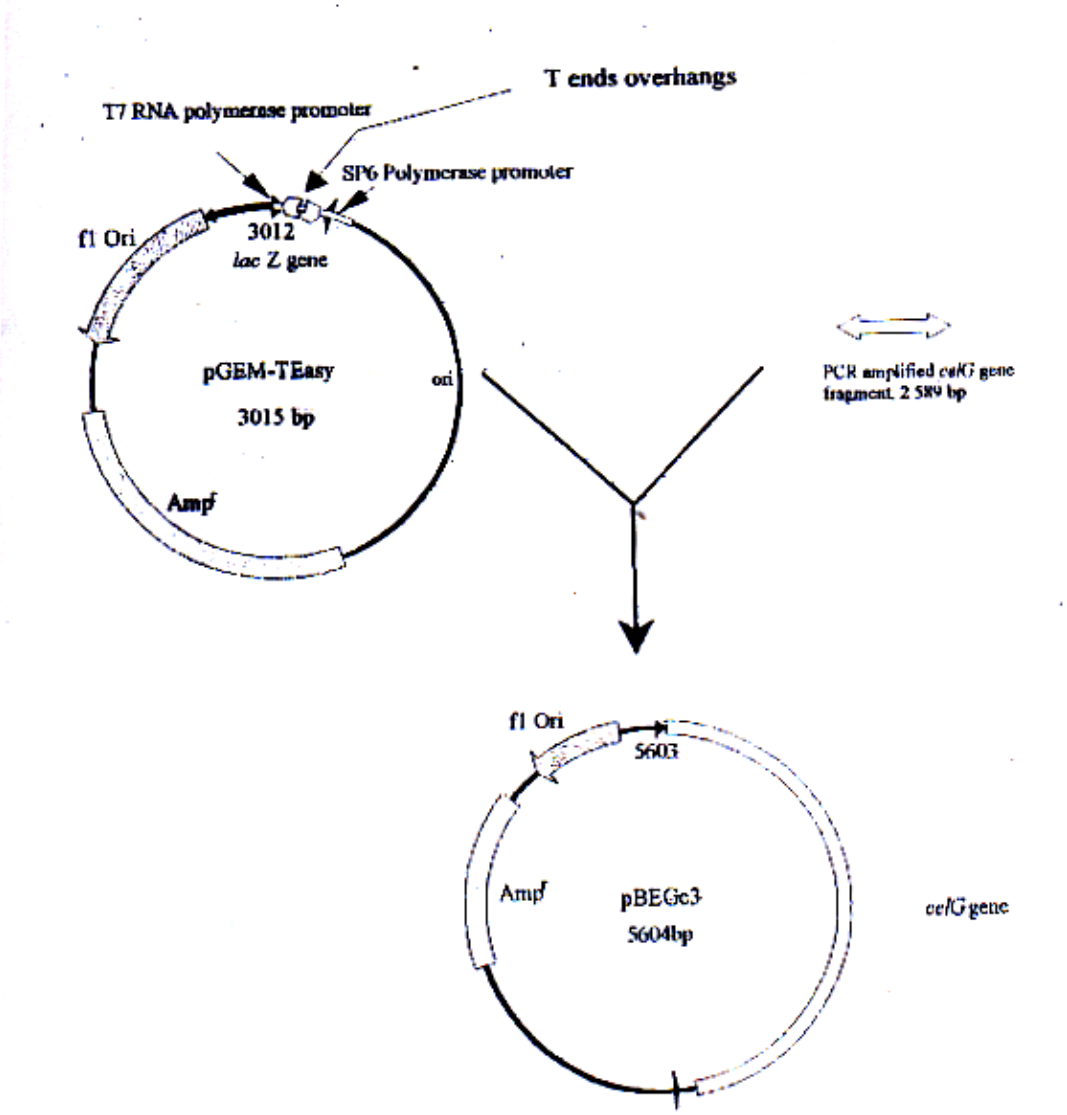


Figure 30: Schematic representation of the strategy used to clone pBEGc3 with *celG* DNA fragment obtained by PCR amplification. pGEM-T Easy is a linear plasmid with T-nucleotide overhangs (denoted by arrow fittings) that will ligate with A-nucleotide overhangs (denoted by arrows) added by *Taq* polymerase during *celG* amplification.

The T7 promoter primer was used for determining the orientation of the DNA fragment insertion in pBEGc3 clone and this confirms the orientation *celG* gene. Sequencing with SP6 primer showed that the secretory signal terminal end of the *celG* gene fragment was ligated in *cis* configuration and hence the expression of the endoglucanase could be induced with IPTG in *E. coli* DH5α.

4.5.3 Analysis of *celG* gene

Sequencing for this full-length fragment gave a size of 2 589 bp, which confirms very well with the size obtained by agarose gel analysis. The cloned endoglucanase gene sequence is shown in figure 31.

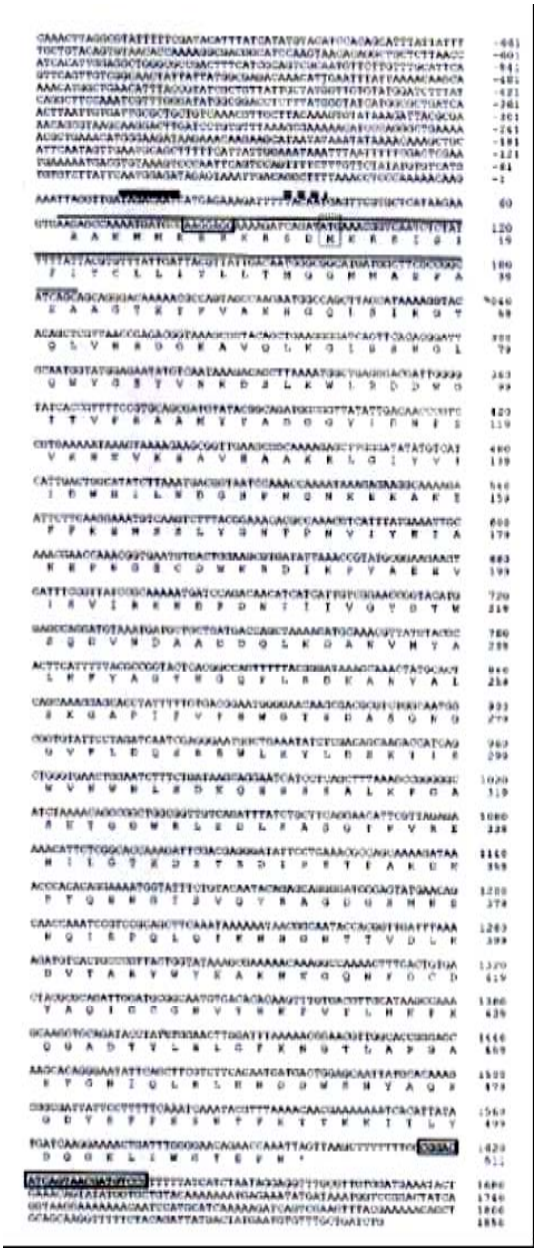


Figure 31: The nucleotide and the deduced amino acid sequence of the endoglucanase from *B. subtilis* CHZ1. The –35 and –10 promoter regions are marked by double solid and dashed lines respectively. A single solid line indicates the predicted secretory signal sequence. The three initiation codons are shown in dashed line boxes. A solid-line box within the secretory signal sequences indicates the most likely ribosomal binding site. The inverted repeat sequence is indicated in a box with sequences in bold text.

This *celG* sequence was deposited in GenBank database under accession no. AY044252. The BLASTN alignment search in the available databases revealed that the nucleotide sequence of *celG* had 98 % which the endoglucanase of *B. subtilis* (accession no. X04689). The *celG* gene also showed homology to other *Bacillus* endoglucanase genes. The fragment contains the conserved regions similarly with other *Bacillus* endoglucanase genes. A computer analysis of the sequence revealed an open reading frame (ORF) of 1 524 bp encoding an endoglucanase precursor protein with 508 amino acid residues and an apparent molecular weight of 56 472 daltons. Given a secretory signal peptide length of 38 amino acid residues, and a predicted molecular weight of 4 271, the size of the mature protein was estimated to be 52 kDa. This value is in the molecular weight range of other endoglucanases from some *Bacillus subtilis* species (Khanongnouch *et al.,* 1999; Robson & Chambliss, 1987; Nakamura *et al.,* 1987; Park *et al.,* 1991;Murphy *et al.,* 1984; Mackay *et al.,* 1986; Gormely *et al.,* 1988). When compared with other endoglucanases, the polypeptide shows the common modular structure with other cellulases comprising a catalytic domain (CD) with 278 amino acid residues linked to a cellulose binding domain (CBD-3) with 83 residues. Comparison of the deduced amino acid sequence with the primary structure of other microbial endoglucanase sequences was conducted using the SWISSPRO computer program. The protein sequence was shown to have the structural features of family-5A glycosyl hydrolases. The consensus putative promoter sequences at –35 region (-TAGACA-) and –10 region (-TACAAT-) were identified. The regions are interspaced with a nonconsensus promoter sequence containing 15 nucleotides.

A ribosome binding site –AAGGAGG- is located between positions 82-90. The *celG* gene has three potential ATG initiation codons but the nearest codon downstream of the ribosome binding site can be assumed to be the most likely initiation codon. The other two possible binding sites are at nucleotide positions 74-76 and 7-79. There is no recognizable ribosomal binding site upstream of any one of the other two initiation sites to assume translation. Beyond the ORF there is a stop codon TAG at position 1538-1540. An inverted repeat sequence of the *celG* gene, -CGGACATCAGTAACGATGTCCG-, which could play a role, as a transcription-termination signal sequence was identified 15 bp downstream of the TAG stop codon. Similar structures are observed downstream of *Bacillus* strain endoglucanase genes (Simonen & Palva, 1993).

4.5.4 Analysis of structural and functional domains in CelG primary protein structure

The crystal structures of all endoglucanases of *Bacillus* closely resemble each other (Sakon *et al.,* 1996; Davies et al., 1998; Coutinho & Henrissat, 1999; Czjzek *et al.,* 2000; Henrissat & Davies, 2000). Generally, endoglucanases are divided into families depending on their structural domains

and mode of β-glycosidic bond hydrolysis. Alignment of an unknown endoglucanase protein's primary sequence can allow identification of conserved and important residues for a defined understanding of the enzyme's structure and function. Any differences obtained in certain amino acids position can explain the notable differences in their efficiency of catalysis and other chemical or physical properties e.g. temperature or pH-optima and glycosylation. A SWISSPRO motif alignment of CelG with other members of family-5A glycosyl hydrolases is shown in figure 32. The seven conserved motifs of *Bacillus* alkaline endoglucanase enzymes were identified on the CelG enzyme from *Bacillus subtilis* CHZ1.

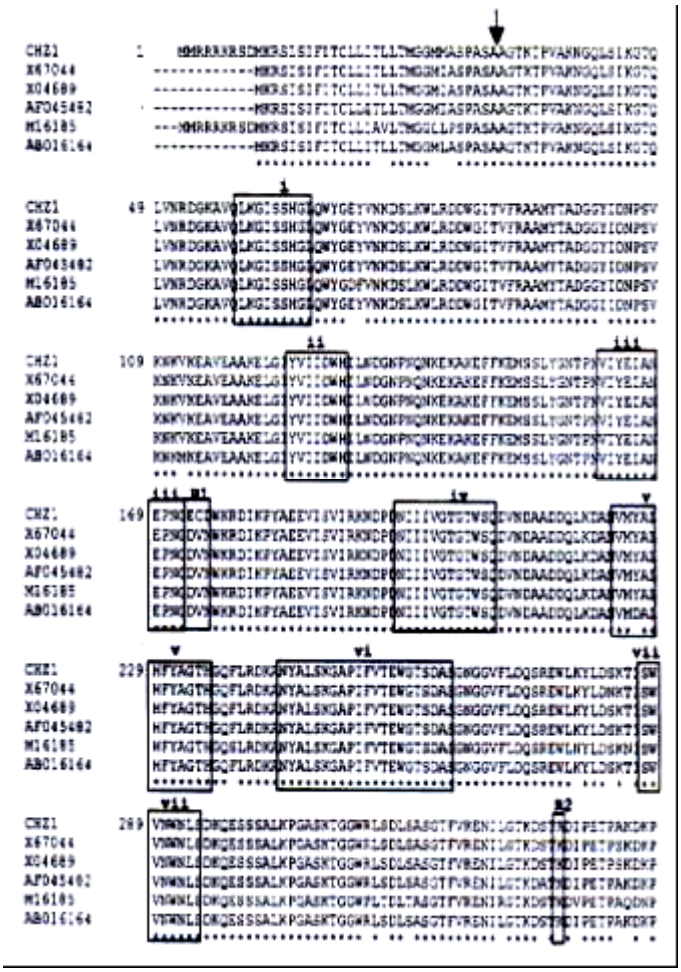


Figure 32: Full length alignment of the predicted amino acid sequence of the *B. subtilis* CHZ1 cellulase and other *Bacillus* cellulase. The arrow shows the cleavage site for all the secretory signal peptide sequences. The selected species of family-5A glycosyl hydrolases sequences were obtained from the GenBank database. The strains were *B. subtilis* strains; X04689, X67044 and M16185 and *Bacillus* sp.; AF044582 and AB16164. The seven endoglucanases conserved motifs are indicated as shown from i-vii. Below the alignments are asteristiks showing identical amino acids for all the strains. The unshaded box indicates the amino acid residues for CHZ1 strain that are different from all the other cellulases.

A tertiary structure prediction by homology model building was performed by superimposing CelG sequence onto the endoglucanase of *Bacillus agaradherens* using SPDV computer program. There is not much literature on family-5A endoglucanases tertiary structural information besides that of *Bacillus agaradherens* endoglucanase.

The conserved domain sequence alignment displayed 99.6 % homology of the CD comprising of 278 amino acid residues stretching from position 18 to 272 of the precursor protein. The linker region is located between positions 273 to 327 and the CBD had 100 % homology stretching from positions 328 to 408. The domains showed homology with several *Bacillus* species. It was interesting to note that a major difference in amino acid residues lies within the active site. The DVN residues at positions 182-184 present in the most endoglucanases were replaced by ECD in celG endoglucanase. The amino acid residue exchange V183C was an observed isosteric replacement to stabilize the active site region via hydrogen bonding with the sulphur side group of this substituted cysteine residue.

4.5.5 Expression of celG gene *E. coli* DH5α

The cloned *celG* gene was expressed in *E. coli* DH5α suggesting that this cellulase promoter is recognized in *E. coli* DH5α and the protein was not toxic to the cells due to high-levels of expression as was reported with other several *Bacillus* genes and other foreign genes in *E. coli* DH5α hosts (Sanchez-Torres *et al.,* 1996; Kurland & Dong, 1996; Wicher *et al.,* 2001). The *E. coli* DH5α cell free supernatants had very low endoglucanase activity whilst the wild type strain had no cell bound endoglucanase activity. Following induction of the *celG* gene by 1.0 mM IPTG, the enzyme was overexpressed by a 10 fold magnitude. In the presence of this inducer after 25 h of growth, the enzyme activity was 450 nkat/ml compared to 45 nkat/ml with no induction (figure 33).

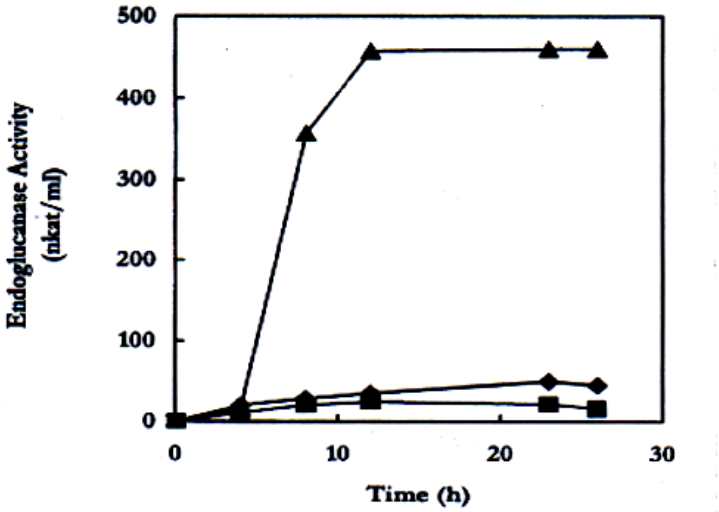


Figure 33: Comparison of endoglucanase production profile in *B. subtilis* CHZ1 and *E. coli* DH5α cloned with pBEGc3 after cell homogenization. Cultures were fermented in 200 ml of Luria-Bertani broth supplemented with 1 % (w/v) carboxymethyl cellulose. (■) CHZ1, (◆) clone uninduced, (▲) clone induced with IPTG.

There was no drastic reduction in endoglucanase activity during cultivation of the clone with *celG* gene as was observed with the wild type strain, probably due to low protease production from host *E. coli* DH5α cells used. The endoglucanase activity was assayed from crude supernatant at different pH values and temperatures to compare with the wild type. The cloned endoglucanase enzymatic properties were the same as those described from the wild type strain. Similar optimum conditions for the wild type endoglucanase enzyme (pH 6.0 and temperature of 70°C) were obtained with the cloned gene. The expression levels of the uninduced clone were similar to the wild *B. subtilis* CHZ1 strain. It was however observed that, about 70 % of the activity was cell bound. There was very low extracellular expression with the clones. There was no endoglucanase activity from sonicated *B. subtilis* CHZ1 cells after resuspending them in buffer, showing that the wild type strain has very low or negligible cell bound endoglucanase.

CHAPTER 5

5 DISCUSSION

5.1 SCREENING AND CHARACTERISATION OF CELLULASE MICROBES

5.1.1 Screening of cellulase producing strains

The media used supported growth of several microorganisms both endoglucanase and non-endoglucanase producers. Of the total number of strains isolated only one produced an endoglucanase enzyme. No strains producing other components of the cellulase besides endoglucanase were isolated. The media had a neutral pH value, and the strains isolated grow well in the neutral to alkaline pH range. Screening for microorganisms based on culturing methods requires the use of different media in order to optimize, broaden selection and support growth for the wide spectrum of microorganisms.

Although *Bacillus* species have been among the most common bacteria that appeared on culturing plates from hot spring samples (Sharp *et al.,* 1992) however, this does not indicate their dominance in these samples as proven by the uncultivable bacteria that have shown the contrary (Reysenbach *et al.,* 1994; Hugenholtz *et al.,* 1998b; Hjorleifsdottir *et al.,* 2001; Skirnisdottir *et al.,* 2000). Hot springs have been proven to be habitats of many different coexisting bacteria as evidenced by molecular diversity samples rather than culturing techniques (Baker *et al.,* 2001; Barns *et al.,* 1994 & 1996b; Reysenbach *et al.,* 1994 & 2000; Ward *et al.,* 1997 & 1998; Hugenholtz *et al.,* 1998). Some microorganisms are very difficult or even impossible to culture using the standard cultivation techniques yet they might produce enzymes that scientists are striving to get through mutagenesis. Analysis of nucleic acid extracted directly from the samples is now increasingly used to describe microbial diversity of a sample rather than culturing, circumventing the problems of uncultivable microorganisms (Ward *et al.,* 1990; Hjorleifsdottir *et al.,* 2001).

5.1.2 Characterisation and identification of the cellulase producer

The morphological, biochemical and molecular techniques used in this study have been used to identify *Bacillus* species from hot spring waters and various environmental and food sources (Markossian *et al.,* 2000; Nielsen *et al.,* 1995, Claus & Berkeley, 1986; Madigan, 1997; Cowan & Steel, 1993; Ronimus *et al.,* 1997). The results from the current study identified the microorganism of interest as a *Bacillus* strain.

The physiological and biochemical tests of the endoglucanase producer strain together with the amplification of the 16S rRNA gene from its DNA, confirmed that the strain was a *Bacillus subtilis*. The 16S rRNA gene size and sequence homology conforms very well to other established *Bacillus* strains (Weisburg *et al.,* 1991; Black *et al.,* 1997; Daffonchio *et al.,* 1998; Daffonchio *et al.,* 1998; Stetten *et al.,* 1998; Watanabe *et al.,* 2001; Hansen & Hendriksen, 2001). The comparison of 16S rRNA sequences is now used as a reliable method for the classification and identification of bacterial species. This is because rRNA genes are highly conserved and present in large copy numbers within bacterial cells (Scheinert *et al.,* 1992; Nagpal *et al.,* 1998; Moffett *et al.,* 2000; Jeng *et al.,* 2001). A lot of bacterial species have their 16S rRNA sequences deposited in GenBank databases, therefore the sequences can be accessed and used in homology searches as to characterise and classify unknown microorganisms. All the identification results obtained with the current microorganism has genotype and phenotype similarity with the *Bacillus subtilis* group. Hence the strain used in this study was designated *Bacillus subtilis* CHZ1.

Analysing restriction fragment length polymorphism of rRNA operons can do further identification of the strain to subspecies level as well as the 16S-23S rRNA intergenic regions (ISR) sequences then compare with other *Bacillus* strains, because analysis of 16S rRNA only cannot differentiate closely related species (Shaver *et al.,* 2002).

During cultivation of the strain, the cells elongate in the early exponential phase. This has been reported in other *Bacillus* strains (Sturr *et al.,* 1994; Aono, 1995). This characteristic is quite compatible with the nature of the membrane lipids that are found in *Bacillus* species (Kaneda, 1991 & 1997; Kates, 1993). The *Bacillus subtilis* CHZ1 was observed to have a varied Gram reaction. The *Bacillus* showed Gram-positive reaction during exponential phase and Gram negative in the stationary phase.

5.2 OPTIMISING CONDITIONS FOR GROWTH AND ENDOGLUCANASE PRODUCTION BY *B. SUBTILIS* CHZ1

5.2.1 Effect of temperature and initial pH on the growth and endoglucanase production

Bacillus subtilis CHZ1 had highest growth rate at 40^{o}C and no growth beyond a temperature of 50^{o}C, and can be classified as a moderate thermophile (Madigan *et al.,* 1997; Baker *et al.,* 2001). Although the term thermophile was controversial, now scientists prefer a cut off of growth temperature value above 55-60^{o}C (Kristjansson & Hreggvidsson, 1995; Kristjansson *et al.,* 2000). Production of endoglucanase enzyme was biomass depended however highest enzyme levels were observed at 50^{o}C although this temperature had low biomass. Au and Chan (1987) also reported similar results. *B. subtilis* CHZ1 produced endoglucanase during the exponential phase. Other

workers have reported endoglucanase production in the exponential phase (Robson & Chambliss, 1986; Chan & Au, 1987; Lee & Pack, 1987; Sharma *et al.,* 1987; Sharma *et al.,* 1990; Mawadza *et al.,* 1996). Although another *Bacillus* studied by Aa *et al.,* 1994 produced endoglucanase in the stationary phase in three different media.

The temperature 50°C was also the highest temperature for culturing *B. subtilis* CHZ1 strain. Culturing the *Bacillus* strain using batch fermentation at 50°C for endoglucanase production required downstream processing of the culture supernatant to recover high enzyme levels after 5 h, since there was a rapid drop in enzyme levels. At 50°C, it is an unfavourable growth temperature and the *Bacillus* cells tend to quickly sporulate as a response to attain a thermoadaptable form. The sporulation process causes the *Bacillus* cells to produce proteases that have to prepare the cells for spore formation (Pero & Sloma, 1993; Jan *et al.,* 2000). However, proteases in some cases are produced for protein turnover, germination and enzyme modifications, nutrition and regulation of gene expression.

The onset of stationary phase in batch cultivation is a sign of nutrient depletion. The sigma factors of the *Bacillus* cells are then activated to initiate protease production and sporulation (Haldenwang, 1995; Pero & Sloma, 1993; Jan *et al.,* 2000). Proteolytic inactivation of enzymes, leads to irreversible loss of *in vivo* catalytic activity and is an important physiological event because this is what contributes to the observed rapid loss in endoglucanase enzyme levels. Culturing at low temperatures e.g. 30°C had low endoglucanase enzyme production, about 20 nkat ml^{-1}.

The *B. subtilis* CHZ1 had maximal endoglucanase production in M162 medium with an initial pH of 6.0 and a temperature of 50°C. But optimal conditions for culturing were an initial pH value of 8.0 and a temperature of 40°C. A high endoglucanase level obtained at an initial medium pH value of 6.0 is probably because of the stability of the enzyme at this pH. The protease enzyme is also not stable at this pH. The extracellular protease produced by the *Bacillus subtilis* CHZ1 is active in alkaline pH range and has very low activity in acidic pH range.

The pH value of the batch fermentor was observed to drop for the first 2 h and then increased to alkaline values. This pH profile has been reported with other workers (Svensson, 1996; Mawadza *et al.,*, 1998). This trend has been attributed to acidic products formation from carbon sources. The change in pH conditions from acidic to alkaline during cultivation and the concomitant depletion of carbon and nitrogen nutrients activates protease production essential for sporulation process (Pero & Sloma, 1993; Driks, 1999). The alkaline conditions are favourable for proteolytic activity and accumulation; hence the drop in endo-β-1,4-glucanase levels by proteolysis.

5.2.2 Effect of carbon source on growth and endoglucanase production

The *Bacillus subtilis* CHZ1 could utilise all the used carbon sources. Our results suggest that endoglucanase production from the carbon sources by the *Bacillus* strain was constitutive. The endoglucanase production response to carbon source can be used to determine the regulation of the enzyme synthesis. Our results suggest that cellobiose is not an inducer as was shown in *Trichoderma reseei* and *Provetella bryantic* (Kuhad *et al.,* 1997). The lack of catabolite repression by glucose or cellobiose on the synthesis and activity of the endoglucanase enzyme produced by *B. subtilis* CHZ1 has been reported by other workers (Aa *et al.,* 1995; Dhillion *et al.,* 1985; Robson & Chambliss, 1986; Chan & Au, 1987). The regulatory mechanism on endoglucanase production varies among the *Bacillus* genus as in some strains glucose was observed to strongly inhibit synthesis (Fukumori *et al.,* 1985; Au & Chan, 1986). The property of constitutive production of endoglucanase by *B. subtilis* CHZ1 is advantageous because the enzyme can be used in conjunction with other cellulases for the bioconversion of cellulose to energy and as an animal feed stock additive. Aa and co-workers (1995) reported the production of endoglucanase enzyme in media without cellulose.

Growth on several carbohydrate substrates shows the capability of the strain to produce several enzymes that can hydrolyse the polysaccharides. *B. subtilis* has been shown by other workers to be a ubiquitous bacterium. The strain is capable of degrading a variety of natural substrates hence it can exploit a wide spectrum of carbon and nitrogen sources (Nicholson *et al.,* 2000; Priest, 1993; Claus & Berkeley, 1986; Cowan & Steel, 1993; Ash *et al.,* 1991; Nielsen *et al.,* 1995). The biodiversity in metabolising different carbon sources by the *Bacillus* genus permit them to therefore inhabit diverse environments.

5.2.3 Effect of protease enzyme on endoglucanase production

Since the endoglucanase enzyme produced by CHZ1 strain is prone to proteolysis from the same producer strain, it is therefore necessary to reduce extracellular protease production. Other workers have reported similar effects of protease enzyme on endoglucanase production (Sharma *et al.,* 1990; Kunst & Rapoport, 1995; Mawadza *et al.,* 1996; Hirose *et al.,* 2000) Reduction of proteolytic activity can be accomplished by including protease inhibitors in the medium during culturing of the *Bacillus subtilis* CHZ1. It has been shown that the addition of 3 mM PMSF, a serine-protease inhibitor, to a *Bacillus subtilis* culture in the late exponential growth phase inhibits proteolytic activity (Hirose *et al.,* 2000). Protease production can also be greatly reduced by using a high salt medium (Kunst & Rapoport, 1995). High salt concentration medium leads to a strongly decreased sporulation efficiency and decreased expression of the alkaline protease gene (*aprE*).

The workers used a medium that had high salt concentration of 1M NaCl and very low yeast extract, yielding very low protease activity and microbial growth could be sustained for two days without rapidly attaining stationary phase.

5.2.4 Effect of temperature and pH on endoglucanase activity

The endoglucanase enzyme of *Bacillus subtilis* CHZ1 had an optimum pH and temperature of 6.0 and 70°C respectively. The CHZ1 strain endoglucanase is classified as an acidic type. A number of purified or studied *Bacillus* endoglucanase enzymes to date are either acidic with pH optima between 5.5 and 6.8 (Au & Chan, 1987; Sharma *et al.,* 1990; Gilkes *et al.,* 1991; Hoon *et al.,* 1997) or neutral to alkaline type with pH optima between 7.0 and 10.0 (Ozaki *et al.,* 1990; Yoshimatsu *et al.,* 1990; Okoshi *et al.,* 1990; Ito, 1997; Ito, 1998; Horikoshi 1999). All these endoglucanase have temperature optima in the range 40 – 70°C. Generally, endoglucanases show activity within a broad range of pH from 6.0 to 10.0 and in the temperature range 30 – 70°C.

5.2.5 Applications of CelG enzyme

Although the CelG enzyme has no dramatic characteristics, its properties are amenable as a potential animal feeds additive. The optimised conditions for the endoglucanase of CHZ1 strain are suitable properties for the enzyme to be applied as an animal feedstock additive. Cellulosic materials fed to ruminants are first pretreated with steam to increase digestibility. The wet materials resulting from this pretreatment method produce acidic cellulosic extracts (as a result of pectin and hemicellulose glucans hydrolysis). The acidic pH attained by the cellulose mixture is suitable for this *Bacillus*'s endoglucanase enzyme. The CelG enzyme can be used in crude form as an animal feedstock additive. Although it is susceptibility to proteolytic action, its degradation can be stabilised by adding protein stabilisers like albumin or soybean protein to the cellulose mixture (Morgavi *et al.,* 2000). It has been concluded that the stability of cellulases in rumen fluid is unlikely to be a limiting factor in their application as feedstock additives. If thermostability of these enzymes is improved then they can enhance cellulolytic material pre-digestion before fed to ruminants. Since the optimum pH of CelG's activity is 6.0, improving its thermal stability will make it amenable with the acidic pre-processing conditions of agricultural cellulolytic material fed to livestock (Lehman *et al.,*, 2000).

The CelG enzyme has a cellulose binding domain (CBD), therefore it cannot be used in cotton textile industry or in biopolishing of fabrics unless it is cloned truncating the CBD domain. The CBD unit by affinity will bind to the cloth fibres and even after washing will proceed in hydrolysing the fabric after laundry. CelG enzyme cannot be used as a detergent additive because

it is susceptible to proteases and detergents have a mixture of hydrolytic enzymes, proteases being one of them.

5.3 EFFECT OF FOOD WASTES ON PRODUCTION OF HYDROLYTIC ENZYMES

5.3.1 Shake flask experiments

The formulated media based on food wastes was able to support growth of the *B. subtilis* CHZ1 producing the following hydrolase enzymes: amylase, endoglucanase, protease, xylanase and polygalacturonase. Free reducing sugars were observed to increase after 10 h of cultivation. The increase in sugars observed could be as a result of glycosidic hydrolases acting on soluble polysaccharides from the malt flours. The biomass levels obtained in the shake flask and fermentor experiments indicate that spent yeast and sorghum malt flour are essential components for CWW medium preparation to culture the *Bacillus* strain. There was no remarkable influence on growth when either defatted soya or barley malt flour was excluded. Low levels of enzymes were observed in the media prepared without spent yeast. Therefore, brewers spent yeast contributes very essential factors for growth and enzymes production by *B. subtilis* CHZ1. All the components except sorghum malt flour were observed to be essential for maximum endoglucanase enzyme production.

Similar work with food wastes enriched with fish waste flour rich in nitrogen source and ammonium salts showed improved production of several hydrolase enzymes by *Aspergillus* sp. (Farid *et al.,* 1984; Shaker *et al.,* 1985). These workers optimised conditions for enzyme production by the microorganisms in shake flask experiments. Fujuwara and Yamamoto (1987) produced a protease enzyme from a soya bean meal based low cost medium. Other food wastes have been studied for production of hydrolytic enzymes in our laboratory using different *Bacillus* species (Mawadza & Zvauya, 2001). Ellouz and co-workers (2001) produced results that showed that fish waste flour promote biomass and protease enzyme synthesis by a *Bacillus subtilis* strain. The compositions of the food wastes used in this study have been studied. Spent yeast has been established to be rich in proteins, minerals, vitamins e.g. biotin, pantothenic acid, niacin and has high amount of essential amino acids (Miller & Churchill, 1986). Study on sorghum malt showed that it is rich in carbohydrates (soluble cellulosic and sugar substrates), defatted soya has high protein content and barley malt is a very good source of choline, niacin and essential amino acids (Massey *et al.,* 2001; William *et al.,* 1984). Brewery wastewater has been shown to be quite rich in mineral salts mainly derived from the malt flours (Zvauya *et al.,* 1994).

Shake flask experiments show that each component of CWW medium contributes one or more nutrients essential for either optimal microbial growth or maximum enzyme production. All these food waste products are available in large quantities from the respective food industries in developing and developed countries for large-scale production of the hydrolytic enzymes (Wiseman, 1993).

5.3.2 Fermentor experiments

Use of the fermentor optimises hydrolytic enzymes production with the formulated medium. Fermentor conditions improves dissolution and oxygen transfer to the cells. Coupled with the medium that is quite rich in nutrients, the *Bacillus subtilis* CHZ1 showed rapid growth and high hydrolytic enzymes levels in fermentor experiments. Higher enzyme levels and biomass were produced in fermentor experiments within 5 h. All the hydrolytic enzymes except the protease enzyme lost activity during the stationary phase. Other workers have reported the observed rapid drop in enzyme levels during cultivation and this has been attributed to the associated increased levels of proteolytic activity (Au & Chan, 1987; Sharma *et al.,* 1990; Mawadza *et al.,* 1996). This can be attributed to the very high proteolytic activity obtained in fermentor experiments.

The CWW medium is a potential cheap bacteriological medium for culturing microbes. It has been used to culture other microorganisms in our laboratory. This cheap medium therefore is rich in minerals and nutrients utilizable by other microorganisms. Each of the nutrient factors contributes to the microbial growth and enzyme production. Taking into account the current market costs of synthetic media, microbial research work can be carried out using this medium to culture various microbes. Besides producing hydrolytic enzymes, the microorganisms can be optimized for treatment of industrial wastes.

Sorghum malt has high levels of polyphenolic compounds and these have been observed to affect endoglucanase activity and microbial growth (Bae *et al.,* 1993). Extraction of the hydrolysates during medium preparation from the food wastes has to be limited to reduce solubilising high levels of polyphenolic compounds. Polyphenolic compounds have been shown to have anti-microbial activities that affect biomass and enzyme production negatively. Terpenes such as resin acids, phenolic derivatives that include lignin and stilbene derivatives have been noted to be the most likely candidates to inhibit extracellular cellulase activity and fungal growth during culturing (Woodward & Pearce, 1988; Lindberg *et al.,* 1993) and presumably can affect bacterial growth.

5.4 PURIFICATION OF A PROTEASE ENZYME FROM THE *B. SUBTILIS* CHZ1

5.4.1 Effect of nitrogen source on protease production

Peptone and yeast extract are good inducers of protease synthesis. However, it is possible that other components in the organic nitrogen source could have contributed to the observed increased protease production. The readily available and ease to assimilate nitrogen from the organic sources could have contributed to the high biomass and protease production observed with *B. subtilis* CHZ1 strain. Several researchers have focused on formulation of synthetic media that improve protease enzyme production (Beshay & Moreira, 2001; Farid *et al.,* 1984; Shaker *et al.,* 1985). However, Ferrero and coworkers (1996) showed that organic nitrogen on *B. licherniformis* MIR 29 reduced protease production by 80 – 90 %. Nitrate source increased by 45 % the production of alkaline protease. Regulating concentrations of carbon, nitrogen and phosphorous sources can increase secretion of protease enzymes. The repression of proteolytic synthesis in the presence of excess ammonium has been reported (Heineken & O'Connor, 1972; Kole *et al.,* 1988; Beshay & Moreira, 2001). An initial phosphate concentration of 24 g/l was necessary for maximal protease levels (Beshay & Moreira, 2001). Kim and coworkers (2001) produced protease enzyme using poultry feather waste as nitrogen source supplemented to a synthetic medium. In another study, *B. pumilis* and *B. cereus* were shown to produce proteolytic enzymes in the late exponential phase (Kim *et al.,* 2001). The enzyme production in both strains was inducible with feathers but constitutively in *B. subtilis*. In another study, Ellouz and coworkers (2001) showed that low-cost industrial fish waste flour, rich in nitrogen source (45.5 g N/100 g material), supplemented to YT medium had more protease enzyme production by 112 U/ml compared to peptone supplementation. The cultivation was carried out using a *B. subtilis* strain.
Optimum medium formulation including nitrogen, carbon, phosphate sources and concentration has to be ascertained for maximal protease production by the *Bacillus subtilis* CHZ1. This may reduce the number of ion exchange chromatography runs performed to get a reasonable quantity of the enzyme to run on SDS-PAGE gel and it also optimises on enzyme production. However, cloning of this gene and using immobilized cells has shown better and higher alkaline protease expression (Zaghloul *et al.,* 2002; Kumar & Schügerl, 1990).

5.4.2 Purification of the protease enzyme

The purification protocols for *Bacillus* proteases that have been widely used to purify the enzymes to homogeneity by other researchers are basically concentrating the culture, gel filtration and or lastly performing an ion exchange chromatography (Asakara *et al.,* 1998; Kole *et al.,*, 1988; Ferrero *et al.,*, 1996; Kobayashi *et al.,* 1996).

Purification of the protease enzyme from *B. subtilis* CHZ1 culture was carried out at 4°C to minimise autodigestion and proteolysis that could mislead as multiple forms of the enzyme after a SDS-PAGE gel analysis. Enzyme multiplicity as a result of proteolytic activity has been observed in purifying enzymes from *Bacillus* culture supernatants (Kobayashi *et al.,* 1996). The products from proteolytic digestion have been observed to show activity on a zymogram stain and are resolved as different forms or isozymes of proteases. Although some workers have managed to show that certain *Bacillus* strains produced three proteases with different distinct pH and temperature optima properties (Kim *et al.,* 2000). Ferrero and coworkers obtained results that showed that a protease enzyme from a *B. licherniformis* had two bands of Mr 25 and 45 kDa on a denaturing gel. Multiplicity of proteases has been attributed to different gene products (Strydom *et al.,* 1986; Kobayashi *et al.,* 2001) or in vivo physiological deamination and phosphorylation (Green, 1985; Ederibigbe & Odunfa, 1988).

5.4.3 Properties and characterisation of the protease enzyme

Alkaline proteases have been purified from several sources that include *Bacillus* strains (Kole *et al.,*, 1988; Ferrero *et al.,*, 1996; Kobayashi *et al.,* 1996; Kobayashi *et al.,* 2001), *Thermoactinomyces* (Kleine *et al.,* 1980), *Conidiobolus* sp. (Bhosale *et al.,* 1995), squid muscle (Rodger *et al.,* 1984) and from shipworm bacterium (Griffin *et al.,* 1992).

It has been shown that a number of microbial alkaline proteases require Ca^{2+} ions to be thermostable at higher temperatures than their optimal temperature as a result of increased ionic binding. Therefore the activation of this protease by Mn^{2+}, Mg^{2+} and Fe^{2+}, could possibly imply that the purified protease might have enhanced stability in the presence of Ca^{2+} ions at elevated temperatures like other alkaline serine proteases (Takami *et al.,* 1989; Tsuchiya *et al.,* 1992; Kleine *et al.,* 1981; Asawaka *et al.,* 1998; Bhosale *et al.,* 1995; Griffin *et al.,* 1992).

Kobayashi and coworkers (2001) purified three proteases from a *Bacillus* strain that were designated the names H, M and N proteases. H had Mr of 28 kDa with optima pH and temperature of 11.0 and 55^{o}C respectively. N had Mr of 27 kDa with two chains on an SDS-PAGE gel, with pH and temperature optima of 11.0 and 60^{o}C respectively. M was a product of auto proteolytic hydrolysis of H protease at pH 8.0. However, the enzyme retained only 20 % residual activity after 10 min at 70^{o}C.

Other workers got optimal conditions of pH 12.0 and a temperature of 60^{o}C using *B. licherniformis* strain. The enzyme was quite stable at 60^{o}C retaining 100 % activity after 10 min incubation. However, EDTA did not inhibit the protease at 5 mM. Meanwhile CHZ1 protease was inhibited to 64 % at 2.5 mM EDTA and Ca^{2+} ions did not affect the protease activity from the two

Bacillus strains. A number of these alkaline proteases lose activity within 1 h at temperatures above 50°C. Some alkaline proteases isolated from *Bacillus* genus have high optimal activity of pH ranging from 9.0 to 12.0 (Takami *et al.,* 1989; Tsai *et al.,* 1988; Durham *et al.,* 1987). The enzymes have high thermostability, which is an important characteristic for laundry detergents. Some of the genes coding for the protease enzyme have been cloned, sequenced and their potential biotechnological applications determined. Most of these alkaline proteases are active in the pH range 8 to 13 and have 420 to 480 amino acid residues. The protease enzymes have very low homology among themselves compared to endoglucanase enzymes. However, amino acid residues on their active sites are highly conserved. This criterion is used to classify proteases. A common feature with alkaline proteases is that almost all of them are stabilised by divalent metal ions particularly Ca^{2+} ions. The *B. subtilis* CHZ1 protease share the optimum catalytic conditions with other *Bacillus* proteases but did not show a change in activity in the presence of Ca^{2+} ions. It has the same inhibition properties compared to other *Bacillus* proteases. However, *B. subtilis* CHZ1 protease showed considerable thermal stability retaining 90 % activity for 5.5 h at 50°C.
Several *Bacillus* species secrete two types of proteases that is subtilisin or alkaline proteases and metalloproteases or neutral proteases. Most of these enzymes have been cloned and expressed in *E. coli* and *Bacillus* hosts (Horikoshi, 1999; Takami & Horikoshi, 2000; Rao *et al.,* 1998; Ito *et al.,* 1998; Horikoshi & Akiba, 1982; Jorgensen *et al.,* 2000; Kobayashi *et al.,* 2001).
The purified protease enzyme of the *B. subtilis* CHZ1 strain belongs to the serine protease class as shown by its maximum activity at pH 8.0, and its full inactivation by PMSF inhibitor. These characteristics are consistent with alkaline serine proteases. The protease enzyme's stability in the presence of chelators, oxidising agents and heavy metals and high thermostability can be of valuable use in detergent industry.

5.5 CLONING OF ENDOGLUCANASE GENE OF *BACILLUS SUBTILIS* CHZ1

The potential application of cellulases in diverse applications has motivated increasing interest in this group of enzymes. Some of the interesting application areas include improvements in brewing, wine making, fruit juice production, fabric softness and brightness, laundry detergents, nutritional quality and digestibility of animal feeds, production of stone-washed denims and de-inking of paper (Galante *et al.,* 1998; Ito, 1997; Cavaco-Paulo, 1998; Bhat & Bhat, 1997; Srinivasan *et al.,* 1999). Conversion of renewable cellulosic materials to commodity chemicals remains a challenging area. An investigation of the structure-function properties of the cellulases has helped to provide an insight of the mechanism of plant carbohydrate polymers hydrolysis.

Most of the cellulases for industrial applications come from fungal sources. However, several cellulases from aerobic and anaerobic bacteria are also known to contribute to the diversity of the cellulase family.

5.5.1 Endoglucanase amplification and cloning

Several endoglucanase genes have been cloned using the conventional methods of creating a genomic library followed by screening with the Congo Red assay for enzyme expression on a carboxymethylcellulose plates (Teather & Wood, 1982). A few of the endoglucanase genes have been cloned using the PCR technique. The conventional method is preferred because it recovers an original wild type gene whilst PCR amplification can incorporate a mutation(s). The PCR reaction however can be performed with much reliability yielding an original endoglucanase gene if run with blended *Taq*/*Pfu* DNA polymerase mixture or *Pfu* DNA.

The truncated endoglucanase gene yielded 75 % of the mature enzyme localised in region of the gene coding for the catalytic domain of the mature enzyme. This truncated gene did not express an active enzyme product as reported for the other endoglucanase genes lacking a carboxyl terminal end (Kim *et al.,* 1995; Din *et al.,* 1994; Park *et al.,* 1993). The carboxyl terminal possesses the linker region and cellulose binding domain hence these two regions have no effect on the activity of the catalytic domain of cellulases. Cellulase activity from the cloned truncated *celG* gene was confirmed by assaying both extracellular and intracellular endoglucanase activities after whole cell culture sonication. This could imply that there was expression of an inactive product or there was no expression. The remaining carboxyl region of the gene might be essential for an expression of an active CelG enzyme. Another possible explanation could be that there was no expression as a result of the missing promoter region and its upstream region that might contain transcriptional enhancers. In another study, a *Bacillus* endoglucanase gene has been reported to require about 200 bp of the upstream region of the promoter for transcription (Sumitomo *et al.,* 1995). The upstream region contained transcriptional enhancers which have been observed to be important in stimulating or communicating with RNA polymerases via DNA loops to enhance open complex formation and regulating the transcription of genes (Weiss *et al.,* 1992).

5.5.2 Endoglucanase (*celG*) gene amplification and cloning

The nucleotide sequence of the 1200 bp CelG gene fragment gave the highest homology with the *Bacillus* sp. (AF045482). The amplified DNA fragment with the full endoglucanase gene showed 98 % homology with *Bacillus* endoglucanase genes in the GenBank databases. Since the *celG*

gene fragment was generated from the highly conserved motifs of *Bacillus* endoglucanase genes and has high sequence homologies with those in the database, it can be used for detection of endoglucanase genes in *Bacillus* microbial sources. Although this will have to be confirmed by Southern blot hybridization with several members of a large population of known positive controls. This will allow for testing the sensitivity and specificity of this *celG* fragment with strains that have slightly different gene sequences. Eventually, the *celG* gene fragment could be regarded as a *Bacillus* endoglucanase gene-specific probe (molecular marker targeting the CMCase gene). This marker can be used as a hybridization marker or PCR endoglucanase specific marker. Such an application can be equivalent to the Jeffery's probes, 33.6 and 33.15 (Jefferys *et al.,* 1985) used in comparing genetic relatedness obtained from microsatellite fingerprints that show allelic differences; 16S rRNA gene primers for microbial strains identification purposes (Weisburg *et al.,* 1991) or the developed fluorogenic probe-based PCR assay for detection of *Bacillus cereus* in non-fat dry milk (Kim *et al.,* 2000). The probe can be used to identify fragments coding for an endoglucanase gene from digested genomic DNA of other members of the *Bacillus* family. This approach can be of widespread application for functional cloning. The primers for this fragment can as well have potential application as universal endoglucanase specific primers of *Bacillus* species, however they require verification with other *Bacillus* species.

5.5.3 Analysis of the *celG* gene

The *B. subtilis* CHZ1 *celG* gene structure is similar to other established *Bacillus* endoglucanase genes. This result showed the reliability of PCR in cloning endoglucanase genes and can be used to amplify genes from other *Bacillus* strains. Most *Bacillus* endoglucanase genes are encoded by a single structural gene. The upstream of the *Bacillus subtilis* CHZ1 endoglucanase gene sequence has the common features found in other promoter regions of *Bacillus* alkaline endoglucanase genes (Johnson *et al.,* 1983; Simonen & Palva, 1993). The promoter regions are, the –35 and –10 regions upstream from the potential transcription start codons, the spacer DNA separating –35 and –10 regions and an AT rich UP element (75 %) between –40 and –60 positions of the gene. This AT region is known to enhance or promote formation of 'open' promoter complexes via the α– subunit of the RNA polymerase (deHaseth *et al.,* 1998). The existence of UP elements has been reported to be an additional determinant for an active promoter. The consensus putative promoter sequences at –35 region (-TAGACA-) and –10 (-TACAAT-) were identified. The regions are interspersed with a nonconsensus promoter sequence containing 15 nucleotides. The promoter sequence of *celG* gene has high homology with promoter sequences recognized by *B. subtilis* σ^{43} factor and *E. coli* σ^{70} RNA polymerase holoenzyme as is reported in a number of alkaline cellulase

genes of *Bacillu*s strains (Khanongnuch *et al.,* 1999; Robson & Chambliss, 1987; Nakamura *et al.,* 1987; Park *et al.,* 1991; Lindahl *et al.,* 1994).

Bacillus endoglucanase genes share a conspicuous characteristic of existence of multiple ATG codons in the signal sequences (Tezuka *et al.,* 1989; Simonen & Palva, 1993; Murphy *et al.,*, 1984; Mackay *et al.,* 1986; Gormley *et al.,*, 1988). Probably this could be the possible explanation for the expression of these enzymes in other hosts that can make use of the putative ribosomal binding sites and any one of the initiation codons. This has enabled the expression of *Bacillus* endoglucanase genes in diverse hosts i.e. yeasts, *E. coli*, other *Bacillus* strains, mammalian cells and plants (Lo *et al.,* 1988; Olsen & Thomsen, 1991; Soole *et al.,* 1993; Meldgaard & Svendsen, 1994; Zhang *et al.,* 1997; Dai *et al.,* 2000). An inverted repeat sequence has a role in transcription-termination signal as was observed downstream of *Bacillus* strain endoglucanase genes.

Improved cellulolytic activity towards several cellulose substrates can be achieved by cloning bifunctional cellulolytic enzyme genes that can be expressed by the same promoter e.g. fusing cellulolytic and xylanolytic catalytic domains sharing a CBD domain (Endo *et al.,* 2001; Warren *et al.,* 1987; Tomme *et al.,* 1994; Boisset *et al.,* 2001). The fused enzymes had similar catalytic properties to their respective substrates as compared to one hydrolytic individual enzyme but with more substrates to hydrolyze. Tomme and co-workers (1994) showed that the bifunctional enzyme had improved enzyme activities towards the microcrystalline cellulose and hydrolysis rate (V_{max}) than the individual enzymes. Also low enzyme quantity was required to achieve a similar hydrolysis rate.

5.5.4 CelG protein structure analysis

The predicted protein structure that could be expressed by the cloned *Bacillus* endoglucanase gene further confirmed the amplification and cloning of the *Bacillus subtilis* CHZ1 endoglucanase gene. The hydropathic profile and the predicted amino acid sequence alignment with CLUSTALW computer program were used to assign the protein as belonging to the family-5A glycosyl hydrolases. The CHZ1 CelG enzyme has the seven conserved regions of alkaline *Bacillus* endoglucanase enzymes. Therefore, the endoglucanase enzyme expressed by the *B. subtilis* CHZ1 was named CelG, according to the suggestions of Henrissat *et al.,* 1998. The linker sequence for CelG domains is rich in proline, alanine, glycine and hydroxyamino acids, a common feature of endoglucanase enzymes (Gilkes *et al.,* 1991; Wicher *et al.,* 2001; Cazemeir *et al.,* 1999). The arrangement of these amino acids is meant to impart the flexibility that is essential for the function of these enzymes (Tomme *et al.,* 1995). The highly conserved Glu residues involved in hydrolysis

of cellulose were identified at positions 178 and 195 (Henrissat *et al.,* 1989; Gilke*s et al.,* 1991; Kawaminami *et al.,* 1998) although there was a notable amino residue sequence difference at positions 182ECD184 this sequence is DVN in other enzymes and at position 347, K is substituted by R. The amino acid substitution of CelG enzyme at position 183 with C stabilises the active site region by hydrogen bonding involvement of the sulphur side group on the enzymes outer active site cleft. The mechanism of action is determined by the location of functional residues within the 3-D structure and the sequence of the enzyme (Davies & Henrissat, 1995). Catalysis is conserved within each family and can be inferred for all members of the family hence the CelG mechanism is similar to that of the family-5 glycoside hydrolases.

5.5.5 Expression of the celG in *E. coli* host

The cloned *Bacillus* endoglucanase enzyme levels in uninduced *E. coli* represent basal expression levels from the resident promoter. When IPTG was used to induce endoglucanase expression, a 10-fold increase in enzyme production was observed. Since the *Bacillus* endoglucanase gene was cloned in *cis* with the SP6 promoter it could be induced with IPTG. This orientation was further confirmed by sequencing the clone using the T7 promoter primer to check for the orientation of the gene in the clone.

Expression in *E. coli* hosts occurs under the control of the *Bacillus* promoter and signal sequence. There is about 40 – 80 % periplasmic or intracellular protein expression. Although, *E. coli* has the extracellular transporting components, it will fail to secrete these cloned enzymes because of the difference in the signal peptides. The signal peptide is a stretch of hydrophobic amino acids that are known to integrate and span the cell membrane for extracellular transportation (Simonen & Palva, 1993; Tjalsma *et al.,* 2000).

The S-complex export protein machinery in *Bacillus* strains is different from that of *E. coli* hence secretion of cloned gene products from one species to another can be a problem. Extracellular expression is further complicated by the difference in amino acid sequences of signal sequence. The proteases involved in *E. coli* for cleaving signal sequences might not recognise the cleavage site on foreign products cloned in them. The lipoprotein signal sequence will then anchor the cloned proteins to the cell membrane giving rise to high cell bound *Bacillus* endoglucanase protein (Soole *et al.,* 1993).

Although much effort has been expended to overcome low enzyme yields from producer strains, the main problem in *E. coli* is in the extracellular expression. The expression and production of an active endoglucanase in different hosts implies that the chaperones in these hosts recognise these enzymes. Hence several homologous endoglucanase from *Bacillus* strains could be expressed in

several hosts. Some full structural genes have been shown to be toxic to *E. coli* because their products form aggregates/clumps inside the cell due to failure of the *E. coli* cells to secrete the expressed protein thereby causing cell death (Sanchez-Torres *et al.,* 1996; Kurland & Dong, 1996; Wicher *et al.,* 2001; Mierendorf *et al.,* 1994; McMurray *et al.,* 1998). The problem of overexpression is particularly observed when Gram-positive bacterial genes are cloned into Gram-negative hosts. However, using low copy number vectors that will express basal levels or using *E. coli* strains that can lower the high copy number plasmids can circumvent this problem. Generally, Gram-positive bacteria use ribosomal binding sequences that are close to the consensus sequence for efficient translation initiation (Farwell *et al.,* 1992; Isono & Isono, 1976).

Cloning of endoglucanases enzymes has realised overproduction in eukaryotic hosts. The other advantage in using eukaryotic hosts is posttranslational process of glycosylation that has led to improved thermostability and reduction in proteolysis (Langsford *et al.,* 1987; Olsen & Thomsen, 1991; Zhang *et al.,* 1997).

Though cloning of endoglucanase enzyme can enhance production levels, efficient hydrolysis of cellulolytic materials is improved by employing non-enzymatic and enzymatic hydrolysis methods. The non-enzymatic processes (physical or chemical) are responsible for conversion of much of the crystalline cellulose to amorphous form. Then, the synergy of cellulases enhances enzymatic hydrolysis of cellulose materials as was observed on alfalfa fibre after liquid hot water pretreatment (Sreenath *et al.,* 1999).

5.5.6 Site-directed mutagenesis of CelG to improve on thermostability

In an attempt to increase thermostability of the endoglucanase enzyme from *B. subtilis* CHZ1, an alignment of its protein sequence was performed with a homologous thermostable enzyme from *Thermotoga neopolitana* using the CLUSTLAW tool (Bok *et al.,* 1998). The mutation at position 1168 was confirmed by sequencing within the site after mutagenesis. Mutation at position 1168 resulted in expression of an inactive endoglucanase enzyme because assaying for the enzyme activity produced no reduction of DNS reagent.

CHAPTER 6

6 CONCLUSIONS

Thirteen isolates were isolated from the Chimanimani hot springs. Only one isolate was shown to produce endoglucanase enzyme. The microorganism isolated was identified to be a *Bacillus*

subtilis strain. Identification of this microbe was confirmed with the conversional classification identifying tools and 16S rRNA DNA amplification, sequencing and homology analysis from GenBank databases.

Optimal growth conditions of this *B. subtilis* CHZ1 strain, when cultured in shake flask experiments using M162 medium (Degryse *et al.,* 1978) are pH of 8.0 and temperature of 40ºC. The bacteria did not grow at pH values less than 4.0 and above 10. The optimal conditions for endoglucanase production are pH 6.0 and 50ºC. The endoglucanase activity optimal conditions are a pH of 6.0 and 70ºC. Batch cultivation for maximum enzyme production of endoglucanase enzyme by this *Bacillus* strain has to be performed at 50ºC but downstream recovery of the enzyme has to be performed after 5 h of culturing due to loss in enzyme activity. The fall in endoglucanase enzyme levels during cultivation could be due to proteolytic degradation. The proteolytic enzyme produced by the strain was characterised and the optimal conditions determined. The CHZ1 strain did not show any catabolite repression on endoglucanase production with cellobiose or glucose as carbon sources. Endoglucanase production was constitutive.

The developed food waste media were able to support growth of the *B. subtilis* CHZ1 strain with the production of hydrolytic enzymes. The media proved to be rich in nutrients and can support microbial growth and production of amylase, protease, polygalacturonase, xylanase and endoglucanase enzymes to equivalence levels compared to synthetic media. Above all, the developed CWW medium is quite inexpensive. A plethora of thermophilic enzymes with outstanding characteristics have not been translated into concrete applications because of the major restricting reason of the costs of the production process. Several studies have shown that the use of inexpensive media supplemented with various nitrogen sources can support microbial growth. The food wastes are so rich in nutrients such that there is a potential of producing secondary products although the end products might be compromised on the quality after using these undefined media.

The optimal pH for the protease enzyme production was 8.0. Highest enzyme levels were produced when M162 medium was supplemented with organic nitrogen sources compared to inorganic nitrogen sources. Although, the protease showed high activity towards the azocasein substrate, the enzyme was produced in small amounts. These are too low for any economical application hence the need to clone the enzymes' gene to overproduce the enzyme. The protease enzyme was observed to have high enzyme activity in the pH range 6.0 – 10.0 with maximum activity at 8.0. The protease has Mr value of 35 kDa. Optimum temperature for the activity was found to be 60-80ºC and the enzyme had considerable thermostability for 5.5 h retaining about 90 % activity. Metal ions that include Mg^{2+}, Fe^{2+} and Mn^{2+} increased the enzymes activity by 20 % meanwhile Zn^{2+}, Cu^{2+} and Ca^{2+} had no effect. PMSF a serine protease inhibitor completely

inhibited the activity of the enzyme. Inhibition of the protease enzyme by sulfhydryl agents that include iodoacetamide showed that there are tryptophan and or cysteine residues within the active site of the enzyme. The characteristics of the protease enzyme produced by the *B. subtilis* CHZ1 are consistent with those of the alkaline serine proteases. The enzyme has good properties for example optimal activity and stability in the alkaline range and thermostability hence the enzyme has potential applications in detergent industry.

Cloning of the endoglucanase gene of the *Bacillus subtilis* CHZ1 strain produced *E. coli* clones that were overexpressing the enzyme by 10 fold after 1.0 mM IPTG induction. The cloned enzyme is of the family-5A glycosyl hydrolases as confirmed by 98 % sequence homology with alkaline endoglucanases in that family using a BLASTN search tool. Also the SWISSPRO database alignment of the ORF of the gene further confirmed the structural features of family-5A glycosyl hydrolases. The enzyme was analysed and observed to have the seven regular conserved motifs of *Bacillus* alkaline endoglucanase enzymes. The conserved glutamate amino acid residues involved in catalysis of cellulose hydrolysis were identified at positions E178 and E195. There was a notable difference on the amino acid residues within the active site cleft at positions 182-184. This sequence is DVN in other endoglucanases, meanwhile it was replaced by ECD in CelG endoglucanase. Another major difference was at position 346 whereby CelG had R residue instead of K.

The cloned endoglucanase (CelG) enzyme properties were same as the wild protein, however about 70 % of the enzyme was cell bound. There was low extracellular expression with the clones. Recovery of endoglucanase enzyme from the cloned plasmids can be improved by cloning in vectors that can be transformed into developed *Bacillus* hosts that have extracellular transportation mechanisms compatible with the *celG* gene. The *Bacillus* hosts have been developed and they do not express proteolytic enzymes that will affect CelG levels at any stage of the growth phase. Cloning in *Bacillus* hosts can also possibly allow use of the optimised conditions in the wild producer strain because *Bacillus* microbes are quite adaptable and ubiquitous.

Site-directed mutagenesis showed that the mutation at point 1168 resulted in an inactive mutant. Hence this residue cannot be used for any mutation to improve thermostability of CelG enzyme. The inactivation of the enzyme might be due to improper folding of polypeptide chain as a result of the instability introduced by the mutation.

CHAPTER 7

7 FURTHER WORK

An in depth study of cellulase producing microbial strain diversity from Zimbabwean hot springs and other sources needs to be carried out. The work can be made cheaper with the use of the developed low-cost CWW medium. Further work needs to be done in culturing various microbes with the medium such that it can be used for bacteriological applications like enumeration of microbes and screening purposes.

Expression of the cloned endoglucanase enzyme in *E. coli* can further be improved by optimizing fermentation conditions. High enzyme activity has been reported by using fed-batch and a new type of microfiltration (MF) bioreactor (Schiraldi *et al.,* 2001). These bioprocesses have confirmed achievements of quite high biomass yield that has productivity ranging from 6 to 500 folds (Shiang *et al.,* 1991; Shene *et al.,* 2000). Since CelG production by *E. coli* without IPTG induction is biomass related the technique might improve on the enzyme production. An evaluation on cost-effectiveness on enzyme production with these culturing tools in comparing use of synthetic media and food waste medium can be an interesting aspect to study too. This will give an insight into the need to exploit these untapped reserves of foodstuff wastes to prepare low-cost media.

CHAPTER 8

8 REFERENCES

Aa, K., Flengsrud, R., Lindahl, V. and Tronsmo, A. 1994 Characterization of production and enzyme properties of an endo-β-1,4-glucanase from *Bacillus subtilis* CK-2 isolated from compost soil. *Antonie van Leewenhoek*, **66**, 319 –326.

Ahn, H. D., Kim, H. and Pack, M.-Y. 1993 Cleavage of *Bacillus subtilis* endo-β-1,4-glucanase by *B. megateruim* protease. *Biotechnology Letters*, **15**, 127 – 132.

Akhtar, M. S., Saleem, M., Malik, N. N. Akhtar, M. W. 1997 Cloning and expression of *Bacillus subtilis* endoglucanase genes in *E. coli*. *Journal of Biochemistry and Molecular Biology*, **30**, 27 – 32.

Alfredsson, G. A, Kristjansson, J. K., Hjorleifsdottir, S. and Stetter, K. O. 1988 *Rhodothermus marinus*, gen. Nov., sp. Nov., a Thermophilic, Halophilic Bacterium from submarine hot springs in Iceland. *Journal of General Microbiology*, **134**, 299 – 306.

Ali, S. M. and Akhand, A. A. 1992 Cellulase from *Trichoderma* isolate. *Journal of Basic Microbiology*, **32**, 259 – 268.

Angelides, K. J. and Fink, A. L. 1979a Mechanism of action of papain with a specific anilide substrate. *Biochemistry*, **18**, 2355 - 23662.

Angelides, K. J. and Fink, A. L. 1979b Mechanism of thiol proteases catalysis detection and stabilisation of a tetrahedral intermediate in papain catalysis. *Biochemistry*, **18**, 2363 - 2369.

Aono, R., Ito M., Joblin, K. N. and Horikoshi, K. 1995 A high cell wall negative charge is necessary for the growth of alkaliphilic *Bacillus lentus* C-125 at elevated pH. *Microbiology*, **141**, 2955 – 2964.

Armand, S., Drouillard, S., Schelin, M., Henrissat, B. and Drigeuz, H. 1997 A bifunctional fluorogenic tetrasaccharide as a substrate to study cellulases, *The Journal of Biologicval Chemistry*, **275**, 2709 – 2713.

Asawaka, M., Sadakata, Y., Araki, T., Sumi, T. and Hiroki, N. 1998 Purification and characterisation of the alkaline serine protease produced by *Bacillus* sp. strain from fish skin mucus. *Fisheries Science*, **64**, 793 – 797.

Ash, C., Farrow, F. A., Wallbanks, S. and Collins, M.D. 1991 Phylogenetic heterogeneity of the genus *Bacillus* revealed by comparative analysis small subunits ribosomal RNA sequences. *Letters in Applied Microbiology*, **13**, 202 – 206.

Aspinall, G.O. 1980 Chemistry of cell polysaccharides. In Preiss J. (Editor) *The Biochemistry of plants*. Academic Press. New York. 473 - 497.

Au, K. S. and Chan, K-Y. 1986 Carboxymethylcellulase production by a *Bacillus subtilis. Microbios*, **48**, 93 – 108.

Bacic, A, Harris, P. J. and Stone, B. A. 1988 Structure and function of plant cell walls. In Preiss J. (Editor) *The Biochemistry of plants.* Academic Press. New York. pg 297 - 358.

Bae, H. D., McAllister, T. A., Janke, J., Cheng, K. and Muir, A. D. 1993 Effects of condensed tannins on endoglucanase activity and filter paper digestion by *Fibrobacter succinogens* S85. *Applied and Environmental Microbiology*, **59**, 2132 – 2138.

Barns, S. M., Delwiche, C-F., Palmer, J. D. and Pace, N. R. 1996 Perspectives on archael diversity, thermophily and monophyly from environment rRNA sequences. *Proceedings of National Academy of Sciences*, **93**, 9188 – 9193.

Baik, H. B. and Park, M. Y. 1990 Expression of *Bacillus subtilis* endoglucanase gene in *Lactobacillus acidophilus*. *Biotechnology Letters*, **12**, 919 – 924.

Bailey, M. J. and Poutanen, K. 1989 Production of xylanolytic enzymes by strains of *Aspergillus. Applied Microbiology and Biotechnology*, **30**, 5 – 10.

Bailey, M. J. and Viikari, L. 1993 Production of xylanases by *Aspergillus fumigatus* and *Aspergillus oryzae* on xylan-based media. *World Journal of Microbiology and Biotechnology*, **9**, 80 – 84.

Baird, S. D., Herfford, M. A., Johnson, D. A., Sung, W. L., Yaguchi, M. and Seligy, V. L. 1990 The Glu residue in the conserved Asn-Glu-Pro sequence of two highly divergent endo-β-1,4-

glucanases is essential for enzyme activity. *Biochemical and Biophysical Research Communications,* **169**, 1035 – 1039.

Baird, S. D., Johnson, D. A. and Seligy, V. L. 1990 Molecular cloning, expression, and characterisation of endo-β-1,4-glucanase genes from *Bacillus polymyxa* and *Bacillus circulans*. *Journal of Bacteriology*, **172**, 1576 – 1588.

Baker, G. C., Gaffar, S., Cowan, D. A. and Suharto, A. R. 2001 Bacterial community of Indonesian hot springs. *FEMS Microbiology Letters*, **200**, 103 – 109.

Barns, S. M., Fundyga, R. E., Jeffries, M. W. and Pace, N. R. 1994 Remarkable archael diversity detected in a Yellowstone National Park hot spring environment. *Proceedings of the National Academy of sciences of the United States of America,* **91**: 1609 – 1613.

Béguin, P. 1990 Molecular biology of cellulose degradation. *Annual Review of Microbiology*, **44**, 219 – 248.

Belaich, J.-P., Tardif, C., Belaich, A. and Gaudin, C. 1997 The cellulolytic systems of *Clostridium cellulolyticum*. *Journal of Biotechnology*, **57**, 3 –14.

Belghith, H., Ellouz-Chaabouni, S. and Gargouri, A. 2001 Biostoning of denims by *Penicillum occitanis* (Pol6) cellulases. *Journal of Biotechnology*, **89**, 257 – 262.

Bertrand, J. L., Morosoli, R., Sharek, F. and Kluepfel, D. 1989 Expression of the xylanase gene of *Streptomyces lividians* and production of the enzyme on natural substrates. *Biotechnology and Bioengineering*, **33**, 791 - 799.

Beshay, V. and Moreira, A. 2001 Effect of medium composition on the production of alkaline protease by *Teredinobacter turnirae.* Presented at *Advances in Fermentation Technology Conference*, Baltimore, Maryland, USA.

Bhat, M. K. and Bhat, S. 1997 Cellulose degrading enzymes and their potential industrial applications. *Biotechnology Advances*, **15**, 583 – 620.

Bhosale, S. H., Rao, M. B., Deshpande, V. V. and Srinivasan, M. C. 1995 Thermostability of high activity alkaline proteases from *Conidiobolus coronatus*. *Enzyme Microbial Technology*, **17**, 136 – 139.

Bishop, D. G., Rutberg, L. and Samuelson, F. 1975 The chemical composition of the cytoplasmic membrane of *Bacillus subtilis*. *European Journal of Biochemistry*, **2**, 448 - 453.

Black, G. E., Snyder, A. P. and Heroux, K. S. 1997 Chemotaxonomic differentiation between the *Bacillus cereus* group and *Bacillus subtilis* by phospholipid extracts analyzed with electrospray ionization tandem mass spectrometry. *Journal of Microbiological Methods*, 28, 187 – 199.

Blanco, A., Diaz, P., Martinez, J., Vidal, T., Torres, A. L. and Pastor, F. I. J. 1998 Cloning of a new endoglucanase gene from *Bacillus* sp. BP-23 and characterisation of the enzyme: Performance in paper manufacture from cereal straw. *Applied Microbiology and Biotechnology*, 50, 48–54.

Boisset, C., Petrequin, C., Chanzy, H., Henrissat, B. and Sculein, M. 2001 Optimised mixtures of recombinant *Humicola insolens* cellulases for the biodegradation of crystalline cellulose. *Biotechnology Bioengineering*, **72**, 339 – 345.

Bomio, M, Sonneleitner, B. and Fiechter, A. 1989 Growth and biocatalytic activities of aerobic thermophilic populations in sewage sludge. *Applied Microbiology and Biotechnology*, **32**, 356 – 362.

Bok, J. D., Yernool, D. A. and Eveleigh, D. E. 1998 Purification, characterisation and molecular analysis of thermostable cellulases CelA and CelB from *Thermotoga neopolitana. Applied and Environmental Microbiology*, **63**, 4774 – 4781.

Brownell, J. E. and Nakas, J. P. 1991 Bioconversion of acid-hydrolysed poplar hemicellulose to acetic acid by *Clostridium thermoaceticum*. *Journal of Industrial Microbiology*, **7**, 1 – 6.

Cantwell, B. A. and McConnell, D. J. 1983 Molecular cloning and expression of a *Bacillus subtilis* β–glucanase gene in *Escherichia coli. Gene*, **23**, 211 – 219.

Cantwell, B., Brazil, G., Hurley, J. and McConnell, D. 1985 Expression of the gene for the endo-β–1,3-1,4-glucanase from *Bacillus subtilis* in *Saccharomyces cerevisiae*. In *Proceedings of the 20th European Brewing Convention Congress*, IRL Press Ltd., Oxford.. Pp 259 – 266.

Cavaco-Paulo, A., 1998 Mechanism of cellulase action in textile process. *Carbohydrate Polymers*, **37**, 273 – 277.

Cazemier, A. E., Verdoes, J. C., Op den Camp, J. H. M., Hackstein, J. H. P. and van Ooyen, A. J. J. 1999 A β-1,4-endoglucanase encoding gene *from Cellulomonas pachnodae*. *Applied Microbiology and Biotechnology*, **52**, 232 – 239.

Chan, K. and Au, K. 1987 Studies on cellulase production by a *Bacillus subtilis*. *International Journal of General and Molecular Biology*, **53**, 125 – 136.

Chaudhuri, B. K. and Sahai, V. 1993 Production of cellulase enzyme from lactose batch and continuous cultures by a partially constitutive strain of *Trichoderma reesei*. *Enzyme Microbiology and Technology,* **15**, 513 – 518.

Chen, S. and Wayman, M. 1992 Novel inducers derived from starch for cellulase production by *Trichoderma reesei*. *Process Biochemistry*, **27**, 327 – 334.

Chen, H., Li, X. L., Dhum, D. L. and Ljungdahl, L. G. 1998 Two genes of anaerobic fungus *Orpinomyces* sp. Strain PC-2 encoding cellulases with endoglucanase activities may have arisen by gene duplication. *FEMS Microbiology Letters*, **159**, 63 – 68.

Cheung, S. W. and Anderson, B. C. 1997 Laboratory investigations of ethanol production from municipal primary wastewater. *Bioresource Technology*, **59** 81 – 96.

Christovkopoulos, P., Hatzinikolaou, D. G., Fountoukidis, G., Kekos, D., Cleyssens M. and Macris, B. J. 1999 Purification and mode of action of an alkali-resistant endo-β-1,4-glucanase from *Bacillus pumilus*. *Archives of Biochemistry and Biophysics*, **364**, 61 – 66.

Chun, S., Kim, Y., Chung, Y., Kim, K., and Sung, N. 1995 High–level expression of *Pseudomonas* sp. LBC505 endoglucanase gene in *Escherichia coli. Journal of Microbiology and Biotechnology*, **5**, 14 – 17.

Claus, D. and Berkeley, R. C. W. 1986 Genus *Bacillus*. In Sneath, P. H. A (Chief-Editor) *Bergey's Manual of Systematic Bacteriology*. Williams and Wilkins co., Baltimore, USA. 1105 – 1139.

Colvin, J. R. 1980 The biosynthesis of cellulose. In Preiss J. (Editor) *The Biochemistry of plants.* Academic Press. New York. 544 - 567.

Coutinho, J. B., Gilkes, N. R., Kilburn, D. G., Warren, R. A. J. and Miller, R. C. Jr. 1993. The nature of the cellulose-binding domains affects the activities of a bacterial endoglucanase on different forms of cellulose. *Federation of European Microbiological Societies,* **113**, 211 – 218.

Coutinho, P. M. and Henrissat, B. 1999 The modular structure of cellulases and other carbohydrate-active enzymes: an integrated database approach. In *Genetics, Biochemistry and Ecology of Cellulose Degradation,* K. Ohmiya, K.Hayashi, K. Sakka, Y. Kobayashi, S. Karita & T. Kimura, (Editors), Uni Publishers Co., Tokyo, 15-23.

Cowan, T. S. and Steel, K. K. 1993 Manual for the Identification of Medical Bacteria. Barrow G. I. and Feltham R. K. A. (Editors). Cambridge University Press. Cambridge. UK.

Creighton, E. T. 1993 Biosynthesis of Proteins. In *Proteins: Structures and Molecular Propertie*s. W. H. Freeman and Company. New York. 49 - 104.

Czjzek, M., Darbon, H., Mosbah, A., Receveur, V., Roigzamboni, V. and Henrissat, B. 2001 Structure, mechanism and function of glycoside hydrolases. *AFMB Activity Report*, 47 – 52.

D'Elia, J. and Chesbro, W. 1992 Maintenance energy demand affects biomass synthesis but not cellulase production by a mesophilic *Clostridium. Journal of Industrial Microbiology*, **10**, 123 – 133.

Daffonchio, D., Borin, S., Consolandi, A., Mora, D., Manachini, P. L. and Sorlini, C. 1998 16S-23S rRNA internal transcribed spacers as molecular markers for the species of the 16S rRNA group 1 of the genus *Bacillus*. *FEMS Microbiology Letters*, **163**, 229 – 236.

Daffonchio, D., Borin, S., Frova, G., Manachini, P. L. and Sorlini, C. 1998 PCR fingerprinting of whole genomes: the spacers between the 16S and 23S rRNA genes and of intergenic tRNA regions reveal a different intraspecific genomic variability of *Bacillus cereus* and *Bacillus licherniformis*. *International Journal of Systematic Bacteriology*, **48**, 107 – 116.

Dai, Z. Y., Hooker, B. S., Anderson, D. B. and Thomas, S. R. 2000 Expression of *Acidothermus cellulolyticus* endoglucanase (E1) in transgenic tobacco: biochemical characteristics and physiological effects. *Transgenic Research*, **9**, 43 – 54.

Davies, G. J., Dauter, M., Brzozcvski, A. M., Bjornvad, M. E., Andersen, K. V. and Schulein, M. 1998 Structure of the *Bacillus agaradherens* Family 5 endoglucanase at 1.6 Å and its cellobiose complex at 2.0 Å resolution. *Biochemistry*, **37**, 1926 – 1932.

Davies, G. and Henrissat, B. 1995 Structures and mechanisms of glycosyl hydrolases. *Structure*, **3**, 853 – 859.

de Mendoza, D., Grau, R. and Cronan, J. E. (Jnr) 1993 Biosynthesis and function of membrane lipids. In Sonenshein L.A, Hoch J.A. and Losick R. (Editors) *Bacillus subtilis and other Gram positive bacteria*. American Society for Microbiology. Washington DC. 411 - 420.

Degryse, E., Glandsdorff, N. and Pierard, A. 1978 A comparative analysis of extreme thermophilic bacteria belonging to the genus *Thermus*. *Archives of Microbiology,* **117**, 189 – 196.

deHaseth, L. P., Zupancic, M. L. and Record, T. (Jnr) 1998 RNA Polymerase-Promoter interactions: The comings and goings of RNA Polymerase. *Journal of Bacteriology*, *180*, 3019–3025.

Dekker, R. F. H. and Richards, G. N. 1976 Hemicellulases: their occurrence, purification, properties, and mode of action. *Advances in Carbohydrate Chemistry and Biochemistry*, **32**, 277 – 352.

Dhillon, N., Chabber, S., Saxena, M., Pajni, S. and Vadehra, D. V. 1985 A constitutive endoglucanase (CMCase) from *Bacillus licherniformis*-1. *Bacteriology Letters.* **7**, 695 – 697.

Din, N., Damude, H. G., Gilkes, N. R., Miller, R. C. Jr., Warren, R. A. J. and Kilburn, D. G. 1994 C□-Cx revisited: Intramolecular synergism in a cellulase. *Proceedings of the National Academy of sciences of the United States of America*, **91**, 11383 – 11387.

Doi, R. H., Goldstein, M., Hashida, S., Park, J. S. and Takagi, M. 1994 The *Clostridium cellulovorans* cellulosome. *Critical Reviews in Microbiology*, **20**, 87 – 93.

Doran, J. B. and Ingram, L. O. 1993 Fermentation of crystalline cellulose to ethanol by *Klebsiella oxytoca* containing chromosomally integrated by *Zymomonas mobilis* genes. *Biotechnolog***y**, **9**, 533 – 538.

Driks, A. 1999 *Bacillus subtilis* spore coat. *Microbiology and Molecular Biology Reviews*, **63**, 1 – 20.

Dunning, J. W. and Lathrop, E. C. 1945 The saccharification of agricultural residues: a continuous process. *Industrial and Engineering Chemistry*, **37**, 24 - 29.

Durham, D. R., Stewart, D. B. and Stellwag, R. 1987 Novel alkaline and heat-stable serine proteases from alkalophilic *Bacillus* sp. Strain GX6638. *Journal of Bacteriology*, **169**, 2762 – 2768.

Ederibigbe, E. Y. and Odunfa, S. A. 1988 Purification and characterisation of extracellular proteinases excreted by a strain of *Bacillus subtilis* BS2, isolated from fermented African locust bean '*iru*'. *Journal of Applied Bacteriology,* **65**, 361 – 369.

Endo, K., Hakamada, Y., Takizawa, S., Kubota, H., Sumitomo, N, Kobayachi, T. and Ito, S. 2001 A novel alkaline endoglucanase from an alkaliphilic *Bacillus* isolate: enzymatic properties and nucleotide and deduced amino acid sequences. *Applied Microbiology and Biotechnology*

Ellouz, Y., Bayoudh, A., Kammoun, S., Gharsallah, N. and Nasri, M. 2001 Productiopn of protease by *Bacillus subtilis* grown on sardinelle heads and viscera flour. *Bioresource Technology*, **80**, 49 – 51.

Fanutti, C., Ponyi, T., Black, G-W., Hazelwood, G. P. and Gilbert, H. J. 1995 The conserved non-catalytic 40-residue sequence in cellulases and hemicellulases from anaerobic fungi functions as a protein docking domain. *Journal of Biological Chemistry*, **270**, 20314 – 29322.

Farid, M. A., Shaker, H. M. and El-Refai, A-M. H. 1984 Productivity *of Trichoderma viridie* 253 cellulase in relation to the composition of the fermentation medium. *Food Chemistry Microbiology Technology*, **8**, 161 – 163.

Farwell, M. A., Roberts, M. W. and Raninowtitz, J. C. 1992 The effect of ribosomal protein S1 from *Escherichia coli* and *Micrococcus luteus* on protein synthesis in vitro by *E. coli* and *Bacillus subtilis*. *Molecular Microbiology*, **6**: 3375 – 3383.

Ferrero, M. A., Castro, G. R., Abate, C. M., Baigorí, M. D. and Siñeriz, F. 1996 Thermostable alkaline proteases of *Bacillus licherniformis* MIR 29: isolation, production and characterization. *Applied Microbiology and Biotechnology*, **45**, 327 – 332.

Fukumori, F., Kudo, T. and Horikoshi, K. 1985 Purification and properties of a cellulase from an alkalophilic *Bacillus* sp. No. 1139. *Journal of General Microbiology*, **131**, 3339 – 3345.

Fujuwara, N. and Yamamoto, K. 1987 Production of alkaline protease in low cost medium by alkalophilic *Bacillus* sp. and properties of the enzyme. *Journal of Fermentaion Technology*, **65**, 345 – 348.

Galante, Y. M., De Conti, A. and Monteverdi, R. 1998 Application of *Trichoderma* enzymes in the textile industry. P 311 – 325. In Harman, G. E., Kubicek, C. P. (eds) *Trichoderma* and *Gliocladium* vol 2. London. Tylor & Francis.

Gardener, K. H. and Blackwell, J. 1974 The structure of Native cellulose. *Biopolymers*, **13**, 1975 - 2001.

Gebler, J., Gilkes, N. R., Claeyssens, M., Willson, D. B., Béguin, P., Wakarchuk, W. W., Kilburn, D. G., Miller, R. C. Jnr., Warren, R. A. J. and Withers, S. G. 1992 Stereoselective hydrolysis catalysed by related β-1,4-gluanases and β-1,4-xylanases. *Journal of Biological Chemistry,* **267**, 12559 – 12561.

Gerhardt, P., Murray, R. G. E., Wood, W. A. and Krieg, N. R. 1994 Physicochemical factors in growth. In *Methods for General and Molecular Bacteriology.* American Society for Microbiology. Washington DC. 137 – 154.

Ghanghas, S. G. and Wilson, D. B. 1987 Expression of a *Thermonospora fusca* cellulase gene in *Streptomyces lividians* and *Bacillus subtilis*. *Applied and Environmental Microbiology*, **53**, 1470 – 1475.

Ghose, T. K. 1987 Measurement of cellulase activities. *Pure and Applied Chemistry*, **59,** 257–268.

Gilbert, H. J., Jenkins, G., Sullivan, D. A. and Hall, J. 1987 Evidence of multiple carboxymethylcellulase genes in *Pseudomonas fluorescens* subsp. *cellulosa*. *Molecular General Genetics*, **210**, 551 – 556.

Gilkes, N. R., Henrissat, B., Kilburn, D. G., Miller, R. C. Jnr and Warren, R. A. J. 1991 Domains in microbial β-1,4-endoglucanase: sequence conservation, function and enzyme families. *Microbiological Reviews,* **55**, 303 – 315.

Gilkes, N. R., Kilburn, G. J., Miller, R. C. and Warren, R. A. J. 1991 Bacterial cellulases. *Bioresource Technology*, **36**, 21 – 35.

Gill, D. M. 1982 Bacterial Toxins: A table of lethal amounts. *Microbiological Reviews* **46**: 86 – 94.

Giraud, E., Brauman, A., Keleke, S., Lelong, B. and Raimbault, M. 1991 Isolation and physiological study of an amylolytic strain of *Lactobacillus plantarum. Applied Microbiology and Biotechnology*, **36**, 379 – 383.

Gong, C. S., Cao, N. J., Du, J. and tsao, G. T. 1999 Ethanol production from renewable rsources. In Scheper, T. and Tsao, G. T. (Editors) *Advances in Biochemical Engineering/Biotechnology*. Springer-Verlage, Berlin. pp 207 – 239.

Gormley, E. P., Cantwell, B. A., Barker, P. J., Gilmour, R. S. and McConnell, D. J.1988 Secretion and processing of the *Bacillus subtilis* endo-β-glucanase in *Escherichia coli*. *Molecular Microbiology*, **2**, 813–819.

Green, R. S. 1985 Characterisation of certain proteinases isoenzymes produced by benign and virulent strains of *Bacteriodes nodosus*. *Journal of General Microbiology*, **24**, 2871 – 2876.

Griffin, H. L., Greene, R. V. and Cotta, M. A. 1992 Isolation and characterisation of an alkaline protease from the marine shipworm bacterium. *Current Microbiology*, **24**, 111 – 117.

Gutierrez-Correa, M. and Tengerdy, R. P. 1997 Production of cellulase on sugarcane bagasse by fungal mixed culture solid substrate fermentation. *Biotechnology Letters*, **19**, 665 – 667.

Gusek, T-W. and Kinsella, J. E. 1988 Properties and potential applications of a Unique heat-stable protease. *Food Technology*, **42**, 102 – 107.

Gusek, T-W. and Kinsella, J. E. 1987 Purification and characterisation of the heat-stable serine protease from *Thermonospora fusca* YX. *Biochemical Journal*, **246**, 511 – 516.

Haggkvist, M., Li, T. and Odberg, L.1998 Effects of drying and pressing on the pore structure in the cellulose fibre wall studied by 1H and 2H NMR relaxation. *Cellulos*e, **5**, 33 – 49.

Hahn, M., Olsen, O., Politz, O., Borriss, R. and Heinemann, U. 1995 Crystal structure and site-directed mutagenesis of *Bacillus macerans* endo-β-1,3-1,4-glucanase. *The Journal of Biological Chemistry*, **270,** 3081 – 3088.

Hakamada, Y., Hatada, Y., Ozawa, T., Ozaki, K., Kobayashi, T. and Ito, S., 2001, Identification of thermostabilizing residues in a *Bacillus* alkaline cellulase by construction of chimeras from mesophilic and thermostable enzymes and site-directed mutagenesis. *FEMS Microbiology Letters,* **195**, 67 – 72.

Haldenwang, G. W., 1995 The sigma factors of *Bacillus subtilis*. *Microbiological Reviews*, **55**, 1 – 30.

Haltrich, D. and Steiner, W. 1994 Formation of xylanase by *Schizophyllum commune*: effect of medium components. *Enzyme Microbial Technology*, **16**, 229 – 235.

Han, S. J., Yoo, Y. J. and Kang, H. S. 1995 Characterisation of a bifunctional cellulase and its structural gene: The cel gene of *Bacillus* sp. D04 has exo- and endoglucanase activity. *Journal of Biological Chemistry*, **270**, 26012 – 26019.

Han, Y. W. and Callihan, C. D. 1974 Cellulose Fermentation: Effect of substrate pretreatment on Microbial growth. *Applied Microbiology*, **27**, 159 - 165.

Handelsman, J., Raffel, S., Mester, E. H. and Raffel, S. 1990 Biological control of damping off of alfalfa seedlings with *Bacillus cereus* UW85. *Applied and Environmental Microbiology*, **57**, 2767 – 2770.

Hansen, B. M. and Hendriksen, B. N. 2001 Detection of enterotoxic *Bacillus cereus* and *Bacillus thuringiensis* strains by PCR analysis. *Applied and Environmental Microbiology*, **67**, 185 – 189.

Hansen-Sonne, J., Mathrani, M. and Ahring, B. K. 1993 Xylanolytic anaerobic thermophilic from Icelandic Hot Springs. *Applied Microbiology and Biotechnology*, **38**, 537 – 541.

Harmova, M., Biely, P. and Vrsanska, M. 1986 Specificity of cellulase and β–xylanase induction in *Trichoderma reesei* QM 9414. *Archives of Microbiology*, **144**, 307 – 311.

Heinken, F. G. and O'Connor, R. J. 1972 Continuous culture studies on the biosynthesis of alkaline protease, neutral protease and amylase by *Bacillus subtilis* NRRL-B3411, *Journal of General Microbiology*, **73**, 35 – 44.

Hemila, H., Glode, L. M. and Palva, I. 1989 Production of diphtheria toxin CRM228 in *Bacillus subtilis*. *FEMS Letters*, **65**: 193 – 198.

Hergyi, H. and Gerstein, M. 1999 The relationship between protein structure and function: a comprehensive survey with application to the yeast genome. *Journal of Molecular Biology*, **288**, 147 – 164.

Henrissat, B. and Davies, G. 2000 Glycoside hydrolases and glycosyltransferases. Families, modules and Implications for Genomics. *Plant Physiology*, **124**, 1515 – 1519.

Henrissat, B. and Bairoch, A. 1993 New families in the classification of glycosyl hydrolases based on amino acid sequence similarity. *Biochemistry Journal*, **293**, 781 – 788.

Henrissat, B., Claeyssens, M., Tomme, P., Lemesle, L. and Mornon, J-P. 1989 Cellulase families revealed by hydrophobic cluster analysis. *Gene*, **81**, 83 – 89.

Henrissat, B. and Davies, G. 1997 Structural and sequence-based classification of glycoside hydrolases. *Current Reviews on Structural Biology,* **7**, 637 – 644.

Henrissat, B., Teeri, T. T. and Warren, R. A. J. 1998 A scheme for designating enzymes that hydrolyze the polysaccharides of the plant cell walls of plants. *FEBS Letters,* **425,** 352 – 354.

Her, S., Lee, H.-S., Choi, S.-J., Choi, S.-W., Choi, H.-J., Yoon, S.-S. and Oh, D.-H 1999 Cloning and sequencing of β-1,4-endoglucanase gene (*celA*) from *Pseudomonas* sp. YD-15. *Letters in Applied Microbiology*, **29**, 389 – 395.

Herna'ndez, A and Marin, M. 2002 New turbidimetric method for estimating bacterial growth in heterogenous media. *Protein Biochemistry*, **37**, 1125 – 1128.

Hinchliffe, E., Edward, B. and Wendy, G. 1984 Expression of the cloned endo-β-1,4-glucanase gene of *Bacillus subtilis* in *Saccharomyces cerevisiae*. *Current Genetics*, **8**, 471 – 475.

Hirose, I., Sano, K., Shioda, I., Kumano, M., Nakamura, K. and Yamane, K. 2000 Proteome analysis of *Bacillus subtilis* extracellular proteins: a two dimensional protein electrophoretic study. *Microbiology,* **146**: 65 – 75.

Hjorleifsdottir, S., Skirnisdottir, S., Hreggvidsson G. O., Holst, O. and Kristjansson, J. K. 2001 Species composition of cultivated and noncultivated bacteria from short filaments in an Icelandic hot spring at 88 °C. *Microbial Ecology*, **42**: 117 – 125.

Ho, N. W. Y., Chen, Z., Brainard, A. P. and Sedlank, M. 1999 Successful design and development of genetically engineered Saccharomyces yeasts for effective cofermentaion of glucose and xylaose from cellulosic biomass to fuel ethanol. In Scheper, T. and Tsao, G. T. (Editors) *Advances in Biochemical Engineering/Biotechnology*. Springer-Verlage, Berlin. pp 163 – 192.

Hohn, L. and Saunder, C. 1994 Searching protein structure databases has come of age. *Protein*, **19**, 165 – 173.

Holtz, C., Kaspari, H. and Klemme, J.-H. 1991 Production and properties of xylanases from thermophilic actinomycetes. *Antonie van Leewenhoek*, **59**, 1 – 7.

Holtzapple, M. T. and Humphrey, A. E. 1984 The effect of organoslov pretreatment on the enzymatic hydrolysis of poplar. *Biotechnology and Bioengineering*, **26**, 670 – 676.

Holtzapple, M. T., Jun, J. H., Ashok, G., Patibandla, S. R. and Dale, B. E. 1991 The ammonia freeze explosion (AFEX) process: a practical ligninocellulose pretreatment. *Applied Biochemistry and Biotechnology*, **29**, 59 – 74.

Horani, H. K. and Priest, F. G. 1994 Thermotolerant varieties of *Bacillus licherniformis* isolated from desert environments. *Journal of Applied Bacteriology*, **77**, 392 – 400.

Horikoshi, K. 1972 Production of alkaline enzymes by alkalophilic microorgnisms, Part III. Alkaline Pectinase of *Bacillus* No. P-4-N, *Agricultural Biology and Chemistry*, **36**, 285 – 293.

Horikoshi, K. 1999 Alkalophilic: Some applications, their products for Biotechnology. *Microbiology and Molecular Biology Reviews*, **63**, 735 – 750.

Horikoshi, K. and Akiba, T. 1982 Alkalophilic microorganisms, a new microbial world. Japan Scientific Societies Press and Springer-Verlag. Berlin. Heidelberg. 93 – 172.

Horikoshi, K. and Akiba, T. 1982 Enzymes of alkalophilic bacteria. In *Alkalophilic Microorganisms*. Springer-Verlag. New York. 93 – 101.

Hotten, P. M., Jones, K. L. and Grainger, J. M. 1983 The application of a mathematical model to an Appraisal of the Cellulose-Azure method for determining cellulase activity. *Applied Microbiology and Biotechnology*, **18**, 344 – 349.

Hugenholtz, P., Pitulle, C., Hershberger, K. L. and Pace, N. R. 1998a Novel division level bacterial diversity in a Yellowstone hot spring. *Journal of Bacteriology*, 180: 366 – 376.

Hugenholtz, P., Goebel, B-M. and Pace, N. R. 1998b Impact of culture-independent studies on the emerging phyolgenetic view of bacterial diversity. *Journal of Bacteriology*, **180**, 4765 – 4774.

Igarashi, K., Hatada, Y., Ikawa, K., Araki, H., Ozawa, T., Kobayashi, T., Ozaki, K. and Ito, S. 1998 Improved thermostability of a *Bacillus* α–amylase by deletion of an arginine-glycine residue is caused by enhanced calcium binding. *Biochemical and Biophysical Research Communications*, **248**, 372 – 377.

Ihde, D. C. and Armstrong, D. 1973 Clinical spectrum of infection due to *Bacillus* species. *American Journal of Medicine*, **55**: 839 – 845.

Ikemura, H. and Inouye, M. 1988 In vitro processing of pro-subtilisin produced in *Escherichia coli*. *Journal of Biological Chemistry*, **263**, 12959 – 12963.

Isono, K. and Isono, S. 1976 Lack of ribosomal protein S1 in *Bacillus stearothermophilus. Proceedings of National Academy in Sciences in United States of America*, **73**: 767 – 770.

Ito, S. 1997 Alkaline cellulases from alkaliphilic *Bacillus*: Enzymatic properties, genetics, and application to detergents. *Extremophiles*, **1**, 61 – 66.

Ito, S., Kobayashi, T., Ara, K., Ozaki, K., Kawai, S. and Hatada, Y. 1998 Alkaline detergent enzymes from alkaliphiles: enzymatic properties genetics, and structures. *Extremophiles*, **2**, 185 – 190.

Jan, J., Valle, F., Bolivar, F. and Merino, E. 2000 Characterisation of the 5´ subtilis in (aprE) regulatory region from *Bacillus subtilis*. *FEMS Microbiology Letters*, **183**, 9 – 14.

Jefferys, A. J., Wilson, V. and Thein, S. L. 1985 Hypervariable "minisatellite" regions in human DNA. *Nature* **314**: 67-73.

Jeng, S. R., Svircev, A. M., Myers, A. L., Beliaeva, L., Hunter, D. M. and Hubbes, M. 2001 The use of 16S and 16S-23S rRNA to easily detect and differentiate common Gram-negative orchard epiphytes. *Journal of Microbiological Methods*, **44**, 69 – 77.

Jørgensen, P. L., Tangney, M., Pedersen, P. E., Hastrup, Sdiderichsen, B and Jørgensen, S. T. 2000 Cloning and sequencing of an alkaline protease gene from *Bacillus lentus* and amplification of the gene on the *B. lentus* chromosome bby an improved technique. *Applied and Environmental Microbiology*, **66**, 825 – 827.

Johnson, W. C., Moran, P. C. and Losick, R. 1983 Two RNA polymerase sigma factors from *Bacillus subtilis* discriminate between overlapping promoters for a developmentally regulated gene. *Nature*, **302**, 800 – 803.

Joseph, P., Fantino, J. R., Herbaud, M.-L. and Denizot, F. 2001 Rapid orientated cloning in a shuttle vector allowing modulated gene expression in *Bacillus subtilis. FEMS Microbiology Letters***, 205**, 91 – 97.

Julian, G. St., Bothast, R. J. and Krull, H.L. 1990 Glycerol accumulation while recycling thin sillage in corn fermentation to ethanol. *Journal of Industrial Microbiology*, **5**, 391 – 394.

Jung, K. H., Chun, Y. C., Lee, J. C., Kim, J. H. and Yoon, K. H. 1996 Cloning and expression of a *Bacillus* sp. 79-23 cellulase gene. *Biotechnology Letters*, **18**, 1077–1082.

Kai, Y.1991 Chemistry of extractives. In Hon D. N. S. and Shirashi N. (Editors) *Wood and cellulosic chemistry*. Marcel Dekker Inc. New York. 215 – 255.

Kalia, A., Rattan, A. and Chopra, P. 1999 A method for extraction of high-quality and high-quality genomic DNA generally applicable to pathogenic bacteria. *Analytical Biochemistry*, **275**, 1 – 5.

Kaneda, T. 1991 Iso- and anteiso-fatty acids in bacteria: biosynthesis, function and taxonomic significance. *Microbiological Reviews*, **55**, 288 - 302.

Kaneda, T. 1997 Fatty acids in the genus *Bacillus*: an example of branched-chain preference. *Bacteriology Review*, **41**, 391 – 418.

Karita. S., Sakka, K. and Ohmiya, O. 1997 Cellulosomes, cellulases complexes of anaerobic microbes: Their structure models and functions. *In*: Onodera, R., Habashi, H., Ushida, K., Yano, H. and Sasaki, Y. (Editors), *Rumen Microbes and digestive Physiology in Ruminants*. Japanese Scientific Society Press and S. Karger, Tokyo/Basle, 14 edition, pp 47 – 57.

Kates, M. 1993 Membrane lipids of archae. In *The Biochemistry of Archae (archaebacteria)*. Elsevier. Amsterdam. 261 – 295.

Kawaminami, S., Takahashi, H., Ito, S., Arata, Y. and Shimada, I. 1998 A multinuclear NMR study of active site of an endo-β–1,4-glucanases from a strain of *Bacillus*. *The Journal of Biological Chemistry*, **274**, 19823 – 19828.

Keay, L., Moseley, M. H., Anderson, R. G., O' Connor, R. J. and Wildi, B. S. 1972 Production and isolation of microbial proteases. *Biotechnology and Bioengineering Symposium*, **3**, 63 – 92.

Keay, L., Moser, P. W. and Wildi, B. S. 1970 Proteases of the genus *Bacillus* II: Alkaline proteases. *Biotechnology and Bioengineering*, **12**, 213 – 249.

Kennedy, M. J., Thakur, M. S., Wang, D-I. C. and Stephananopoulos, G. N. 1992 Estimating cell concentration in the presence of suspended solids: a light scatter technique. *Biotechnology and Bioenegineering*, **40**, 875 – 887.

Khanongnuch, C., Ooi, T. and Kinoshita, S. 1999 Cloning and nucleotide sequence of β-mannanase and cellulase genes from *Bacillus* sp. 5H. *World Journal of Microbiology and Biotechnology*, **15**, 249-258.

Kim, J. M., Lim, W. J. and Suh, H. J. 2001 Feather-degrading *Bacillus* species from poultry waste. *Process Biochemistry*, **37**, 87 – 291.

Kim, H. J. and Pack, M. Y. 1993 Overproduction of extracellular endoglucanase by generating engineered *Bacillus subtilis*. *Biotechnology Letters*, **15**, 138 – 138.

Kim, H. J., Kim, S. F., Ahn, D. H., Lee, J. H. and Pack, M. Y. 1995 Internal cleavage of *Bacillus subtilis* BSE616 endoglucanase expressed in *E. coli*. *Journal of Microbiology and Biotechnology*, **5**, 26 – 30.

Kim, H. J., Lee, B. and Pack, M. Y. 1998 Overproduction and secretion of β-glucosidase in *Bacillus subtilis*. *Journal of Microbiology and Biotechnology*, **8**, 141 – 145.

Kim, H., Goto, M., Jeong, H. J., Jung, K. H., Kwon, J. and Furukawa, K. 1998 Functional analysis of a hybrid endoglucanase of bacterial origin having a cellulose binding domain of a fungal exolucanase. *Applied Microbiology and Biotechnology*, **75**, 193 – 204.

Kim, Y-R., Czajka, J. and Batt, A. C. 2000 Develppment of a fluorogenic Probe-based PCR assay for detection of *Bacillus cereus* in non-fat dry milk. *Applied and Environmental Microbiology*, **66**, 1453 – 1459.

Kleine, R., Rothe, U., Kettmann, U. and Schelle, H, 1980, Purification, properties and application of thermitase; a microbial protease. Presented at *Proteinases: Their Inhibitors International Symposium*, Mladinska Knjiga, Ljubljana, Yugoslavia.

Kloepper, J. W., Lifshitz, R. and Zablotocz, R. M. 1989 Free-living bacterial inocula for enhancing crop productivity. *Trends in Biotechnology*, **7**, 39 – 44.

Kluepfel, D. 1988 Screening of prokaryotes for cellulose and hemicellulose-degrading enzymes. In Wood W.A and Kellogg S.T. (Editors) *Methods in Enzymology*. Academic Press. New York. 180 – 186.

Kobayashi, T., Hakamada, Y., Hitomi, J., Koike, K. and Ito, S. 1996 Purification of alkaline proteases from a *Bacillus* strain and their possible interrelationship. *Applied Microbiology and Biotechnology*, **45**, 63 – 71.

Kobayashi, T., Higaki, N., Suzumatsu, A., Sawada, K., Hagihara, H., Kawai, S. and Ito, S. 2001 Purification and properties of a high molecular weight, alkaline exopolygalacturonase from a strain of *Bacillus. Enzyme and Microbial Technology*, **29**, 70 – 75.

Kole, M. M., Draper, I. and Gerson, F. D. 1988 Protease production by *Bacillus subtilis* in oxygen controlled, glucose feed batch. *Applied Microbiology and Biotechnology*. **28**, 404 – 408.

Kormos, J., Johnson, P. E., Brun, E., Tomme, P., McIntosh, L. P., Haynes, C. A. and Kilburn, D. G. 2000 Binding site analysis of cellulose binding domain CBD_{N1} from endoglucanase C of *Cellulomonas fimi* by site-directed mutagenesis. *Biochemistry*, **39**, 8844 – 8852.

Kraut, J.1977 Serine proteases: structure and mechanism of catalysis. *Annual Review of Biochemistry*, **46**, 331 – 358.

Kristjansson, J. K. and Hreggvidsson, G. O. 1995 Ecology and habitats of extremophiles. *World Journal of Microbiology and Biotechnology*, **11**, 17 - 25.

Kristjansson, J. K., Hreggvidsson, G. O. and Grant, W. D. 2000 Taxonomy of extremophiles. In Priest F.G. and Goodfellow M. (Editors) *Applied Microbial systematic*. Kluwer Academic Publishers. Dordrecht. The Netherlands. 231 - 291.

Krulwich, T. A. and Guffanti, A. A. 1989 Alkalophilic bacteria. *Annual Review of Microbiology*, **43**, 435 – 436.

Kubicek, C. P., Eveleigh, D. E., Esterbauer, H., Steiner, W. and Kubicek-Franz, E. M. 1991 Action of purified *Trichoderma reesei* cellulases on cotton fibers and yarn. *Journal of Biotechnology,* **89**, 247 – 255.

Kubicek, C. P. and Pentilla, M. E. 1998 Regulation of production of plant polysaccharide degrading enzymes by *Trichoderma*. *In* Harman, G. E. and Kubicek, C. P. (Editors). *Trichoderma nd Gliodadium, Enzymes, Biological control and Commercial Applications*, vol 2, Tylor and Francis, London, pp 49 – 72.

Kuhad, R. C., Singh, A and Eriksson, K.-E. L. 1997 Microorganisms and enzymes involved in the degradation of plant fibre cell walls. In Scheper, T. and Eriksson, K.-E. L. (Editors) *Advances in Biochemical Engineering/Biotechnology*. Springer-Verlag, Berlin. pp. 45 – 125.

Kulkarni, N., Shendye, A. and Rao, M. 1999 Molecular and biotechnological aspects of xylanase. *FEMS Microbiology Reviews*, **23**, 411 – 456.

Kunst, F. and Rapoport, G. 1995 Salt stress is an environmental signal affecting degradative enzyme synthesis in *Bacillus subtilis*. *Journal of Bacteriology*, **177**, 2403 – 2407.

Kumar, P. K. and Schügerl, K. 1990 Immobilisation of genetically engineered cells: a new strategy for higher stability. *Journal of Biotechnology*, **14**, 255 – 272.

Kurland, C. G. and Dong, H.1996 Bacterial growth inhibition by overproduction of protein. *Molecular Microbiology*, **21**, 1-4.

Langan, P., Nishiyama, Y. and Chanzy, H. 1999 The crystal structure and hydrogen bonding system in cellulose from neutron fibre diffraction data. *Fibre Diffraction Review*, **8**, 42 – 47.

Langsford, M. L., Gilkes, N. R., Singh, B., Moser, B., Miller, R. C., Warren, R. A., Kilburn, D. G. 1987. Glycosylation of bacterial cellulases prevents proteolytic cleavage between functional domains. *FEBS Letters*, **225**, 163 – 167.

Langworthy, T. A. 1978 Membrane and lipids of extremely thermoacidophilic microorganisms In Friedman S. M. (Editor) *Biochemistry of Thermophily*. Academic Press. New York. 11 – 30.

Lee, Y. Y., Iyer, P. and Torget, R. W. 1999 Dilute Acid hydrolysis of lignocellulosic biomass. In Scheper, T. and Tsao, G. T. (Editors) *Advances in Biochemical Engineering/Biotechnology*. Springer-Verlage, Berlin. pp 276 – 299.

Lee, J.-W, Kang, D.-O, Kim, B.-Y, Oh, W.-K, Mheen, T.-I, Lee, S.-W, Pyun, Y.-R and Ahn, J.-S 2000 Improvement of bacterial endo-β-1,4-glucanase (CMCase) secretion in yeast by mutagenesis of glucoamylase signal sequence. *Sarop Misaengmul Hkhoechi*, **28**, 195 – 201.

Lee, N. M. 1996 Parameters affecting microorganisms and the process performance in biological wastewater treatment. PhD Thesis, Lund University, Sweden.

Lee, D. S. and Pack, M. Y. 1987 Use of bacilli for overproduction of exocellular endo-β-1,4-glucanase encoded by cloned gene. *Enzyme Microbial Technology,* **9**, 594 – 597.

Lehman, M., Pasamontes, L., Lassen, S. F. and Wyss, M. 2000 The consensus concept for thermostability engineering of proteins. *Biochimica et Biophysica Acta*, **1543**, 408–415.

Leisola, M. and Linko, M. 1976 Bioconversion of acid-hydrolysed poplar hemicellulose to acetic acid by *Clostridium thermoacetium*. *Journal of Industrial Microbiology*, **7**, 1 – 6.

Lennarz, W. J. 1970 Bacterial lipid. In Waki J. J. (Editor) *Lipid metabolism.* Academic Press. New York. 210 - 270

Lin, E and Wilson, D. B. 1987 Regulation of β-1,4-endoglucanase synthesis in *Thermonospora fusca*. *Applied and Environmental Microbiology*, **53**, 1352 – 1357.

Lindahl, V., Aa K. and Tronsmo, A. 1994 Nucleotide sequence of an endo-β-1,4-glucanase gene from *Bacillus subtilis* CK-2. *Antonie van Leeuwenhoek*, **66**, 327 – 332.

Lindberg, M., Lundegren, L., gref, L., Johansson, T. and Welinder, K. G. 1995 Infection frequencies through bark of *Picea abies* and *Pinus sylvestris* seedlings and *in vitro* growth rates at different oxygen levels. *European Journal for Pathogenecity*, **22**, 41 –45.

Linder, C., Stulke, J. and Hecker, M. 1994 Regulation of xylanolytic enzymes in *Bacillus subtilis*. *Microbiology*, **140**, 753 – 757.

Linder, M, and Teeri, T. T. 1997 The roles and function of cellulose binding domains. *Journal of Biotechnology,* **57**, 15 – 28.

Lo, A. C., Mackay, R. M., Seligy, V. L. and Willick, G. E. 1988 *Bacillus subtilis* β-1,4-endoglucanase products from intact and truncated genes are secreted into the extracellular medium by *Escherichia coli. Applied and Environmental Microbiology*, **54,** 2287 – 2292.

Lüthi, E., Love, D. R., McAnulty, J., Wallace, C., Caughey, P. A, Saul, D. and Bergquist, P. L. 1990 Cloning, sequence analysis, and expression of genes encoding xylan-degrading enzymes from the thermophilic "*Caldocellum saccharolyticus*". *Applied and Environmental Microbiology*, **56**, 1017 – 1024.

Mackay, R. M., Lo, A., Willick, G., Zuker, M. Baird, S. Dove, M., Moranelli, F. and Seligy, V. 1986 Structure of a *Bacillus subtilis* endo-β-1,4-glucanase gene. *Nucleic Acid Research*, **14**, 9159 - 9170.

Madigan, M. T., Martinko, J. M. and Parker, J. 1997 Brock Biology of Microbiology. Prentice Hall International Inc. Upper Saddle River. New Jersey. USA,

Malek, M. A., Chowdhury, N. A., Youssouf, Q. M. and Chowdhury, N. 1988 Bacterial cellulases and saccharification of lignocellulosic materials*. Enzyme Microbial Technology*, **10,** 750 – 753.

Manachini, P. L., Fortina, G. M. and Parini, C. 1988 Thermostable alkaline protease produced by *Bacillus thermoruber*-a new species of *Bacillus. Applied Microbiology and Biotechnology,* **28**, 409 – 413.

Mandels, M., Andreotti, R. and Roche, C. 1976 Measurement of saccharifying cellulase. *Biotechnology and Bioengineering Symposium*, **6**, 21 – 33.

Manonmani, H. K. and Sreekantiah, K. R. 1987 Saccharification of sugar-cane bagasse with enzymes from *Aspergillus ustus* and *Trichoderma viridie*. Enzyme Microbiology and Technology, **9**, 487 – 488.

Markossian, S., Becker, P., Märkl, H. and Antranikian, G. 2000 Isolation and characterisation of lipid-degrading *Bacillus thermoleovorans* IHI-91 from an icelandic hot springs. *Extremophiles*, **4**, 365 – 371.

Martins, R. E., Davids, W., Abu Al-Soud, W., Levander, F., Rådström, A. and Hatti-Kaul, R. 2001 Starch-hydrolyzing bacteria from Ethiopian soda lakes. *Extremophiles*, **5**, 135 – 144.

Massey, K. L., Palmer, R. G. and Hormer, H. T. 2001 Oxalate content of soyabean seeds (Glycine max: Leguminosae Soyfoods, and other edible legumes. *Journal of Agricultural and Food Chemistry*, **49**, 4262 – 4266.

Mathrani, I. M. and Ahring, B. K. 1992 Thermophilic and alkalophilic xylanases from several *Dictyoglomus* isolates. *Applied Microbiology and Biotechnology*,**38**, 23 – 27.

Mawadza, C. 1998 Production and purification of endo-β-1,4-glucanases produced by *Bacillus* strains isolated from Zimbabwean hot springs. PhD Thesis, University of Zimbabwe, Harare, Zimbabwe.
Mawadza, C. and Zvauya, R. 1996 Some factors affecting endo-β-1,4-glucanase production by two *Bacillus* strains isolated from Zimbabwean hot springs. *Journal of Basic Microbiology*, **36**, 177 – 185.

Mawadza, C. and Zvauya, R. 2001 Production of cellulases by *Bacillus subtilis* strains cultured on wastewater. *Advances in Food Science*, **23**, 15 – 19.

Mawadza, C., Hatti-Kaul, R., Zvauya, R. and Mattiasson, B. 2000 Purification and characterisation of cellulases produced by two *Bacillus* strains. *Journal of Biotechnology*, **83**, 177 – 187.

McMillan, J. D. 1994 Pretreatment of lignocel;lulosic biomas. In Himmel, M. E., Baker, J. O. and Overend, R. P. (Editors) *Enzymatic conversion of biomass for fuels production*. American Chemical Society. Washington, DC, pp. 292 – 324.

McMurray, A. A., Sulston, J. E. and Quail. M. A. 1998 Short-insert libraries as a method of problem solving in genome sequencing. *Genome Research*, **8**: 562 – 566.

Meinke, A., Damude, H. G., Tomme, P., Kwan, E., Kilburn, D. G., Miller, R. C. Jnr, Warren, J. A. R. and Gilkes, N. R. 1995 Enhancement of the endo-β-1,4-glucanase activity of an exocellobiohydrolase by deletion of a surface loop. *The Journal of Biological Chemistry*, **270**, 4383 – 4386.

Meldgaard, M and Svendsen, I. 1994 Different effects of N-glycosylation on the thermostability of highly homologous bacterial (1,3-1,4)-beta-glucanases secreted from yeast. *Microbiology*, **140(Pt 1):** 159-66.

Meyer, P. S., du Preez, J. C. and Kilian, S. G. 1992 Chemostat cultivation of *Candida blankii* on sugar cane bagasse hemicellulose hydrolysate. *Biotechnology and Bioengineering*, **40**, 353 – 358.

Meyer, P. S., Preez, J. C. and Kilian, S. G. 1992 Cultivation of *Candida blankii* in simulated bagasse hemicellulose hydolysate. *Journal of Industrial Microbiology*, **9**, 109 – 113.

Mierendorf, R., Yeager, K. and Novy, R. 1994 The pET system: your choice for expression. *Novogen Newsletter*, **1**, 1 – 3.

Miller, J. F. 1994 Bacterial transformation by electroporation. *Methods in Enzymology*, **235**, 375-385

Miller, T-L. and Churchill, B. W. 1986 Substrates for large-scale fermentations. In Demain A. L. and Solomon N. A. (Editors) *Manual of Industrial Microbiology and Biotechnology.* American Society for Microbiology. Washington DC.122 – 136.

Millet, M. A. and Baker, A. J. 1975 Pretreatments to enhance chemical, enzymatic and microbiological attack of cellulosic materials. *Biotechnology and Bioengineering Symposium*, **5**, 193 – 219.

Min, H., Choi, Y., Cho, K., Ha, J. and Woo, J. 1994 Cloning of the endoglucanase gene from *Actinomyces* sp. 40 in *Escherichia coli* and some properties of the gene products. *Journal of Microbiology and Biotechnology*, **4**, 102 – 107.

Mishra, C., Deshpande, V. and Ro, M. 1983 Immobilisation of *Penicillum funiculosum* cellulase on a soluble polymer. *Enzymes in Microbiology and Technology*, **5**, 342 – 344.

Mittendorf, V. and Thompson, J. A. 1993 Cloning of an endo-β-1,4-glucanase, *celA*, from the rumen bacterium *Clostridium* sp. (*'C. longisporum'*) and characterization of its product, CelA, *in Escherichia coli. Journal of General Microbiology,* **139**, 3233 - 3242

Miyatake, M. and Imada, K. 1997 A gene encoding endo-β-1,4-glucanase from *Bacillus* sp. 22-28. *Bioscience Biotechnology and Biochemistry*, **61**, 362 – 2364.

Moffet, B. F., Walsh, K. A., Harris, J. A. and Hill, T. C. J. 2000 Analysis of Bacterial Community Structure using 16S rDNA Analysis. *Anaerobe*, **6**, 129 – 131.

Morgavi, D. P., Newbold, C. J., Beever, D. E. and Wallace, R. J. 2000 Stability and stabilization of potential additive enzymes in rumen fluid, *Enzyme and Microbial Technology*, **26**, 171 – 177.

Morikawa, Y., Karube, I. and Suzuki, S. 1980 Continuous production of bacitracin by immobilised living whole cells of *Bacillus* sp. *Biotechnology and Bioengineering*, **22**, 1015 – 1023.

Morohoshi, N. 1991 Chemical characterisation of wood and its components, In Hon D. N. S. and Shirashi N. (Editors) *Wood and Cellulosic Chemistry*. Marcel Dekker Inc. New York. 331 – 392.

Mosbach, K., Birnbaum, S., Hardy, K., Davies, J. and Bulow, L., 1983, Formation of proinsulin by immobilized *Bacillus subtilis*, *Nature*, **302**: 543 – 545.

Mosier, N. S., Hall, P., Ladisch, M. C. and Ladisch, M. R. 1999 Reaction kinetics, Molecular Action, and Mechanisms of Cellulolytic proteins. In Scheper, T. and Tsao, G. T. (Editors) *Advances in Biochemical Engineering/Biotechnology*. Springer-Verlag, Berlin, Germany. 23 – 40.

Mullings, R. and Parish, J. H., 1984, Mesophilic aerobic Gram negative cellulose degrading bacteria from aquatic habitats and soils*, Journal of Applied Bacteriology*, **57**: 455 – 468.

Muronde, W. and Zvauya, R. 1992 Isolation and partial characterisation of moderate thermophilic (*Pseudomonas*) strains from alkaline hot springs in Zimbabwe. (*unpublished data*)

Murashima, K., Nishimura, T., Nakamura, Y., Koga, J., Moriya, T., Sumida, N., Yaguchi, T. and Kono, T. 2002 Purification and characterisation of new endo-β-1,4-glucanase from *Rhizopus oryzae. Enzyme and Microbial Technology*, **30**, 319 – 326.

Murphy, N., McConnell, D. J. and Cantwell, B. A., 1984, The DNA sequence of the gene and genetic control sites for the excreted *B. subtilis* enzyme β-glucanase, *Nucleic Acid Research*, **12**: 5355–5367.

Murtinho, D., Lagoa, A. R., Garcia, F. A. P. and Gil, M. H. 1998 Cellulose derivative membranes as support for immobilisation of enzymes. *Cellulose*, **5**, 299 – 308.

Nagpal, M. L., Fox, F. K. and Fox, A. 1998 Utility of 16S-23S rRNA spacer region methodology: how similar are interspace regions within a genome and between strains for closely related organisms? *Journal of Microbiological Methods*, **33**, 211 – 219.

Nakamura, A., Uozumi, T. and Beppu, T., 1987, Nucleotide sequence of a cellulase gene of *Bacillus subtilis*, *European Journal of Biochemistry*, **164**: 317 – 320.

Nakamura, K. and Kitamura, K. 1982 Isolation and identification of crystalline cellulose hydrolysing bacterium and its enzymatic properties. *Journal of Fermentation and Technology*, **60**, 343 – 348.

Navarro, A., Chebrou, M. C., Beguin, P. and Aubert, J. P. 1991 Nucleotide sequence of the cellulase gene celF of *Clostridium thermocellum*. *Research in Microbiology*, **142**, 927 – 936.

Ness, J. E., Welch, M., Giver, L., Bueno, M., Cherry, J. R., Borchert, T. V., Stemmer, W. P. C. and Minshull, J. 1999 DNA shuffling of subgenomic sequences of subtilisin, *Nature*, **17**, 893 – 896.

Nicholson, W. L., Munakata, N., Horneck, G., Melosh, H. J. and Setlow, P. 2000 Resistance of *Bacillus* endospores to extreme terrestrial and extraterrestrial environments. *Microbiology and Molecular Biology Reviews*, **64**, 548 – 572.

Nicolaus, B., Manca, M. C., Lama, L., Esposito, E. and Gambacorta, A. 1995 Effects of the growth temperature on the polar lipid pattern and fatty acid composition of seven thermophilic isolates from the Antartic Continent. *Systematics Applied Microbiology*, **18**, 32 – 36.

Nielsen, P., Fritze, D. and Priest, F. G. 1995 Phenetic diversity of alkaliphilic *Bacillus* strains: proposal for nine new species. *Microbiology*, **141**, 1745 – 1761.

Nishihara, M., Nagahama, S., Ohga, M. and Koga, Y. 2000 Straight-chain fatty alcohols in the hyperthermophilic archaeon *Purococcus furiosus*. *Extremophiles*, **4**, 275 – 277.

O'Sullivan, A. C. 1997 Cellulose: The structure slowly unravels. *Cellulos*e, **4**, 173 – 207.

Oakley, B., Kirsch, D. and Morris, N. R. 1980 A simplified ultrasensitive silver stain for detecting proteins in polyacrylamide gels. *Analytical Biochemistry,* **105**, 361 – 363.

Okoshi, H., Ozaki, K., Shikata, S., Oshino, K.-K and Ito, S. 1990 Purification and characterisation of multiple carboxymethylcellulases from *Bacillus* KSM-522. *Applied Biology and Chemistry*, **54**, 83 – 89.

Oksanen, T., Pere, J., Paavilainen, L., Buchert, J. and Viikari, L. 2000 Treatment of recycled kraft pulps with *Trichoderma reesei* hemicellullases and cellulases. *Journal of Biotechnology*, **78**, 39 – 48.

Olsen, O., and Thomsen, K. K. 1991 Improvement of bacterial β-glucanase thermostability by glycosylation. *Journal of General Microbiology*, **137**, 579 – 585.

Oshima, T. 1978 Novel polyamines of extremely thermophilic bacteria. In Friedman S. M. (Editor) *Biochemistry of Thermophily*. Academic Press. UK. 211 – 220.

Ozaki, K., Shikata, S., Kawai, S, Ito, S. and Okamoto, K. 1990 Molecular-cloning and nucleotide-sequence of a gene for alkaline cellulase from *Bacillus* sp. KSM-635. *Journal of General Microbiology*, **136**, 1327 – 1334.

Ozaki, K., Sumitomo, N. and Ito, S. 1991 Molecular cloning and nucleotide sequence of the gene encoding an endo-β-1,4-glucanase from *Bacillus* sp. KSM-330. *Journal of General Microbiology*, **137**, 2299 – 2705.

Ozaki, K., Sumitomo, N., Hayashi, Y., Kawai, S. and Ito, S. 1994 Site-directed mutagenesis of the putative active site of endoglucanase K from *Bacillus* sp. KSM-330. *Biochimica et Biophysica Acta,* **1207**, 159 – 164.

Paice, M. G., Bergnier, R. and Jurasek, L. 1988 Viscosity enhancing bleaching of hardwood kraft pulp with xylanase from a cloned gene. *Biotechnology and Bioengineering*, **32**, 235 - 239.

Park, J. S., Nakamura, A., Horinouchi, S. and Beppu, T. 1993 Identification of the cellulose binding domain of a *Bacillus subtilis* endoglucanase distinct from its catalytic domain. *Bioscience Biotechnolöogy and Biochemistry*, **57**, 260 – 264.

Park, S. H., Kim, H. K. and Pack, M. Y. 1991 Characterisation and structure of the cellulase gene of *Bacillus subtilis* BSE616. *Agricultural Biology and Chemistry*, **55**, 441 – 448.

Paul, J. and Varma, A. K. 1993 Hydrolytic enzyme(s) production in *Micrococcus roseus* growing on different cellulosic substrates. *Letters in Applied Microbiology*, **16**, 167 – 169.
Pavlostathis, S.G., Miller, T. L. and Wolin, M. J. 1990 Cellulose fermentation by continuous cultures of *Ruminococcus albus* and *Methanobrevibacter smithii*. *Applied Microbiology and Biotechnology*, **33**, 109 - 116.

Pere, J., Piolakka, A., Noisiainen, P. and Buchert, J. 2001 Action of purified *Trichoderma reesei* cellulases on cotton fibers and yarn. *Journal of Biotechnology*, **89**, 247 – 255.

Pero, J. and Sloma, A. 1993 Proteases. In Sonenshein A. L., Hoch J. A. and Losick R. (Editors) *Bacillus subtilis and other Gram-positive Bacteria*. American Society for Microbiology. Washington DC. 939 – 952.

Person, I., Tjerneld, F. and Hahn-Hagerdal, B. 1991 Fungal cellulolytic enzyme production: A Review. *Process Biochemistry*, **26**, 65 – 74.

Prasertsan, P. and Doelle, H.W. 1986 Separation and characterisation of endoglucanases from culture filtrates of *Cellulomonas* sp. *Applied Microbiology and Biotechnology*, **24**, 326 – 333.

Presutti, D. G., Hughes, T. A. and Stutzenberger, F. J. 1991 Cloning of three endoglucanase genes from *Thermonospora curvata* into *Escherichia coli. Journal of Biotechnology*, **17**, 177 – 188.

Priest, F. G. 1977 Extracellular enzyme synthesis by the genus *Bacillus*. *Bacteriological Review*, **41**, 711 – 753.

Priest, F. G. 1993 Systematics and ecology of *Bacillus.* In Sonenshein A. L., Hoch J. A. and Losick R. (Editors) *Bacillus subtilis and other Gram-positive Bacteria.* American Society for Microbiology. Washington DC. 3 – 16.

Priest, F. G., 1981 DNA homology in the genus *Bacillus*. In Berkeley, R. C. W. and Goodfellow, M. (Editors) *The aerobic endspore-forming bacteria*: Classification and identification. Academic Press, Inc., London. 33 – 57.

Rahkamo, L., Viikari, L., Buchert, J., Paakkari, T. and Suortti, T. 1998 Enzymatic and alkaline treatments of hardwood dissolving pulp. *Cellulos*e, **5**, 79 - 88.

Rainey, F. A, Ward-Rainey, N., Kroppenstedt, R. M. and Stackebrandt, E. 1996 The genus *Nocardiopsis* represents a phylogenetically coherent taxon and distinct actinomycete lineage: proposal of *Nocardiopsaceae* fam. No. *International Journal of Systematic Bacteriology*, **46**, 1088 – 1092.

Ramamurthy, V., Thacker, S. P. and Kothari, R.M. 1992 Optimised protocol for the pilot-scale preparation of fungal cellulase. *Journal of Industrial Microbiology,* **9**, 121 –125.

Rao, M. B., Tanksale, A. M., Ghatge, M. S. and Deshpande, V. V. 1998 Molecular and Biotechnological Aspects of Microbial Proteases. *Microbiology and Molecular Biology Reviews*, **62**, 597 – 635.

Ratledge, C. 1988 Biotechnology group meeting on extremophiles. *Journal of Chemical Technology and Biotechnology*, **42**, 289 – 322.

Reynolds, P. S., Sissons, C. H., Daniel, R. M. and Morgan, H. W. 1986 Comparison of cellulolytic activities in *Clostridium thermocellum* and three thermophilic, cellulolytic anaerobes. *Applied and Environmental Microbiology*, **51**, 12 – 17.

Reysenbach, A. L., Wickham, G. S. and Pace, N. R. 1994 Phylogenetic analysis of the hyperthermophilic pink filament community in Octopus spring, Yellowstone National Park. *Applied and Environmental Microbiology*, **60**: 2113 – 2119.

Reysenbach, A. L., Wickman, G. S. and Pace, N. R. 2000 Microbial diversity at 83 degree C in Calcite Springs, Yellowstone National Park: another environment where the Aquificales and "Korarchaeota" coexist. *Extremophiles*, **4**: 61 – 67.

Robson, L. M. and Chambliss, H. G. 1987 Endo-β-1,4-glucanase gene of *Bacillus subtilis* DLG. *Journal of Bacteriology,* **169**, 2017 – 2025.

Robson, L. M. and Chambliss, H. G. 1986 Cloning of the *B. subtilis* DLG β–1,4-glucanase gene and its expression in *Escherichia coli* and *B. subtilis*. *Journal of Bacteriology*, **165**, 612 – 619.

Roche Molecular Biochemicals, 1999 PCR Applications Manual. Roche Diagnostics, GmbH, Mannhein, Germany.

Rodgers, G., Weddle, R. B. and Hastings, R. R. 1984 Effect of alkaline protease activity on some properties of communuted squid. *Journal of Food Science*, **49**, 117 – 123.

Rogers, C. J., Coleman, E., Spino, D. F., Scarpino, P. V. and Purcell, T. C. 1972 Production of fungal protein from cellulose and waste cellulosics. *Environmental Science and Technology*, **6**, 715 – 719.

Ronimus, S. R., Parker, L. E. and Morgan, W. H. 1997 The utilization of RAPD-PCR for identifying thermophilic and mesophilic *Bacillus* species. *FEMS Microbiology Letters*, **147**, 75 – 79.

Rothman, J. E. and Kennedy, E. P. 1977 Asymmetrical distribution of phospholipids in the membrane of *Bacillus megaterium*. *Journal of Molecular Biology*, **110**, 603 - 618.

Royer, J. C. and Nakas, J. P. 1987 Production of mycelial protein and hydrolytic enzymes from paper mill sludges by cellulolytic fungi. *Journal of Industrial Microbiology*, **2**, 9 – 13.

Ryu, D. D. Y. and Mandels, M. 1980 Cellulases: biosynthesis and applications. *Enzyme and Microbial Technology*, **2**, 91 – 102.

Sakata, M., Ooshima, H. and Harano, Y. 1985 Effects of agitation on enzymatic saccharification of cellulose. *Biotechnology Letters*, **7**, 689 – 694.

Sakon, J. 1997 Crystal structure of endo/exocellulase E4, Presented at the Biophysical Society Meeting. Kansas City. USA.

Sambrook, J., Fritsch, E. F. and Maniatis, T. 1989 Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press. Cold Spring Harbor. New York.

Sami, A. J., Akhtarar, M. W., Malik, N. N. and Naz, B. A 1988 Production of free and substrate-bound cellulases of *Cellulomonas flavigena. Enzyme Microbiology and Technology*, **10**, 626 – 631.

Sánchez-Torres, J., Pérez, P. and Santamaria, R. I. 1996 A cellulase gene from a new alkalophilic *Bacillus* sp. (strain N186-1). Its cloning, nucleotide sequence and expression in *Escherichia coli*. *Applied Microbiology and Biotechnology*, **46**, 148 – 155.

Saris, E. J., Airaksinen, U., Nurmiharju, S., Runeberg-Nyman, K. and Palva, I. 1990 Expression of *Bordatella-pertussis* toxin subunits in *Bacillus subtilis*. *Biotechnology Letters*, **12**, 873 – 878.

Saris, P., Taira, S., Airaksinen, U., Palva, A., Sarvas, M., Palva, I. and Runeberg-Nyman, K. 1990 Production and secretion of pertussis toxin subunits in *Bacillus subtilis*, *Federal European Microbiology Society and Microbiology Letters*, **56**, 143 – 148.

Saxena, A., Garg, S. K. and Verma, J. 1992 Simultaneous saccharification and fermentation of waste newspaper to ethanol. *Bioresource Technology*, **42**, 13 – 15.

Scaman, C. 2000 Gums and stabilizers for the food industry. In Williams P. and Phillips G. O. (Editors). Royal Society of Chemistry. Cambridge. UK.

Schafner, D. W. and Toledo, R. T. 1991 Cellulase production in continuous culture by *Trichoderma reesei* on xylose-based media. *Biotechnology and Bioengineering*, **39**, 865 – 869.

Scheinert, P., Krausse, R., Ullmann, U., Söller, R. and Krupp, G. 1996 Molecular differentiation of bacteria by PCR amplification of the 16S-23S rRNA spacer. *Journal of Microbiological Methods*, **26**, 103 – 117.

Schiraldi, C., Acone, M., Giuliano, M., Carteni, M. and De Rosa, M. 2001 Innovative fermentation strategies for the production of extremophilic enzymes. *Extremophiles*, **5**, 193 – 198.

Schmidhalter, D. R. and Canevascini, G. 1992 Characterisation of the cellulolytic enzyme system from the brown-rot fungus *Coniophora puteana. Applied Microbiology and Biotechnolog*y, **37**, 431 – 436.

Schmidhalter, D. R. and Canevascini, G. 1993 Purification and characterisation of two exocellobiohydrolases from the brown-rot fungus coniophora puteana. *Archives of Biochemistry and Biophysics*, **300**, 551 – 558.

Schomburg, D. 1994 Rational Design of Proteins with New Properties. In Wrede P. & Schneider G. (Editors) *Concepts in Protein Engineering and Design.* Walter de Gruyter. Germany. 169 – 208.

Schülein, M. 2000 Protein engineering of cellulases. *Biochimica et Biophysica Acta*, **1543**, 239 – 252.

Schwan, R. F. and Rose, A. H. 1994 Polygalacturonase production by *Kluyveromyces marxianus*: effect of medium composition. *Journal of Applied Bacteriology*, **76**, 62 – 67.

Selinger, L. B., Forsberg, C. W. and Cheng, K.-J 1996 The Rumen: A unique source of enzymes for enhancing livestock production. *Anaerobe*, **2**, 263 – 284.

Shaker, H. M., Farid, M. A. and El-Diwany, A. I. 1985 Optimisation of the composition of the nutrient medium for cellulase and protein biosynthesis by thermophilic *Aspergillus* spp. *Food Chemistry Microbiology Technology*, **9**, 1 – 5.

Sharma, P., Gupta, J. K., Vadehra, D. V. and Dube, D. K. 1987 Molecular cloning and expression in *Escherichia coli* of a thermophilic *Bacillus* sp. PDV endo-β-1,4-glucanase gene. *Enzyme and Microbial Technology*, **9**, 602 – 605.

Sharma, P., Gupta, J. K. and Vadehra, D. V. 1990 Purification and properties of an endoglucanase from a *Bacillus* isolate. *Enzyme and Microbial Technology*, **12**, 132 – 137.

Sharp, R. J., Riley, P. W. and White, D. 1992 Heterotrophic thermophilic Bacilli. In: Kristjansson, J. K., editor. *Thermophilic Bacteria*. Boca Raton, Fla, CRC Press, NY, pg 19 – 50.

Shaver, Y. J., Nagpal, M. L., Rudner, R., Nakamura, L. K., Fox, K. F. and Fox, A. 2002 Restriction fragment length polymorphism of rRNA operons for discrimination and intergenic spacer sequences for cataloging of *Bacillus subtilis* subgroups. *Journal of Microbiological Methods*, **50**: 215 – 223.

Shene, C., Mir, N., Andrews, B. A. and Asenjo, J. A. 2000 Effect of the growth conditions on the synthesis of a recombinant β-1,4-endoglucanase in continuous and fed-batch culture. *Enzyme and Microbial Technology*, **27**, 248 – 258.

Shiang, M., Linden, C. J., Mohagheghi, A., Grohmann, K. and Himmel, M. E. 1991 Enhanced production of cellulase using *Acidothermus cellulolyticus* in fed-batch culture. *Applied Microbiology and Bacteriology*, **34**, 591 – 597.

Shima, S., Igarashi, Y., and Kodama, T. 1991 Molecular cloning of a new endoglucanase gene from *Clostridium cellulolyticum* and its expression in *Escherichia coli*. *Applied Microbiology and Biotechnology*, **35**, 233 – 236.

Simonen, M. and Palva, I. 1993 Protein Secretion in *Bacillus* species. *Microbiological Reviews*, 57, 109–137.

Smith, P. K., Krohn, R. i. and Hermanson, E. K. 1986 Measurement of protein using bicichoninic acid. *Analytical Biochemistry*, **150**, 76 – 85.

Sinha J., Dey, P. K. and Panda, T. 2000 Extractive fermentation for improved production of endoglucanase by an intergeneric fusant of *Trichoderma reesei/Saccharomyces cerevisae* using aqueous two-phase system. *Biochemical Engineering Journal***, 6**, 163 – 175.

Skirnisdottir, S., Hreggvidsson G. O., Hjorleifsdottir, S., Marteinsson, T. V., Petursdottir, K. S., Holst, O. and Kristjansson, J. K. 2000 Influence of sulfide and temperature on species composition and community of hot spring microbial mats. *Applied and Environmental Microbiology*, **66**: 2835 – 2841.

Soutschek-Bauer, E. and Staundenbauer, W. L. 1987 Synthesis and secretion of a heat-stable carboxymethylcellulase from *Clostridium thermocellum* in *Bacillus subtilis* and *Bacillus stearothermophilus*. *Molecular General Genetics*, **208**, 537 – 541.

Soole, K. L., Hirst, B. H., Hazlewood, G. P., Gilbert, H. J., Laurie, J. L. and Hall, J. 1993 Secretion of prokaryotic cellulase in bacterial and mammalian cells. *Gene*, **125**, 85 – 89.

Soundar, S. and Chandra, T. S. 1987 Cellulose degradation by a mixed bacterial culture. *Journal of Industrial Microbiology*, **2**, 257 – 265.

Sreenath, H. K., Kroegel, R. G., Moldes, A. B., Jeffries, T. W. and Straubs, J. R. 1999 Enzymatic saccharification of alfalfa fibre after liquid hot water pretreatment. *Process Biohemistry*, **35**, 33 – 41.

Srinivasan, M. C., Rele, M. V. and Ingale, S. 1999 Production and application of enzymes stable to and active under extreme environments: an overview. *Pinsa, **B65***, 143 – 162.

Srivastava, K. K., Verma, P. K. and Srivastava, R. 1999 A recombinant cellulolytic *Escherichia coli*: Cloning of the cellulase gene and characterisation of a bifunctional cellulase. *Biotechnology Letters*, **21**, 293 – 297.

Stellwagen, E. and Wilgus, H. 1978 Thermostability of proteins. In Friedman S. M. (Editor) *Biochemistry of Thermophily*. Academic Press. UK. 223 - 232.

Stetten, von F., Francis, K. P., Lechner, S., Neuhaus, K. and Scherer, S. 1998 Rapid discrimination of psychrotolerant and mesophilic strains of the *Bacillus cereus* group by PCR targeting of 16S rDNA. *Journal of Microbilogical Methods*, **34**, 99 – 106.

Strongin, A., Ya, D. I., Gorodetsky, A. I., Kuznetsova, V. V., Yanonis, Z. T., Abramov, Z. T., Belyanova, L. P:, Baratova, L. A. and Stepanov, V. M. 1979 Intracellular serine protease of *Bacillus subtilis*: sequence homology with extracellular subtilisins. *Journal of Bacteriology*, **179**, 333 – 339.

Strydom, E., Mackie, R. I. and Woods, D. R. 1986 Detection and characterisation of extracellular proteases in *Butyrivibrio fribrisolvens* H17c. *Applied Microbiology and Biotechnology*, **24**, 214 – 217.

Sturr, M. G., Guffanti, A. A., Krulwich, T. A. 1994 Growth and bioenergetics of alkaliphilic *Bacillus firmus* OF4 in continuous culture at high pH. *Journal of Bacteriology*, **176**, 3111 – 3116.

Subramaniyan, S. and Prema, P. 2000 Cellulase-free xylanases from *Bacillus* and other microorganisms. *FEMS Microbiology Letters*, **183**, 1 – 7.

Sumitomo, N, Hakamata, Y., Hayashi, Y., Ozaki, K., Kobayashi, T. and Kawai, S. 2000 Plasmid vectors based on *Bacillus* KSM-64 alkaline cellulase gene upstream regulatory region for efficient protein expression. Patent no. JP 99-95948 19990402, Kao Corp., Japan.

Sumitomo, N., Ozaki, K., Hitomi, J., Kawaminami, S., Kobayashi, T., Kawai, S. and Ito, S. 1995 Application of the upstream region of *Bacillus* endoglucanase gene to high-level expression of foreign genes in *Bacillus subtilis*. *Bioscience Biotechnology and Biochemistry*, **59**, 2172 –2175.

Sumitomo, N., Ozaki, K., Kawai, S. and Ito, S. 1994 Regulatory sequences of alkaline cellulase gene of *Bacilus* KSM-64 for improving gene expression. Patent no. JP 06217781 A2 19940809, Kao Corp., Japan.

Sumitomo, N., Ozaki, K., Kawai, S. and Ito, S. 1992 Nucleotide sequence of the gene for an alkaline endoglucanase from an alkalophilic *Bacillus* and its expression in *Escherichia coli* and *Bacillus subtilis*. *Bioscience Biotechnology and Biochemistry*, **56**, 872 – 877.

Sun, Y. and Cheng, J. 2002 Hydrolysis of lignocellulosic materials for ethanol production: a review. *Bioresource Technology*, **83**, 1 – 11.

Svennsson, S. 1996 Aspects of *Bacillus* as an additive for galactooligosaccharide degradation in animal-feed. Licentiate Thesis, Lund University, Sweden.

Schwarz, H. W. 2001 The cellulosome and cellulose degradation by anaerobic bacteria. *Applied Microbiology and Biotechnology*, **56**, 634 – 649.

Taherzadeh, M. J., Niklasson, C., Gustafsson, L., Lidén, G. 1998 Fermentation of strongly inhibiting wood hydrolyzates using fed-batch technique. Bioenergy '98, *Proceedings of National Bioenergy conference*, 8th, Madison, Wisconsin, **2**: 872-880

Takami, H. and Horikoshi, K. 2000 Analysis of the genome of an alkaliphilic *Bacillus* strain from an industrial point of view. *Extremophiles*, **4**, 99 – 108.

Takami, H., Akiba, T. and Horikoshi, K. 1989 Production and characterisation of an extremely thermostable alkaline protease from *Bacillus* sp. no, AH-101. *Applied Microbiology and Biotechnology*, **30**, 120 – 124.

Teather, R. M. and Wood, P. J. 1982 Use of Congo red polysaccharide interactions in enumeration and characterisation of cellulolytic bacteria from bovine rumen. *Applied and Environmental Microbiology*, **43**, 777 – 780.

Tengborg, C., Stenberg, K., Galbe, M., Zacchi, G., Larsson, S., Palmqvist, E., and Hahn-Hägerdal, B. 1998 Comparison of SO_2 and H_2SO_4 impregnation of softwood prior to steam pretreatment on ethanol production. *Applied Microbiology and Biotechnology,* **70-72**, 3-15

Teunissen, M. J., Camp, H. J. M. O., Orpin, C. G., Veld, J. H. and Vogels, G. D. 1991 Comparison of growth characteristics of anaerobic fungi isolated from ruminant and non-ruminant herbivores during cultivation in a defined medium. *Journal of General Microbiology*, **137**, 1401 – 1408.

Teunissen, M. J., Baerends, J. R. S., Knelissen, R. A. G., Op den Camp, H. J. M. and Vogels, D. G. 1992 A semi-continuous culture system for the production of cellulolytic and xylanolytic enzymes by the anaerobic fungus *Piromyces* sp. strain E2. *Applied Microbiology and Biotechnology*, **38**, 28 – 33.

Tewari, H. K., Marwaha, S. S., Kennedy, J. F. and Singh, L. 1988 Evaluation of acids and cellulase enzyme for the effective hydrolysis of agricultural ligninocellulosic residues. *Journal of Chemical Technology and Biotechnology*, **41**, 261 – 275.

Tewari, H. K., Singh, L., Marwaha, S. S. and Kennedy, J. F. 1987 Role of pretreatments on enzymatic hydrolysis of agricultural residues for reducing sugar production. *Journal of Chemical Technology and Biotechnology*, **38**, 153 – 165.

Tezuka, H., Yuuki, T. and Yabuuchi, S. 1989 Construction of a β–glucanase gene hyperproducing *Bacillus subtilis* using the cloned β–glucanase gene and a multi-copy plasmid. *Agricultural and Biological Chemistry*, **53**, 2335 – 2339.

Thompson, J. D., Higgins, D. G. and Gibson, T, J, 1994 Clustal W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. *Nucleic Acid Research*, **22**, 4673 – 4680.

Tjalsma, H., Bolhuis, A., Jongbloed, Jan D. H., Bron, S. and van Dijl, Jan M. 2000 Signal peptide-dependent protein transport in *Bacillus subtilis*: a genome-based survey of the secretome. *Microbiology and Molecular Biology reviews*, **64**, 515 – 547.

Tomme, P., Gilkes, N. R., Miller, R. C. Jnr, Warren, A. J. and Kilburn, D. G. 1994 An internal cellulose-binding domain mediates adsoprtion of an engineered bifunctional xylanase/cellulase. *Protein Engineering*, **7**, 117 – 123.

Tolan, J. S. and Foody, B. 1999 Cellulase from submerged fermentation. In Scheper, T. and Tsao, G. T. (Editors) *Advances in Biochemical Engineering/Biotechnology*. Springer-Verlage, Berlin. pp 41 – 67.

Tothill, I. E. and Seal, K. J. 1993 Biodeterioration of waterborne paint cellulose thickeners. *International Biodeterioration and Biodegradation*, **31**, 241 – 254.

Tsai, Y. –C., Juang, R.-Y., Lin, S.-F., Chen, S.-W., Yamasaki, M. and Tamura, G. 1988 Production and further characterisation of an alkaline elastase produced by alkalophilic *Bacillus* strain Ya-B. *Applied and Environmental Microbiology*, **54**, 3156 - 3161.

Tsao, G. T., Cao, N. J., Di, J. and Gong, C. S.1999 Production of multifunctional organic acids from renewable resources. In Scheper, T. and Tsao, G. T. (Editors) *Advances in Biochemical Engineering/Biotechnology*. Springer-Verlage, Berlin. pp 243 – 280.

Tsuchiya, K., Nakamura, Y., Sakashita, H. and Kimura, T. 1992 Purification and characterisation of a thermostable alkaline protease from alkalophilic *Thermoactinomyces* sp. HS682. *Bioscience Biotechnology and Biochemistry,* **56**, 246 – 250.

Tsuru, D., Kira, H., Yamamoto, T. and Fukumoto, J. 1966 Studies on bacterial protease Part XVI Purification, crystallisation and some enzymatic properties of alkaline protease of *Bacillus subtilis var amylosacchariticus*. *Agricultural Biology and Chemistry*, **30**, 1261 – 1268.

Varrot, A., Schülein, M. and Davies, J. G. 2000 Insights into Ligand–induced conformational change in Cel5A from *Bacillus agaradhaerens* revealed by a catalytically active crystal form. *Journal of Molecular Biotechnology*, **297**, 819 – 828.

Vieille, C. and Zeikus, G. J. 2001 Hyperthermophilic Enzymes: Sources, Uses, and Molecular Mechanisms for Thermostability. *Microbiology and Molecular Biology Reviews*, **65**, 1 – 43.

Waldron, G. and Eveleigh, D. E. 1986 Saccharification of cellulosics by *Microbispora bispora*. *Applied Microbiology and Biotechnology*, **4**, 487 – 492.

Walker, J. M. 1994 The Bicichoninic acid (BCA) assay for protein quantitation. *Methods in Molecular Biology*, **32**, 5 – 8.

Wang, H. and Jones, R. W. 1997 Site-directed mutagenesis of a fungal β-1,4-endoglucanase increases the minimum size required for the substrate. *Applied Microbiology and Biotechnology*, **48**, 225 – 231.

Ward, D. M., Santegoeds, C. M., Nold, S. C., Ramsing, N. B., Ferris, M. J. and Bateson, M. M. 1997 Biodiversity within hot spring microbial mat communities: molecular monitoring of enrichment cultures. *Antonie van Leeuwenhoek* **71**: 143 – 150.

Ward, D. M., Weller, R. and Bateson, M. M. 1990 16S rRNA sequences reveal numerous uncultured microorganisms in a natural community. *Nature*, **345**: 63 – 65.

Ward, O. P. 1983 Proteinases. In Forgarty, W. M. (Editor) *Microbial enzymes and Biotechnology*. Applied Science Publications. New York. 51 – 317.

Warren, R. A. J., Gerhard, B., Gilkes, N. R., Owolabi, J. B., Kilburn, D. G. and Miller Jr. R. C. 1987 A bifunctional exoglucanases-endoglucanase fusion protein. *Gene*, **61**, 421 – 427.

Watanabe, K., Kodama, Y. and Harayama, S. 2001 Design and evaluation of PCR primers to amplify bacterial 16S ribosomal DNA fragments used for community fingerprinting. *Journal of Microbiological Methods*, **44**, 253 – 262.

Wayman, M., Chen, S. and Doan, K. 1992 Bioconversion of waste paper to ethanol. *Process Biochemistry*, **27**, 239 – 245.

Weisburg,. W. G., Barns, S. M., Pelletier, D. A. and Lane, D. J. 1991 16S Ribosomal DNA amplification for Phylogenetic study. *Journal of Bacteriology*, **173**, 697 – 703.

Weiss, D. S., Klose, K. E., Hoover, T. P.,, north, A. K., Porter, S. C., Wedel, A. B. and Kustu, S. 1992. Prokaryotic transcriptional enhancers. In MacKnight, S. L. & Yamamoto, K. R. *Transcriptional Regulations*. Cold Spring Harbours, Laboratory press, New York, USA. 667 – 694.

Wicher, K. B., Abou-Hachem, M., Halldórsdóttir, S., Thorbjarnadóttir, S. H., Eggerrtsson, G., Hreggvidsson, G. O., Karlsson, E. N. and Holst, O. 2001 Deletion of a cytotoxic, N-terminal putative signal peptide results in a significant increase in production yields in *Escherichia coli* and improved specific activity of Cel12A from *Rhodothermus marinus*. *Applied Microbiology and Biotechnology*, **55**, 578 - 584.

William, P. C., Preston, K. R., Norris, K. H. and Starkey, P. M. 1984 Determination of amino acids in wheat and barley by near-infrared reflectance spectroptroscopy. *Journal of Food Science*, **49**, 17 – 20.

Wiseman, A. 1993 Designer enzyme and cell application in industry and in environmental monitoring. *Journal of Chemical Technology and Biotechnology*, **56**, 3 – 13.

Woodward, S. and Pearce, R. B. 1988 The role of stilbenes in resistance of Stika spruce (*Picea sitchenisis* (Bong) Carr. To entry of fungal pathogens. *Physiology and Molecular Plant Pathology*, **33**, 127 – 149.

Yazdi, M. T., Woodward, J. R. and Radford, A. 1990 Cellulase production by *Neurospora crassa*: The enzymes of the complex and their regulation. *Enzyme Microbial Technology*, **12**, 116 – 119.

Yoshimatsu, T., Ozaki, K., Shikata, S., Ohta, Y., Koike. K., Kawai, S. and Ito, S. 1990 Purification and characterisation of alkaline endo-β-1,4-glucanases from alkalophilic *Bacillus* sp. KSM-635. *Journal of General Microbiology*, **136**, 1973 –1979.

Yumoto, I., Yamazaki, K., Hishinuma, M., Nodasaka, Y., Inoue, S. and Kawasaki, K. 2000 Identification of facultatively alkaliphilic *Bacillus* sp. strain YN-2000 and its fatty acid composition and cell-surface aspects depending on culture pH. *Extremophiles*, **4**, 285 – 290.

Yutami, K., Ogasahara, K., Suzuki, M. and Sugino, Y. 1978 Effect of single amino acids substitution on conformational stability of the tryptophan synthase alpha-subunit of *E.coli.* In Friedman S. M. (Editor) *Biochemistry of Thermophily*. Academic Press. UK. 1 - 10.

Zaghloul, T. I., Hendawy, H. M., Assar, E. S. and Mostafa, M. H. 2002 Enhanced stability of the cloned *Bacillus subtilis* alkaline protease gene in alginate-immobilised *B. subtilis* cells. *Enzyme and Microbial Technology*, **30**, 862 – 866.

Zeikus, J. G., Vielle, C. and Savchenko, A. 1998 Thermozymes: Biotechnology and structure-function relationship. *Extremophiles*, **2**, 179 – 183.

Zhang, J. X., Krell, P. J., Phillips, J. P. and Forsberg, C. W. 1997 Expression of a bacterial endo-(1,4)-β-glucanase gene in mammalian cells and post translational modification of the gene product. *Biochimica et Biophysica Acta*, **1357**, 215 – 224.

Zhang, J.-X, Meidinger, R., Forsberg, C. W., Krell, P. J. and Phillips, J.-P 1999 Expression and processing of a bacterial endoglucanase in transgenic mice. *Archives of Biochemistry and Biophysics*, **367**, 317 – 321.

Zvauya, R. and Zvidzai, C. J. 1995 Constitutive production of endoglucanase by a *Bacillus* sp. isolated from a Zimbabwean hot spring. *World Journal of Microbiology and Biotechnology*, **11**, 658 – 660.

Zvauya, R. and Zvidzai, C. J. 1996 Production of hydrolytic enzymes by a *Bacillus* sp. grown on opaque beer brewery wastewater supplemented with spent yeast and defatted soya. *Advanced Food Sciences*, **18**, 11 – 17.

Zvauya, R., Parawira, W. and Mawadza, C. 1994 Aspects of aerobic thermophilic treatment of Zimbabwean Traditional opaque-beer brewery wastewater. *Bioresource Technology*, **48**, 273 – 274.

Zvidzai, C. J. and Zvauya, R. 2001 Purification of a protease enzyme from an alkalophilic *Bacillus subtilis* CHZ1 isolated from a Zimbabwean hot spring. *Journal of Food Biochemistry*, **25**, 1 – 13.

Zvidzai, C. J., Delgado, O., Sithole-Niang, I, Zvauya, R. and Hatti-Kaul, R. 2002 Cloning, sequencing and expression of an endo-β-1,4-glucanase gene from a *Bacillus subtilis* CHZ1. *Journal of Applied Sciences in Southern Africa*.**8**: 65 – 75.

Appendix

THESIS PUBLICATIONS

This thesis is based on the following papers:

I Zvauya, R. and **Zvidzai, C. J.** 1995 Constitutive production of endoglucanase by a *Bacillus* sp. isolated from a Zimbabwean hot spring. *World Journal of Microbiology and Biotechnology*, **11**, 658-660.

II Zvauya, R. and **Zvidzai, C. J**. 1996 Production of hydrolytic enzymes by a *Bacillus* sp. grown on opaque beer brewery wastewater supplemented with spent yeast and defatted soya. *Advanced Food Sciences*, **18**, 11-17.

III **Zvidzai, C. J.** and Zvauya, R. 2001 Purification of a protease enzyme from an alkalophilic *Bacillus subtilis* CHZ1 isolated from a Zimbabwean hot spring. *Journal of Food Biochemistry*, **25**, 1-13.

IV **Zvidzai**, **C. J.,** Hatti-Kaul, R., Sithole-Niang, I., Zvauya, R. and Delgado, O. 2003 Cloning, sequencing and expression of an endo-β-1,4-glucanase (*celG*) gene of a *Bacillus subtilis* CHZ1. *Journal of Applied Sciences in Southern Africa*. **8**: 65 – 75.