SCREENING AND ASSESSMENT OF CELLULASE FROM *BACILLI SP* ISOLATED FROM A KENYAN SODA LAKE

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DECLARATION

Declaration by the student

I hereby declare that this research dissertation is my original work and has not been presented to any other university for examination or any other award.

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ABBREVIATIONS

cm = Centimetre

CMC = Carboxymethyl cellulose

Conc = Concentration

DNS = Dinitrosalicyclic acid

Fig = Figure

h = hour

IU = International Unit

KCl = Potassium Chloride

K_2HPO_4 = Di-potassium Hydrogen Phosphate

LBW = Lake Bogoria Water

M = Molarity

mg = milligram

$MgSO_4$ = Magnesium Sulphate

min = minutes

ml = millilitre

Pa = Pascal

Na^+ = Sodium ion

NaCl = Sodium Chloride

Na_2CO_3 = Sodium Carbonate

$NaNO_2$ = Sodium Nitrite

NaOH = Sodium Hydroxide

rpm = Revolutions per minute

sec = seconds

sp. = species

w/v = weight per volume

µl = microlitre

µM = micromole

ºC = Degree centigrade

Abstract

Alkaline cellulases have wide spread industrial and commercial applications due to their stability at pH values higher than 9.5. They can be applied in place of chemicals during detergent formulation for cleaning and colour clarification in detergent industries as well as de-inking and fibre modification in pulp and paper industries (Kochavi et *al*., 1990; Tyndall, 1990). Another application would be in the replacement of pumice stone and harsh chemicals in bio stoning process in textile industries. These cellulases can be extracted from alkaliphilic bacteria i.e. bacteria growing in alkaline/basic habitats. Use of alkaline cellulases is especially attractive in industrial applications as they are able to withstand the harsh conditions associated with such processes.

In this study, 5 *Bacillus sp* designated *Bacillus sp* LBW 213, *Bacillus sp* LBW 2 7 19, *Bacillus sp* LBW 35 *Bacillus sp* LBW 33 and *Bacillus sp* 313 previously obtained from Lake Bogoria water samples were used as a source of alkaline cellulases. The isolates were screened for cellulase production on CMC agar plates at pH 10.4 and 37ºC using the Congo red clearing zone assay. All five were found to be excellent cellulase producers with a halo size of approximately 2.5. Due to this remarkable outcome, all the *Bacilli sp* were then assessed for production of Endoglucanase and Fpase.

Both endoglucanase and Fpase activities of the cells were followed for a period of 36 hours, with cultivation carried out at 37°C in liquid broth. Endoglucanase activities were observed in the range of 0 - 0.106 U/ml with maximum production recorded after 4 h and subsequent decrease in activity for all the bacteria, reaching undetectable levels after 36 h. Fpase activities were observed within a range of 0 - 0.009 U/ml with maximum production after 4 hours and decline in activity throughout the remaining 36 hour incubation. Low enzyme production was attributed to un-optimized culture conditions.

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CHAPTER 1

1.0 Introduction

Extremophiles are organisms known to live in extreme environments. These include areas of extremely high and low pH and temperature, high salt concentrations, high levels of radiation or toxic compounds among others. Enzymes produced by extremophiles are known as extremozymes. Extremozymes, have been of keen interest in industrial applications since they are stable and active at the harsh conditions presented by industrial applications. Some examples of extremozymes used in industries include proteasese, amylases and cellulses in detergent industries, amylases, pectinases and cellulases in textile industries, proteases in leather industries etc.

An example of extremophile and which is of key interest in this study is the alkaliphile. Alkaliphiles are organisms living in areas of pH >9. These organisms can be isolated from various sources including rotting organic waste, fecal matter, some industrial affluents and soda lakes. Of these habitats, soda lakes present the best alkaline conditions because their pH values are persistently between pH 10 and 12. Therefore, micro-organisms from soda lakes would be a good source for studies of alkaliphilic extremozymes.

Alkaliphiles produce extracellular enzymes which are stable and show optimum, activity at alkaline pH. Some of the enzymes produced by alkaliphiles include amylases, pectinases, lipases, xyalanases, chitinases and cellulases. These enzymes are suitable for use in certain industrial applications carried out at alkaline pH values. Alkaline cellulases for example have found massive use in the detergent industries and biostoning of denim in textile industry.

Cellulases are a group of hydrolytic enzymes distinguished by their ability to hydrolyze β 1.4 glycosidic linkages in cellulose to smaller oligosaccharides and eventually to glucose. The cellulose enzyme system has 3 major enzymes i.e. endoglucanase (EC 3.2.1.4), exoglucanaes (EC 3.2.1.91) and cellobiase (EC 3.2.1.21) whose synergistic effect leads to the hydrolysis of cellulose to glucose. The main source of cellulases is fungi and bio-prospecting for bacterial species is underway.

Bacilli species have been of keen interest with respect to production of alkaline cellulases which possess high activity.

1.1 Statement of Problem

Biotechnological application of enzymes in industrial processes has gained popularity over the past few years. This is due to environmentally clean and safe practices employed by enzymes as opposed to the harsh chemicals used in conventional practices. Cellulose is the most abundant biopolymer on earth accounting to about 80% of natural biopolymers. Despite the fact that many micro-organisms are capable of degrading cellulose, only a few produce significant quantities of cell-free enzymes capable of completely hydrolysing crystalline cellulose in vitro. Alkaline cellulases have found major applications in various industries including the paper and pulp industries, detergent and textile industries. However, the yield of enzyme liberated by certain micro-organisms is not sufficient for industrial purposes. Continued bio-prospecting for bacterial species exhibiting high producing alkaline cellulase with high activity is still in place.

1.2 Justification

Cellulose is commonly degraded by the cellulases. These enzymes are produced by several micro-organisms, commonly bacteria and fungi (Lederberg, 1992) which produce it either being cell associated or in a free form so as to metabolise the insoluble cellulose (Walsh, 2002). Fungi are the main cellulose producing micro-organisms though a few bacteria and actinomycetes have also been reported to yield cellulose activity (Jadhav *et al,* 2013). The fungal sources are predominantly used for cellulase production. But they take longer growth period for enzyme production. Compared to fungi the bacterial cellulase producers are less effective but they produce enzymes quicker and can be genetically manipulated effectively (Ponnambalam et al, 2011). Cellulase enzymes of *Bacillus* spp. have been the focus of much attention because of their potential use in the conversion of agricultural wastes into useful products and in other biotechnological applications. (Ponnambalam et al, 2011)

1.3 Hypothesis

Bacillus sp LBW 213, *Bacillus sp* LBW 2 7 19, *Bacillus sp* LBW 35, *Bacillus sp* LBW 33 and *Bacillus sp* LBW 313 produce alkaline cellulases with potential biotechnological application.

1.4 Research Objectives

1.4.1 General Objective

The purpose of this study is to screen and assess for cellulase enzyme production in *Bacillus sp* designated *Bacillus sp* LBW 213, *Bacillus sp* LBW 2 7 19, *Bacillus sp* LBW 35 *Bacillus sp* LBW 33 and *Bacillus sp* 313.

1.4.2 Specific Objectives

1. To screen for alkaline cellulase producing *Bacillus sp* from Lake Bogoria water samples.
2. To assay for Fpase and Endoglucanase activity.
3. To compare enzyme production in relation to bacterial growth.

CHAPTER 2

2.0 Literature Review

2.1 Extremophiles

Life is diverse and extremophiles best depict this truth. Extremophiles are organisms known to live in extreme environments. Extreme is relative since environments that are extreme for one organism may be essential for the survival of another (Gomes & Steiner, 2004). This relativity is therefore, measured with respect to mesophilic organisms. Interestingly, these organisms not only grow or survive in theses environments but they thrive and reproduce. Conditions sub-optimal to their extreme range would consequently result in their death e.g. *Pyrolobus fumarii*, a hyperthermophile reproduces best in an environment of about 105 ℃ and can multiply in temperatures of up to 113 ℃ but it stops growing at temperatures below 90 ℃ (Madigan, Mars 1997). Extreme environments would therefore include areas of either high (55 to 121 °C) or low (–2 to 10 °C) temperatures, high salinity (2–5 M NaCl) and either high alkalinity (pH>8) or high acidity (pH<4), high pressure, high levels of radiation or toxic compounds, or conditions that we consider unusual, such as living in rocks deep below the surface of the earth or living in extremely dry areas with very low water and nutrient supply (Gomes & Steiner, 2004)

2.1.1. Diversity of Extremophiles

Extremophiles colonise all three domains of life i.e. archaea, bacteria and eukarya but the main group to thrive in extreme environments are archaea (Rampelotto, 2013). Of these three domains, archaea are known to inhabit the most extreme of environments, characterised by extremely low or high pH and temperatures, extremes of salt concentrations etc. *Picrophilus torridus*, an example of archaea is known to inhabit areas of as low as pH 0.06 (Rampelotto, 2013). Within the bacteria domain, cyanobacteria are the most common extremophiles (Rampelotto, 2013). They live in areas characterised by high salt and metal concentrations, low water activity and extreme temperatures such as Antarctic and continental hot springs. In such areas, they are found in association with other bacteria, forming microbial mats. Cyanobacteria can also be found living in desert rocks, forming endolithic

communities. However, they are rarely found in acidic environments at pH values lower than 5–6 (Rampelotto, 2013). Eukarya less often fall under this category. Fungi are the most encountered eukaryotic extremophiles. They can be found living alone or in symbiotic associations with algae (forming lichens) or cyanobacteria (Rampelotto, 2013) Fungi are not hyperthermophiles but they adapt well to other extreme environments. Fungi live in acidic and metal-enriched waters from mining regions, alkaline conditions, hot and cold deserts, deep oceans and in hyper-saline regions such as the Dead Sea. Of keen interest is the tardigrade, a microscopic invertebrate which is also a polyextremophile. Tardigrades can go into a hibernation mode, called the tun state, whereby it can survive temperatures from −272 °C (1 °C above absolute zero!) to 151 °C, vacuum conditions (imposing extreme dehydration), pressure of 6,000 atm as well as exposure to X-rays and gamma-rays. Furthermore, even active tardigrades show tolerance to some extreme environments such as extreme low temperature and high doses of radiation (Rampelotto, 2013).

2.1.2. Classification of Extremophiles

Extremophiles may be divided into two broad categories: extremophilic organisms which require one or more extreme conditions in order to grow and extremotolerant organisms which can tolerate extreme values of one or more physicochemical parameters though growing optimally at normal conditions (Rampelotto, 2013). Extremophiles are classified according to the conditions in which they grow i.e.

- Thermophiles- are the most studied extremophiles. Thermophiles are generally classified into moderate thermophiles (growth optimum 50-60 ºC), extreme thermophiles (growth optimum 60-80 ºC) and hyperthermophiles (growth optimum 80-110 ºC) (Gomes & Steiner, 2004). In contrast to thermophiles, no multicellular animals or plants have been found to tolerate temperatures above about 50 ºC and no microbial eukarya yet discovered can tolerate long-term exposure to temperatures higher than about 60ºC (Madigan & Marrs, 1997) .
- Acidophiles – they live in acidic environments. These organisms cannot tolerate acidic conditions in their cytoplasm and hence have ways to maintain neutral internal pH (Madigan & Marrs, 1997). They do this by maintaining a

large chemical proton gradient across the membrane. Proton movement into the cell is minimized by an intracellular net positive charge; the cells have a positive inside membrane potential caused by amino acid side chains of proteins and phosphorylated groups of nucleic acids and metabolic intermediates that act as titrable groups. As a result, the low intracellular pH leads to protonation of titrable groups and produces net intracellular positive charge. (Satyanarayana, Raghukumar, & Shivaji, 2005).

- Alkaliphiles- organisms which can easily thrive at pH values exceeding 10.
- Metallophiles – organisms which can survive in high metal concentrations.
- Halophiles – organisms which are completely dependent on salt concentrations exceeding the salinity of sea water by more than a factor of 10.
- Barophiles – also known as piezophiles. These organisms live under conditions of hydrostatic pressure of greater than 1000 atmospheres. This environment is representative of deep sea conditions (Satyanarayana et al., 2005). In addition, barophiles are sensitive to UV and require darkness or reduced light for their growth (Satyanarayana et al., 2005).
- Radiophiles – these are microorganisms that are highly resistant to ionising radiation such as UV. They have potential application in disposal of radioactive waste (Gomes & Steiner, 2004).

The molecular strategies employed for survival of extremophiles in their habitats is yet to be fully elucidated (Rampelotto, 2013). However, it is believed that their biomolecules have been adapted for the harsh environments. In addition, a possibility of peculiar biochemical pathways has also been suggested. All these coupled together have shown potential use in biotechnological processes. Due in part to the fact that exremozymes, enzymes produced by exremophiles are stable and active at extreme conditions as opposed to the labile mesophilic ones (Rampelotto, 2013).

Production of extremozymes for large scale biotechnological applications can prove to be an expensive affair if it were to be done in conditions similar to their natural habitats. After a particular organism has been screened for an enzyme of interest and sufficient quantities are produced, the gene encoding for that enzyme is cloned and produced in a mesophile. This has proved cheaper in the long run (Madigan & Marrs, 1997).

Some of the applications of bioactive compounds from extremophiles are shown in Table 1

Table 1: Biotechnological Applications of Bioactive Compounds of Extremophiles?

Bioactive Compounds of Extremophiles	Application in Biotechnology
Thermostable DNA polymerases and restriction enzymes (Thermophiles)	Molecular Biology and Genetic Engineering
Alkaline Phosphatases (Psychrophiles)	Molecular Biology
Compatible Solutes (Halophiles)	DNA and Protein Stabilisers
Lipids (Hyperthermophiles)	Liposomes for drug administration

2.2 Alkaliphiles

There is no clear cut definition of what an alkaliphile is (Horikoshi, 1999; RIkizo, 1997). This is largely due to the fact that microbes have pH ranges in which they thrive. In addition, growth is not only dependent on pH but also nutrient availability, water activity, temperatures etc. Therefore the pH showing optimal growth will also depend on these other growth factors (Horikoshi, 1999). However, micro-organisms that thrive at pH values of > 9 are generally referred to as alkaliphiles or alkali-tolerant (RIkizo, 1997). Despite their habitats, alkaliphiles maintain neutral pH within their cells (Madigan & Marrs, 1997). According to Gomer and Steiner, 2004, alkaliphiles can be divided into 2 groups i.e. alkaliphiles and haloalkaliphiles. Alkaliphiles require an alkaline pH of 8 or more for their growth and have an optimal growth pH of around 10, whereas haloalkaliphiles require both an alkaline pH (pH > 8) and high salinity (up to *m*/*V* NaCl = 33 %).

2.2.1 Distribution of Alkaliphiles

Alkaliphiles are mainly found in neutral environments and in some cases others have been isolated from acidic environments as well as in faecal matter. Haloalkaliphiles on the other hand have been isolated from extremely saline environments (Horikoshi, 1999). Such environments may include those observed in Lake Magadi in the east African Rift valley, Owens Lake in California, the Wadi Natrum Lake in Egypt, several saline soda lakes, and soils in Tibet, Pakistan, India and Russia (Satyanarayana et al., 2005). The lakes are often coloured red due to large numbers of haloalkalophilic archaea such as *Natronobacterium pharaonis*, *N. gregoryi* and *Natronococcus occultus* (Madigan & Marrs, 1997). Naturally, alkaliphilic aerobes

have been found to coexist with neutrophiles or to colonise extreme environments. In the former case, they are isolated by addition of Na_2CO_3 to the growth media and adjusting the pH to 10 (Horikoshi, 1999). Calcium springs in Oman yielded aerobic species of *Bacillus, Vibrio, Flavobacterium, Pseudomonas* and members of *enterobacteria* (Satyanarayana et al., 2005). Some alkaliphiles have been isolated in man-made alkaline environments e.g. *Exiguobacterium aurantiacum* from potato processing waste while *Ancyclobacterium sp.* was described from Kraft paper and board process effluent (Satyanarayana et al., 2005).

2.3 East African Rift Valley and Lakes Found Therein

Soda lakes and soda environments are the most stable highly alkaline environments on earth. Soda lakes in particular are a normal occurrence in the Rift valley which is an enormous volcanic stretch from the north of Africa with an eastern branch through Kenya and Tanzania (Grant, 2004) (Fig 1).

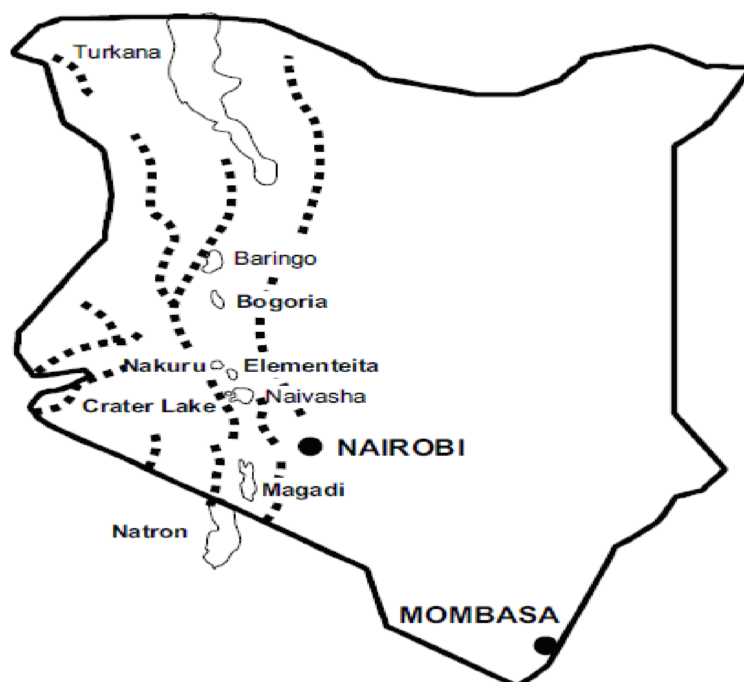


Fig 1: Outline map of Kenya showing the major rift systems and location of major soda lakes

The floor of the rift valley has a considerable number of highly alkaline soda lakes as a result of volcanic activity, which is still active in some areas. The alkalinity is a consequence of high Na^+, low Mg^{2+} and Ca^{2+} (Grant, 2004). The climate in the rift valley is majorly arid or semi-arid. High evaporation rates are therefore prevalent and

due to low Ca^{2+} and Mg^{2+} concentrations, coupled with high Na^{+} concentrations, alkaline sodium carbonate brines develop. These brines are a key feature associated with East African soda lakes (Grant, 2004). The concentrations of some salts in some of the Rift valley lakes and subsequent pH values are shown in Table 2

Table 2: Concentrations of salts in Rift valley lakes and pH values

Lake	Na^{+}	K^{+}	Ca^{2+}	Mg^{2+}	SiO_2	PO_4^{3-}	Cl^{-}	SO_4^{2-}	CO_3^{2-}	pH
Elmenteita	195.7	3.6	0.07	<0.004	2.9	0.03	65.1	2.0	68.0	10.5
Nakuru	326.1	5.6	0.15	<0.004	3.3	0.15	57.5	0.5	198.3	10.5
Bogoria (north)	734.8	5.5	0.21	0.008	2.2	0.09	100.9	1.0	476.7	11.0
Bogoria (south)	795.7	6.8	0.19	0.008	2.0	0.17	115.5	1.1	516.7	11.0
Sonachi	140.4	9.0	0.05	0.008	2.1	0.04	12.4	0.8	90.0	10.0
Oloidien	8.7	1.8	0.28	0.65	1.0	0.003	4.8	0.5	<10.0	9.0
Magadi (lake brines)	7000.0	57.0	<0.01	<0.01	14.9	1.82	3154.9	17.5	3900.0	>11.5
Magadi (lagoon brines)	2826.1	26.1	0.03	<0.01	7.1	0.23	1123.9	12.8	1816.7	>11.5
Little Magadi	4626.1	61.1	0.02	0.03	7.5	0.31	1856.3	13.1	2433.3	>11.5
Natron	4521.7	43.7	0.04	0.03	3.1	4.21	1464.8	1.7	2666.7	>11.5

All concentrations given in mM

2.3.1 Microbial Diversity of Soda Lake Micro-organisms

Soda lakes often exhibit blooms of phototrophs such as *Cyanospira* (*Anabaenopsis*) sp.,*Chlorococcum* sp. and *Pleurocapsa* sp. East African lakes contain *Spirulina platensis* and other lakes harbour *Spirulina maxima*. The photosynthetic yield of *Spirulin* surpasses that of others in the terrestrial environment. Significant blooms of red pigmented *Ectothiorhodospira mobilis* and *E. vacuolata* have been found together with cyanobacteria in soda lakes. They play an important role in sulphur cycle in these lakes by utilizing H_2S as an electron donor in photosynthesis. Black anoxic lake sediments, rich in methylamine-utilizing methanogens such as *Methanohalophilus*, also occur. Highly saline and alkaline environments such as those observed in Lake Magadi in Rift Valley, Owens Lake in California, the Wadi Natrum Lake in Egypt, several saline soda lakes, and soils in Tibet, Pakistan, India and Russia harbour different populations of prokaryotes. The lakes are often coloured red due to large numbers of haloalkalophilic archaea such as *Natronobacterium pharaonis*, *N. gregoryi* and *Natronococcus occultus*. Moderately haloalkaliphilic methanotrophs such as *Methylomicrobium alcaliphilum* were reported from Kenyan soda lake sediments. Alkaliphilic spirochetes such as *Spirochaeta alcalica* and haloalkalophilic *S. asiatica* have been isolated from Lake Magadi and Lake Khadyn, respectively. An alkaliphilic sulphate-reducing bacterium *Desulfonatronovibrio hydrogenovorans* was

reported from Lake Magadi. Anaerobic alkalithermophiles *Clostridium, Thermoanerobacter* sp. and *Thermopallium natronophilum* were recovered from lake sediments (Satyanarayana et al., 2005).

2.3.2 Special Feature for Adaptation to Alkaline Environments

Most alkaliphiles have a growth optimum at pH 10 and yet seem to maintain a cytoplasmic pH of neutral or slightly alkaline (Satyanarayana et al., 2005). Various mechanisms are employed in order to achieve this. They include

2.3.2.1 Cell Walls

Bacterial cell walls have been suggested to play a critical role in protecting the cell from alkaline environments. *Bacillus* protoplasts lose their stability in alkaline environments (Horikoshi, 1999). In addition to the peptidoglycan, bacterial cell walls contain acidic polymers such as glutamic acid, aspartic acid, phosphoric acid, galacturonic acid and gluconic acid. These acidic polymers function by conferring net negative charges which in turn enable the cell surface to adsorb sodium and hydronium ions, consequently repulsing hydroxide ions and enabling the cells to grow in alkaline environments (Horikoshi, 1999). In addition, alkaliphilic *bacillus* species contain an abundance of hexosamines such as glucosamine, muramic acid, alanine and glutamic acid.

2.3.2.2 Na^+ Ions and Membrane Transport

Alkaliphiles require Na^+ ions for growth. The presence of Na^+ in the surrounding environment has proved to be essential for effective solute transport through the membranes of alkaliphilic *bacillus* species. According to the chemiosmotic theory, the proton motive force in the cells is generated by the electron transport chain or by excreted H^+ derived from ATP metabolism by ATPase. H^+ is then reincorporated into the cells with co-transport of various substrates. In Na^+-dependent transport systems, the H^+ is exchanged with Na^+ and ATPase by the Na+/H+ antiporter systems, thus generating a sodium motive force, which drives substrates accompanied by Na^+ into the cells (Horikoshi, 1999).

2.3.3 Biotechnological Application of Alkaliphiles and their Enzymes

Alkaliphiles are good sources of alkaliphilic enzymes like cellulases, xylanases, amylases, proteases, lipases, pectinases, chitinases, catalases, peroxidases and oxidoreductases (Gomes & Steiner, 2004). Thermoalkaliphilic enzymes have great bio-catalytic potential in processes that are performed at alkaline pH and higher temperatures. For example, proteases, lipases and cellulases are used as additives in laundry and dishwashing detergents. Proteases are also used for dehairing of hides and skins and to improve smoothness and dye affinity of wool. Cellulase-free xylanases are used for bio-bleaching of pulp and paper, pectinases are used in degumming of ramie fibers and catalase and peroxidase or oxidoreductase may be used to remove residual hydrogen peroxide from effluent streams of the textile processing industry. Due to their versatile uses, alkaliphilic enzyme-producing bacteria and archaea have received great attention in recent years. A summary of some of the enzymes produced by alkaliphiles is shown in Table 3

Table 3: Table showing examples of alkaliphiles and the enzymes they produce

Alkaliphile	**Enzyme**
Alkalimonas amylolytica	Amylase
Streptomyces sp.	Endocellulase
Bacillus firmus	Xyalanases
Bacillus halodurans strains	Pullulanases/Amylases
Bacillus subtilis	Amylase
Bacillus isolate KSM-K38	Amylase
Nesterenkonia sp. AL-20	Alkaline protease
Bacillus pumilus	Alkaline protease
Arthrobacter ramosus, Bacillusalcalophilus	Alkaline protease
Nocardiopsis sp.	Alkaline protease, Keratinase
Pseudomonas sp. LBA34, *Halomonas* sp. LBB1	Lipase
Bacillus sp.	Azoreductase
Bacillus sp.	Catalase-peroxidase
Thermus brockianus	Catalase
Bacillus alcalophillus	Pectate lyase
Thermomonospora (Actinomycete)	Endocellulase

2.3.4 Alkaliphilic *Bacillus sp.* and their alkaline active enzymes

2.3.4.1 Alkaliphilic *Bacillus sp.*

Bacillus refers to a specific genus of bacteria. The family *Bacillaceae* are all Gram positive, rod-shaped bacteria which form endospores, Characteristically, *Bacillus* cultures are Gram positive when young, but may become Gram negative as they age. *Bacillus* species are aerobic, sporulating, rod-shaped bacteria. *Bacillus sp* colonise wide ecological niches. They may be found in alkaline environments, acidic areas, in soils, areas of high salt concentrations etc. Alkaliphilic *Bacillus species grow* in environments with high pH. Examples of alkaliphilic *Bacillus* include *B. alcalophilus, Bacillus cellulosilyticus, Bacillus wakoensis, Bacillus hemicellulosilyticus*, *Bacillus mannanilyticus*, *Bacillus akibai* etc (Nogi, 2005). Alkaliphilic *Bacillus* species have important industrial applications due to their ability to produce alkaline enzymes such as protease, cyclomaltodextrin glucanotransferase (CGTase) and cellulase. These species produce extracellular enzymes that are resistant to high pH and/or high temperature conditions (Nogi, 2005).

2.3.4.2 Alkaline active enzymes from alkaliphilic *Bacillus sp.*

Studies of alkaliphiles have led to the discovery of many types of enzymes that exhibit interesting properties. Below are examples of alkaline active enzymes from alkaliphilic bacillus

Alkaline Proteases

In 1971, Horikoshi reported the production of an extracellular alkaline serine protease from alkaliphilic ***Bacillus*** sp. strain 221. This strain, isolated from soil, produced large amounts of alkaline protease. The optimum pH of the purified enzyme was 11.5, and 75% of the activity was maintained at pH 13.0. Fujiwara et al. purified a thermostable alkaline protease from a thermophilic alkaliphile, ***Bacillus*** sp. strain B18. The optimum pH and temperature for the hydrolysis of casein were pH 12 to 13 and 85°C, both of which are higher than those for alkaline proteases.

Pectinases

The first paper on alkaline endopolygalacturonase produced by alkaliphilic *Bacillus* sp. strain P-4-N was published in 1972 . The optimum pH for enzyme action was 10.0 for pectic acid. Fogarty et al 1977 then reported that *Bacillus* sp. strain RK9 produced endopolygalacturonate lyase. The optimum pH for the enzyme activity toward acid-soluble pectic acid was 10.0.

Xyalanases

Dey et al. 1992 isolated an alkaliphilic thermophilic *Bacillus* strain (NCIM 59) that produced two types of cellulase-free xylanase at pH 10 and 50°C. Khasin et al. 1997 reported that alkaliphilic *B. stearothermophilus* T-6 produced an extracellular xylanase that optimally bleached pulp at pH 9 and 65°C.

Lipases

A thermophilic lipase-producing bacterium was isolated from a hot-spring area of Yellowstone National Park . The organism, characterized as a *Bacillus* sp., grew optimally at 60 to 65°C and in the pH range from 6 to 9. The partially purified lipase preparation had an optimum temperature of 60°C and an optimum pH of 9.5. It retained 100% of the original activity after being heated at 75°C for 30 min. It was active on triglycerides containing fatty acids having a carbon chain length of $C_{16:0}$ to $C_{22:0}$, as well as on natural fats and oils.

2.3.4.3 Cellulases from alkaliphiles

Industrial application of cellulases has mainly focused on fungal enzymes in terms of the potential of the use in saccharification of cellulosic material. Several species of bacteria such as *Clostridium*, *Cellulomonas* and *Ruminococcus* have also been studied for their ability to produce cellulolytic enzymes. Park et al. 1991 and Damude et al. 1993 studied a semialkaline cellulase produced by alkaliphilic *Streptomyces* strain KSM-9. Dasilva et al. 1993 reported two alkaliphilic microorganisms, *Bacillus* sp. strain B38-2 and *Streptomyces* sp. strain S36-2. The optimum pH and temperature of the crude enzyme activities ranged from 6.0 to 7.0 at 55°C for the *Streptomyces* strain and 7.0 to 8.0 at 60°C for the *Bacillus* strain. However, the results indicated that the properties of these enzymes were not appropriate for industrial purposes.

2.4 Cellulose and cellulose hydrolyzing enzymes

Cellulose is a structural polysaccharide and the most abundant natural polymer. It is hydrophilic and highly crystalline (Kuutti & Valtion teknillinen tutkimuskeskus, 2013). Cellulose is composed of linear β-1, 4-linked D-glucopyranose chains. While β-1,4-linked glucose is the chemical repeating unit, the structural repeat is β-cellobiose (Andersen, 2007) (Fig 2)

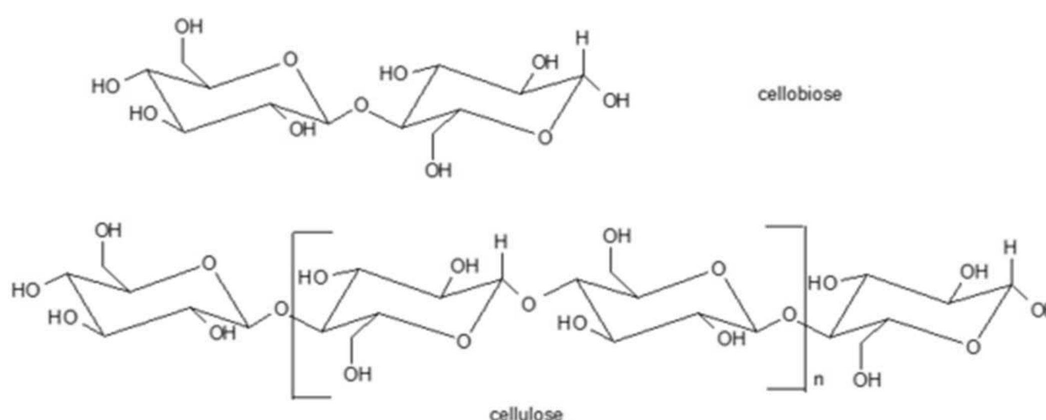


Fig 2: Structure of cellulose, (adapted from Kuuti 2013)

1. Show the β-1, 4-linkages in both the cellulose chain and cellobiose in the fig.

In cellulose, glucose chains are tightly bound to each other by van-der-Waals forces and hydrogen bonds into crystalline structures called elementary fibril. Aggregates of elementary fibrils, of essentially an infinite length, and a width of approximately 250 Å, are called microfibrils (Andersen, 2007). It has been shown that the -d-glucopyranose adopts the chain conformation, the lowest free energy conformation of the molecule. Consequently, the hydroxyl groups are positioned in the ring plane (equatorial), while the hydrogen atoms are in the vertical position (axial) (Klemm, Schmauder, & Heinze, 2005). Regions of high order microfibrils are termed crystalline, and less ordered regions are said to be amorphous (Andersen, 2007).

2.4.1 Supramolecular Structure

Cellulose exists in seven crystal structures (polymorphs) designated celluloses Iα, Iβ, II, IIII, IIIII, IVI, and IVII (O'Sullivan, 1997). These polymorphs can be inter-converted. Cellulose I, the native form is found in nature. Cellulose II, is obtained from cellulose I by any of the 2 processes i.e. regeneration, which is the solubilization

of cellulose I in a solvent followed by re-precipitation by dilution in water to give cellulose II or mercerization, which is the process of swelling native fibres in concentrated sodium hydroxide, to yield cellulose II on removal of the swelling agent (O'Sullivan, 1997). Celluloses III1and III11 are formed, in a reversible process, from celluloses I and II, respectively, by treatment with liquid ammonia or some amines, and the subsequent evaporation of excess ammonia (O'Sullivan 1997). Polymorphs IV1 and IV11 may be prepared by heating celluloses III1 and III11 respectively, to 206 ^{o}C in glycerol (O'Sullivan, 1997).

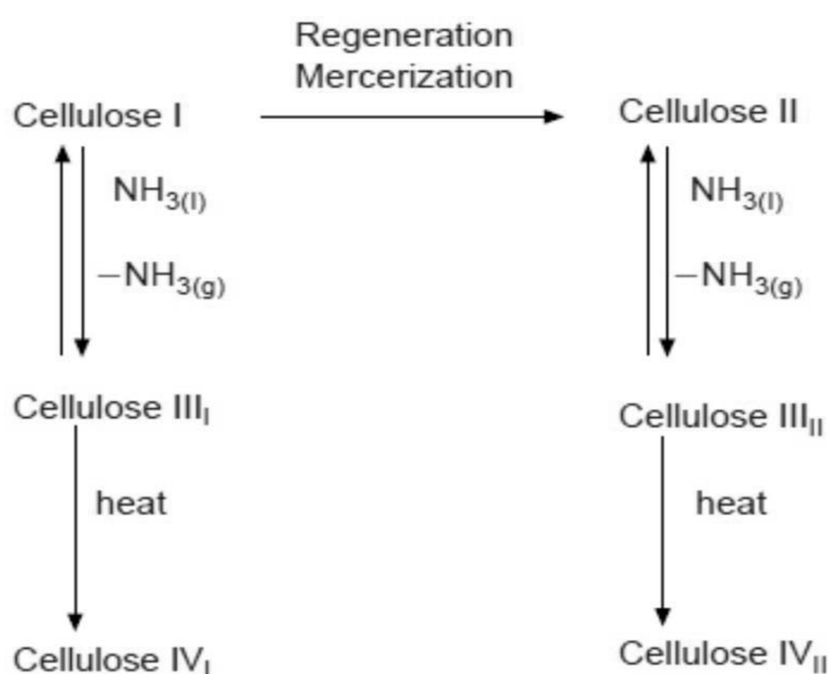


Fig 3: Interconversion of cellulose polymorphs (O'Sullivan 1997)

Cellulases are enzymes that hydrolyze β -1, 4 linkages in cellulose chains. They are produced by many organisms including fungi, bacteria, protozoans, plants, and animals (Zhang & Zhang, 2013). Cellulases are composed of independently folded, structurally and functionally discrete units, referred to as domains or modules. Most cellulases consist of one catalytic domain (CD) and one carbohydrate binding module (CBM), which is usually joined to the CD by a linker peptide (Andersen, 2007). The major role of CBM is to allow physical contact between the enzyme and substrate i.e. cellulose. Consequently, this increases the effective concentration of the enzyme as

well as time spent by the enzyme in close proximity to the substrate (Andersen, 2007). Some cellulases lack the CBM thus they have only one domain i.e. the CD. These enzymes have been shown to nonetheless have the ability to adsorb cellulose but with lower affinity compared to their counterparts (Andersen, 2007).

Complete hydrolysis of cellulose is due to the synergistic effect of 3 main types of cellulases. They include Endoglucanases (EC 3.2.1.4), exoglucanases (EC 3.2.1.91) and β -glucosidase (EC 3.2.1.21). To hydrolyse and metabolize insoluble cellulose, the micro-organisms must secrete cellulases either in free form or cell surface bound (Zhang, 2013). Cellulose producing microbes may produce the enzymes in discrete non-complexed forms or as complexed cellulases. These multi-enzyme complexes are referred to as cellulosomes (Zhang, 2013). Most aerobic micro-organisms produce a set of individual cellulases each containing a CBM linked to the CD whereas most anaerobes produce large multi-enzyme complexes which are usually bound to the cell surface of the micro-organism. Only a few of the enzymes in cellulosomes contain a CBM, but most of them are attached to the scaffoldin protein that contains a CBM (Zhang, 2013).

Endoglucanase or CMCase, randomly cut β -1, 4-bonds of cellulose chains, generating new ends (Zhang, 2013). They are, thus, primarily responsible for decreasing degree of polymerization of cellulosic substrates (Andersen, 2007). Below is a schematic diagram showing the action of endoglucanase on cellulose.

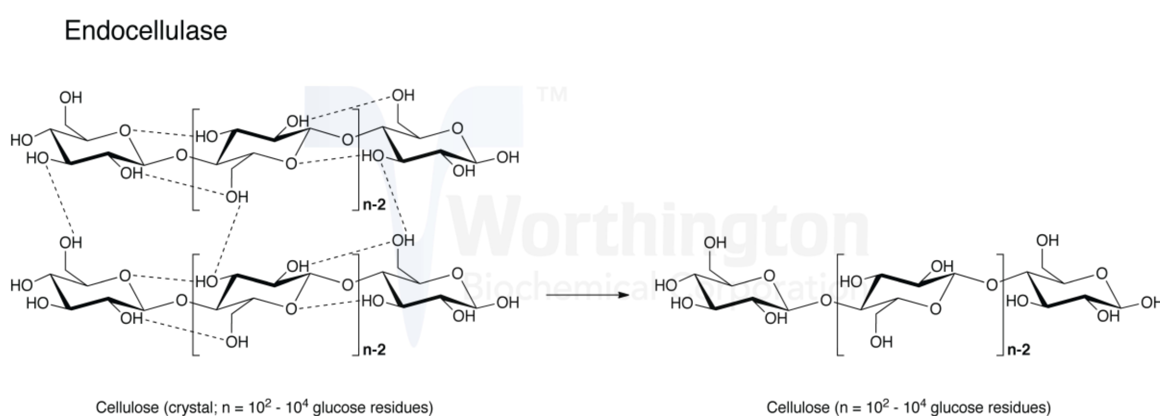


Figure 4: Diagram showing endonuclease hydrolysis of cellulose

Exoglucanases cleave cellulose on both reducing and non-reducing ends. The action of exoglucanases liberates either cellobiose or glucose as the major products (Zhang, 2013). Below is a schematic diagram showing the action of exoglucanases on cellulose.

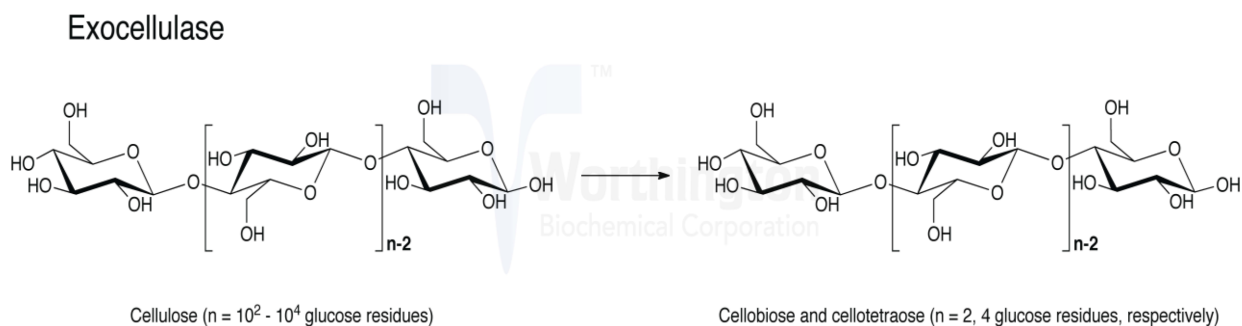


Figure 5: A long chain cellulose molecule is cleaved into cellotetraose and cellobiose by action of exoglucanase

β –glucosidase (cellobiase), the final enzyme in the cellulases complex hydrolyse soluble cellulodextrins and cellobiose to glucose. This hydrolytic reaction takes place in the liquid phase as opposed to exo- and endo- glucanase activities which occur on the surface of the insoluble cellulose particles (Andersen, 2007). The activity of β –glucosidase on cellulose is negligible since they only degrade cellobiose. Removal of cellobiose by the activity of β –glucosidase is a critical step in enzymatic hydrolysis of cellulose (Andersen, 2007). This is because cellobiose is an inhibitor of endoglucanase and exoglucanase (Zhang, 2013). β –glucosidase has often been found to be the rate-limiting step during cellulose hydrolysis and due to this commercial cellulase enzyme preparations are often supplemented with β –glucosidase. β –glucosidases do not have a CBM (Zhang, 2013). Below is a schematic diagram showing β –glucosidase activity on cellobiose.

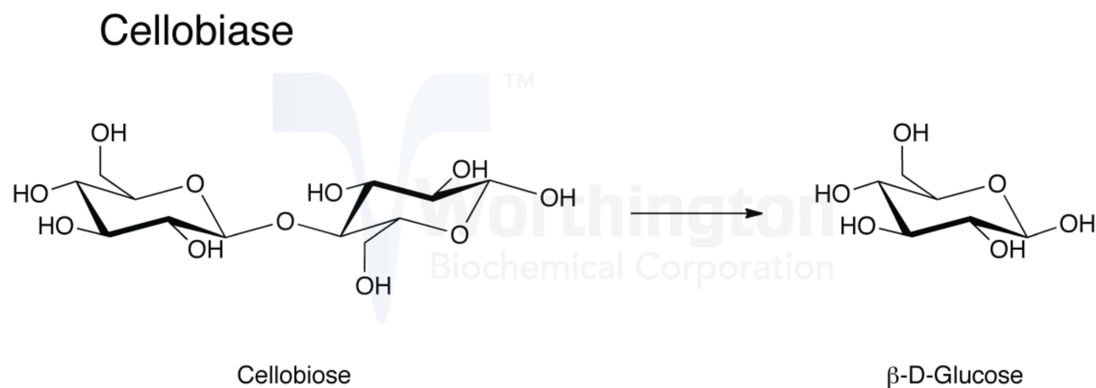


Figure 6: Cellobiase action on cellobiose to form glucose

2.4.2 Screening of Cellulase Producing Bacteria

There are several methods employed in screening of cellulase producing microbes i.e.

2.4.2.1 Cellulose agar clearance (cellulose agar)

This assay uses ball-milled, acid-swollen or microcrystalline cellulose such as Avicel in the agar at a final concentration of 0.1-0.5 %(w/v). Incorporation of the cellulose into solid agar media results in an opaque substrate due to the insolubility of the cellulose. Clearance indicates cellulolysis. Positive reactions indicate simultaneous action of all cellulolytic enzymes, although rates of clearance vary according to substrate. Generally microcrystalline cellulose is degraded more slowly than ball-milled or acid swollen cellulose (Pointing, 1999). However, the diameter of the clearing zone may not accurately reflect the true cellulase activity (Sadhu, Saha, Sen, Mayilraj, & Maiti, 2013).

2.4.2.2 Dye diffusion from a cellulose-dye complex (cellulose azure agar)

This assay utilises cellulose-azure into the upper two layers of agar tubes. The dye released from the substrate is determined densitometrically(Sadhu et al., 2013). The use of dyed cellulose yields less ambiguous data than either of the above methods since results are more visual. This method also tests for simultaneous action of all cellulase enzymes. Degradation of cellulose results in the release of a bound dye, the vertical migration of which can be observed. Dye decolorization generally follows migration due to cellulolysis (Pointing, 1999).

2.4.2.3 Dye staining of carboxymethylcellulose agar (CMC agar)

For rapid screening of cellulase producing bacteria, cells are inoculated in media containing 0.5% (W/V) carboxymethyl cellulose (CMC) as sole carbon source for a suitable incubation period.The resultant is flooded with 0.1% (W/V) Congo red and after 20 minutes, the dye is decanted and the plates are again flooded with 1M NaCl which is decanted after 20-30 minutes. Positive colonies are detected to be surrounded by a pale orange to clear zone against red background (Sadhu et al., 2013). Kasana et al. 2008 found that Gram's iodine for plate flooding in place of hexadecyltrimethyl ammonium bromide or Congo red, gave a more rapid and highly discernable result. However,it should be noted that plate-screening methods using dyes are not quantitative method for the poor correlation between enzyme activity and halo size. This problem has been solved by the development of short cellooligosaccharides possessing modified reducing terminal with chromogenic/fluorogenic groups e.g. fluorescein, resorufin and 4-methylumbelliferone for higher sensitivity and quantification..A major limitation of the use of fluorescent substrates into agar plates is the tendency for hydrolysis products to diffuse widely and therefore are not as readily used such compounds (Sadhu et al., 2013).

2.4.2.4 Esculin plus iron agar (esculin agar)

The hydrolysis of cellobiose to glucose is achieved by ~-glucosidase. This enzyme is probably ubiquitous among cellulolytic fungi producing hydrolytic endoglucanases or cellobiohydrolases. Activity of p-glucosidase can be detected by growth of the test fungus on agar containing esculin (6,7dihydroxycoumarin 6-glucoside) as the sole carbon source. Splitting of the substrate by the enzyme yields glucose, and a coumarin product that react with iron sulphate to produce a black colour in the growth medium(Pointing, 1999).

CHAPTER 3

3.0 Materials and Methods

3.1 Materials

Five alkaliphilic *Bacilli sp.* (*Bacillus sp.* LBW213, *Bacillus sp.* LBW 2 7 19, *Bacillus sp.* LBW33, *Bacillus sp.* LBW35 and *Bacillus sp.* LBW313) were graciously donated by Mr. Oluoch K. R. The bacteria had previously been isolated from water samples collected from Lake Bogoria, a soda lake found within the east African Rift valley in Kenya, and identified as belonging to alkaliphilic *Bacillus sp.* (personal communication from Mr. Oluoch K. R).

The reagents and chemicals used in this study were of analytical grade. Na_2CO_3, Ethanol, Glycine, $MgSO_4$, $NaNO_2$, Congo Red, KCl, Yeast Extract and Potassium Sodium Tartarate were obtained from Sigma Aldrich (Louisiana, USA). Agar, NaOH, K_2HPO_4 and NaCl were purchased from (Kobian Nairobi, Kenya), CMC was from Laborama Chemicals Nairobi, Kenya, while Glucose and DNS were from BDH Chemicals, Poole, England and Acros, New Jersey USA.

3.2 Methods

3.2.1 Screening for cellulase producers

Screening for cellulase producers among the five alkaliphilic *Bacilli sp.* was carried out by the method described by Bai *et al,* (2012), but with a slight modification. Briefly, the five bacteria were inoculated separately in enriched agar medium in petri dishes containing (g/L): CMC 5g, $NaNO_2$ 1g, K_2HPO_4 1g, KCl 1g, $MgSO_4$ 0.5g, yeast extract 0.5g, agar 16g, glucose 1g and, Na_2CO_3 200g. The Na_2CO_3 was prepared and autoclaved (121 ^oC, 20 Psi for 15min) separately, after which 50ml of it was added asceptically to the rest of the medium to raise the pH to 10.5. 20 ml of the sterile alkaline medium was asceptically poured in sterile petri dishes and allowed to solidify at room temperature. The five alkaliphilic bacillus species. were then inoculated in the plates and the latter incubated at 37 ^oC in an incubator for 96 h for colony formation. Plates were finally developed by flooding with 0.1 % (w/v) Congo red dye for 30 min, followed by decanting of the dye, and de-staining of the plate with 1M NaCl for 30

min. Cellulase-producing colonies were detected by the appearance of clearance zones or halos around the colonies.

3.2.2 Cultivation of cellulose producers in broth medium

Selected cellulase-producing bacteria were cultivated aerobically in separate 100 ml Erlenmeyer flasks containing 20 ml culture media prepared as described above, but without agar. Cultivation was carried out in a shaker incubator at 37 ^{o}C, 100 rpm, during which samples were withdrawn after 4, 12, 24 and 36 h for cell growth and cellulase activity determination as described below.

3.3 Analytical methods

3.3.1 Plate screening for cellulase producers

Selection for cellulase producing bacteria was carried out by determining the size of halos they produced during cultivation on agar plates. This was carried out by determining the ratio of halo to colony for each isolate by measuring the diameters of the colonies and the halos (in cm) they formed. Two diameter measurements (at right angles) were taken for each colony and its halo, averaged and used to determine the size of halo. Colonies producing the biggest halos were selected for further experiments.

3.3.2 Cell growth determination

Absorbance readings of the samples withdrawn after 4, 12, 24 and 36 h of cultivation were measured at 600 nm using a spectrophotometer, and characteristic bacterial growth curves generated.

3.3.3 Enzyme assays

Cellulase activity in the culture supernatants obtained after 4, 12, 24 and 36 h of cultivation was determined by centrifuging 19 ml of each culture at 5000 rpm and 4°C for 30 min in a centrifuge (Hanil Science Industry, Korea) and then assaying the cell-free culture supernatants for total cellulases (FPases) and endoglucanase using a glucose standard curve, prepared as described below:

NB: a) It was not possible to assay for exoglucanase and cellobiase, since the necessary reagents were not available at the time the research was conducted.

b) Cell-free culture supernatants were stored at – 80 ^{o}C when not in use.

3.3.3.1 Glucose standard curve

A stock glucose solution (2.5 mg/ml) was prepared by dissolving 250mg D-glucose (w/v) in a minimum volume of 50 mM NaOH-Glycine buffer pH 10.4 and then topping up to 100ml using the same buffer. A working glucose solution was prepared from the stock solution by aliquoting 10ml of the stock solution into a clean conical flask and then topping up to 100ml using the same buffer. Standard glucose solutions were prepared by pipetting the working glucose solution into test tubes in the range of 0-1ml at intervals of 0.2ml, in triplicates, and the final volumes in the test tubes made up to 1ml with the buffer. A summary of how the glucose standard solutions were prepared is shown in Table 1. The absorbance exhibited by the various amounts of reducing sugars in each test tube was then determined by the DNS method as described by Miller (1972). Briefly, 1 ml of DNS reagent (DNS 10g, Phenol 2g, Na_2SO_3 0.5g, and NaOH 10g made up to 1 litre) was added to each of the glucose samples in the test tubes. (To avoid loss of the samples due to evaporation, the test tubes were covered with paraffin film). Samples were heated at 90 ^{0}C in a water bath (Memmert, Germany) for 15 minutes to develop the characteristic red-brown colour, after which 0.3 ml of 40% (w/v) potassium sodium tartrate (Rochelle salt) solution added to stabilize the colour. After cooling to room temperature in tap water for 30 min, the absorbance of the coloured products was measured at 540 nm in a spectrophotometer, and a standard curve generated (Table 4 and Fig 7).

Table 4: Preparation of glucose standards

	Test tube number					
	1	**2**	**3**	**4**	**5**	**6**
Glucose concentration (mg/ml)	0	0.2	0.4	0.6	0.8	1.0
Vol. of 50 mM glycine-NaOH buffer added	1.0	0.8	0.6	0.4	0.2	0
Final concentration of glucose (mg/ml)	0	0.05	0.1	0.15	0.2	0.25
Absorbance 540 nm	0	0.223	0.351	0.405	0.511	0.542

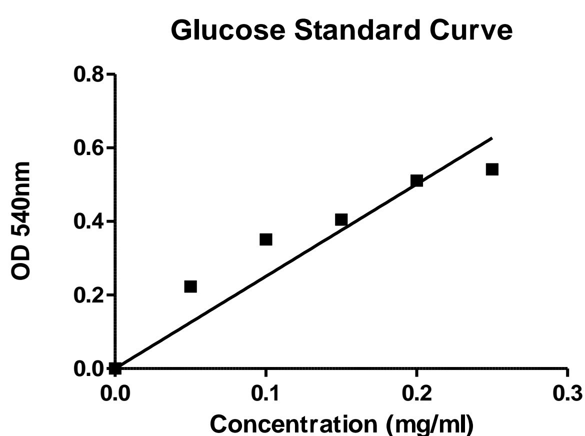


Fig 7: Glucose standard curve

3.3.3.2 FPAse Activity

FPase activity in the cell-free culture supernatants was determined by the method described by Romero et al., 1999. Briefly, 50 µl of crude enzyme was added to 1.95 ml of 50 mM NaOH-glycine buffer, pH 10.4 containing 50 mg Whatman no. 1 filter paper. The mixture was incubated at 50 °C for 60 min to allow the enzyme hydrolyse the substrate. The reaction was then stopped by the addition of 1 ml DNS solution followed by heating at 90 ^{0}C for 15 min to develop the characteristic red-brown colour. 0.3 ml of 40% (w/v) potassium sodium tartrate (Rochelle salt) solution was then added to stabilize the colour. Sample was then cooled in tap water for 30 min and the absorbance read at 540 nm. The readings were then used in the standard curve to

determine the amount of reducing sugars formed which were then used to calculate the activity of the enzyme. 1 U of enzyme activity was defined as the amount of enzyme releasing 1 μmol reducing sugars per min under the standard assay conditions.

The experiment was done in triplicates and heat inactivated enzyme (boiled at 100 ^{o}C for 15 min) served as control.

3.3.3.3 Endoglucanase activity

Endoglucanase activity in the cell-free culture supernatants (crude enzyme) were determined by the method described by Lopez et al., 2007. Briefly, 500 μl of crude enzyme was added to 500 μl of 50mM NaOH - Glycine buffer, pH 10.4, containing 1% (w/v) CMC. The mixture was then incubated at 50 ^{o}C for 10 min to allow the enzyme hydrolyse the substrate. The reaction was then stopped by the addition of 1 ml DNS reagent followed by heating at 90 ^{o}C for 15 min in a water bath (Memmert, Germany). 0.3 ml 40% (w/v) potassium sodium tartrate (Rochelle salt) solution was then added to stabilize the characteristic red-brown colour. Sample was then cooled to room temperature in tap water for 30 min, after which its absorbance was measured at 540 nm in a spectrophotometer. Finally, the absorbance reading was used with the standard curve to determine the amount of reducing sugars formed from which the activity of the enzyme was calculated. 1 U of enzyme activity was defined as the amount of enzyme releasing 1 μmol reducing sugars per min under the standard assay conditions. The experiment was done in triplicates and heat inactivated (boiled at 100 ^{0}C for 15 min) enzyme served as control.

CHAPTER 4

4.0 Results

4.1 Plate test assay for screening for cellulase producers

Five alkaliphilic *Bacilli* sp. were cultivated for 96 h in alkaline agar-medium containing CMC-cellulose as carbon source. All five bacteria were able to grow in the medium. Morphologically, the colonies were circular with flat smooth entire margins. LBW 213 and LBW 2 7 19 were observed to be white, opaque and dull. LBW 33 and LBW 313 were shown to be creamy, translucent and shiny whereas LBW 35 was white, translucent and shiny. Similar results were obtained for these bacterial species (personal communication from Mr. Oluoch, K. R). The colonies also showed cellulase activities as depicted by formation of general clearance zones or halos (yellow) around them (Fig 8).

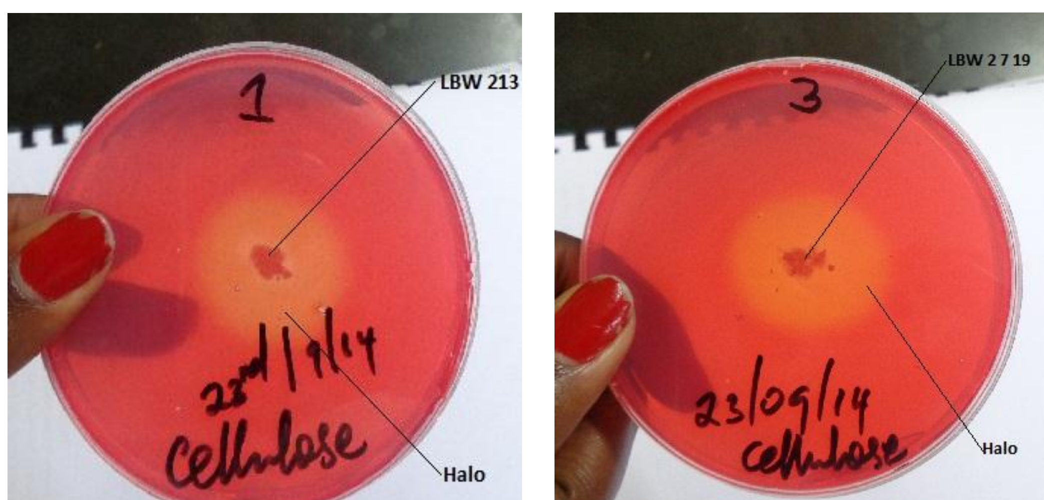


Fig 8:Picture of the 5 alkaliphilic bacterial colonies grown on agar plates (pH 10.4)containing CMC as carbon source, followed by assay of depolymerized cellulose (depicted by halos formed around the colonies after flooding plates with 0.1 % (w/v) Congo red dye and destaining with 1 M NaCl solution)

In addition to the general yellow clearance zones, colonies formed by *Bacillus sp.* 21 3 and *Bacillus sp.* 3 3, exhibited white clearance zones immediately around them, suggesting that they may have produced the highest amounts of cellulases.

The size of halo formed by each colony was calculated by determining the ratio of halo diameter to colony diameter. This ratio was then used as a semi-quantitative method for classifying the isolates as excellent cellulase producers (halo size > 1.5),

very good producers (halo size >1 but < 1.5), good producers (halo size > 0.5 but < 1), weak producers (halo size > 0 but < 0.5) and poor producers where no clear zones were observed. Based on this analysis, all five bacteria exhibited halo sizes of about 2.5 and were therefore classified as excellent cellulase producers (Table 5) and for this reason, considered for further experiments.

Table 5: Classification of the 5 alkaliphilic bacillus species based on their halo sizes (calculated from the ratio of diameter of halo to that of bacterial colony)

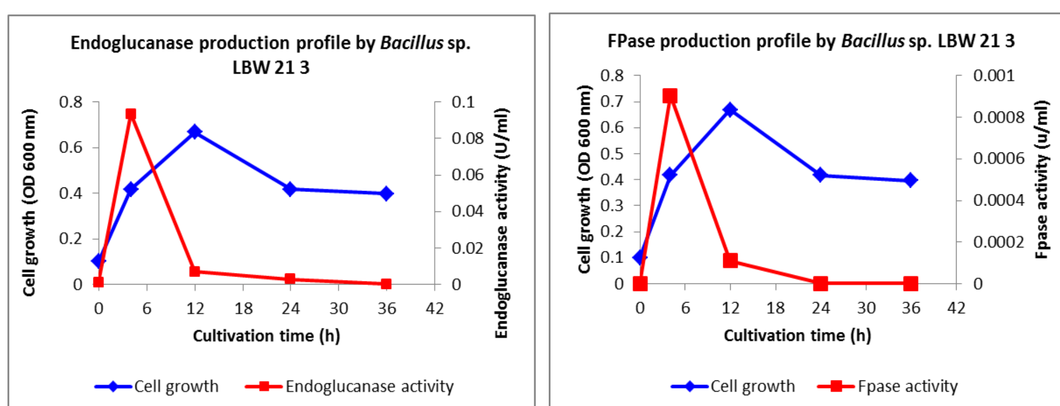
Isolate	Diameter of colony (cm)	Diameter of halo (cm)	Halo Size	Classificati on
LBW213 (1)	1.25	3.1	2.48	Excellent
LBW 2 7 19 (3)	1.15	3.05	2.65	”
LBW33 (4)	1.3	3.1	2.38	”
LBW35 (5)	1.3	3.3	2.54	”
LBW313 (6)	0.9	2.2	2.44	”

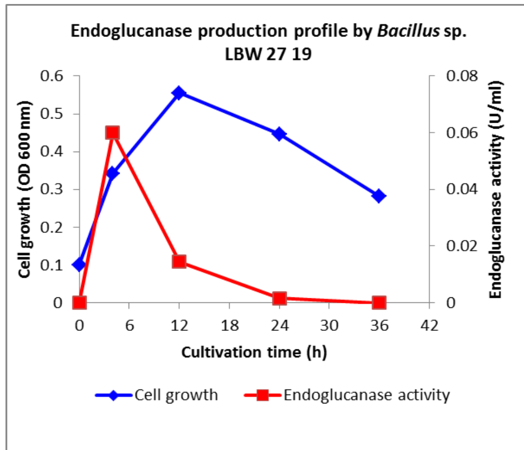
4.2 Cell growth and enzyme production

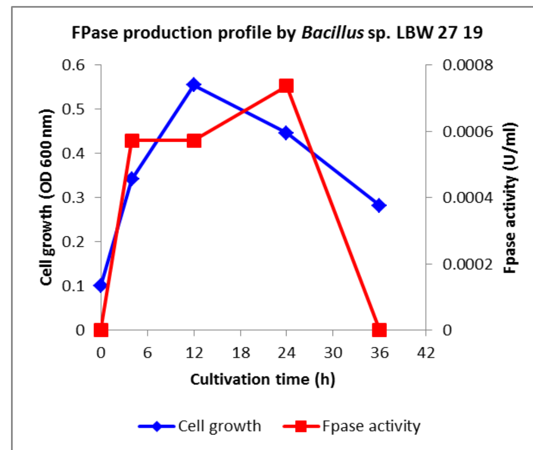
All the 5 bacteria were considered as excellent cellulase producers and were therefore cultured separately (37°C and 100 rpm) in alkaline (pH 10.4) in broth media containing CMC as carbon source in order to study the relationship between cell growth and enzyme production. During the 36 h incubation period, samples were withdrawn periodically and cell growth determined by measuring their optical densities at 600 nm. Calculation of enzyme activities was done by determining the amounts of reducing sugars formed at the end of enzymatic reactions with the aid of the glucose standard curve.

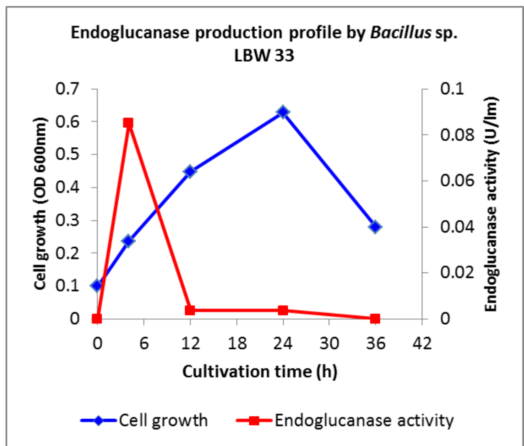
During the cultivation period, all bacteria exhibited growth and enzyme production. Individual cells of each bacteria reached their stationary phases after 12 h of growth, reaching maximum OD 600 nm of 0.668, 0.555, 0.574 and 0.8 for *Bacillus* sp. LBW

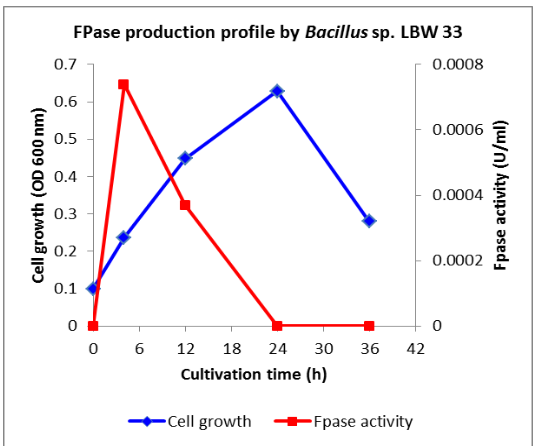
213., *Bacillus sp*. LBW 27 19., *Bacillus* sp. LBW 3 5 and *Bacillus* sp. 313, respectively, and 0.555 for *Bacillus* sp. 27 19 after 24 h of growth (Fig 9). Both endoglucanase and Fpase activities of the cells were followed during the cultivation. Basal endoglucanase activities of 0 U/ml were observed from the start of growth for all the bacteria which increased to a maximum of 0.093 U/ml, 0.064 U/ml, 0.085 U/ml, 0.091 U/ml and 0.106 U/ml after 4 h of cultivation for *Bacillus* sp. LBW 213, *Bacillus* sp. 27 19, *Bacillus* sp. LBW 3 3, *Bacillus* sp. LBW 3 5 and *Bacillus* sp. LBW 313, respectively. Thereafter, there was a decrease in the activity for all the bacteria, reaching undetectable levels after 36 h of cultivation for *Bacillus* sp. LBW 213, *Bacillus* sp. 27 19 and *Bacillus* sp. LBW 33, and after 12 h of cultivation for *Bacillus* sp. LBW 3 5 and *Bacillus* sp. LBW 3 13. Basal Fpase activities of 0 U/ml were also observed from the start of growth for all the bacteria which increased to a maximum of 0.009 U/ml, 0.0007 U/ml, 0.0004 U/ml and 0.0004 U/ml after 4 h of cultivation for *Bacillus* sp. LBW 21 3, *Bacillus* sp. LBW 33, *Bacillus* sp. LBW 35, and *Bacillus* sp. LBW 313, respectively, and 0.0007 U/ml after 24 h of cultivation for *Bacillus* sp. LBW 27 19. Thereafter, there was a decrease in the activity for all the bacteria, reaching undetectable levels after 24 h of cultivation for *Bacillus* sp. LBW 213, *Bacillus* sp. LBW 33 and *Bacillus* sp. LBW 35, and 36 h for *Bacillus* sp. LBW 2 7 19. For *Bacillus* sp. LBW 313, no FPase activity was detected after 36 h of cultivation in the medium.

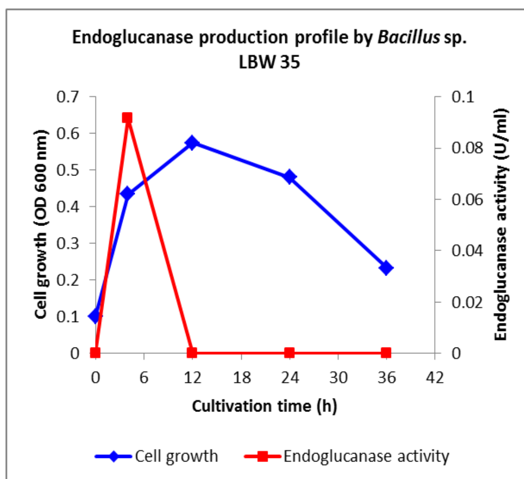


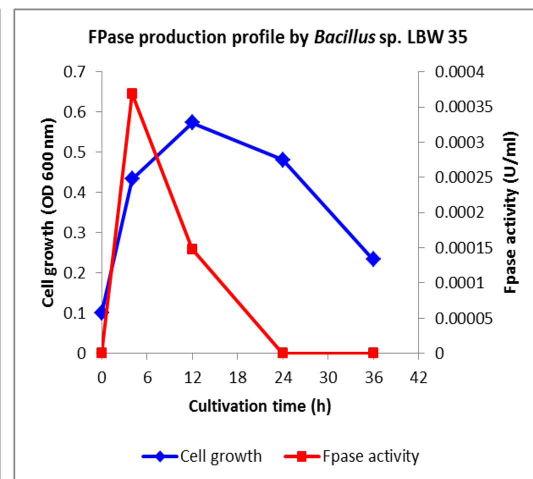

Endoglucanase production profile by Bacillus sp. LBW 27 19
Cell growth (OD 600 nm)
Endoglucanase activity (U/ml)
Cultivation time (h)
Cell growth
Endoglucanase activity


FPase production profile by Bacillus sp. LBW 27 19
Cell growth (OD 600 nm)
Fpase activity (U/ml)
Cultivation time (h)
Cell growth
Fpase activity


Endoglucanase production profile by Bacillus sp. LBW 33
Cell growth (OD 600nm)
Endoglucanase activity (U/lm)
Cultivation time (h)
Cell growth
Endoglucanase activity


FPase production profile by Bacillus sp. LBW 33
Cell growth (OD 600 nm)
Fpase activity (U/ml)
Cultivation time (h)
Cell growth
Fpase activity


Endoglucanase production profile by Bacillus sp. LBW 35
Cell growth (OD 600 nm)
Endoglucanase activity (U/ml)
Cultivation time (h)
Cell growth
Endoglucanase activity


FPase production profile by Bacillus sp. LBW 35
Cell growth (OD 600 nm)
Fpase activity (U/ml)
Cultivation time (h)
Cell growth
Fpase activity

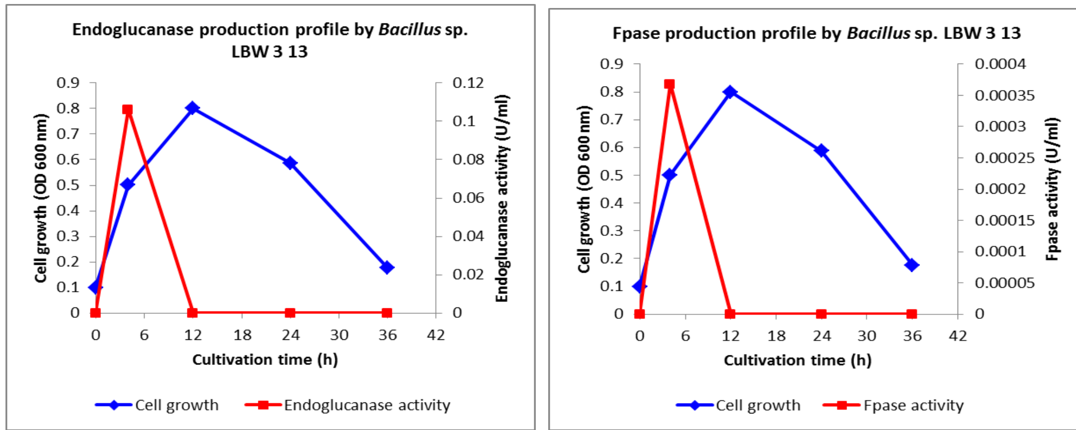


Fig 9 : Cell growth and enzyme production profiles for the 5 alkaliphilic *Bacillus* sp.

CHAPTER 5

Discussion

Five alkaliphilic *Bacilli* sp., previously obtained from Lake Bogoria – a soda lake found in the east African Rift valley, were screened for alkaline active cellulase producers on agar plates containing CMC as carbon source, pH 10.4 at 37°C. All the bacteria tested positive for the enzyme and were classified as excellent producers of the same, since they exhibited halos whose sizes were about 2.5. Consequently all the 5 bacteria were subjected to cultivation in alkaline broth media under the same conditions, and both cell growth and cellulase production monitored periodically. I was only able to monitor for Fpases and endoglucanases, since reagents required to assay for both exoglucanases and cellobiases were unavailable. All the five bacteria exhibited maximum alkaline-active endoglucanases in the range 0.06 U/ml–0.106 U/ml during their exponential phases of growth. The bacteria also exhibited maximum FPase activities in the range 0.000369 U/ml –0.00904 U/ml during their exponential phases of growth, except for *Bacillus* sp. 27 19, which did so during its death phase. This may be attributed to the fact that the bacteria preferred to utilize glucose which was present in the medium instead of CMC, and only started utilizing the latter after the glucose got exhausted. Enzyme production by the 5 alkaliphilic bacillius sp. is low compared to those of other known alkaliphilic bacillus species. *Bacillus circulans* has been shown to exhibit an endoglucanase activity of 4.9 U/ml at pH 9 (Niralma and Sindhu, 2011) while *Bacillus subtilis* exhibited 0.34U/ml of the same enzyme (Deka et al., 2011) Comparison data for alkaline Fpase activity from bacterial species was not easily found in literature. The low production levels by may be attributed to un-optimized culture conditions. Kun et al (2012) have shown that *Marinomicrobium* species has an alkaline cellulase activity of 2.5IU/ml under optimized conditions which was 3.1 times greater than that obtained before optimisation of the culture conditions. Decka et al., 2011 have also shown that under optimized conditions, there was a 6 fold increase in enzyme production by *Bacillus subtilis*.

CHAPTER 6

6.0 Conclusion

All 5 alkaliphilic bacillus species under this study possess extracellular alkaline-active cellulase activities, with *Bacillus sp.* LBW21 3 and *Bacillus* sp. LBW 33 producing the highest amounts of both endoglucanase and Fpase

6.1 Recommendations

1. Assay for exoglucanase and cellobiase in the frozen culture supernatants
2. Optimize culture conditions for maximum enzyme yields
3. Characterize the crude enzyme with respect to the harsh conditions it is expected to encounter during its application in the detergent, textile or pulp and paper industry
4. Evaluate the efficacy of the enzyme with regard to application in the detergent, textile or pulp and paper industry
5. Establish strong collaborations with potential partners in industry
6. Identify the alkaliphilic cellulase-producing *Bacilli* sp. used in this study

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