Isolation And Characterization Of Cellulolytic Bacteria From Soils Excavated From Geothermal Wells Along The Kenyan Rift Valley

M.Sc. Thesis by:

Kiio Irene Kateve

Reg. No. H56/71121/2011

A thesis submitted in partial fulfillment of the requirements for the award of a Master of Science degree in Biochemistry Department of Biochemistry School of Medicine, College of Health Sciences University of Nairobi

2014.

DECLARATION

This thesis is my original work and has not been presented for a degree in any other university

KIIO IRENE KATEVE

Signature …… Date ……

This thesis has been submitted for examination with our approval as university supervisors.

DR. EDWARD MUGE

Department of Biochemistry

University of Nairobi

Signature ……. Date ……

DR. BENSON WAMALWA

Department of Chemistry

University of Nairobi

Signature ……. Date……

DEDICATION

To my family and Sila

ACKNOWLEDGEMENT

This task has been accomplished with the grace of God. I am grateful for the wisdom and perseverance that He has bestowed upon me during this research project, and indeed, throughout my life.

I offer my profound gratitude to my supervisors Dr. Muge, Biochemistry department, University of Nairobi and Dr. Wamalwa, Chemistry Department University of Nairobi, for their invaluable support and guidance throughout this project. I also thank the University of Nairobi for awarding me a Scholarship thus enabling me to pursue a Master of Science degree in Biochemistry.

To all my colleagues in the laboratory, classmates and technical staff in Biochemistry Department, I thank you for your kind cooperation, suggestions and encouragement. I'm greatly indebted to you all.

No one walks alone in this journey of life, to my family and friends thanks for walking beside me and urging me to move on even when I had no will to.

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LIST OF ABBREVIATIONS

ACEI	Activator of cellulase expression proteins I
ACEII	Activator of cellulase expression proteins II
BGI	β-glucosidase I
BGVII	β-glucosidase VII
CBD	Cellulose Binding Domain
CBHI	Cellobiohyrolase I
CBHII	Cellobiohyrolase II
CBHA	Cellobiohyrolase A
CBM	Cellulose Binding Module
Cip A	Cellulose intergrating protein A
CMC	Carboxymethylcellulose
CMCase	Carboxymethylcellulase
CREI	Catabolite repressor protein I
CREII	Catabolite repressor protein II
CTAB	Cetyl trimethyl ammonium bromide
CbP	Cellobiose phosphorylase
CdP	Cellodextrin phosphorylase
CV	Crystal Violet
DP	Degree of Polymerization
EGI	Endoglucanase I
EGVIII	Endoglucanase VIII
DNS	Dinitrosalicylic acid

dNTPs	deoxynucleotide triphosphates
GRAS	Generally recognized as Safe
LicB	Lichenase B
ManA	Mannose-6-phosphate isomerase
XynA	Xylanase A
XynB	Xylanase B
XynV	Xylanase V
XynY	Xylanase Y
XynZ	Xylanase Z

ABSTRACT

Cellulolytic microorganisms such as fungi and bacteria are responsible for much of the cellulose degradation in soils. Despite this vast number of cellulase producers, there is a deficiency of microorganisms that can produce significant amount of the three cellulase enzyme specifities i.e. endoglucanases, exoglucanases and cellobiases to efficiently degrade cellulose to fermentable products. Little emphasis has been given to cellulase production from bacteria despite their extremely high natural diversity, which endows them with the capability to produce stable enzymes. Soil samples were collected from Hell's gate and from a geothermal well in Eburru hill at depths of 155 Metres, 156 Metres and 157 Metres in the Kenyan Rift valley. The soil samples were inoculated separately and from each, only a single bacterial isolate was obtained. The four isolates were screened for cellulolytic activity using Congo red stain on Carboxymethylcellulose (CMC) agar plates inoculated with the isolates. All the isolates were found to hydrolyze Carboxymethylcellulose. A Gram stain test carried out identified the four isolates as Gram-positive rods. Phylogenetic analysis indicated that they all associated mainly with members of the *Bacillus licheniformis*. Eburru 156 isolate (Isolate 3) selected for further functional studies bore the three enzyme specificities of a cellulase enzyme system. A crude enzyme extract was found to hydrolyse Cellobiose, Avicel and CMC with enzyme activities of 0.46878U/mg, 0.18784U/mg and 0.13571U/mg respectively. Optimum temperature for activity measured over 60 minutes was found to be 60^{o}C with relatively high activity at both 70^{o}C and 80^{o}C. The optimum pH at the predetermined optimum temperature was found to be pH 5. This thermotolerance in addition to production of the three cellulase enzyme activities makes the isolate attractive for potential application in the biorefinery industry.

CHAPTER 1: INTRODUCTION

Microbial communities have been found in the most diverse environmental conditions such as extreme temperatures, pressure, salinity and pH suggesting that microbial life is not limited to any specific environment (Khalil, 2011). Such microorganisms are referred to as extremophiles and are classiffied into several categories; psychrophiles, halophiles, alkalophiles, acidophiles, piezophiles, thermophiles, metallophiles, radiophiles and microareophiles according to the conditions they thrive in (Burg, 2003). These microorganisms are adapted to survive in such the extreme conditions.

This ability is associated with their features such as novel enzymes and biochemical pathways that enable them to remain active or mechanisms that temper the intracellular milieu from the harsh external environment (Liszka *et al*., 2012). Such microorganisms are a source of enzymes that can be applied in industrial processes, which take place under conditions similar to those of their natural habitats (Kirk *et al*., 2002; Straathof *et al*., 2002; Burg, 2003; Vieille & Zeikus, 2001; Cherry & Fidantsef, 2003; Taylor *et al*, 2004; Robertson & Steer, 2004).

On realizing this, industries have been fuelling research on novel enzymes to serve as biocatalysts from extremophiles (Herbert, 1992; Khalil, 2011). Biocatalysts are associated with better chemical precision due to their chemical selectivity, which confers a number of benefits to the industries. These include production of better stereoisomers, fewer side reactions and less environmental pollution (Rozzell, 1999). Application of biocatalysts in industries is however limited due to the fact that the bulk of enzymes used have been isolated from mesophilic organisms and cannot withstand harsh industrial conditions. At temperatures above 40^{o}C or

below 25^{o}C, mesophilic enzymes are less effective due to decreased enzyme stability. This leads to a shorter half life and lower activity (Liszka *et al*., 2012). Enzymes from extremophiles are thus an attraction due to their stability and activity under conditions that are considered harsh for enzymes from mesophilic organisms (Madigan & Mars, 1997). Researchers have consequently delved on characterization of organisms thriving in extreme environments in search for extremozymes (Khalil, 2011). Despite their usefulness as biocatalysts, genes from extremozymes contribute immensely to the genetic pool used for protein engineering and directed evolution (Ness *et al*., 1999; Richardson, 2002).

Various enzyme types from extremophiles have been used in industries; these include proteases, lipases, oxidases, dehydrogenases, esterases and polymer degrading enzymes amongst others. Amongst the extremophiles, enzymes from thermophilic organisms have received the most attention possibly due to their stability at elevated temparatures. Thermostable enzymes are also believed to be more resistant to other denaturing factors. This correlation however does not hold for denaturing processes that do not depend on the folding stability of a protein e.g oxidation of surface residues (Wang *et al*., 2000; D'Amico *et al*., 2006; Burg *et al*., 1998). Thermostable enzymes are quite useful in degradation of polymeric substrates such as starch, chitin and cellulose since their solubility is highly improved at elevated temperatures and the risk of contamination reduced (Burg, 2003). In addition to this, performing hydrolysis at elevated temparatures reduces the viscocity and increases miscibility (Liszka *et al*., 2012). Chitinases and amylases degrade chitin and starch respectively while complete hydrolysis of the cellulose requires the activity of a group of enzymes collectively referred to as cellulases (Tengerdy & Szakacs, 2003).

With increased industrialization combined with the fact that cellulose is the most abundant renewable raw material and energy source, cellulases have found a wide range of application in numerous industries such as agriculture, pulp and paper, textile, food and beverage, animal feed, detergent industry and bioconversion of cellulosic material into solvents such as ethanol amongst others (Kuhad *et al*., 2011; Karmakar & Ray, 2011). Of interest, is the conversion of lignocellulosic biomass to ethanol used as biofuel.

For many years crude oil has been the major resource for meeting the ever-increasing energy demands (Sun & Cheng, 2002). The gap between the demand and supply is slowly widening since existing oil fields are declining and new ones are not yet well established (NREL, 2006). Considering that economies all over the world are highly dependent on energy, the consequences of inadequate oil availability would be quite severe (Sun & Cheng, 2002). In addition, burning fossil fuels releases additional carbon dioxide (CO_2) into the atmosphere over and above that released in the normal cycle. This CO_2 accumulation is a major cause of global warming (Wyman, 2007). Research on alternative, sustainable and environment friendly energy sources to provide power and fuel for transport is therefore necessary and the focus is now on nuclear energy and biofuels (Wyman, 2007). In countries such as United States of America, gasoline fuels contain up to 10% ethanol by volume (Sun & Cheng, 2002). Ethanol blended fuel for automobiles significantly reduces petroleum use and green house gas emission (Wang *et al*., 1999).

Biofuels are energy sources that are derived from biological materials i.e. plant and animal materials and can be solid, liquid or gaseous (Demirbas, 2008). Depending on the feedstock

used, biofuels can be classified into first, second and third generation biofuels. First generation biofuels are made from sugars, starch and vegetable oils, while second generation biofuels are made from sustainable feedstocks such as lignocellulose. Third generation biofuels are produced from crops that require further research and development such as fast growing trees and algae (Petrobras, 2010). Lignocellulose is considered to be a highly sustainable feedstock due to its abundance and the fact that it does not divert food away from man and animal food chain (Petrobras, 2010).

Production of ethanol from lignocellulosic biomass involves the hydrolysis of cellulose by cellulases to produce reducing sugars, which are further fermented to produce ethanol. In order for the chemical reactions to be more rapid, temperatures of 70^{o}C or even 80^{o}C are required (Caltech, 2009). Cellulose swells at these temperatures making it easier to degrade. This implies that thermostability is a major requirement for efficient hydrolysis of cellulose by cellulases. Thermostable enzymes last longer and this translates to more cellulose being broken down hence lowering the cost of production (Caltech, 2009). Known cellulases from nature cannot function at temperatures higher than about 50^{o}C (Caltech, 2009). This makes the cellulose hydrolysis stage a major challenge in the production of biofuel from lignocellulosic biomass. Consequently there is an increasing demand for stable, highly active and specific cellulases at a minimal cost (Chinedu *et al*., 2008).

There are several approaches that can be used to obtain enzymes with improved stability. These include; directed evolution (Turner, 2003; Cherry & Fidantsef, 2003; Zhao *et al*., 2002; Lutz & Patrick, 2004; Robertson & Steer, 2004; Arnold *et al*., 2001), protein engineering (Bornscheuer,

2005) and bioprospecting i.e. screening for enzymes from organisms living in the appropriate extreme environment (Vieille & Zeikus, 2001; Schiraldi & De Rosa, 2002; Burg, 2003). Kenya is endowed with extreme environments especially along the Rift Valley. Sinking of geothermal wells for energy production may provide worthwhile research areas for cellulolytic microorganisms that may offer a priceless solution in the quest to achieve a sustainable and economically feasible lignocellulosic biofuel production.

In this study traditional microbiological isolation techniques were used to screen for isolates bearing cellulolytic activity. The isolates obtained were identified using both morphological and molecular methods. Functional studies were carried out to determine the optimal pH, temperature and time for enzyme activity for one of the isolates. Temperature and pH are amongst the factors that affect the stability of enzymes. Other factors include binding of metal ions, oxidative stress, solvents and presence of surfactants (Eijsink *et al*., 2005).

CHAPTER 2: LITERATURE REVIEW

2.1. Cellulose

Anselme Payen (1795-1871) coined the term cellulose and introduced it to scientific literature in 1839 after isolating a fibrous substance mostly found in wood, cotton and other plants (Payen, 1838). Higher plant tissues such as trees, cotton, flax, cereal straw represent the main sources of cellulose i.e. it makes up 35-50% of dry plant weight (Lynd *et al*., 1999). Algae such as *Valonia ventricosa* and *Microdicyan* are representatives of lower plants that synthesize cellulose (Boisset, *et al*., 1999; Fierobe, *et al*., 2002). In addition to plants, non-photosynthetic organisms such as bacteria i.e. aerobic *Acetobacter xylinum*, marine invertebrates from the ascite family i.e. tunicates, fungi, slime moulds and amoebae also produce cellulose (Tomme *et al*., 1995a; Lynd *et al*, 2002).

Cellulose is a crystalline polymer of β (1-4) linked D glucopyranose residues. Adjacent residues are arranged such that the glucosidic oxygens of adjacent monomers point in opposite direction to form cellobiose; the repeating unit of cellulose. Cellobiose units are then linked to form an extended, straight chain as shown below (Brown *et al.,* 1996).

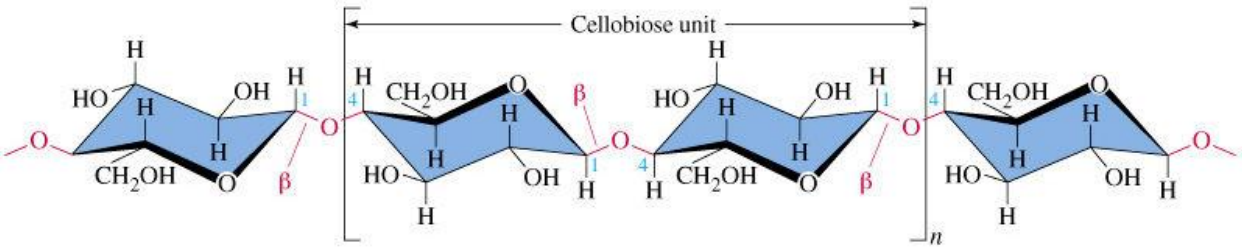
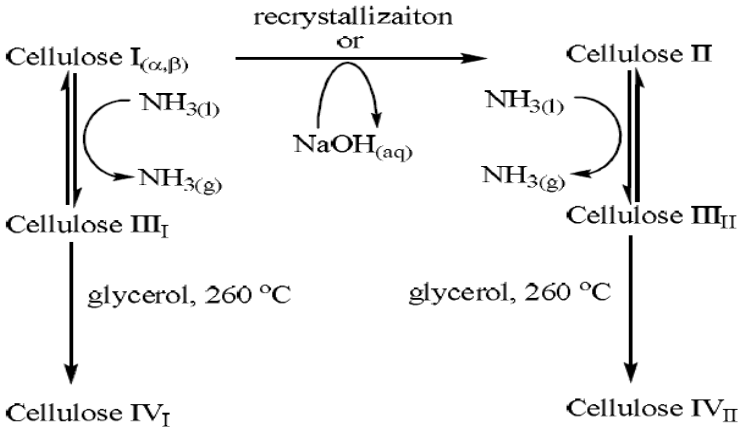


Figure 1: Structure of a cellulose chain. The repeating unit, cellobiose, is indicated in brackets while glucosidic oxygens are marked by a "β" (Adapted from Brown *et al.,* 1996).

One chain end consists of a D - glucopyranose residue in which the anomeric carbon atom is involved in a glycosidic linkage while the residue on the other chain end has a free anomeric carbon thus the two chain ends are chemically different giving the molecule a polarity (Pe´rez & Mazeau, 2005).

The chain length denoted as the degree of polymerization (DP) is approximately 10,000 - 15,000 glucopyranose residues. These parallel chains are held together by intrachain and interchain hydrogen bonds that aggregate the cellulose molecules to form microfibrils. Microfibrils consist of crystalline and amorphous regions. Crystalline regions arise due to tight packing of the chains while amorphous regions arise due to loose packing (Warren, 1996). These are eventually assembled into cellulose fibers (Lynd *et al*, 2002). This compact structure makes cellulose to be highly inert and resistant (Sjöström, 1993).

Native cellulose from nature is referred to as Cellulose I. Other forms of cellulose include cellulose II, III_I, $III_{II,}$ IV_I, IV_{II}. These are modifications produced by chemical interconversion of native cellulose (O'Sullivan, 1997).



Polymorphs of cellulose (Adapted from O' Sullivan, 1997)

Figure 2: Polymorphs of cellulose (Adapted from O' Sullivan, 1997)

Cellulose I is a mixture of two allomorphs; cellulose Iα and cellulose Iβ (Atalla & Vanderhart, 1984). Cellulose Iα is predominant in algae and bacteria celluloses while the later is predominant in cotton - ramie types (Horii *et al*., 1987).

In most cases, cellulose fibers are found embedded in a matrix of lignin and hemicelluloses. This forms lignocellulose, the solid framework of the plant cell wall. Lignin and hemicellulose comprise 5-30% and 20-35% respectively (Lynd *et al*., 1999; Marchessault & Sundararajan, 1993; Van Soest, 1994).These matrix interactions vary with plant cell type and plant maturity (Wilson, 1993). In cotton balls however, cellulose fibers are present in nearly pure state (Lynd *et al*., 2002).

2.2. Cellulose degrading enzymes

Plant cell materials are composed of intertwined polysaccharides chains (cellulose) of varying crystallinity, hemicellulose and pectin embedded in lignin (Lynd *et al*., 2002). In order to degrade them, microorganisms produce multiple enzymes known as enzyme systems (Warren, 1996). Complete degradation of cellulose requires a suite of enzymes collectively referred to as cellulases. These are hydrolytic enzymes that hydrolyze the β (1-4) glucosidic linkages to smaller oligosaccharides and eventually glucose. This ability to hydrolyse β (1-4) glucosidic linkages distinguishes cellulases from other hydrolases.

Cellulases are produced by a large diversity of microorganisms during their growth on cellulolytic substrates (Kubicek, 1993; Sang-Mok & Koo, 2001). Their structure is composed of independently folding, structurally and functionally discrete units called domains (Henrissat *et al*.,1998). A free cellulase enzyme structure is composed of two domains, a catalytic domain and a cellulose binding domain (CBD) also referred to as a carbohydrate binding module (CBM). The two are linked by flexible linker peptide. The catalytic domain is classified into a glycosyl hydrolase family with other members according to amino acid sequence homology. The positions of the catalytic residues are usually conserved within a given hydrolase family (Bayer *et al*., 1998). The CBD adsorbs to accessible sites of cellulose containing substrates to form an enzyme cellulose complex held together by specific non-covalent interactions. Formation of this complex is a prerequisite for cellulose hydrolysis (Klyosov, 1990). Nonspecific interactions have been noted between cellulases and lignin (Ooshima *et al*., 1990; Tatsumoto, *et al*., 1988).

2.3. Mechanism of cellulose hydrolysis

A cellulase enzyme system comprises of three classes of enzymes; endoglucanases (EC 3.2.1.4), exoglucanases (EC 3.2.1.91) and β glucosidase (EC 3.2.1.21). Exoglucanases are further grouped into glucanohydrolases (cellodextrinases) and cellobiohydrolases. These categories are based on their structural properties and mode of action (Henrissat *et al*., 1998; Henrissat & Davies, 2000). Endoglucanases randomly cleave at the internal sites of cellulose to yield oligosaccharides of various lengths. Exoglucanases on the other hand act on the reducing or non-reducing end of cellulose to liberate glucose, cellobiose or cellooligosaccharides, which are finally hydrolysed to glucose by β glucosidases (Sukumaran *et al*., 2005).

This enzyme system exhibits synergy, a phenomenon in which the collective enzyme activity is higher than the sum of activities of individual enzymes. Four forms of synergy have been reported. Exo-exo synergy between exoglucanase attacking the reducing and the non-reducing ends of cellulose; endo-exo synergy between endoglucanases and exoglucanases; Exo-β glucosidase synergy and intramolecular synergy between the catalytic domain and the CBMs (Din *et al*., 1994; Driskill *et al*.,1999).

Cellulolytic anaerobes have an extracytoplasmic cellodextrinase for hydrolyzing cellodextrins and intracellular cellodextrin and cellobiose phosphorylases (CdP and CbP). These phosphorylases catalyse Pi mediated phosphorylation of cellodextrins and cellobiose respectively to yield glucose 1 monophosphate (G-1-P) which is converted to Glucose 6 Phosphate (G-6-P), the entry point to Embden-Meyerhoff pathway (Lynd *et al*., 2002). Other bacteria produce intracellular β glucosidases which cleave cellobiose and cellodextrins to produce glucose which

is assimilated by the microbes (Karmakar & Ray, 2011). Simultaneous presence of extracellular cellodextrinases, intracellular CbP and CdP activities, and intracellular β glucosidases in cellulolytic microorganisms suggest that metabolism of cellobiose and cellodextrins probably occurs through several pathways. (i) Extracellular hydrolysis of the substrates i.e. cellobiose and cellodextrins and subsequent uptake and metabolism. (ii) Direct uptake followed by intracellular phosphorolytic cleavage and subsequent catabolism. (iii) Direct uptake by the organism followed by hydrolytic cleavage and metabolism (Lynd *et al*., 2002). Cellulosic substrates occurring in nature contain hemicellulose and lignin which impedes the access of cellulase components to β (1-4) glucosidic linkages thus other hydrolytic enzymatic activities distinct to those of cellulases are required. Enzymatic cleavage of the β 1 - 4-glucosidic linkages in cellulose proceeds through an acid hydrolysis mechanism, using a proton donor and nucleophile or base (Lynd *et al*., 2002).

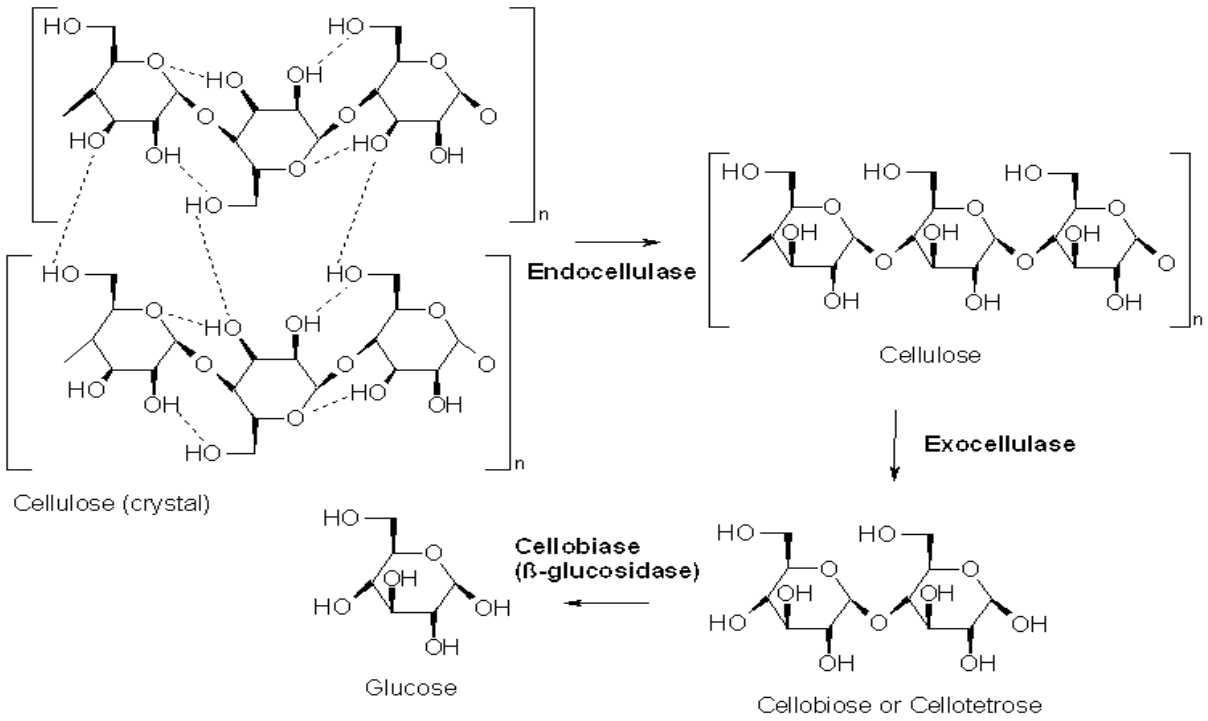


Figure 3: A schematic representation of cellulolysis

The three types of reactions catalyzed by cellulases: (1) Breakage of the noncovalent interactions present in the amorphous structure of cellulose by endoglucanase (2) Hydrolysis of chain ends to break the polymer into smaller sugars by exoglucanase (3) Hydrolysis of disaccharides and tetrasaccharides into glucose by β-glucosidase (Adapted from Karmakar & Ray 2011).

2.4. Cellulase enzyme systems

Cellulose utilization takes place in both aerobic and anaerobic microorganisms. Members of the genus *cellulomonas* are the sole facultatively anaerobic degraders reported so far (Bagnara, *et al* 1985; Bagnara *et al*.,1987; Clemmer & Tseng, 1986; Dermoun & Belaich, 1988).

Cellulase enzyme systems are generally classified into two; complexed (Shoham *et al*.,1999; Schwarz, 2001) and non complexed (Stutzenberger, 1990; Teeri, 1997). This classification is dependent on whether the microorganism is aerobic or anaerobic (Lynd *et al*., 2002).

2.4.1. Non complexed systems

In non complexed cellulase systems components are free and mostly secreted thus can be recovered from the culture supernatant. These are normally found in aerobic cellulose degraders i.e. both fungi and bacteria (Rapp & Beerman, 1991). Cellulases from aerobic fungi are by far the most studied group (Lynd *et al*., 2002). Representatives in this category include *Trichoderma reesei* previously *Trichoderma viride. T. reesei* produces cellobiohydrolases CBHI and CBHII, eight endoglucanases EGI-VIII and seven β- glucosidases BGI-VII (Pakula & Penttila, 2005). The cellulase enzyme system from *Humicola insolens* is homologous to that of *T. reesei* with at least seven cellulases i.e. Two cellobiohydrolases CBHI and CBHII and five endoglucanases EGI, EGII, EGIII, EGV and EGVI (Schülein, 1997).

Most aerobic bacteria species are found in soil. They fall in genera that are known for non growth associated metabolism (secondary metabolism) that include formation of dormant states (*Bacillus, Miromonospora* and *Thermobifida* and production of secondary metabolites such as antibiotics *(Bacillus* and *Micromonospora* (Lynd *et al.,* 2002). Most aerobic bacteria adhere to cellulose but the physical contact is not necessary for cellulose hydrolysis (Kauri & Kushner, 1985).

2.4.2. Complexed system

Anaerobic cellulose degraders degrade cellulose via a complexed system; a cellulosome (Schwarz, 2001). Cellulosomes are protuberances on bacterial cell wall that harbor enzyme complexes. These enzyme complexes are firmly bound on to the cell wall but flexible enough to bind cellulose. Cellulosomes from different *Clostridia* (*Clostridium thermocellum*, *Clostridium cellulolyticum*, *Clostridium cellulovorans*, and *Clostridium josui*) and *Ruminococcus* species in the rumen have been studied. Cellulosome enzyme sub units are not any different from free cellulases. Both have catalytic domains from the same glycosyl hydrolase families. The major difference between these two enzyme types is that all cellulosomal enzymes have a dockerin domain which mediates the integration of the enzyme into the cellulosome complex. Free cellulases however lack a dockerin domain but have a catalytic binding module that helps binding of a given catalytic domain to the substrate (Bayer *et al*.,1994; Tomme *et al*., 1995b; Be´guin & Lemaire, 1996).

The cellulosome structure from *C. thermocellum* was resolved through a combination of biochemical, immunochemical, ultra structural and genetic techniques. It consists of a large noncatalytic and multimodular scaffoldin protein (CipA) of 197kDa which is anchored to the cell wall via type II cohesin domains. A total of 22 catalytic modules have dockerin moieties that can associate with the cohesins of the CipA protein to form the cellulosome. Nine of these catalytic modules exhibit endoglucanase activity (CelA, CelB, CelD, CelE, CelF, CelG, CelH, CelN, and CelP), four exhibit exoglucanase activity (CbhA, CelK, CelO, and CelS), five exhibit hemicellulase activity (XynA, XynB, XynV, XynY and XynZ), chitinase activity is exhibited by ManA and lichenase activity is exhibited by LicB (Bayer *et al*., 1994).

The cellulosome is thought to bring enzyme activity in close proximity to the substrate thus facilitating optimum synergy by the cellulases present in the cellulosome and also to minimize the distance over which hydrolysis products diffuse thus allowing for efficient uptake of oligosaccharides by the cell (Bayer *et al*., 1994; Schwarz, 2001).

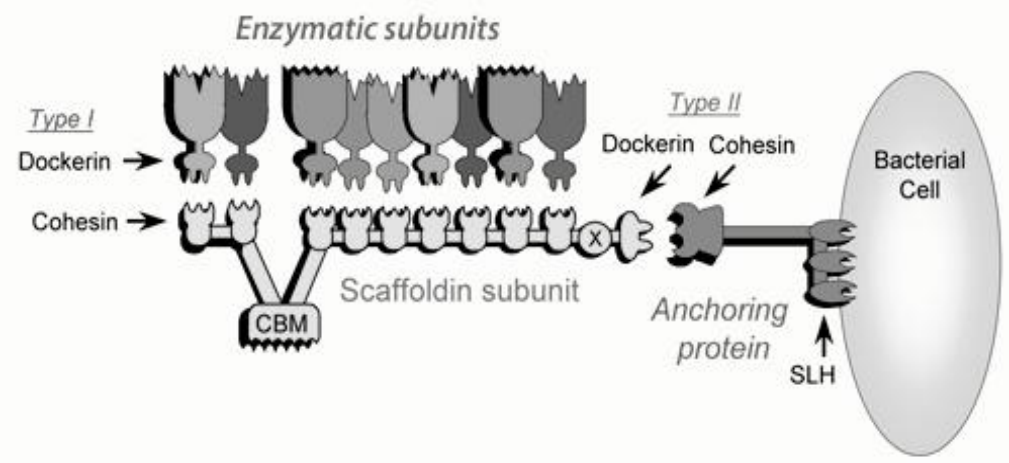


Figure 4: Schematic representation of the structure of the cellulosome present in bacteria (Adapted from Karmakar & Ray, 2011).

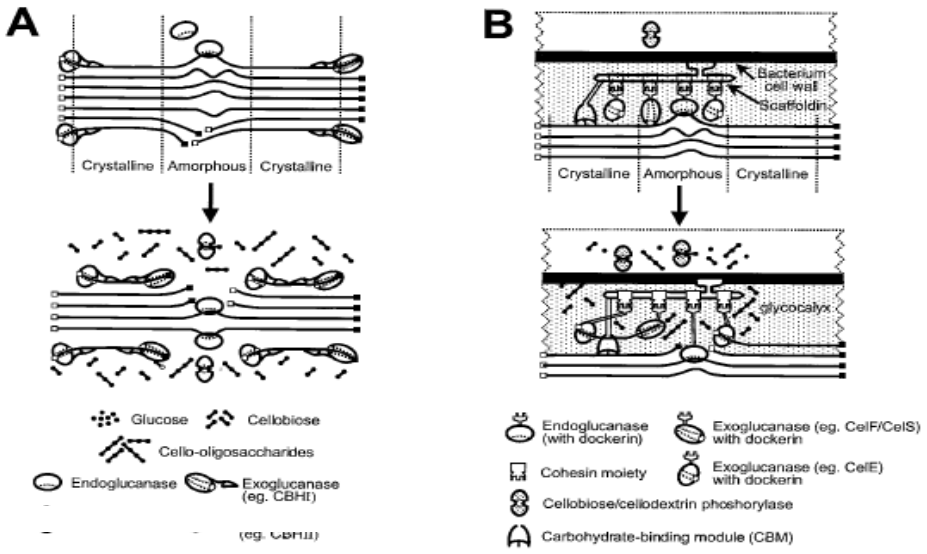


Figure 5: A schematic representation of the hydrolysis of amorphous and crystalline cellulose by (A) non complexed (B) Complexed cellulase systems (Adapted from Lynd *et al*., 2002).

2.5. Taxonomic diversity of cellulolytic microorganisms

Ability to degrade cellulose is widely distributed in several fungal and bacterial genera. In addition to these two, the domain eubacteria has a considerable distribution of cellulytic capability. Members in the aerobic order *Actinomyctes* and anaerobic order *Clostridiales.* Fungi are the main agents of decomposition of organic matter in general and especially cellulosic substrates (Lynd *et al*., 2002; Montegut *et al*., 1991) and it's no surprise that cellulolytic capability is distributed across the entire kingdom from the advanced *Basidiomycetes* to the primitive *Chytridomycetes* (Lynd *et al*., 2002). Chytridomycetes are known to degrade cellulose in gastrointestinal tracts of ruminant animals (Orpin, 1977).

Cellulolytic capability is however not exclusive to microorganisms. Species such as termites and cray fish produce their own cellulases that are different from those produced by their indigenous micro flora (Orpin, 1977). There is a broad distribution of cellulolytic capability and it's possible that a primordial ancestor acquired it early in the evolutionary development. This however may not be the case because cellulose biosynthesis capability evolved much later with the development of land plants, algae amongst others (Lynd *et al*., 2002).

Table 1: Fungi and bacteria with cellulolytic capability (Adapted from Kuhad *et al.,* 2011)

Fungi	Soft rot fungi *Aspergillus niger, A. nidulans, A. oryzae, A. terreus; Fusarium solani, F.oxyspourm; Humicola insolens, H.grisea; Melanocarpus albomyces; Penicillium brasilianum, P.occitanis, P.decumbans, P. janthinellum ; Trichoderma reesei, T. harzianum, T. longibrachiatum, T.atroviride; Chaetomium cellulyticum, C. thermophilum; Thermoascus aurantiacus; Mucorcircinelloides; Paelomyces inflatus, P. echinolatum.*
	White rot fungi *Phanerochaete chrysosporium; Sporotrichum thermophile; Trametes versicolor; Agaricus arvensis; Pleurotus ostreatus; Phlebia gigantea.* *Brown rot fungi* *Coniophora puteana; Lanzites trabeum; Poria placenta, Tyromyces palustris; Fomitopsis sp*

Bacteria	Aerobic bacteria *Acinetobacter junii, A. amitratus; Acidothermus cellulolyticus; Anoxybacillus sp; Bacillus subtilis, B.pumilus, B. licheniformis, B. amyloliquefaciens, B. circulans, B. flexus; Bacteroides sp; Cellulomonas biazotea; cellvibrio gilvus; Eubacterium cellulosolvens, Geobacillus sp; Microbispora bispora; Paenibacillus curdlanolyticus; Pseudomonas cellulose; Salinivibrio sp; Rhodothermus marinus.* Anaerobic bacteria *Acetovibrio cellulolyticus; Butyrivibrio fibrisolvens; Clostridium thermocellum; C. cellulolyticum; C. acetobutylium; C. papyrosolvens; Fibrobacter succinogenes; Ruminoccus albus*

2.6. Ecological aspects of cellulose degraders

Cellulose is widely available in many habitats that differ in their characteristics i.e. water availability, oxygen availability, redox potential and temperature among others. These different habitats call for development of different cellulose utilization strategies such as enzyme architecture and presentation, extend and rate of cellulolysis, fate of hydrolytic products and interactions between cellulolytic and non-cellulolytic microorganisms (Lynd *et al*., 2002).

In soils, cellulose is available in the form of dead plant material, which is highly recalcitrant due to the high lignin content. In addition to this, deficiency of fixed nitrogen and other nutrients together with low moisture content limit microbial growth. This favors the growth of fungi as the predominant cellulose degraders (Lynch, 1988). In a study by Vardavakis, (1989) cellulolytic

activity and production of $^{14}CO_2$ from [^{14}C] cellulose was found to decrease with depth implying that cellulose utilization is a majorly an aerobic process. The primary bacterium isolates included *Cytophaga, Bacillus* and *Cellulomonas* species. Decomposing cattle manure on the other hand was found to have very few fungal isolates while *Micromospora, Cytophaga* and *Sporocytophaga* bacterial genera were abundant (Godden & Penninckx, 1984).

Plant biomass produced in aquatic environments has low lignin content and is mainly degraded by bacteria, which are poor lignin degraders but better adapted to the aquatic environments. Plant detritus settles at the bottom and establishes an enrichment zone which is anoxic resulting in the proliferation of anaerobic cellulolytic degraders. Cellulolytic enzymes are presented as a polycellulosome complex by cells adhering to the substrate to maximize hydrolysis and capture of hydrolytic enzymes (Lynd *et al.,* 2002).

The digestive system of herbivorous animals resembles the anaerobic aquatic habitats. It's characterized by retention of plant fiber in the rumen to allow growth of cellulolytic microbes which include bacteria and *Chytridomycetes* fungi. Both are destroyed in the acidic abomasum due to the acidic conditions and lysozyme to yield amino acids which are absorbed in the small intestines. This contributes protein to the nutrition of the ruminant (Van Soest, 1994).

2.7. Regulation of cellulase production

Cellulase is an inducible enzyme system where enzyme production is regulated by activation and repression mechanisms (Sukumaran *et al*., 2005). In *T. reesei*, production of cellulase genes is regulated at the transcriptional level. The expression of cellulase genes (cbh1, cbh2, egl1, egl2

and egl5 in *T. reesei* strain QM9414 is coordinated by transcriptional factors (Ilme´n, *et al*., 1997). Cellulase genes have binding sites for both transcriptional activators i.e. Activator of cellulase expression proteins I & II (ACEI & ACEII) and catabolite repressor protein I (CRE I) in addition to the CCAAT sequence which binds general transcription activator complexes designated 'HAP' proteins (Narendja *et al*., 1999). Genes encoding the transcriptional factors ACEI (Saloheimo *et al*, 2000) and ACEII (Aro *et al*., 2001) were identified due to their ability to bind to the *T. reesei* cbh1 promoter region.

Several carbon sources have been tested to find the best inducer (Mandels & Reese, 1960; Mandels, 1975). Substrates are known to induce the synthesis of the enzymes that catalyze their hydrolysis (Reese *et al*., 1969). Products of hydrolysis can often induce their respective polysaccharasses; galacturonic acid for polygalacturonases in *Peni**cillium chrysogenum* (Phaff, 1947), xylose for pentonase in several molds (Simpson, 1954), maltose for amylase in *Aspergillus niger* (Mandels & Reese, 1957), N-acetylglucosamine for chitinase in *Aspergillus fumigatus* and *Myrothecium verrucaria* (Mandels & Reese, 1957). The use of a product as an inducer often leads to lower enzyme yields than what is obtained with the substrate (Mandels & Reese, 1957).

Production of cellulolytic enzymes in *T. reesei*, production is induced in the presence of cellulose and repressed by the availability of easily utilizable sugars (Sukumaran *et al*., 2005). In order to serve as an inducer, a substance must access the site of enzyme production (Mandels & Reese, 1960). The insolubility and size of cellulose makes it hard to enter the cell. This probably implies that the hypothesis that an inducer has to access the enzyme production site is not sufficient to

explain why cellulose is the best inducer (Mandels & Reese, 1960). Other inducers are sophorose, cellobiose, δ-cellobiose-1-5- lactone and lactose (Mandels & Reese, 1957; Vaheri *et al*., 1979; Nogawa *et al*., 1979). When organisms are grown on dimmers like cellobiose, enzyme yields are much lower than those obtained from growth on the polymeric cellulose because the soluble sugars are rapidly metabolized and therefore repress enzyme formation. Similar results can be obtained by using metabolites that are not inducers (Mandels & Reese, 1960). Repression can be avoided when using soluble induces to obtain high enzyme yields. This is achieved by supplying the inducer continuously in low quantities, retarding metabolism by unfavorable growth conditions such that the inducer is slowly consumed, supplying a modified soluble substrate which is slowly broken down by the organism to release the inducer (Mandels & Reese, 1960). Production of cellulases was found to be increased by use of non ionic surfactants (Reese & Maguire, 1969).

2.8. Applications of cellulases in the industries

For years, cellulases have been a target for academic and industrial research and are currently being applied in many industries (Singh *et al.*,2007).

2.8.1. Cellulases in the textile industry

The textile industry has been revolutionized by introduction of enzymes that are slowly replacing the conventional chemical processes, which are generally severe and lead to fiber damage (Bhat, 2000; Kuhad *et al*., 2011). Cellulases have the ability to modify cellulosic fiber in a controlled and desired manner thus improving the fabric quality (Mojsov, 2012). They are mostly used during wet processing to improve fabric properties. Processes that involve cellulase activity

include biostoning of jeans and biopolishing cellulosic fibers**.** Denim is heavy grade cotton and when dyed, the dye is mainly adsorbed on the surface of the fibre. When cellulases are used during the biostoning process, they break off small fiber ends on the yarn surface to loosen the dye, which is consequently easily removed during the wash cycle by mechanical abrasion. This enzyme based treatment replaced pumice stone biostoning hence less damage to the fiber, increased productivity and a safe working environment (Christian *et al*., 2006; Karmakar & Ray, 2011).

Fading can be achieved without loss of fabric strength. Fabrics made from cellulosic fibers such as cotton, linen, ramie, viscose and lyocell are normally characterized by short fibers protruding from the surface (fuzz formation) and 'pilling' i.e. loosened fuzz attached to the surface. This often decreases their market value and in order to prevent this, a process called biopolishing is done. Biopolishing is usually done during the wet processing stage and includes desizing, scouring, bleaching, dyeing and finishing. Cellulase mixtures usually rich in endoglucanases are used in this process to remove the small protruding fibers from the fabric surface without using chemicals. The fabric attains a smooth and a glossy appearance, improved brightness and uniformity. Biopolishing is a key procedure in the production of high quality garments (Bhat, 2000; Kuhad *et al*., 2011).

2.8.2. Cellulases application in the wine and brewing industry

Enzyme technology plays a crucial role in the beer and wine industries. Wine making requires the extraction of juice from grapes and subsequent fermentation by yeast while beer brewing involves malting of barley and fermentation of the resulting wort (Bhat, 2000). Brewing of beer

is based on activity of the enzymes activated during malting and fermentation stages. Malting is dependent on seed germination, which initiates biosynthesis and activation of α and β amylases, carboxy peptidases and β glucanase that hydrolyses the seed reserves (Bamforth, 2009). Under optimal conditions all the three enzymes act in synergy to produce high quality malt. However due to seasonal variations and or poor harvest, brewers end up using poor quality barley which contains low levels of endogenous β glucanase activity. This results in the presence of a 6 -10 % of non starch polysaccharide mainly soluble β glucan which forms a gel during the brewing process leading to poor filtration of the wort, slow run off time, low extract yield and development of a haze in the final product (Galante *et al*., 1998b). The viscosity of the wort is usually reduced by addition of microbial β glucanases, which hydrolyse β glucan. Commonly used microbial β glucanases are obtained from *Penicillium emersonii, Aspergillus niger, Bacillus subtilis* and *Trichoderma reesei* (Galante *et al*., 1998b).

A study carried out by Oksanen *et al*., (1985) observed that endoglucanase II and cellobiohydrolase II of the *Trichorderma* cellulase system were responsible for most activity in reduction of the degree of polymerization and wort viscosity thus they are best suited for the production of high quality beer from low quality barley. In wine making, pectinases, β glucanases and hemicellulases comprise the main exogenous enzymes added. These enzymes give a better skin maceration, improved colour extraction, easy must clarification and filtration and improved wine quality and stability (Galante *et al*., 1998b). In order to improve the wine's aroma, β glucosidase is added to modify glycosylated precursors that are naturally present (Caldini *et al*., 1994; Gunata, *et al*., 1990).

2.8.3. Cellulases in the detergent industry

Recent innovations in the detergent industry have seen the incorporation of enzymes such as cellulases, proteases and lipases in detergents (Singh *et al*., 2007). Due to repeated washing, cotton and cotton blend fabrics become dull and fluffy due to the presence of detached microfibrils. Cellulase containing detergents are capable of degrading the cellulose microfibrils to restore a smooth surface and original colour to the garment. In addition, the degradation softens the fabric and removes dirt particles trapped in the microfibril network (Sukumaran *et al*., 2005; Singh *et al*., 2007). Cellulase preparations from *H. insolens* that are active under mild alkaline conditions (pH 8.5 - 9) and temperatures over 50^{o}C are added to detergents. Such cellulases active under alkaline conditions increase the cleaning capacity of detergents by selective contraction fibers hence facilitating the removal of oil from inter fiber space (Karmakar & Ray, 2011).

2.8.4. Cellulases in pulp and paper industry

Application of enzyme preparations comprising cellulases, xylanases and lignases in the pulp and paper industry has increased in the last decade (Mai *et al*., 2004; Karmakar & Ray, 2011). Pulping starts with the conversion of woody raw material into a flexible fiber that can be made into paper. Depending on the application of the paper, various methods of pulping can be used (Bajpai, 2012). Mechanical pulping usually involves mechanical grinding of the woody material to give fibers that can be used in the production of different grades of paper. This method is usually characterized by high energy consumption and gives paper with incompletely ground fiber bundles, low strength and tends to yellow with time due to little removal of lignin a weakness associated with the process (Bhat, 2000).

Bio pulping using cellulases and allied enzymes reduces the energy required to achieve the desired strength and freeness of the pulp hence it's a better alternative to mechanical pulping (Karmakar & Ray, 2011). Cellulases containing enzyme mixtures are also useful in the hydrolysis of 'fines' smal particles produced during refining of primary or secondary fibres. These particles usually reduce the drainage rate of pulp during the paper making process. Hydrolysis of these particles improves the pulps drainage property which inturn determines the paper mill's speed. Addition of these preparations before refining is either done to improve the beatability response or modify the fiber properties (Noe *et al*., 1986; Pommier *et al*., 1989; Pommier *et al*., 1990).

Deinking process is crucial during paper recycling. Enzymatic deinking using cellulases reduces the need for deinking chemicals and also results to little or no loss in paper strength. Enzymatic deinking is usually combined with mechanical agitation in order to improve the efficacy of the process (Karmakar & Ray, 2011).

2.8.5. Cellulases in Agriculture

Cellulolytic fungi including, *Trichoderma sp*, *Geocladium sp*, *chaetomium sp* and *Penicillium sp* are known to play an important role in agriculture by facilitating enhanced seed germination, rapid plant growth and flowering, improved root system and increased crop yields (Bailey & Lumsden, 1998; Harman & Kubicek, 1998).

Cellulases and related enzymes from certain fungi are capable of degrading the cell wall of plant pathogens hence controlling plant diseases. The β-1, 3- glucanase and N-acetyl- glucosaminidase

from *Trichoderma harzianum* were reported to synergistically inhibit the spore germination and germ tube elongation of *B. cinerea* (Lorito *et al*.,1994; Bhat, 2000). The β- 1, 3- glucanase from *Trichoderma harzianum* CECT 2413 induced morphological changes such as hyphal tip swelling, cytoplasm leakage and the formation of numerous septae and also inhibited the growth of *R. solani* and *Fusarium sp* (Benitez *et al.*,1998).

Cellulases are also important in soil quality improvement. In order to reduce overreliance on mineral fertilizers, farmers incorporate straw in soil. Microbial routes to hasten straw decomposition using organisms such as *Aspergillus*, *Chaetomium* and *Trichoderma* and *actinomycetes* have shown promising results (Ortiz Escobar & Hue, 2008; Tejada, *et al*., 2008).

2.8.6. Cellulases in the food industry

Industries producing fruit juices in the 1930s encountered challenges such as low yield and a poor clarity of the product (Uhlig, 1998). Research on industrially suitable enzymes such as cellulases, hemicellulases and pectinases from food grade microorganisms such as *Aspergillus niger, Trichoderma sp* and increased knowledge of fruit components led to the overcoming of these challenges and led to improved methods of extraction, clarification and stabilization (Singh *et al.*, 2007). Cellulases along with xylanases and pectinases are the macerating enzymes that serve to increase the yield and process performance without any additional cost. Macerating enzymes are usually used in two steps; after crushing, the fruit pulp is macerated to either partial or complete liquefaction. After the extraction, pectinases are then used for its clarification and this lowers viscosity of fruit juice prior to its concentration and further increases the filtration rate and the stability of the juice. Macerating enzymes also improve the cloud stability, texture,

decrease viscosity and facilitate easy concentration of nectars and purees (Grassin & Fauquembergue, 1996a).

There is a growing demand for natural pigments for food colorants such as carotenoids. In their natural state, carotenoids remain bound to proteins thus preventing pigment oxidation. When solvents are used to extract carotenoids, they disrupt that association thus making the pigments insoluble in water and oxidation. This can be prevented by use of enzymatic methods. Cellulases hydrolyze cellulose in the cell walls hence the structural rigidity is interfered with exposing intracellular materials for extraction. These pigments remain bound to proteins and are more stable than those obtained through traditional methods that involve use of solvents (Bassi *et al*., 1993).

2.8.7. Cellulases in the Biorefinery

Bioconversion of lignocellulosic biomass to produce biofuel is the most popular area of cellulase application being investigated recently (Sukumaran *et al*., 2005). Potential lignocellulosic feedstocks sources include agricultural crop residues such as stover and straw, the perennial prairie grass, municipal waste, packaging and construction debris, agricultural or forest processing by products e.g. food processing residues, pulping liquor from paper mills and forest woody biomass either logging residues from conventional harvest operations or removal of excess biomass from timberlands (NREL, 2006).

Lignocellulosic biomass consists of cellulose tightly linked to lignin and hemicellulose (Kuila *et al*., 2011). Cellulose must be separated from lignin and hemicelluloses in order to make it more

accessible to the hydrolytic enzymes through pretreatment. Pretreatment methods used can be physical, chemical or biological but the latter are not yet intensely developed as the physical and chemical methods (Kuila *et al*., 2011). Conversion takes place in two phases; hydrolysis of cellulose into fermentable reducing sugars by cellulases and fermentation of the sugars to ethanol, a process carried out by yeast or bacteria (Sun & Cheng, 2002). The cost of ethanol production from lignocellulosic materials is relatively high with the main challenges being low yield and a high cost of hydrolysis. Studies on optimization of the cellulase enzymes and enzyme loading can be done in order to improve the hydrolytic process (Sun & Cheng, 2002). Screening for cellulolytic microorganisms from extreme environments will also enrich the current databanks.

2.9. Enzyme assays

Characterization of cellulase enzyme systems poses a challenge rarely encountered on other enzyme studies to enzymologists. The insolubility and structural variability of cellulose, synergistic activities between endoglucanases and exoglucanases and various mechanisms of feedback control make cellulase enzyme kinetics difficult (Ghose, 1987). Assays for determining cellulase activity have been classified into three main groups (Zhang *et al*., 2006). The 1st group involves assays in which accumulation of products after the hydrolytic process is targeted, the 2nd groups are assays in which the reduction of the substrate concentration is monitored and the 3rd groups are for assays in which change in physical properties of the substrate are monitored.

The 1st group of assays is preferred when measuring individual cellulase activities in a short time and the 3rd group is preferred for total enzyme activities within a given time (Wu *et al*., 2006;

Zhang *et al*., 2006). For soluble enzyme the sample is filtered or centrifuged to remove solids and the filtrate or the supernatant is analyzed for enzyme activity while for cell bound enzymes, the cells are homogenized in an appropriate buffer such as 0.05M citrate, pH 4.8 and enzyme powders dissolved at 1-5mg/ml in buffer (Ghose *et al*., 1987).

Assay activities may not reflect potential saccharification performance since other factors such as end product inhibition, addition of cellobiase activity, reactor process configuration come into play in commercial cellulose hydrolysis. These factors vary with different cellulase systems and significantly affect the conversion efficiency (Ghose *et al*., 1987).

2.10. Problem statement

Successful utilization of lignocellulose for biofuel production depends on the development of economically feasible technologies for hydrolysis of lignocellulose into low molecular products and their subsequent fermentation into commercial products such as ethanol (Chinedu *et al*., 2008; Mathew *et al*., 2008). This is however not the case with the current technologies which are characterized by high costs and low yields of the hydrolysis process (Sun & Cheng, 2002). Cellulases currently applied in this industry have been isolated from various species of plant decaying filamentous fungi and are slow and unstable and this makes their utilization expensive (Caltech, 2009). The extreme environmental resistance of bacteria thus permits screening and isolation of novel cellulases to overcome existing challenges in biorefinery (Maki *et al*., 2011).

2.11. Research objectives

2.11.1. Main objective

The overall objective of this study is to isolate and characterize bacteria isolates with cellulolytic activity for potential application in biorefinery from Hells gate and Eburru ecosystem of Kenya's Rift valley.

2.11.2. Specific objectives

i. To characterize using morphological and molecular methods, isolates bearing cellulolytic activity from Hells gate and Eburru.
ii. To determine the evolutionary relationship between characterized isolates from Hells gate and Eburru with other isolates in the DNA databases.
iii. To determine enzyme specificities of the cellulases (endoglucanase, exoglucanase and β-glucosidases) produced by selected isolate
iv. To determine the optimum conditions (pH, temperature and optimum time) for isolate's endoglucanase enzyme activity.

2.12. Justification

The application of enzymes in industries is quite attractive because biocatalysts have a better biochemical precision and pose a low environmental burden (Rozzell, 1999). The main challenge to their application is the harsh conditions prevalent in industries that these enzymes cannot withstand.

Screening for microorganisms in extreme environments (bioprospecting) is a powerful technique for discovering robust enzymes since extremophilic enzymes are often active under conditions similar to those under which the host resides. Screening for cellulose degraders in extreme environments may provide new isolates whose cellulase genes form the foundation for protein engineering and protein modeling strategies that will see the biorefinery industry get enzymes with improved activity (Maki *et al*., 2011).

In addition each small step will make ethanol production more economically feasible and discourage the overreliance on fossil fuels and also allow progression towards a renewable energy production era.

CHAPTER 3: METHODOLOGY

3.1. Sample collection

Four soil samples were collected from Hells gate and 155, 156 and 157 metres deep in a geothermal well located in Eburru hill. The soil samples were transported to the laboratory in ziplock bags and stored in a refrigerator at 4^{o} C for subsequent use.

3.2. Isolation of cellulolytic bacteria

One gram of the each soil sample was suspended in 9.0 mL of physiological saline (0.85% NaCl) to suspend the cells and an aliquot of this mixture transferred to a selective medium containing 5g/L Carboxymethylcellulose (CMC) (Sigma, St. Louis, USA), 2.2g/L, 1.5g/L KH_2PO_4, 1.3g/L $(NH_4)_2SO_4$, 0.1g/L $MgCl_2$, 0.02g/L $CaCl_2$ and 0.001g/L $FeSO_4.7\ H_2O$ pH 6.9 (Modified Bagnara *et al*., 1985). Incubation was done in a thermoshaker (Gallenkamp, London, England) at 45^{o}C for 7 days. Further isolation was carried out by plating on CMC agar plates (5g/L CMC, selective medium supplemented with 1g/L yeast extract and 15g/L agar). The pH was adjusted to 6.9 and overnight incubation done in a shaker at 45^{o}C, 150 revolutions per minute (rpm).

3.3. Screening for cellulolytic activity

Cellulolytic activity of the bacterial isolates was screened on a solid medium containing 5g/L CMC, 2g/L Yeast extract, 5g/L Malt extract and 15g/L Agar, pH 7.2 (modified, DSMZ medium 65 without $CaCO_3$ and glucose (Wenzel *et al*., 2002)) and incubated for 3 days at 45^{0}C. Cellulase activity was tested by soaking the plates with 1mg/ml Congo red (Sigma, St. Louis, USA) for 15 minutes followed by destaining with 1M NaCl. Clear zones were seen against the red color of Congo red for the positive tests. Positive isolates were used for further studies.

3.4. Preparation of glycerol stocks of the bacteria isolates

Glycerol stocks of the isolated strains were prepared by mixing 500 µL of an overnight culture with 500µL of 60% sterile glycerol (Sigma, St. Louis, USA) in sterile microcentrifuge tubes and the cells dispersed by vortexing briefly before being stored at -80ºC for future use.

3.5. Characterization of the bacteria isolates from soil samples

3.5.1. Morphological characterization

Gram stain test was carried out on the bacterial isolates as described in the manual of veterinary laboratory techniques in Kenya (1981). A bacterial smear from a pure culture was prepared and fixed on a clean glass slide. The slide was flooded with crystal violet for 1 minute, and rinsed with running tap water. The slide was then flooded with Gram's iodine for 1 minute, and again rinsed with running tap water. This was followed by decolourization with 95% ethanol and rinsing with tap water. The slide was counter stained with Safranin for 1 minute, rinsed with running tap water and allowed to air dry. The dry slide was covered with immersion oil and viewed under a Leica ICC 50 microscope (Leica microsystems,Wetzlar,Germany).

3.5.2. Molecular characterization

3.5.2.1. Genomic DNA extraction

Bacteria cells were cultured in nutrient broth (Sigma, St. Louis, USA) containing meat extract 1 g/L, yeast extract 2 g/L, peptone 5 g/L and sodium chloride 5 g/L final pH 7.4±0.2 at 37^{o} C. Nutrient broth was prepared by adding distilled water to 3.25 gm of the powder to make 250 ml and sterilization done by autoclaving at 121^{o} C for 15 minutes. Incubation was done overnight at 45^{o}C and 150 rpm. The procedure described by Kate Wilson (1987) was used to harvest total

genomic DNA from the bacterial isolate. Briefly, 1.5 mL of the culture was spun in a microcentrifuge tube for two minutes at 10,000 rpm (Hettich Zentrifugen,Tuttlingen, Germany). The supernatant was discarded with a sterile tip and the resulting pellet resuspended in 547 µL TE (10 mM Tris HCl, 1 mM EDTA) buffer. To the resuspended pellet, 10 µL of lysozyme (10 mg/mL) was added, thorough mixing done and and resulting mixture incubated further at 37^{o}C for one hour before adding 30 µL of SDS and 3 µL proteinase K (20 mg/mL), mixed thoroughly and incubation done at 37^{o}C for 1 hour. To the mixture, 100 µL of 5 M NaCl and 80 µL of 10% CTAB/NaCl solution were added and mixed well prior to incubation at 65^{o}C for ten minutes. DNA was then extracted sequentially with Phenol/Chloroform/Isoamyl alcohol (25:24:1) and Chloroform/Isoamyl alcohol (24:1). Isopropanol alcohol (0.6 volumes) was used to precipitate the DNA and thereafter, the pellet resuspended in 50 µl of TE buffer (pH 8.0) containing RNAse to get rid of RNA. The amplified products were electrophoresed on a 1% agarose gel in 1× TAE buffer containing 0.5 µg/mL of ethidium bromide and visualized under UV light before storage at -20ºC.

3.5.2.2. DNA analysis by TAE/agarose/EtBr gel electrophoresis

The quality of genomic DNA was analysed on 1% (w/v) agarose (Sigma, St. Louis, USA) gel in 1× TAE buffer. A 1% TAE /agarose/EtBr gel was prepared by boiling 0.7 g agarose in 70 mL of 1× TAE. The hot agarose solution was allowed to cool down to about 60ºC prior addition of ethidium bromide to a final concentration of 0.5 µg/mL. The solution was then poured into the gel casting chamber and a comb placed in position. After polymerization (30 minutes) the gel was transfered into the electrophoresis chamber with the slots facing the cathode and covered with a running buffer (1× TAE buffer). 6× orange DNA loading dye (Fermentas, Pittsburgh,

USA) was premixed with the DNA samples in the ratio 1:5 µL of sample (final concentration: 1×) prior to loading of the samples onto the wells in the gel.

The sample was electrophoresed at 5 V/cm (80V) for genomic DNA and 10 V/cm (120 V) for PCR products for forty five minutes. The current was supplied by an electophoresis power supply (Consort EV265, Holliston, USA). A 1 kb DNA ladder (Fermentas, Pittsburgh, USA) was run alongside DNA samples. The DNA bands were visualised under a UV transilluminator (Herolab,Wiesloch, Germany).

3.5.2.3. PCR amplification of 16S rDNA

An aliquot (1 µL) of genomic DNA extracted from each isolate was used as a template to amplify 16S rDNA gene. Polymerase chain reaction was performed in a TProfessional thermocycler (Biometra,Gottingen, Germany). The 16s rDNA gene was amplified using two bacterial specific primers: 16S F27, forward 5'…AGA GTT TGA TC(AC) TGG CTC AG…3' and 16S R 1492, reverse 5'…TAC GG(CT) TAC CTT GTT ACG ACT T…3'. The PCR reaction was performed in a total volume of 50 µl thin-walled PCR reaction tube (Simport,Quebec,Canada) using 36.5 µL PCR grade water, 5 µL 10× Dream Taq PCR buffer (Fermentas, Pittsburgh, USA), 4 µL $MgCl_2$ (25 mM) 1 µL dNTP mix (10 mM), 1 µL of each primer (200 µM), 0.5 µL Dream Taq DNA polymerase (Fermentas, Pittsburgh, USA), (5 U/µL) and 1 µL of isolated DNA. The thermocycler (Biometra, Göttingen, Germany) conditions for the PCR were as follows: An initial denaturation step at 94ºC for five minutes followed by 35 cycles of 94ºC for one minute, 55ºC for one minute and 72ºC for two minutes and a final extension step of seven minutes at 72ºC. The PCR products were electrophoresed on a 1% agarose gel in 1×

TAE buffer containing 0.5 µg/mL of ethidium bromide and visualized under UV light to determine their sizes and quality. The PCR products were then gel purified using MinElute Gel Extraction Kit (QIAGEN, Venlo, Netherlands) according to manufacturers protocol and sent to ILRI (International Livestock Research Institute, Kenya) for sequencing. A consensus sequence for the each isolate's 16S rDNA was generated using Chromas Lite and sequences deposited in Genebank.

3.5.2.4. Phylogenetic analysis

The 16S rDNA sequences obtained were compared with known 16S rDNA sequences at National Center for Biotechnology Information (NCBI) database using BLAST (Basic Local Alignment Search Tool) algorithm obtained from; http://www.ncbi.nlm.nih.gov/BLAST. All the sequences, including 20 retrieved from the database were then aligned using Multiple Sequence Comparison by Log-Expectation (MUSCLE) algorithm (Edgar, 2004). Phylogenetic trees constructed based on the nucleotide sequences with the Bayesian phylogenetic method in MrBayes software obtained at http://mrbayes.net. The trees were then visualised using fig tree software obtained at http://tree.bio.ed.ac.uk/.

3.6. Enzyme assays

3.6.1. Production of crude enzyme

A volume of 150 mL of nutrient broth media (Sigma, St. Louis, USA) supplemented with 1% CMC (Modified Lin *et al*., 2012) was prepared and sterilized by autoclaving at 121^{o} C for 15 minutes in an autoclave (All American, Hillsville, USA). The bacteria was inoculated and cultured for 24 hours at 45^{o}C and 150 rpm. The culture was then centrifuged at 4000 rpm

(Thermo Scientific, Pittsburgh, Germany) 4^{o}C for ten minutes and the supernatant (crude enzyme extract) stored at -20^{o} C for subsequent use. In all the enzyme assays, one unit of activity was defined as the amount of enzyme producing 1μmol of reducing sugar measured as glucose per mL per minute under assay conditions (Bischoff *et al*., 2006; Dashtban *et al*., 2010).

3.6.2. Determination of crude enzyme concentration

Determination of crude enzyme protein concentration was determined using biuret's test. Bovine serum Albumin (BSA) was diluted with distilled water to a volume of 1 mL and a concentration range of (0-10 mg/mL) in triplicates. To these test tubes, 4 milliliters of biuret reagent was added. The test sample was prepared by adding 4 milliliters of biuret reagent to 1mL of the crude enzyme extract. Incubation was done at room temperature for 30 minutes and absorbances taken at 540 nm. Absorbances obtained were used to plot a standard curve against concentration (mg/mL).

3.6.3. Determination of endoglucanase activity

For this assay, 500μL of crude enzyme was mixed with 500μL substrate (1% CMC prepared in 50mM Sodium acetate buffer pH 5) and incubated in a water bath (Laboratoire Kastler Brossel, Bromma, Sweden) at 60^{o}C for 30 minutes. All procedures were carried out in ice. The quantity of glucose produced was assayed by Dinitrosalicylic Acid test (DNS) (modified Miller, 1959) as described in section 3.6.2.1. A control prepared with heat denatured crude enzyme extract (100^{o}C for 60 minutes) and substrate (1% CMC prepared in 50mM Sodium acetate buffer pH 5) was included.

3.6.3.1. Dinitrosalicylic acid test

An aliquot of 1mL from section 3.6.2 was pipetted into a 15 mL falcon tube, 1 mL of DNS reagent was added to stop the reaction and the resulting mixture boiled at 95^{o} C for 15 minutes until the solution turned brown in color before addition of 333 µl of 40% Rochelle salt (Sodium potassium tartrate). The contents of the falcon tube were cooled, serial dilutions prepared with distilled water and absorbance taken at 540 nm using a digital spectrophotometer (Digilab Hitachi, Tokyo, Japan). Using the established glucose standard curve, the amount of glucose produced was calculated and values obtained used to determine enzyme activity. One enzyme unit (U) was defined as the amount of enzyme producing 1 µmol of reducing sugar measured as glucose per minute per mL.

$$\text{Specific enzyme activity} = \frac{\text{Enzyme units (U/mL)}}{\text{Protein concentration (mg/mL)}}$$

3.6.4. Determination of exoglucanase activity

For this assay, 500 µL of crude enzyme was mixed with 500 µL substrate (1% Avicel prepared in 50 mM Sodium acetate buffer pH 5) and incubated in a water bath at 60^{o}C for 30 minutes. The quantity of glucose produced was assayed by Dinitrosalicylic Acid test (DNS) as described in section 3.6.3.1.

3.6.5. Determination of β-glucosidase activity

For this assay, 500µL of crude enzyme was mixed with 500µL substrate (1% Cellobiose prepared in 50mM Sodium acetate buffer pH 5.5 and incubated in a water bath at 60^{o}C for 30

minutes. The quantity of glucose produced was assayed by Dinitrosalicylic Acid test (DNS) as described in section 3.6.3.1.

3.6.6. Determination of optimum temperature for endoglucanase activity.

To determine the effect of temperature on enzyme activity, an aliquot of 500µl crude enzyme extract was mixed with 500µL of the substrate (1% CMC prepared in 50mM sodium acetate buffer pH 5) and incubated in a water bath at 30^{o}C for 60 minutes. The quantity of glucose produced was assayed by Dinitrosalicylic Acid test (DNS) as described in 3.6.2.1. The procedure was repeated at 40^{o}C, 50^{o}C, 60^{o}C, 70^{o}C, 80^{o}C and 90^{o}C. Using a glucose standard curve, amount of glucose produced was calculated and values obtained used to determine specific enzyme activity.

3.6.7. Determination of optimum pH for endoglucanase activity

The effect of pH on enzyme activity was determined by mixing 500µL of the crude enzyme extract with 500µl substrate at various pH values (1% CMC prepared in 50mM Glycine-HCl pH 3.0, 50mM HAc-NaAc (pH 4.0-5.0), 50mM Na_2HPO_4-NaH_2PO_4 (pH 6.0 - pH 8.0) and 50mM Glycine-NaOH (pH 9.0-11.0) buffers (modified Lin *et al*., 2012). The reaction mixture was incubated in a water bath at pre-determined optimum temperature of 60^{o}C (3.6.2.2) for 60 minutes. Amount of glucose produced was assayed by carrying out a DNS test. Using a standard curve, amount of glucose produced was calculated and values obtained used to determine specific enzyme activity.

3.6.8. Determination of optimum time for endoglucanase activity

To determine the optimum time an aliquot (500µL) of the crude enzyme extract was mixed with an equal volume of substrate (1% CMC in 50mM sodium acetate buffer pH 5 and incubated for 30 minutes at 60^0C. DNS test carried out to determine amount of glucose produced (3.6.3.1). The procedure was repeated for a range of time from 60 to 480 minutes. Using the glucose standard curve, amount of glucose produced was calculated enzyme activity expressed as enzyme units (U/mL). Values obtained were used to plot a bar graph against time.

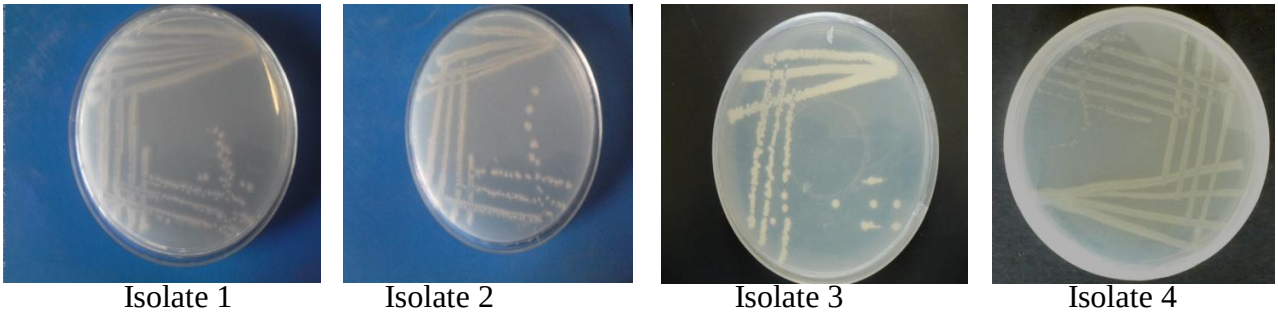
3.9. Data analysis

All analyses were carried out in triplicates and the experimental data analyzed using one way Analysis of variance (ANOVA) using Statistical Package for Social Sciences (SPSS, 2007) version 16.0. Post hoc test (Tukey's test) was used to evaluate differences among mean values for treatments at $P<0.05$. The data was presented as mean ± standard error (SPSS, 2007).

CHAPTER 4: RESULTS

4.1. Isolation of soil bacteria

Figure (6) shows four isolates from soil samples collected from Hells Gate Eburru 155, Eburru 156 and Eburru 157 soils respectively. The isolates were treated as different strains and denoted strain 1, strain 2, strain 3 and strain 4 from Hells gate, Eburru 155, Eburru 156 and Eburru 157 soils respectively.

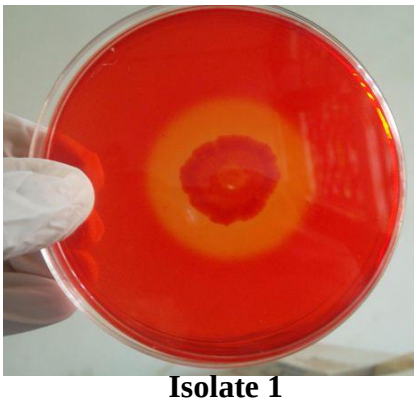


Isolate 1 Isolate 2 Isolate 3 Isolate 4

Figure 6: Photographs of pure bacteria isolates from soils obtained from Hell's gate, Eburru 155, Eburru 156, and Eburru 157 within the Kenyan Rift Valley.

4.2. Screening for cellulolytic activity

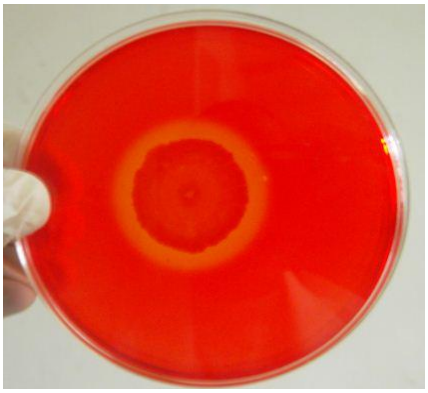
Test for cellulolytic activity was done on CMC medium (modified DSMZ medium 65 without $CaCO_3$ and glucose). CMC degradation was detected by staining the plates with 1mg/ml Congo red dye followed by destaining with 1M NaCl. Degradation of cellulose was indicated by the presence of a clear zone around the bacterial colonies. All the four strains were found to have cellulolytic activity as shown in Figure 7.



Isolate 1

Isolate 2

Isolate 3



Isolate 4

Figure 7: Photographs of CMC containing agar plate depicting cellulolytic activity of the four bacteria isolates

4.3. Characterization of bacteria isolates

4.3.1 Morphological characterization

The isolates' morphological features were determined by Gram stain test. All the isolates retained the purple colour of crystal violet stain implying that they were Gram positive rods as shown in Figure 8.

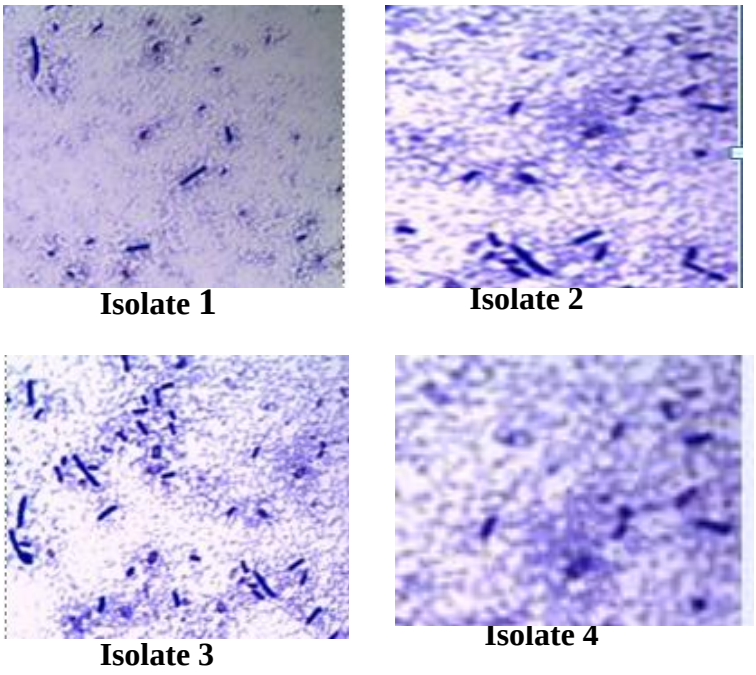


Figure 8: Gram stain photographs of the four cellulolytic bacteria isolates.

4.3.2. Molecular characterization

4.3.2.1. Genomic DNA and 16S rDNA gel analysis

The quality of genomic DNA extracted from the bacterial isolates and their corresponding 16S rDNA PCR amplicons were analysed on a 1% agarose gel stained with ethidium bromide. Figure (9) shows gel photographs of genomic DNA and the 16S rDNA PCR amplicons from the four bacteria isolates.

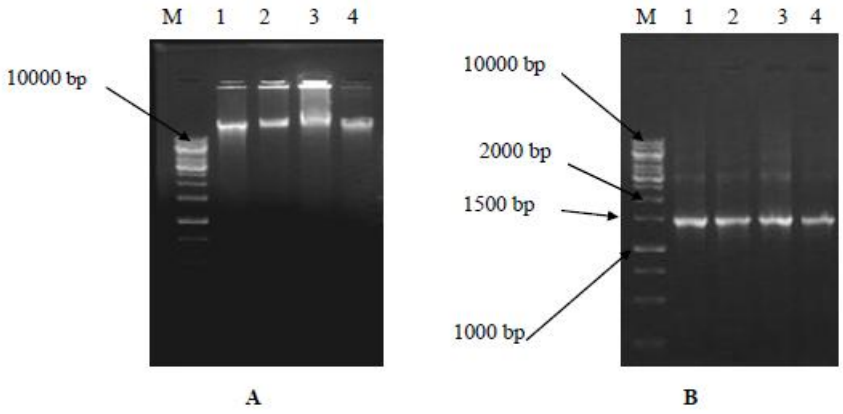


Figure 9: Agarose gel analysis of {(A) genomic DNA from isolates 1, 2, 3 and 4} and {(B) 16S rDNA PCR products for isolates 1, 2, 3 and 4}. Lane M is the molecular ladder (1Kb ladder Fermentas). The 16S rDNA sequences were assigned accession codes KF737352, KF737353, KF737354 and KF737355 for isolates 1, 2, 3 and 4 respectively.

4.3.2.2. Alignment of 16S rDNA sequences

A multiple sequence alignment of the 16S rDNA sequences for isolates 1 to 4 using Jalview 2.8 a software provided by Java Bioinformatics Analysis Web Services as shown in Figure 10.

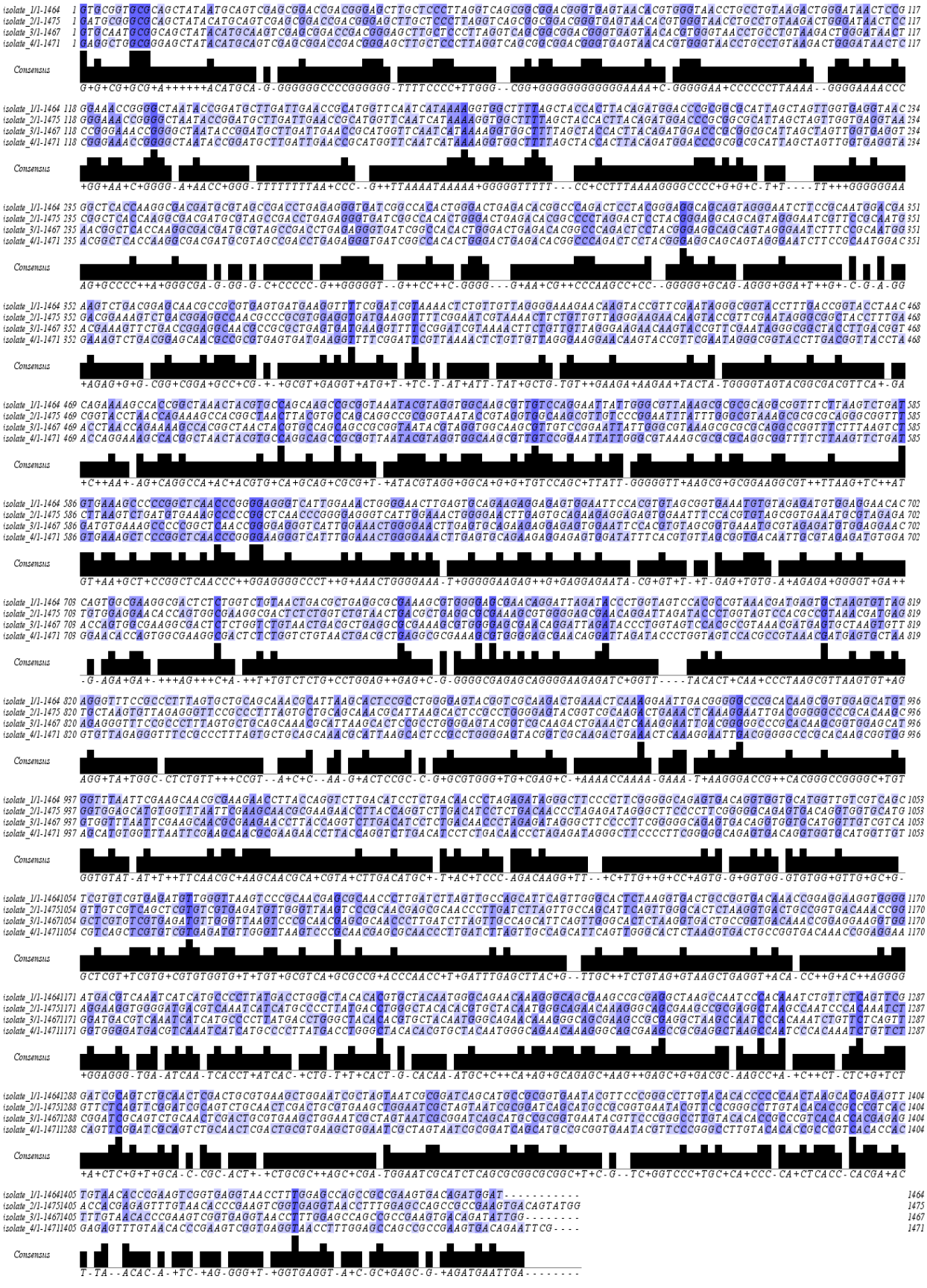


Figure 10: A multiple sequence alignment showing variation in the bases of the isolates 1 to 4 and a consensus. Sections highlighted in blue show similarity in bases for that position.

4.3.2.3. Phylogenetic analysis

A phylogenetic tree based on the blast search was constructed and the topological robustness of the tree was evaluated using percentages of the posterior probabilities. Figure (11) shows the phylogenetic tree for 16S rDNA gene sequences.

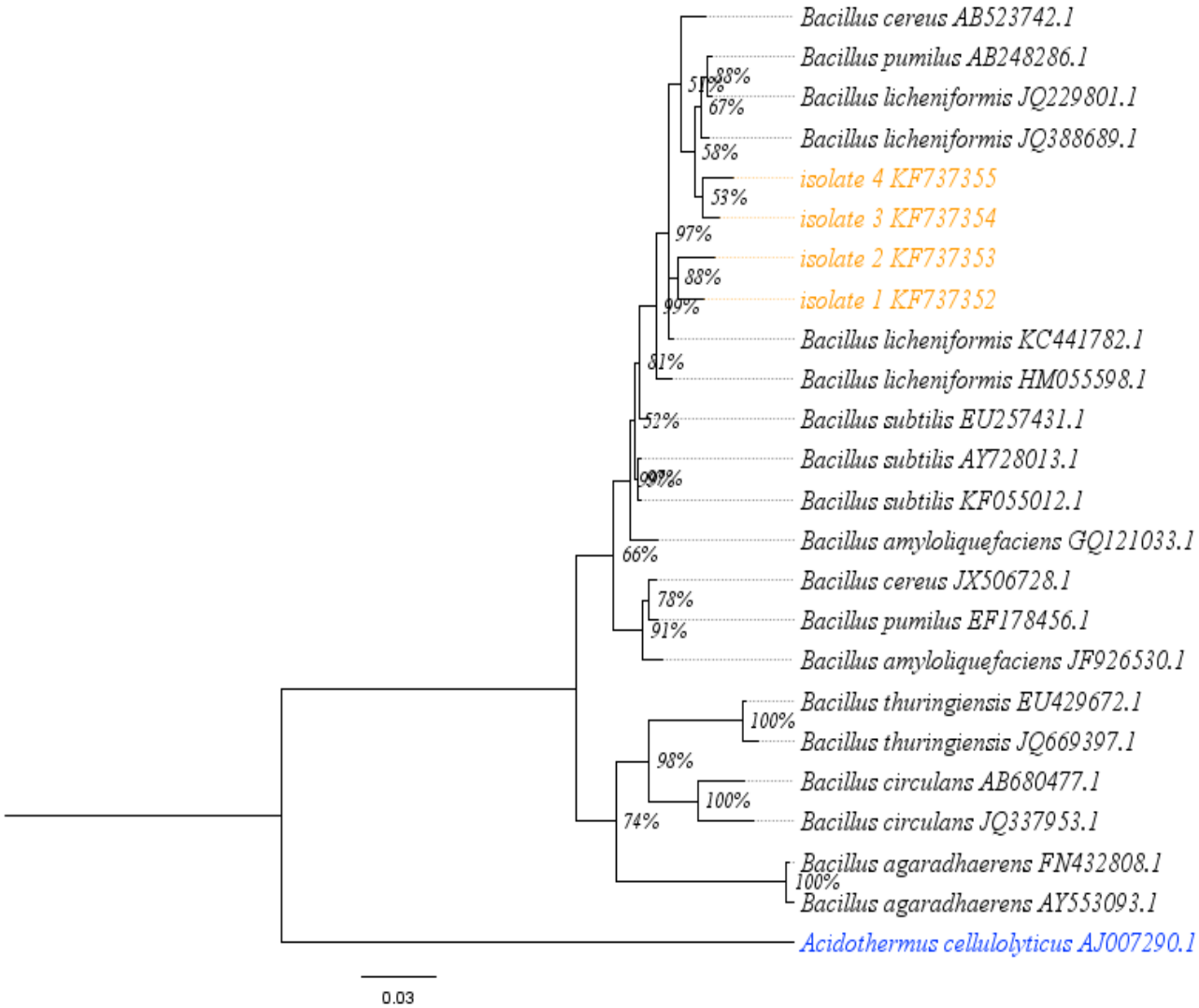


Figure 11: A Phylogenetic tree based on 16S rDNA gene sequences**.**

The tree was constructed in MrBayes, a program for the Bayesian inference of phylogeny that is based on the Markov Chain Monte Carlo (MCMC) method. Numbers at the nodes show percentage of posterior probabilities indicating topological robustness of the Phylogenetic tree. The tree is rooted by a 16SrDNA sequence from *Acidothermus Cellulolyticus.* The four bacterial isolates clustered with *Bacillus licheniformis* strains suggesting they were likely to be *Bacillus licheniformis.*

4.4. Enzyme assays

4.4.1. Generation of a glucose standard curve

A standard curve generated from dinitrosalicylic acid test (DNS) test using a range of D-glucose concentrations (0-0.75mg/ml) is shown in Figure (12).

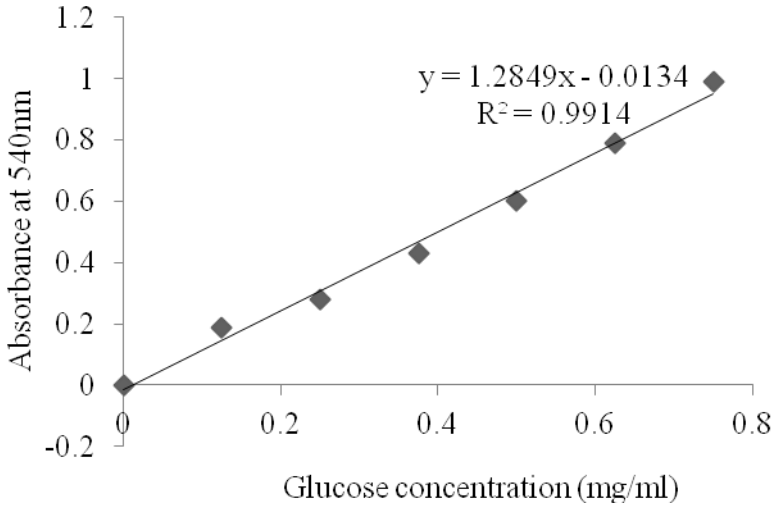


Figure 12: Glucose standard curve for the determination of the quantity of reducing sugar produced from enzyme assays and for determination of optimum temperature, pH and time for endoglucanase enzyme activity.

Absorbances were read at 540 nm. R^2 represents a coefficient of determination. It indicates how well data points fit a line, curve or a statistical model while y represents absorbance of the sample.

4.4.2. Generation of a Bovine Serum Albumin (BSA) standard curve

A standard curve generated from a biuret test using BSA (0-10mg/ml) and biuret reagent for the determination of the protein content in the crude enzyme extract is shown in Figure (13).

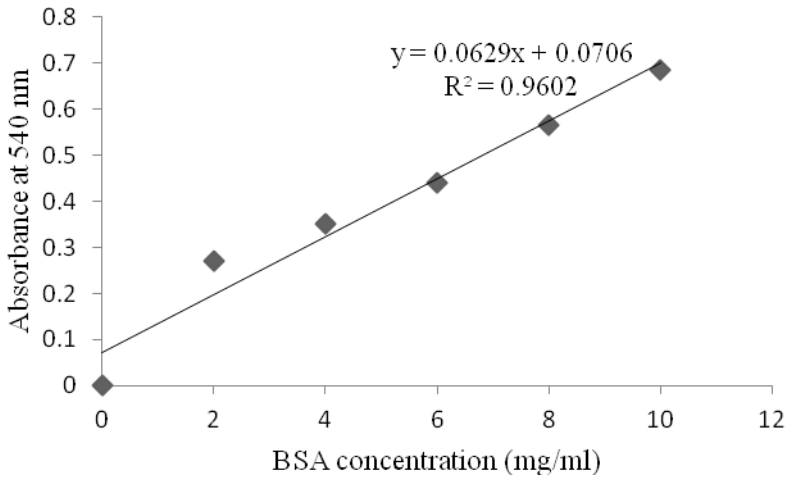


Figure 13: BSA standard curve for the determination of protein quantity in the crude enzyme extract. Absorbances were read at 540 nm.

R^2 represents a coefficient of determination. It indicates how well data points fit a line, curve or a statistical model and y represents absorbance of protein sample. Protein concentration was found to be 1.7122 mg/mL.

4.4.3. Determination of cellulase specificities produced by Isolate 3.

Cellulase specificities were determined using an aliquot of the crude enzyme sample and 1% of substrate (CMC, Avicel and Cellobiose respectively) at pH 5 and 60 ^{o}C. There was a significant difference in enzyme activities for the three enzyme specificities (Figure 14). Post hoc test were carried out using tukey's test at a significance level of 0.05.

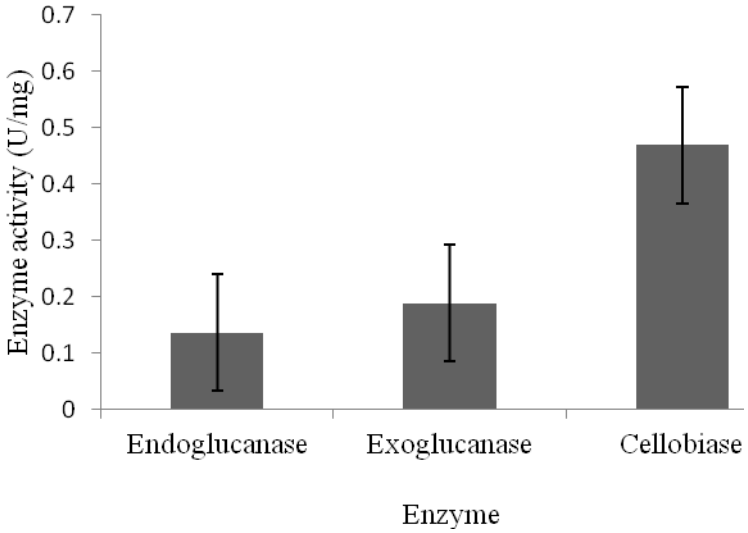


Figure 14: Specific enzyme activity of the different enzyme types produced by isolate 3. Error bars have been displayed using the standard error of the mean. Cellobiase activity was the highest 0.46878 U/mg, exoglucanase 0.18784 U/mg and endoglucanase 0.13571 U/mg.

4.4.4. Determination of optimum temperature for endoglucanase activity

Enzyme assays were carried out using carboxymethylcellulose at temperatures ranging from 30^{o}C to 90^{o}C and pH 5 in order to determine the optimum temperature. There was a significant change in enzyme activity with change in temperature (Figure 15). Post hoc test were carried out using tukey's test at a significance level of 0.05.

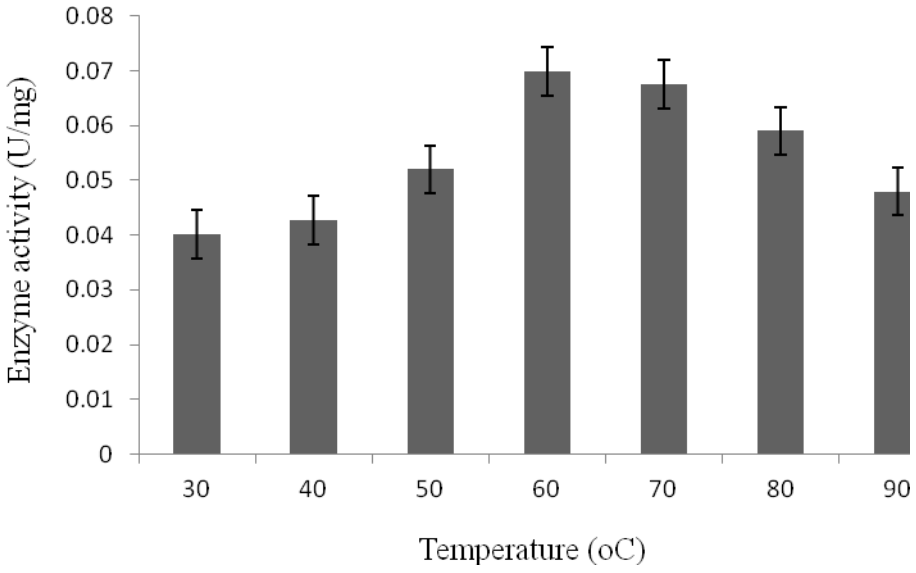


Figure 15: Specific enzyme activity at different temperatures. Error bars have been displayed using the standard error of the mean. Optimum activity was recorded at 60^{o}C

4.4.9. Determination of optimum pH for endoglucanase activity

Enzyme assays were carried out using carboxymethylcellulose at pH values ranging from 3 to 11 in order to determine the optimum pH. There was a significant change in enzyme activity with change in pH (Figure 16). Post hoc test were carried out using tukey's test at a significance level of 0.05.

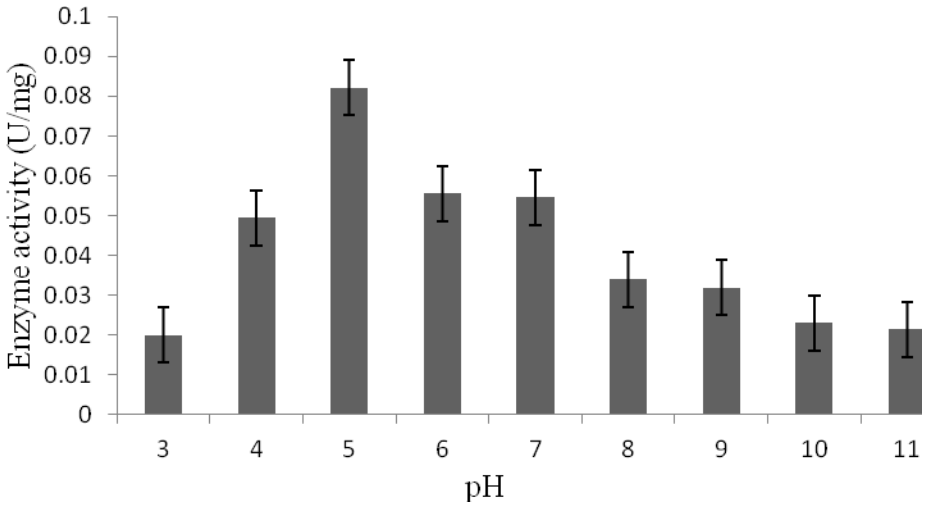


Figure 16: Specific enzyme activity at different pH. Error bars have been displayed using the standard error of the mean. Optimum activity was recorded at pH 5.

4.4.6. Determination of optimum time for endoglucanase activity

Enzyme assays were carried out using carboxymethylcellulose at different time intervals under the predetermined optimum pH and temperature (pH 5 and 60 ^{o}C) to determine the optimum time. There was a significant change in enzyme activity with change in duration of incubation $P<0.05$ (Figure 17). Post hoc test were carried out using tukey's test at a significance level of 0.05.

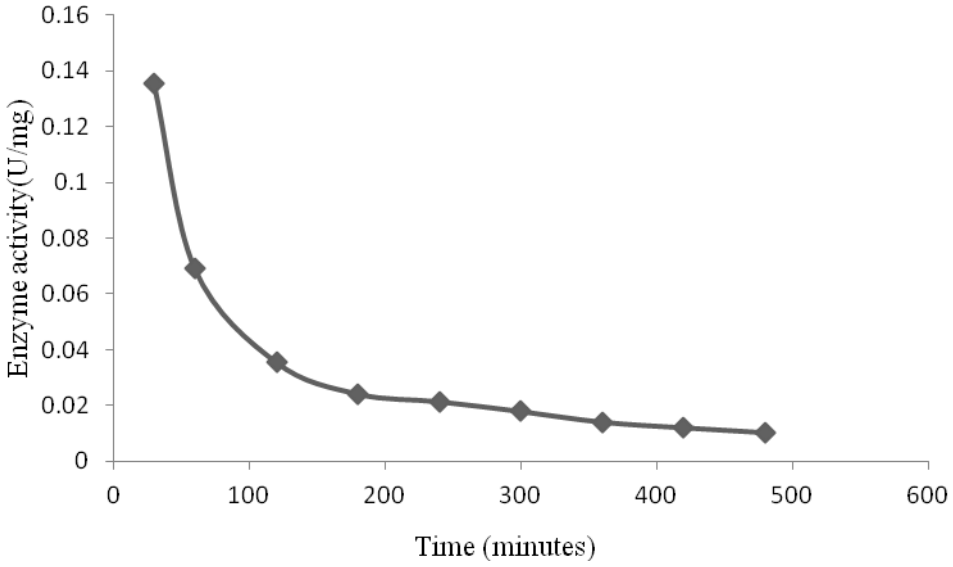


Figure 17: The trend of specific enzyme activity at different incubation periods, at 30 minutes and thereafter 60 minutes to 480 minutes at intervals of 60 minutes. Optimum activity was recorded at 30 minutes.

CHAPTER 5: DISCUSSION

Cellulose is the most abundant plant biomass on earth with an estimated annual production of 4.0×10^7 units (Bakare *et al*., 2005). This abundance confers to cellulose an immense potential as a renewable source of energy. In order to convert cellulose into soluble sugars for fermentation, various bioconversion methods such as pyrolysis, acid hydrolysis and enzymatic hydrolysis can be applied (Cooney *et al*., 1978). The latter is more environmental friendly, and gives a pure product with consumption of less energy (Bakare, 2005; Fennington *et al*., 1982).

Cellulolytic microorganisms such as fungi and bacteria are responsible for much of the cellulose degradation in soils, though some insects, crayfish and mollusks produce their own cellulases to utilize cellulose (Watanabe & Tokuda 2001; Ohkuma, 2003; Breznak, 1982). Despite this vast number of cellulase producers, there is a deficiency of microorganisms that can produce significant amount of the three cellulase enzyme specifities i.e endoglucanases, exoglucanases and cellobiases to efficiently degrade cellulose to fermentable products (Maki *et al*., 2011). In addition to this, majority of the studies have been focused on fungi with less emphasis on bacterial sources for cellulase production. Due to their extremely high natural diversity, bacteria have the capability to produce stable enzymes that can be applied in industries such as biorefinery (Bhat, 2000; Bischoff *et al*., 2006; Camassola *et al*., 2004; Haakana *et al*., 2004). These two factors were the prime motivation in this study; to isolate and characterize cellulolytic bacteria from extreme environments.

5.1. Isolation and characterization of cellulolytic bacteria

Four cellulolytic bacteria isolates were isolated in soils excavated from four different locations within the Kenya's Rift valley (Hells gate, Eburru 155, Eburru 156 and Eburu 157). Attempts to isolate cellulolytic microorganisms from various places have led to the isolation of strong cellulase producers such as *Trichoderma*, *Aspergillus*, *Pellicularia*, *Penicillium*, *Acremonium* and *Humicola*. However, these microorganisms were found to be heat sensitive (Fujimoto *et al*., 2011). To the contrary, the isolates in the current study are thermotolerant cultured at 45^{o}C.

Identification of the four isolates was carried out using the Gram stain procedure. All isolates were found to be Gram-positive rods hence were likely to belong to the *Bacilli* genus. Gram-positive bacteria have a thick mesh like cell wall comprising of 50-90% peptidoglycan while Gram-negative bacteria on the other hand have a thinner cell wall and an additional outer membrane composed of lipids (Hardy, undated). Crystal violet (CV) is the primary stain, it dissociates in aqueous solutions into CV+ and chloride ions. These ions penetrate both the cell wall and cell membrane of the Gram positive and negative bacteria. The CV+ ions interact with the negatively charged components of bacterial cell wall and stain the cells purple. Iodine ions (I^- or I^{3-}) from Grams Iodine interact with the CV+ to form Crystal violet –Iodine complexes (CV-I) within the inner and outer layers of the cell. The decolorization agent (95% ethanol) interacts with lipids on the outer membrane in Gram-negative cells thus loosing it exposing the peptidoglycan layer. The CV-I complexes are washed along with the outer membrane. A Gram-positive bacterium becomes dehydrated from ethanol treatment thus the CV-I complex within the bacteria's cell wall remained trapped due to its thick peptidoglycan layer. Gram-positive

bacterium remains purple while the Gram negative ones pick the positively charged Safranin counterstain to stain pink (Hardy, undated).

5.2 Phylogenetic analysis using 16S rDNA

Classical taxonomy relies on a set of morphological and or biochemical characteristics (Lynd *et al*., 2002), this classification however, is limited by the large diversity and abundance of microorganisms bearing cellulolytic activity. Currently, inferring relatedness between organisms is based on phylogenetic trees that are based on measurements of sequence diversity of chronometers such as 16S ribosomal DNA (16S rDNA). The 16S rDNA has hypervariable regions where the sequences have diverged over evolutionary time. Strongly conserved regions to which primers are designed to bind flank the hypervariable regions enabling their amplification.

From the multiple sequence alignment (Figure 10), nucleotides in positions 105 and 106, 217 to 221, 395 to 398 among others were noted to vary in all the four sequences. For other sections a 50% or 75% similarity in nucleotides was observed in the alignment a strong indication that these four isolates are different from each other.

From the tree topology (Figure 11), the four isolates clustered with members of the Gram positive rods and spore forming genus; *Bacillus* i.e. *B. licheniformis, B. pumilus and B. cereus* in a clade with posterior probability of 97% suggesting the isolates belong to the genus *Bacillus*. Isolates 1 and 2 form a monophyletic clade with a posterior probability of 88%. From the branch length, it appears that isolate 2 has accumulated more mutations hence more variation than

isolate 1. Isolates 3 and 4 form another monophyletic clade with a posterior probability of 53%. From the branch lengths it is evident that more variation has taken place in isolate 4 than in isolate 3.

5.3. Enzyme production

Isolate 3 was cultured in a medium containing 1% CMC; a carbon source to induce the production of cellulases which are inducible enzymes synthesized during the bacteria's growth on cellulosic materials (Sang-Mok & Koo, 2001; Kubicek, 1993). The supernatant was found to bear cellulolytic enzymes (Figure 12) supporting other previous studies that indicated the ability by known members of the *Bacillus* genus to secrete proteins extracellularly (Schallmey *et al*., 2004; Lin *et al.*, 2012).

5.3.1. Determination of cellulase enzyme specificities produced by isolate 3

Cellulase activities are mainly evaluated using a reducing sugar assay to measure end products of the hydrolysis thus assay results are expressed as the hydrolysis capacity of the enzyme (Dashtban *et al*., 2010). The isolate bore significantly different levels of specific enzyme activities ($P<0.05$).

5.3.1.1. Endoglucanase (CMCase) activity

The structural complexity of pure cellulose and difficulty of working with insoluble substrates has led to the wide use of CMC for endoglucanases studies (Lynd *et al*., 2002). CMC is a soluble cellulose derivative with a high degree of polymerization (DP). The isolate was found to bear CMCase activity (0.13571U/mg). This was in line with other studies that have reported on

Bacillus licheniformis strains degrading armophous substrates such as CMC amongst them Fujimoto *et al*., (2011) and Bischoff *et al*., (2006). DNS is reduced to 3-amino-5-dinitrosalicylic acid an aromatic compound that absorbs light strongly at 540nm (Miller, 1959). The disadvantage of using this method is loss of some reducing sugars during the analysis but despite that it's a more convenient test compared to other sugar tests.

5.3.1.2. Exoglucanase (avicelase) activity

Exoglucanases cleave β - (1-4) glycosidic bonds from side chains to release cellobiose or glucose molecules (Béguin & Aubert, 1994). Commercial Avicel (microcrystalline cellulose) is used for measuring exoglucanases activity because it has a low degree of polymerization and inaccessible to attack by endoglucanases despite having some amorphous regions. Avicel contains some quantities of amorphous cellulose and cellodextrins which act as substrate for both exoglucanases and endoglucanases. This implies that there is no highly specific substrate to test exoglucanase activity in cellulase mixtures (Sharrock *et al*., 1988; Wood & Bhat, 1988). However enzymes that show relatively high activity on Avicel and less activity on CMC can be identified as exoglucanases (Maki *et al*., 2009; Makky, 2009).

The isolate was found to display avicelase activity (0.18784U/mg) contrary to some previous research work that indicated that members of the genus *Bacilli* are unable to degrade avicel (Makky, 2009; Bischoff *et al*., 2006).

However during the experiment Avicel was found to settle at the bottom of the assay since the incubation was carried out without agitation. This could have lowered the quantity of reducing

sugar produced. This can be attributed to substrate availability i.e. not all substrate in the reaction mixture was available for attack by the exoglucanases in the cellulase mixture.

5.3.1.3. Cellobiose (β-glucosidase) activity

The isolate was found to bear β-glucosidase activity (0.46878U/mg). From the results, cellobiase activity is higher than the enzyme activities of both endoglucanase and exoglucanase respectively. This can be attributed to the ease of cleaving cellobiose since it has a very low DP i.e. two β 1-4 linked glucose units. High β-glucosidase activity is essential to reduce end product inhibition by cellobiose. β - glucosidases are supplemented during cellulose hydrolysis (Sun & Cheng, 2002) to reduce end product inhibition. This however would be highly unnecessary during hydrolysis with enzymes from this isolate due to the significantly high β-glucosidase activity noted.

A fairly common observation has been that members of *bacillus* genus lack a complete cellulase system, with the primary activity being on CMC and none on avicel (Robson & Chambliss, 1984; Bischoff *et al*., 2006). Contrary to these observations, a crude enzyme extract form isolate 3 was found to bear the three enzyme specificities suggesting presence of a complete cellulase system. Similar observations have been recorded (Flengsrud *et al*., 1994; Kim, 1995; Kim, *et al*., 2011).

5.4. Determination of optimum temperature for enzyme activity

There was a significant change in enzyme activity with change in temperature ($P<0.05$). Optimum temperature for activity was recorded at $60^{o}C$ (Figure 15), however there was no

significant difference (Tukey's test) in enzyme activity at 60^{o}C and 70^{o}C (P>0.05). This implies that the isolate can work optimally in the range of 60^{o}C and 70^{o}C. A similar temperature profile has been observed in a study using *Bacillus licheniformis* (Bajaj *et al*., 2009). Microbial cellulases from different sources have been found to have optimum temperature of approximately 35-50^{o}C (Aygan *et al*., 2011; Bakare *et al*., 2005). Industrial application of the enzyme system from isolate 3 has an advantage over these cellulases due to the high optimum temperature noted.

Enzymes work rapidly at their optimum temperature. Below the optimum temperature, an increase in temperature increases the kinetic energy of the system thus increasing the rate of reaction. Consequently, the number of collisions between the substrate and the active site are increased. At elevated temperatures many enzymes become partly unfolded and inactivated thus rendering them unable to perform their desired tasks (Eijsink *et al.,* 2005). This best explains the trend in Figure 15.

5.5. Determination of optimum pH for enzyme activity

Enzyme activity assays to determine the optimum pH were carried out in reaction mixtures at varying pH values (3-11) at the predetermined temperature (60^{o}C). Each enzyme has its own optimum pH and if the pH increases or decreases beyond the optimum, the ionization groups at the active site may change slowing or preventing the formation of an enzyme substrate complex (Eijsink *et al.,* 2005).

Optimum pH values of 4.5-8.0 have been reported for different microbial cellulases (Bakare *et al*., 2005; Immanuel *et al*., 2007; Dutta *et al*., 2008). For this isolate, there was a significant change in enzyme activity with change in pH ($P<0.05$). Highest activity was recorded at pH 5 (Figure 16) implying that the enzyme is an acid cellulase. Acid cellulases act at a pH range of 3.8 and 5.8 (Mosjov, 2012). Similar observations have been made by Bajaj *et al.*, (2009). There was no significant difference in enzyme activity at pH 6, 7 and pH 3, 11 ($P>0.05$). This may imply that these pH pairs have more or less the same effect on enzyme activity.

5.6. Determination of optimum time for enzyme activity

Enzyme activity was determined for a span period of 8 hours at the predetermined optimum conditions (60^{o}C and pH 5). There was a significant change in enzyme activity with increase in the duration of incubation ($P<0.05$). Maximum activity was recorded after 30 minutes of incubation and thereafter a decline and a platue in enzyme activity (Figure 17). From the curve, enzyme activity at 360, 420 and 480 minutes appears to have reached a platue. Post hoc analyses (Tukey's test) show that there is no significant difference in the enzyme activity ($P>0.05$) at this time range. This phenomenon can be attributed to end product inhibition. Cellulase activity can be inhibited by cellobiose and glucose (Sun & Cheng, 2002). To reduce end product inhibition, supplementation of β- glucosidases during hydrolysis or removal of sugars by ultra filtration can be done (Sun & Cheng, 2002). This however is not feasible in this enzyme assay because the assay conditions do not allow for removal of final products or supplementation of β-glucosidases. The cellulase enzyme system from the isolate was stable for up to 8 hours.

CHAPTER 6: CONCLUSION AND RECOMMENDATION

Four cellulolytic bacteria isolates were obtained from soils excavated from Hells gate and Eburru hills. To the best of our knowledge there are no reports of a cellulolytic organism that has been isolated from geothermal wells along the Kenyan Rift valley. The Gram-positive rods were deduced to be *Bacillus licheniformis* strains from both morphological and molecular phylogenetic analysis.

A crude cellulase mixture from isolate 3, selected for further studies was shown to bear the three types of cellulolytic activities. From the functional tests carried to determine the optimal conditions for cellulolytic activity, the isolate's enzyme activity was found to be high over a range of temperatures i.e. from 50^{o}C to 80^{o}C with the optimum temperature being 60^{o}C. Similarly, enzyme activity was found to be high at the range of pH 4 to pH 7 with an optimum of pH 5 suggesting that the isolate bears acid cellulases.

Application of the isolate 3 in industries has several advantages such as the Generally Recognised as Safe (GRAS) status, high growth rate and ability to secrete proteins extracellularly; features of the *Bacillu*s species. In addition to these, the isolate is capable of producing the three cellulase specificities as well as being thermotolerant.

More studies are however needed before industrial application of this isolate. These include enzyme activity assays of the purified specific cellulases for comparison with the results in this study and with those that have been purified, pilot studies using lignocellulolytic substrates and isolation and expression of the isolate's cellulase genes. These studies would shed more light on

whether to use the whole organism in the industry or harvest the enzymes and carry out downstream processes or whether the gene is to be added to the genetic pool for protein engineering and directed evolutionary studies to come up with super enzymes. Similar studies should be extended to other extreme environments in the country.

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APPENDIX

16S rDNA sequences for the bacteria isolates

>**Isolate 1 KF737352**

GTGCGGTGCGCAGCTATAATGCAGTCGAGCGGACCGACGGGAGCTTGCTCCCTTAGT
CAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACT
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GTGGCTTTTAGCTACCACTTACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGG
TAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACAC
TGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGC
AATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTGGATCGT
AAAACTCTGTTGTTAGGGGAAAGAACAAGTACCGTTCGAATAGGGCGGACCTTGAC
CGGTACCTAACCAGAAAAGCCACCGGCTAAACTACGTGCCAGCAAGCCGCGGTAAA
TACGTAGGTGGCAAGCGTTGTCCAGGAATTATTGGGCGTTAAAGCGCGCGCAGGCG
TTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCCGGGGAGGGTCATTGGAAACT
GGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGTG
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TGAGGCGCGAAAGCGTGGGGAGCGAACAGGATTAGATsACCCTGGTAGTCCACGCC
GTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGCAGCAAACG
CATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGA
CGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAAC
CTTACCAGGTCTTGACATCCTCTGACAACCCTAGAGATAGGGCTTCCCCTTCGGGGG
AGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAG
TCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCAGCATTCAGTTGGGCACCTAAG
GTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCC
CTTATGACCTGGGCTACACACGTGCTACAATGGGCAGAACAAAGGGCAGCGAAGCC
GCGAGGCTAAGCCAATCCCACAAATCTGTTCTCAGTTCGGATCGCAGTCTGCAACTC
GACTGCGTGAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATAC
GTTCCCGGGCCTTGTACACACCCCCCAACTAAGCACGAGAGTTTGTAACACCCGAAG
TCGGTGAGGTAACCTTTGGAGCCAGCCGCCGAAGTGACAGATGGAT

>**Isolate 2 KF737353**

GATGCGGGCGCAGCTATACATGCAGTCGAGCGGACCGACGGGAGCTTGCTCCCTTG
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CTCCGGGAAACCGGGGCTAATACCGGATGCTTGATTGAACCGCATGGTTCAATCATA
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GAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCC
ACACTGGGACTGAGACACGGCCCCTAGGACTCCTACGGGAGGCAGCAGTAGGGAAT
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TCGACTGCGTGAAGCTGGAATCGCTAGTAATCGCGGATCGCATGCCGCGGTGAAGTT
CCCGGGCCTTGTACACACCGCCCGTCACACCACGAGGTTTGTAACACCCGAAGTCGG
TGAGGTAACCTTTGGAGCCAGCCGCCGAAGTGACAGTATGG

>**Isolate 3 KF737354**

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TAACTCCGGGAAACCGGGGCTAATACCGGATGCTTGATTGAACCGCATGGTTCAATC
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GGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCG
GCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAAT
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CCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGG
CAGGCCGGTTTCTTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCAT
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ATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGGCAGAACAAAGGGG
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CAACTCGACTGCGTGAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGG
AATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAACACC
CGAAGTCGGTGAGGTAACCTTTGGAGCCAGCCGCCGAAGTGACAGATATTGG

>**Isolate 4 KF737355**

GAGGCTGGCGGGAGCTATACATGCAGTCGAGCGGACCGACGGGAGCTTGCTCCCTT
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GAAAGCCACGGCTAACTACGTGCCAGGCAGCCGCGGTTAATACGTAGGTGGCAAGC
GTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTTTTCTTAAGTTCTGATC
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TTCTCAGTTCGGATCGCAGTCTGCAACTCGACTGCGTGAAGCTGGAATGCTAGTATG
CGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACA
CCACGAGAGTTTGTAACACCCGAAGTCGGTGAGGTAACCTTTGGAGCCAGCCGCCG
AAGTGACAGAATTCG

Specific endoglucanase activity U/mg used to plot graphs

Table1. Enzyme specifities produced by isolate 3. Specific enzyme activity values are shown as mean ±standard deviation.

Enzyme	Enzyme activity (U/mg)
Endoglucanase	0.13571 ± 0.003^{a}
Exoglucanase	0.18784 ± 0.002^{a}
Cellobiase	0.46878 ± 0.002^{a}

a= $P<0.05$ There was a significant difference in enzyme activities for the three enzyme specificities. Post hoc test were carried out using tukey's test at a significance level of 0.05.

Table 2. Specific enzyme activity for the crude enzyme extract at temperatures ranging from $30^{o}C$ to $90^{o}C$. Values are shown as mean ±standard deviation.

Temperature (^{o}C)	Enzyme activity (U/mg)
30	$0.04017 \pm 0.0006^{b*}$
40	$0.04281 \pm 0.0010^{b*}$
50	0.05201 ± 0.0001^{b}
60	$0.06985 \pm 0.0003^{b*}$
70	$0.06760 \pm 0.0004^{b*}$
80	0.05902 ± 0.0013^{b}
90	0.04797 ± 0.0010^{b}

b= P<0.05 There was a significant change in enzyme activity with change in temperature.

b*= P>0.05 There was no significant change in enzyme activity with change in temperature. Post hoc test were carried out using tukey's test at a significance level of 0.05.

Table 3: Specific enzyme activity of the crude enzyme extract at pH 3 to pH 11. Values are shown as mean ±standard deviation.

pH	Enzyme activity (U/mg)
3	$0.01997\pm0.0005^{c*}$
4	0.04949 ± 0.0003^{c}
5	0.08214 ± 0.0008^{c}
6	$0.05560\pm0.0006^{c*}$
7	$0.05459\pm0.0005^{c*}$
8	0.03390 ± 0.0002^{c}
9	0.03390 ± 0.0001^{c}
10	0.02304 ± 0.0002^{c}
11	$0.0214\pm0.0001^{c*}$

c=P<0.05 There was a significant change in enzyme activity with pH increase.

c*=P>0.05 There was no significant change in enzyme activity with pH increase. Post hoc test were carried out using tukey's test at a significance level of 0.05.

Table 4: Specific enzyme activity for the crude enzyme extract for time intervals ranging from 30 minutes to 480 minutes. Values are shown as mean ±standard deviation.

Time (minutes)	Enzyme activity (U/mg)
30	0.1357 ± 0.0032^{d}
60	0.0692 ± 0.0003^{d}
120	0.0356 ± 0.0001^{d}
180	$0.0242 \pm 0.0002^{d*}$
240	$0.02146 \pm 0.0002^{d*}$
300	0.0179 ± 0.0002^{d}
360	$0.0141 \pm 0.0003^{d*}$
420	$0.0121 \pm 0.0005^{d*}$
480	$0.01034 \pm 0.0002^{d*}$

d=P<0.05 There was a significant change in enzyme activity with change in time

d*=P>0.05 There was no significant change in enzyme activity with change in time. Post hoc test were carried out using tukey's test at a significance level of 0.05.