

**ISOLATION, IDENTIFICATION AND CHARACTERIZATION  
OF ALKALITHERMOPHILES FROM THE HOT SPRINGS  
OF LAKE BOGORIA OF THE KENYAN RIFT VALLEY**

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**Isolation, Identification and Characterization of  
Alkalithermophiles from the Hot Springs of Lake Bogoria of  
the Kenyan Rift Valley**

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**A thesis submitted in partial fulfilment for the degree of Master of  
Science in Biotechnology in the Jomo Kenyatta University of  
Agriculture and technology**

**2009**

## DECLARATION

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

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## **DEDICATION**

I dedicate this work to the people I love. First to my dear parents, Mr. Simasi L. Wafula and Mrs. Felistus N. Wafula, secondly to my uncle and aunt Mr. Ainea Okhoya and Mrs. Loice Okhoya and lastly my sisters Roselyn, Karen and Jacinta and my brothers Lan and Jimmy. You put your heart in all that I do, supported, facilitated and enabled me to achieve all I have.

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## LIST OF ABBREVIATIONS AND ACRONYMS

<b>Bp</b>	Base pairs
<b>BSA</b>	Bovine Serum Albumin
<b>CMC</b>	Carboxymethylcellulose Sodium Salt
<b>dNTP's</b>	Deoxynucleotide Triphosphates
<b>DNA</b>	Deoxyribonucleic Acid
<b>ddH<sub>2</sub>O</b>	Double distilled water
<b>EDTA</b>	Ethylene diamine tetra-acetic acid
<b>EtBr</b>	Ethidium Bromide
<b>G+C</b>	Guanine and Cytosine
<b>JKUAT</b>	Jomo Kenyatta University of Agriculture and Technology
<b>PCR</b>	Polymerase Chain Reaction
<b>rDNA</b>	ribosomal Deoxyribonucleic Acid
<b>RFLP</b>	Restriction Fragment Length Polymorphism
<b>rRNA</b>	ribosomal Ribonucleic Acid
<b>SDS</b>	Sodium dodecyl Sulphate
<b>TBE</b>	Tris Boric acid Ethylene diamine tetra-acetic acid
<b>TE</b>	Tris Ethylene diamine tetra-acetic acid



## ABSTRACT

Hot springs are aquatic environments with high temperatures. They harbor diverse groups of micro-organisms that have developed mechanisms to thrive at wide temperature ranges, according to their optimal growth requirements. Research on these microbes is motivated by their great biotechnological potential such as production of useful secondary metabolites and enzymes with industrial application. The objectives of this research were to determine the physico-chemical characteristics of the hot springs in Lake Bogoria, isolate, characterize and identify alkalithermophilies from the hot springs of Lake Bogoria and then screen the isolates for the production of useful metabolites. Samples were collected from the hot springs of Lake Bogoria. The physico-chemical characteristics were established in the field. Enrichment and isolation was done on modified Horikoshi media, at pH 9, and 55°C. Isolates were identified using morphological, physiological, biochemical and molecular characterization. Molecular analysis of Polymerase Chain Reaction amplicons with 16S rDNA region, were amplified using primers specific for bacteria. Restriction Fragment Length Polymorphism (RFLP), sequencing and phylogenetic analysis were done, with *Hae* III and *Msp* I and a Phylogenetic tree was drawn. The physico-chemical parameters established were temperature 90°C and pH 8. Thirty six isolates were obtained and they grew at varied NaCl concentration (2-20%), temperatures (24°C-65°C) and pH (5.7-9). They were non-fermentative, xylanolytic, non-cellulolytic, amylolytic, and some lipolytic and proteolytic. Best enzyme activity was at 55°C. Molecular analysis of Polymerase Chain Reaction amplicons with 16S rDNA

region, resulted in only three RFLP patterns with *Hae* III and *Msp* I suggesting that the community structure was homogeneous in the sampling areas. Phylogenetic analysis showed that all the isolates belonged to the domain bacteria, phylum *firmicutes*, class *Bacilli*, order *Bacillales*, family *Bacillaceae* and genus *Bacillus*. The hot springs of Lake Bogoria, harbor alkalithermophiles which have the potential to produce useful secondary metabolites such as alkali stable enzymes that can form the basis of bioeconomy if fully exploited. Combining morphological, physiological and molecular approaches and by the use of the modified Horikoshi media, novel isolates related to *B. halodurans* were isolated from the hot springs of Lake Bogoria. Based on the results, the isolates recovered are thermotolerant, alkalitolerant and halotolerant. The isolates also have the potential to produce useful secondary metabolites like enzymes

## **1.0 CHAPTER ONE**

### **1.1 INTRODUCTION**

Alkalithermophiles are alkaliphilic thermophiles; multi-extremophiles that can survive in extreme conditions of high alkaline and elevated temperatures (Kevbrin *et al.*, 1998). Most of the hot springs within the Kenyan lakes harbor extremophiles. Extremophiles are micro-organisms that are well adapted to thrive and grow optimally at environmental and physico-chemical parameters unsuitable for the typical and widely studied mesophilic micro-organisms, such as *Escherichia coli*, *Bacillus subtilis* and *Neurospora crassa* (Kevbrin *et al.*, 1998). Soda lakes represent the naturally occurring environments on earth, with pH values generally greater than 10, occasionally reaching pH 12 and therefore, characterized by the presence of large amounts of  $\text{Na}_2\text{CO}_3$ , (usually as  $\text{Na}_2\text{CO}_3 \cdot 10\text{H}_2\text{O}$  or  $\text{Na}_2\text{CO}_3, \text{NaHCO}_3 \cdot 2\text{H}_2\text{O}$ ). They are depleted of  $\text{Mg}^{2+}$  and  $\text{Ca}^{2+}$  because of the insolubility of carbonates under alkaline conditions (Duckworth 1996). Different definitions have been proposed for both extreme conditions of elevated temperature and high pH (Wiegel, 1986, 1998 and 2002). Most of the described extremophiles are characterized only by one distinctive extreme such as temperature or pH. However, others are multi-extremophiles, for example alkalithermophiles (Kevbrin *et al.*, 1998), which are micro-organisms that can survive high alkaline conditions and elevated temperatures.

Alkalithermophiles are hypothesized to have been among the earliest forms of life. Some geochemical models and geological evidence suggest that the ocean of early

earth was alkaline in nature and capable of supporting primitive alkaliphilic micro-organisms (Kevbrin *et al.*, 1998). These micro-organisms can be regarded to as one type of model organism for the study of possible extraterrestrial life (Kevbrin *et al.*, 1998). They are among the possible types of organisms that could have evolved on Mars (Wiegel and Adams, 1998). Life probably originated not in hyperthermobioc environments but on mineral surfaces in moderate thermobioc (60-85°C range), relatively shallow pools at the edges of the early Earth's oceans (Wiegel and Adams, 1998). Some of the alkalithermophiles can, therefore, function as model organisms for primordial life forms when earth's environment may have been extreme in pH and temperature. Some of the alkalithermophiles, such as chemolithotrophic CO oxidizing iron reducers, could have been one of the earliest microbial life forms on this planet (Kevbrin *et al.*, 1998). In short, many opportunities exist for studies to answer many questions regarding this interesting subgroup of extremophiles (Kevbrin *et al.*, 1998).

The boundaries for life have not yet been found in respect to combined elevated temperatures and pH (i.e. the highest growth temperatures at pH 12 or the most alkaline pH for growth at 100°C). For example, it is possible that micro-organism that can grow optimally at around 100°C and at a pH above 11 or even 12 exist. This extends the boundaries for conditions under which life can thrive on earth (Kevbrin *et al.*, 1998). Subsequently, this leads to new theories of how life could have evolved on early earth and whether it could presently or in the future exist in extraterrestrial habitats. The optimum pH for most alkaliphiles is about 10 (Horikoshi, 1998).

In recent years, awareness of alkaliphiles has blossomed (Yanhe *et al.*, 2004) due to an interest in their physiological adaptations to high pH and their potential uses in biotechnological applications (Horikoshi, 1999). This has led to more systematic studies and a rapid expansion in the numbers and types of alkaliphiles isolated from a variety of environments. Alkaliphilic micro-organisms offer a multitude of actual or potential applications in various fields of biotechnology.

Alkalithermophiles also possess useful physiological properties which can facilitate their exploitation for various purposes; for example, they are alkaline and heat stable and therefore, suitable for exothermic industrial processes. Sodium-dependent transport systems have been reported, which generate a sodium motive force via  $H^+/Na^+$  antiport systems. Internal  $H^+$  is exchanged with  $Na^+$  by the cells, and  $Na^+$  then accompanies substrates into the cells (Horikoshi, 1998). Sodium ions in the surrounding environment are essential for effective solute transport through the membranes of alkaliphilic *Bacillus* spp. 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 cotransport of various substrates. Alkaliphiles have clearly gained large amounts of genetic information by evolutionary processes and exhibit an ability in their genes to cope with particular environments; therefore their genes are a potentially

valuable source of information waiting to be explored and exploited by biotechnologists.

Genes responsible for the alkaliphily of *Bacillus halodurans* C-125 and *Bacillus firmus* OF4 have been analyzed (Horikoshi, 1999). Cell walls of several alkaliphilic micro-organisms also contain a large amount of acidic amino acids. The acidic charges on these components may act as charged membranes, reducing the pH on the cell surface between 8 and 9 (Horikoshi, 1998) allowing the cell to maintain a neutral internal pH. Plasma membranes may also maintain pH homeostasis by using the Na<sup>+</sup>/H<sup>+</sup> antiporter system, the K<sup>+</sup>/H<sup>+</sup> antiporter, and ATPase-driven H<sup>+</sup> expulsion. Recent studies on the critical antiporters in several laboratories have begun to clarify the number and characteristics of the porters that support active mechanisms of pH homeostasis (Horikoshi, 1999)

Genome sequence comparisons between *B. halodurans* and *B. subtilis* reveal that among the total CDSs; 8.8% match sequences of proteins found only in *B. subtilis*. The Shine-Dalgarno (SD) sequence was complementary to the one found at the 3' end of 16S rDNA (*UCU UUC UCC ACU AG*) of alkaliphilic *B. halodurans* C-125 (Takami *et al.*, 2000); as well as that of *B. subtilis*. *B. halodurans* C-125 which is quite similar to *B. subtilis* in terms of genome size, G+C content of the genomic DNA and the physiological properties used for taxonomical identification, except the alkaliphilic phenotype. Furthermore, the phylogenetic placement of *B. halodurans* C-125 based on

16S rDNA sequence analysis indicates that this organism is more closely related to *B. subtilis* than to other members of the genus *Bacillus*. Four types of ATPases were also well conserved between *B. halodurans* and *B. subtilis*. ABC transporter genes are the most frequent class of protein coding genes found in *B. halodurans* genomes as in the case of *B. subtilis*. 16S rDNA sequencing data of 451545 isolates has given new dimensions to microbial taxonomy and is likely to lead to revision of concepts such as species, organism and evolution (Wose *et al.*, 2007). 16S rDNA gene sequencing is often used as an alternative method to define microbes at species level. Protein coding genes having high variability has been successfully used to differentiate taxa that cannot be identified solely on the basis of 16S rDNA sequences (Shalini *et al.*, 2009).

## **1.2 Problem statement**

Enzymes, drugs and other industrial valuable products such as organic acids, antibiotic, enzymes, carotenoids and cholic acid derivatives, are high value products which can form the basis of bioeconomy yet not fully exploited. This project intended to determine the physico-chemical characteristics of the hot springs in Lake Bogoria, isolate, characterize and identify alkalithermophiles from the hot springs of Lake Bogoria, Kenya, and targeted to screen the isolates for the potential to produce valuable enzymes and secondary metabolites such as organic acids.

## **1.3 Justification**

Alkalithermophiles are unique micro-organisms, with great potential for microbiology and biotechnological exploitation. They should therefore, be isolated, characterized and identified for maximum utilization in areas such as; production of industrial

valuable enzymes and metabolites, general genetic resources for applications like production of signals, genetic analysis, mechanisms of membrane transport, pH regulation and in the taxonomy of alkaliphilic micro-organisms (Horikoshi, 1999). Very few alkalithermophiles from soda lakes have been validly published although some alkalithermophilic *Bacillus*-like strains have been isolated (unpublished) and Very little is known about the physiology of the larger proportion of alkalithermophilic micro-organisms that have escaped cultivation. Data generated from this research will provide information for researchers to understand Lake Bogoria's microbial ecology and biotechnological benefits.

#### **1.4 Hypothesis**

The hot springs of Lake Bogoria harbor novel and previously uncultured micro-organisms with biotechnological potential.

#### **1.5 Objectives**

##### **1.5.1 General objective**

To isolate, characterize and identify alkalithermophilic micro-organisms from Lake Bogoria.

##### **1.5.2 Specific objectives**

- 1). To determine the physico-chemical characteristics of the hot springs in Lake Bogoria of the Kenyan Rift Valley.
- 2). To isolate alkalithermophiles from the hot springs of Lake Bogoria.



- 3). To characterize and identify the isolates.
- 4). To screen the isolates for useful secondary metabolites and production of exoenzymes.

## **2.0 CHAPTER TWO**

### **2.1 LITERATURE REVIEW**

#### **2.1.1. General microbial biodiversity of soda lakes in the world.**

It is well understood that saline lakes have a limited species complement in macro organisms in contrast to considerable biodiversity in micro-organisms (Grant *et al.*, 1990; Duckworth *et al.*, 1996). Basic knowledge about molecular mechanisms of alkaliphilic microbes, stem mainly from studies by Horikoshi (1999) on *Bacillus halodurans* C-125 and by Keller and Zengle, (2003) investigating hot springs in Yellowstone National Park (Wyoming, U.S.A), which revealed large archaeal diversity. The archaea are believed to be the dominating micro-organisms in hot springs. An analysis of the bacterial community in the same hot spring samples, revealed remarkable bacterial diversity with twelve new division-level lineages. The same study showed that members of the bacterial domain seemed to outnumber the archaea in this hydrothermal environment (Keller and Zengle, 2003).

A subsequent study of microbial diversity in hot springs of Yellowstone National Park in (Wyoming) U.S.A, showed that although all the hot springs were in close geographical proximity, they had similar temperatures (between 85°C and 95°C) and a comparable pH value (7.8–8.9), but they differed markedly with regard to their overall microbial diversity. This finding indicated that, in common with the complexity of microbial diversity in soil, geochemical variations affect biodiversity and that only

studies that incorporate measurements of geochemical parameters will allow the understanding or begin the prediction of biodiversity (Keller and Zengle, 2003).

Two Gram-positive, endospore-forming, alkaliphilic bacteria were isolated from water samples obtained from the Okuhida hot spa area of Japan. The unknown bacteria were characterized using phenotypic and molecular taxonomic methods. On the basis of phylogenetic evidence and phenotypic distinctiveness, a new species, *Bacillus okuhidensis* sp. nov., was proposed. The type strain of *Bacillus okuhidensis* is GTC 854T (= JCM 10945T = DSM 13666T) (Zhiyu *et al.*, 2002)

Studies of Yanhe *et al.* (2004) on the Baer Soda Lake located in the Hulunbeir area of Inner Mongolia, Autonomous Region of China, showed that with the 16S rDNA phylogenetic analysis, a number of diverse bacteria residing in Baer Soda Lake could be characterized basing on culture and molecular methods. Fifty-three alkaliphilic bacteria were isolated from sediment samples, and 20 of them were subjected to 16S rDNA sequence analysis. Although some of the clones were related to alkaliphilic bacteria from soda lakes such as *Alkalispirillum mobile*, *Thioalcalovibrio denitrificans*, and *Halomonas campisalis*, many of the clones were related to known species (more than 97% similarity) from non-alkaline environments. These isolates were affiliated with the genera of *Bacillus*, *Amphibacillus*, *Gracilibacillus*, *Alkalibacterium*, *Salinicoccus*, *Exiguobacterium*, *Halomonas*, *Pseudomonas*, *Marinospirillum*, and *Cyclobacterium*. Of the 20 isolates, only 4 were Gram-negative,

and Gram positive isolates were diverse and predominant. However, the majority of the clones obtained from Baer Soda Lake were related to *Proteobacteria*, with only about 10% of the clones affiliated with Gram-positive bacteria.

A novel, obligately anaerobic, alkalithermophilic, chemo-organotrophic bacterium was isolated from the sediment of an alkaline hot spring located on Paoha Island in Mono Lake, California, USA. This rod-shaped bacterium was motile via peritrichous flagella. The novel isolate could reduce Fe (III) and Se (IV) in the presence of organic matter. On the basis of physiological properties, 16S rRNA gene sequence and DNA–DNA hybridization data, strain PAOHA-1<sup>T</sup> (=DSM 14826<sup>T</sup>=UNIQEM 227<sup>T</sup>) belongs to the genus *Anaerobranca* and represents a novel species, *Anaerobranca californiensis* sp. nov. The DNA G+C content of the type strain is 30 mol % (Vladimir *et al.*, 2004.)

Northern Egypt has a set of desert alkaline soda lakes in the Wadi Natrun area, which due to their lower surface elevation, are fed by underground water from the river Nile. They have an intensive microbial flora (Imhoff *et al.*, 1979) and are known as a source for the isolation of various mesophilic alkaliphiles. To date, no alkalithermophiles from these lakes have been validly published although alkalithermophilic *Bacillus*-like strains have been isolated (unpublished). Recently, the new alkaliphilic aerobic bacterium *Alkalilimnicola halodurans* was isolated from sediments of Lake Natron which was affiliated with members of the family Ectothiorhodospiraceae (Baumgarte, 2003)

Much of the knowledge of saline lakes in Africa has come from studies of their biodiversity (Jones *et al.*, 1994). Ideal for isolation of alkalithermophilic micro-organisms are the large, warm soda lakes of the East African Rift Valley (Kenya, Tanzania, and Ethiopia), which have high concentrations of sodium carbonate (up to saturation) and pH values of 10-11. They are heated from above by the tropical sun and from below by hot alkaline springs. They represent fairly stable water bodies on a geological time scale (Varnam, 2000). In respect to alkalithermophiles, the less studied soda lakes of Asia have more profound continental and seasonal climatology (Zavarzin *et al.*, 1999).

### **2.1.2. Microbial biodiversity in Kenyan soda lakes**

The best studied soda lakes are those of the East African Rift Valley, which have been investigated since early 1930's (Tindall, 1988). Alkalithermophilic groups of micro-organisms are commonly found in the hot springs of some of the Kenyan Rift Valley lakes such as Bogoria and Elementaita. They have also been isolated from various other habitats such as sewage, alkaline hot springs, manure samples, thermo biotic and slightly acidic to alkaline, pristine to polluted environments over several continents and slightly acidic sugarcane molasses (Engle *et al.* 1996). Alkalithermophiles, represent an important group of extremophile micro-organisms. Their adaptations towards high pH and elevated temperature draw attention not only as a source of industrially valuable enzymes but also for studying adaptive mechanisms to extreme

environmental parameters. The optimum pH for most alkalithermophiles is about 10 (Horikoshi, 1998).

According to Duckworth *et al.* (1996), thermoalkaliphilic bacteria are believed to have biotechnological potential such as sources of alkali-stable enzymes. Around 100 types have previously been randomly isolated from samples of soil, water and sediments in and around the dilute soda lakes of Bogoria, Elementaita, Nakuru and Sonachi and when subjected to a preliminary numerical taxonomic analysis, indicated considerable taxonomic diversity (Duckworth *et al.*, 1996).

Studies on the low saline lakes of the Kenyan Rift Valley such as Bogoria, Crater Lake Sonachi, Elementaita and Nakuru revealed the presence of diverse populations of aerobic sulfur oxidizing bacteria of genera *Thioalkalimicrobium* and *Thioalkalivibrio* (Sorokin *et al.*, 2001). Several *Bacillus* strains such as M8-C22 (FJ 763929), M8-C11 (FJ 764771), M14-C16 (FJ 764778), M4-C7 (FJ 764769), M10-C8 (FJ 764774), M14-C6 (FJ 764777), M1-C6 (FJ 764768), M8-C14 (FJ 764772), M9-C3 (FJ 764773), and M10-C17 (FJ 764775) were isolated from Lake Elementaita (Mwirichia *et al.*, 2009 unpublished). Other alkalithermophilic strains of an autotrophic, carbon dependant and nitriteoxidizing bacteria have also been isolated from Siberian and Kenyan soda lakes (Jones *et al.*, 1998). The strains isolated from diverse locations form a compact species group related to *Nitrobacter* but different from the known species (Sorokin *et al.*, 1998).

Another specific group of thermophiles, including some bacilli, have also been reported from natural and artificial high temperature biotopes (McMullan *et al.*, 2004). These thermophilic bacilli belonging to *Bacillus* genetic group five have been reclassified as members of the recently named genus *Geobacillus* (McMullan *et al.*, 2004). Most *Geobacillus* species are widely distributed and have been successfully isolated from continents where geothermal areas occur (McMullan *et al.*, 2004).

### **2.1.3 Microbial biodiversity in Lake Bogoria**

Lake Bogoria is inhabited by micro-organisms which are known to be of great biotechnological potential due to their ability to grow in high pH and elevated temperature such as alkalithermophiles. A variety of alkaliphiles have been isolated and characterized from this location (Jones *et al.*, 1998; Zavarzin *et al.*, 1999; Martins *et al.*, 2001) including anaerobic alkalithermophiles: *Thermosyntropha lipolytica* (Svetlitsnyi *et al.*, 1996), *Anaerobranca gottschalkii* (Prowe and Antranikian, 2001) and some unidentified strains which are not yet published.

Lake Bogoria, like Lakes Nakuru, Elementaita, Magadi further south in the Rift Valley, and Lake Logipi to the north, is a Ramsar site and has been a protected National Reserve since November 29, 1973. Lake Bogoria also contains the highest concentration of true geysers in Africa; at least 18 are known (Renaut and Tiercelin, 1994). The lake waters are alkaline and saline (up to 100g/L Total Dissolved Salts). The lake has no surface outlet and therefore the water becomes saline mainly through evaporation, since evaporation is high in this semi-arid region. The lake itself is

meromictic (stratified) with less dense surface waters lying on a denser more saline bottom waters. Although the lake is hypersaline, it is also highly productive with abundant cyanobacteria (*Arthrospira fusiformis*). The cyanobacteria are the principal food for the flamingoes and the pelicans, which also inhabit the lake (Renaut and Tiercelin, 1993).

Lake Bogoria is a home at times to one of the world's largest populations of lesser flamingoes, which are a major tourist attraction and hence contribute significantly to the Kenyan economy. Local features include the Kesubo Swamp to the north and the Siracho Escarpment to the East, both within the National Reserve. The reserve is also famous for its geysers and hot springs (Renaut and Tiercelin, 1993).

#### **2.1.4. Molecular characterization of alkalithermophiles**

Duckworth *et al.* (1996) characterized alkalithermophiles in their detailed work on the “Phylogenetic Diversity of Soda Lake Alkaliphiles”, using several different media in enrichment and isolation, under the same specified conditions. 16S rRNA genes from a range of aerobic chemoorganotrophic, alkaliphilic Soda Lake Bacteria and Archaea were sequenced and subjected to phylogenetic analysis. Gram-negative alkaliphiles were found to be confined to the  $\gamma$ 3 subdivision of the Proteobacteria, with many isolates related to the *Halomonas/Deleya* group. Gram-positive alkaliphiles were found in both high % G + C and low % G + C divisions of the Gram-positive lineage, with many isolates being related to the *Bacillus* group, others to *Arthrobacter* spp. Alkaliphilic Archaea were relatively closely related to members of the genera



*Natronococcus* and *Natronobacterium*. An anaerobic, thermophilic isolate was assigned to a new genus within the *Thermotogales*.

The comprehensive molecular work of Vieille and Zeikusl (2001) on the application of results from the discovery of molecular biology and biochemical studies, such as protein purification and characterization, facilitated by the cloning and expressing of genes from hyperthermophiles, the mesophilic hosts. According to Vieille and Zeikusl (2001), the great diversity of archaeal and bacterial hyperthermophiles represents a large pool of enzymes to choose from for developing new biotechnological applications. The stability and activity of thermophilic enzymes can be controlled by separate molecular determinants. Hyperthermophilic enzymes can be used as molecular templates to design highly stable enzymes that have high activity at wide range of temperatures. Such an achievement could greatly enhance the range of applications for hyperthermophilic enzymes in areas including medicine, food, and research reagents.

According to Baumgarte (2003), the strategy of total DNA extraction, amplification of 16S rDNA gene, screening of clone library and sequence determination of cloned 16S rRNA genes enabled detection and recognition of unknown bacterial sequence types from sediment samples of the extreme environment of Lake Magadi and provided new insights into the prokaryotic composition of soda environment.

### **2.1.5. Biotechnological potential of alkalithermophiles**

Alkalithermophiles have a high potential for biotechnological applications, especially as sources of valuable industrial enzymes (Horikoshi, 1999) such as lipases, xylanases, pectinases, pullulanases, chitinases, and amylases among others (Rainey *et al.*, 1994; Demirjian *et al.*, 2001; Maugeri *et al.*, 2001). These enzymes are useful in many industrial exothermic processes because they can withstand high temperatures and pH just like the micro-organisms that produce them. For example Pullulanase enzyme is most active at 55°C and is stable up to 50°C for 15 minutes in the absence of a substrate (Nakamura *et al.*, 1975). It is also documented that extremophiles cultured from samples collected from Lake Nakuru, were isolated and used by Genencor International Company in extraction of enzymes. Puradax enzyme was extracted and used as a critical ingredient in the manufacture of Tide Alternative Bleach detergent. In 1998, an extremophile enzyme, *Puradax cellulase*, derived from a new *Bacillus* species found in the Rift Valley soda lakes of East Africa was commercialized. Genencor had also introduced *Indiage neutra*, an enzyme derived from a bacterium that was isolated from the soda mud flats on the shores of the highly alkaline Lake Nakuru in Kenya ([www.genencor.com](http://www.genencor.com)).

Alkalithermophile micro-organisms can also produce metabolites that are very useful in both the chemical and industrial processes, such as antibiotics, siderophores, cholic acid derivatives and carotenoids (Zeynep and Metin, 2001). It has been documented that the carotenoid pigments of bacteria trap solar radiation, increasing the ambient

temperature and evaporation rates hastening the deposition of sea salt (Tindall, 1988). The bioenergetics that alkaliphiles face in maintaining pH homeostasis in a highly alkaline environment has been increasingly studied in the last two decades (Horikoshi, 1999). Alkalithermophiles also offer various industrial applications such as detergent additives; hide dehairing, starch-degrading and decomposition of the gelatinous coating of X-ray films by alkaline protease, from which silver was recovered among others (Horikoshi, 1999).

### **3.0 CHAPTER THREE:**

#### **3.1 MATERIALS AND METHODS**

##### **3.2 Study site**

Lake Bogoria is located 0° 20'N and 36° 15'E and is one of the soda lakes located in the Kenyan Rift Valley (East Africa). The lake has ca. 5% total salts w/v and a pH of 9.0. It is a saline, alkaline lake that lies in a volcanic region in a half-graben basin south of Lake Baringo, Kenya, a little north of the equator (Fig 3.1). The atmospheric temperature around the lake is 37°C while the hot spring water and stream waterway temperatures of the lake are 90-°C and 76°C respectively.

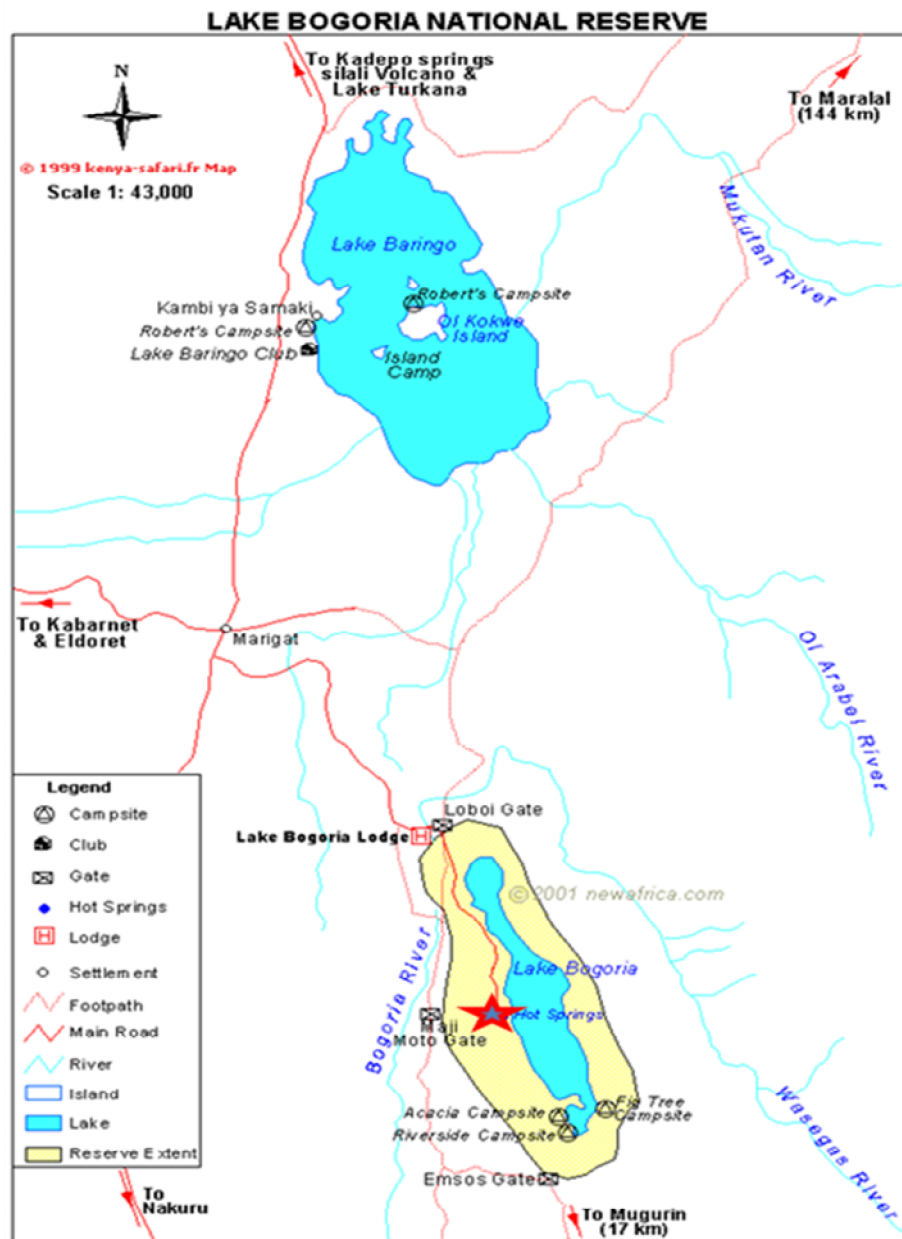
The lake is shallow (about 10 m depth), and is about 34 km long by 3.5 km wide, with a drainage basin of 700 km<sup>2</sup> and a surface elevation of 990 m (Renaut and Tiercelin, 1994). The lake waters are of Na-HCO<sub>3</sub>-CO<sub>3</sub> in composition. They originate from inflow from the Sandai and Emsos rivers, and from about 200 alkaline hot springs that are present at three onshore sites: Loburu, Chemurkeu and a southern group (Ng'wasis, Koibobei, and Losaramat). Other springs discharge directly from the lake floor (Renaut and Tiercelin, 1994).

##### **3.3 Sample collection**

Sampling was done twice, first in October, 2007 during the dry season and the second time in September, 2008 during the rainy season. Three different sites of the thermal

springs were considered for microbial isolation (Figure 3.2). Samples contained in one litre (1L) of thermal water and microbial mats were collected separately (in triplicate) at the three hot springs and main water stream of Lake Bogoria.

Samples from the hot water springs were collected using a water sampler while microbial mats were collected using a shovel and then packed in sterile hotpots; (sterilized by washing with hot water, spraying with 70% ethanol, tightly covered and then preheated with hot spring water at the sampling site) 2 ml of each type of the samples were transferred to two tubes containing 20 ml of each different modified Horikoshi media in the field (Appendix C), as the rest of the samples were transferred to the Institute for Biotechnology Research Laboratory at Jomo Kenyatta University of Agriculture and Technology for analysis.



**Figure 3.1:** A map showing the location of Lake Bogoria at the Lake Bogoria National Reserve.

**Source of the map:** www.kenya. as

★ A general location of the hot springs at Lake Bogoria



**Figures 3.2:** A photograph of one of the hot springs (sampling site) at Lake Bogoria.

**Source:**

Taken at the sampling site on 12/09/08 during the second sampling

### **3.4 Enrichment and isolation**

Media for the enrichment of specific groups of organisms was based on a modified Horikoshi medium consisting, of 10.0 g glucose, xylose, starch or cellulose, 5.0 g (Difco) yeast extract, 5.0 g peptone, 1.0 g dipotassium hydrogen phosphate, 10 g sodium carbonate, 40 g sodium chloride and 0.2 g Magnesium Sulphate in grams per litre, to make broth. The pH was adjusted to 9.0 then dispensed into tubes and autoclaved at 121°C for 20 minutes. Inoculated tubes in duplicate (Appendix C) were incubated at 55°C for 1-2 days. Growth was monitored by withdrawing a sample using

sterile Pasteur pipettes and checking ten slides per sample microscopically and also using eppendoff biophotometer at a wavelength of 600 nm to measure the optical density. The enriched cultures were then transferred to differential agar consisting of 1%  $\text{KH}_2\text{PO}_4$ , 0.01%  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.005%  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 0.14% Agar, 4% NaCl and 1%  $\text{Na}_2\text{CO}_3$  (Appendix A) by the quadrant streak plate method in duplicate and incubated at 55°C in a water bath. Subsequent subcultures were made, to obtain pure cultures, which were all transferred to tryptic soy agar (common media) for further biochemical and molecular analysis.

### **3.5 Morphological characterization of the isolates**

Colony color, shape, size and form of the isolates were observed under the compound and dissecting microscope using slide procedure of Hopwood (1960). Preliminary characterization by Gram staining as well as simple staining (using safranin) of each of the isolates was done in duplicate using the method of Dussault, (1955) and observed under a light microscope at  $\times 100$  (Keast *et al.*, 1984). The Gram staining technique was used to divide isolates on the basis of gram reaction and morphology (Cappuccino and Sherman, 2002). The results were then confirmed by another gram test using 3% potassium hydroxide.



## **3.6 Physiological characterization**

### **3.6.1 Growth at varied sodium chloride concentration**

Nutrient broth (0.13% - diluted ten times) to provide poor nutrient, was supplemented with 1% sodium carbonate and 2%, 5%, 7%, 10% and 20% sodium chloride concentration separately. This was used to determine the ability of isolates to grow at different sodium chloride concentrations. Three separate sets of experiments for each salt concentration, with 36 tubes containing five ml of media, were inoculated with each of the obtained 36 isolates respectively for replication. This was incubated at 55°C then checked for growth after 48 h by measuring the optical density in nm using the eppendoff biophotometer machine at a wavelength of 600. (Nazina *et al.*, 2001). Two uninoculated tubes with the same media served as control. The concentration of salt that allowed for the highest and lowest growth were noted (Korn-Wendisch and Kutzner, 1992),

### **3.6.2. Anaerobic growth**

Nutrient broth (1.3%) was supplemented with 4% sodium chloride and 1% sodium carbonate and used to determine the ability of the isolates to grow in the absence of air according to the protocol of (Harold, 2002). The media was dispensed in tubes and autoclaved. On cooling, the tubes were inoculated with each of the isolates. Experiments were done in duplicate. Without shaking, the tube contents were covered with a thin layer of hot sterile Vaseline and incubated at 55°C for five days then

observed for growth by measuring the optical density in nm using the eppendoff biophotometer machine at a wavelength of 600. Two uninoculated tubes, with same media and exposed to the same conditions served as control (Cappuccino and Sherman, 2002).

### **3.6.3. Effect of pH on growth of isolates.**

To check for the ability of the isolated alkalithermophiles to grow in an acidic, neutral and alkaline pH, 1.3% nutrient broth was supplemented with 3.2% sodium chloride and 1% sodium carbonate. The pH of each set of experiments was adjusted to 5.7, 7.0 and 9.0 respectively. Each medium (3 ml) was then dispensed in tubes, sterilized and inoculated with each of the 36 isolates in triplicate and incubated at 55°C (Nazina *et al.*, 2001). Two uninoculated tubes were used as controls. Positive isolates had growth which was determined by measuring the optical density in nm using the eppendoff biophotometer machine at a wavelength of 600 after 48 h. The pH that generally allowed for the highest and the lowest growth were determined by measuring the optical density (Cappuccino & Sherman, 2002).

### **3.6.4. Growth of isolates at different temperatures**

To determine the ability of the isolates to grow at varied range of temperature, 1.3% nutrient broth was supplemented with 3% sodium chloride and 1% sodium carbonate and 3 ml of this media was then dispensed in four sets of 36 test tubes for each temperature. This was then sterilized, inoculated with each of the 36 isolates respectively and incubated at the four different temperatures (24°C, 40°C, 55°C and

65°C) separately for 48 h (Nazina *et al.*, 2001). Two uninoculated tubes for each temperature were used as controls. Positive isolates had growth detected by measuring the optical density in nm using the eppendoff biophotometer machine at a wavelength of 600 to check the turbidity.

### **3.7 Biochemical characterization of the isolates**

#### **3.7.1 Nitrate reduction test**

Reduction of nitrates by some micro-organisms, occur in the absence of molecular oxygen, whereby anaerobic respiration is an oxidative process. The cell uses inorganic substances such as nitrates as a final hydrogen acceptor during energy formation. Nitrate reacts with hydrogen electrons and it is reduced to nitrite and water. This is indicated by the production of an immediate cherry red color and is used to determine the ability of the isolates to reduce nitrates to nitrites or beyond. On addition of zinc powder, a red color indicates negative results while no color change indicates that reduction was beyond nitrite (to ammonia or molecular nitrogen) (Cappuccino and Sherman, 2002).

Nutrient broth (0.8%) medium containing 1% potassium nitrate was supplemented with 4% NaCl and 1% Na<sub>2</sub>CO<sub>3</sub> according to the protocol of Harold (2002) and that of (Cappuccino and Sherman, 2002). Following inoculation with the 36 isolates in duplicate for replication and incubation at 55°C for 48 h was the addition of 0.2 ml of acetic acid sulfanilic acid and 0.2 ml of dimethyl-alpha-naphthylamine reagent, to the

36 tubes. Those that produced a cherry red coloration indicated positive results for nitrate reduction while those that did not were negative. The results were confirmed by the addition of zinc powder and two uninoculated tubes were used as controls (Korn-Wendisch and Kutzner, 1992; Cappuccino and Sherman, 2002).

### **3.7.2 Methyl Red-Voges- Proskauer (MR-VP) test**

Methyl red (MR) test determines the ability of the isolates to oxidize glucose with production and stabilization of high concentrations of acid end products. The test provides a valuable characteristic for identifying mixed acid fermenting bacteria species that produce strong acids from glucose. pH indicator methyl red detects the large concentration of the acids (Cappuccino and Sherman, 2002). The Voges-Proskauer (VP) test is used to identify bacteria capable of producing non acidic or neutral end products such as acetyl-methyl-carbinol from organic acids that result from glucose fermentation. Addition of Barritts reagent, which produces a deep rose color, indicates positive results while absence of the colour change is negative (Cappuccino and Sherman, 2002).

According to Harold's (2002) protocol, 1.7% MR-VP broth was supplemented with 4% NaCl and 1% Na<sub>2</sub>NO<sub>3</sub>, the initial pH of the media was measured then inoculated with each of the 36 isolates in duplicate as replications and incubated at 55°C for 48 h. Two uninoculated tubes were used as controls. The final pH was measured, and then Methyl red indicator and Barrit's reagent added to aliquots of each culture separately. Negative cultures were indicated by no colour change while the positive cultures were

indicated by colour change to red and deep rose colours respectively (Cappuccino and Sherman, 2002).

### **3.7.3 Gelatin liquefaction**

Gelatin liquefaction demonstrates the hydrolytic activity of gelatinase (Harold, 2002). Gelatin protein produced by hydrolysis of collagen is a major component of the connective tissues in animals. Below 25°C, it gels while above 25°C it turns liquid. Liquefaction is accomplished by some micro-organisms producing a proteolytic extracellular enzyme gelatinase which hydrolyses this protein to amino acids hence not even low temperatures can restore the gel characteristic (Cappuccino and Sherman, 2002).

Nutrient broth (0.8%) was supplemented with 0.4% gelatin, 1.5% agar, 4% sodium chloride and 1% sodium carbonate and autoclaved. On setting, the plates were inoculated with the 36 isolates respectively in duplicate for replication (Cappuccino and Sherman, 2002). After incubation for eight days at 55°C, the cultures were flooded with 1M H<sub>2</sub>SO<sub>4</sub> containing 0.05 g of sodium sulphite. One uninoculated plate was used as control. Cultures that liquefied gelatin were considered positive for gelatin hydrolysis while those which did not liquefy gelatin were negative for gelatin hydrolysis

### **3.7.4 Catalase test**

The catalase test investigates whether the bacteria can produce the catalase enzyme which breaks down hydrogen peroxide into water and oxygen. When hydrogen

peroxide is added to bacteria that contains catalase enzyme, it breaks down hydrogen peroxide producing small air bubbles (oxygen bubbles). Isolates which were positive for catalase test were indicated by the presence of air bubbles while the absence of bubbles indicated a negative catalase test (Cappuccino and Sherman, 2002).

The production of catalase was determined by addition of one drop of 3% hydrogen peroxide to 24 h cultures of each of the 36 isolates in duplicate, grown on 4% Tryptic Soy Agar (TSA) supplemented with 4% sodium chloride and 1% sodium carbonate, based on the methods outlined by Cappuccino and Sherman (2002). A positive reaction was indicated by formation of bubbles and negative by absence of bubbles.

### **3.7.5 Motility, Indole production and Hydrogen sulfide production**

Indole is one of the degradation products from the metabolism of the amino acid tryptophan. Bacteria which possess the enzyme tryptophanase are capable of cleaving tryptophan to produce indole pyruvic acid and ammonia. Indole production is indicated by the formation of a pink to red color after the addition of three to four drops of Kovacs' reagent to the surface of the medium and gentle shaking (Cappuccino and Sherman, 2002).

Sulfur-Indole Motility (SIM) agar media was supplemented with 4% NaCl and 1% Na<sub>2</sub>CO<sub>3</sub>, boiled to mix, dispensed in tubes and then autoclaved. On setting, each of the tubes were inoculated by stabbing with the 36 isolates respectively in duplicate for replication then incubated at 55°C for 48 h. Two uninoculated tubes were used as controls. Presence of indole was detected by addition of Kovac's reagent to 48 h

cultures of the 36 isolates according to the protocol of (Harold, 2002). A negative reaction was indicated by the formation of a yellow color. The ability of isolates to produce hydrogen sulfide from substrates was indicated by a black colour while the absence of black colour in the media indicated negative results. Lack of motility was detected by the confinement of the bacteria along the line of inoculation (Cappuccino and Sherman, 2002).

### **3.7.6 Utilization of skimmed milk**

Milk contains casein as the major protein which is a macro-molecule that contains amino acid subunits linked together by polypeptide bonds. On exposure to proteases, casein degrades step by step into polypeptides, dipeptides and peptones then into its building, amino acids. Proteases cleave the peptide bonds by introducing water into the molecule, liberating the amino acids (Cappuccino and Sherman, 2002).

To determine the ability of the isolates to release proteases, 1% Agar was supplemented with 5% skimmed milk, 1% sodium carbonate and 4% sodium chloride then autoclaved at 115°C for 15 minutes. On cooling, each plate was inoculated with ten spots of the 36 isolates per plate in duplicate, incubated at 55°C and observed after 48 h. Positive isolates for skimmed milk utilization were indicated by clearing zones around the inoculated area (Cappuccino and Sherman, 2000).

### **3.7.7 Sugar fermentation**

To determine the ability of micro-organisms to degrade and ferment carbohydrates with the production of an acid, micro-organisms use certain carbohydrates as sole

carbon source to obtain energy by fermentation. The carbohydrate substrate undergoes anaerobic dissimilation producing organic acids, which may be accompanied by gas production (Cappuccino and Sherman, 2002).

Bromothymol blue dye was added to five ml of a basal media (1%  $\text{KH}_2\text{PO}_4$ , 0.01%  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.005%  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 4%  $\text{NaCl}$ ) and 1%  $\text{Na}_2\text{CO}_3$ ) at pH 8, then dispensed in two sets of 36 tubes for each sugar. The media in tubes was then supplemented with 1% of filter sterilized 10% glucose, xylose, arabinose, fructose and manitol separately, inoculated with each of the 36 isolates respectively and incubated at  $55^\circ\text{C}$  (Nazina *et al.*, 2001). After 10 days, all the sets were checked for growth by measuring the optical density, to determine turbidity using spectrophotometer. Negative results were indicated by a blue colour while for the positive results the colour changed to yellow. Uninoculated tube with the same medium for each setup served as negative control (Williams *et al.*, 1989).

### **3.7.8. L- Arginine, L-Lysine and L-Ornithine decarboxylase**

These tests were used to identify isolates that have decarboxylase enzymes which are capable of decarboxylating specific amino acids in the test medium. Although acids are formed in this reaction, these enzymes remove a molecule of carbon dioxide from an amino acid to form alkaline reacting amines which is indicated by change of colour from red to pink (Cappuccino and Sherman, 2002).



Agar (0.3%) was supplemented with 0.1% peptone, 0.03%  $K_2HPO_4$ , 0.5% NaCl and 1.0% L- Arginine, L-Lysine or L-Ornithine, 5 ml of 0.2% phenol red dye added and the pH adjusted to 8.0. For replication, two sets of 3 ml of each medium were dispensed in tubes and autoclaved. On cooling, inoculation with the 36 isolates was done and then incubated at 55°C for six days (Nazina *et al.*, 2001). Uninoculated tubes with the same medium served as control. Positive isolates for L- Arginine, L-Lysine and L-Ornithine decarboxylase were indicated by colour change from red to pink while the negative isolates remained red (Cappuccino and Sherman, 2002).

### **3.7.9 Growth in Lysozyme 0.001%**

Lysozyme is a basic protein in body fluids of animals, which readily attacks the rigid cell walls of gram positive bacteria, causing them to lyse. The enzyme is therefore known to inhibit growth of gram positive bacteria. This test is used to determine the ability of the isolates to resist lysozyme enzyme from inhibiting their growth, which is indicated by presence of growth.

Nutrient broth (0.1%) was prepared and 3 ml of it dispensed in tubes then autoclaved. After cooling, it was supplemented with filter sterilized 0.001% lysozyme and mixed well. Each tube was then inoculated with one of the 36 isolates in duplicate for replication and incubated at 37°C for three days (Nazina *et al.*, 2001). Two uninoculated tubes were used as controls. Positive isolates were indicated by the presence of growth determined by measuring optical density using spectrophotometer.

### **3.7.10 Tyrosine test**

This test determines the ability of the isolates to remove an amino group from tyrosine amino acid, by oxidative deamination converting it to an organic acid. Tyrosine utilization was determined using Media B containing 1% agar supplemented with 5% L-Tyrosine, 4% NaCl and 1% Na<sub>2</sub>CO<sub>3</sub> and after setting, Media A constituting of 15% agar with 8% nutrient broth was added on the same plate. On setting, inoculation by stabbing with the respective 36 isolates in duplicate followed, then incubation at 55°C for 10 days (Nazina *et al.*, 2001). Positive isolates were indicated by a dirty brown appearance on the lower part of the plate.

### **3.7.11. Utilization of Tween 20 and Tween 80**

Utilization of tween is used for identifying bacteria with lipolytic activity. Around the colonies of bacteria with lipolytic activity, are visible halos due to formation of crystals of calcium salts of the fatty acids liberated by lipolysis process.

To identify the isolates with lipolytic activity, 1.5% agar was supplemented with 1% peptone, 0.01% CaCl, 4% NaCl, 1% Na<sub>2</sub>CO<sub>3</sub> and tween 20 or tween 80 separately and autoclaved. On setting, two sets for each test were inoculated with the 36 isolates, one per plate and incubated at 55°C for eight days (Nazina *et al.*, 2001). Positive isolates were identified by the presence of visible halos around the colonies while the negative ones had no growth or the visible halos were absent.

### **3.7.12 Gas production from glucose**

Fermentative degradation under anaerobic conditions in a fermentation broth in a tube containing a Durham tube inverted in it is done according to the method of Harold (2002). The micro-organisms use the carbon source to obtain energy by fermentation. The carbohydrate substrate undergoes anaerobic dissimilation producing organic acids, which may be accompanied by gas (Cappuccino and Sherman, 2002)

The ability of micro-organisms to degrade and ferment carbohydrates with or without the production of gas was done as follows; 0.2% phenol red indicator was added to basal media (1%  $\text{KH}_2\text{PO}_4$ , 0.01%  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.005%  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 4% NaCl and 1%  $\text{Na}_2\text{CO}_3$ ) at a pH 8 and 5 ml of it dispensed in test tubes and Durham tubes inverted in the test tubes, then autoclaved according to the method of Harold (2002). After cooling, the media was supplemented with 1% of filter sterilized 10% glucose then inoculated with each of the 36 isolates in duplicate and incubated at 55°C for 48 h. Two uninoculated tubes were used as controls. Positive isolates for gas production were indicated by change of the red colour of phenol red to yellow as well as reduction in volume of the media in the Durham tubes due to gas accumulation, while for the negative isolates, the Durham tubes remained full of media and colour remained red (Cappuccino and Sherman, 2002)

### **3.7.13 Citrate utilization**

Some micro-organisms utilize citrate as a sole source of energy in the absence of fermentable glucose. Citrate permease in bacteria facilitates the transportation of

citrate into the cell where it is acted on by citrase enzyme to produce oxalacetic acid and acetate, which are converted to pyruvic acid and carbon dioxide making the medium alkaline due to carbon dioxide combining with sodium and water to form sodium carbonate that changes bromothymol blue from green to blue (Cappuccino and Sherman, 2002).

The ability of the isolates to use citrate as carbon source for their energy was investigated by Simmons citrate agar slants as previously described (Williams *et al.*, 1989 and Harold, 2002). Simmons citrate agar (2.3%) was supplemented with 4% NaCl and 1% Na<sub>2</sub>CO<sub>3</sub>, the pH adjusted to 8 then boiled to mix. The medium was then dispensed in tubes and autoclaved. On cooling, the slants were inoculated by streaking with each of the 36 isolates in duplicate for replication and incubated at 55°C for 48 h. Uninoculated tube with the same medium served as control. Utilization of citrate was indicated by colour change from green to deep blue, while for the negative isolates the colour remained green (Cappuccino and Sherman, 2002).

#### **3.7.14 Phenylalanine deaminase test**

Micro-organisms contain deaminase enzymes that remove the amino group from amino acids to produce keto acids and ammonia. Some bacteria deaminate Phenylalanine into keto acid phenylpyruvic acid and ammonia. On addition of 12% ferric chloride solution on the surface of the media, a green colour indicates formation of the keto acid which is positive while no colour change is negative (Cappuccino and Sherman, 2002).

To determine the ability of the isolates to remove an amino group from phenylalanine amino acid, 2.3% Phenylalanine agar was supplemented with 4% NaCl and 1% Na<sub>2</sub>CO<sub>3</sub>, the pH adjusted to 8 and then boiled to mix. The medium was then dispensed in tubes and autoclaved. On cooling, the slants were inoculated with each of the 36 isolates in duplicate and incubated at 55°C for 48 h, after which, four drops of 12% ferric chloride and four drops of 0.1M HCl were added. Uninoculated tube with the same medium served as control. Isolates that tested positive for phenylalanine deaminase were indicated by change of colour from brown to green while the negative ones remained brown (Cappuccino and Sherman, 2002).

### **3.7.15. Screening the isolates for production of enzymes**

Bacterial isolates were screened for their ability to produce enzymes i.e. gelatinases, amylases, proteases, xylanases, lipases and cellulases and the ability of the isolates to utilize substrates such as gelatin, starch, xylan, cellulose, carboxymethylcellulose (CMC), olive oil, and casein. Positive results were indicated by the potential of the respective isolates to produce useful enzymes that would utilize these substrates while negative results were indicated by the presence of the substrate after growth of the isolates.

- **Extracellular amylase**

The 36 isolates were cultured on basal media (1% KH<sub>2</sub>PO<sub>4</sub>, 0.01% MgSO<sub>4</sub>.7H<sub>2</sub>O, 0.005% CaCl<sub>2</sub>.2H<sub>2</sub>O, 4% NaCl and 1% Na<sub>2</sub>CO<sub>3</sub>) supplemented with 1% starch, as the sole carbon source (Horikoshi, 1971). The medium was then inoculated with smear

spots of 10 isolates per plate in duplicate and incubated at 55°C. After 48 h the plates were then flooded with glucose iodine (Cappuccino and Sherman, 2002). Clear halos indicated extracellular amylase production while negative isolates were indicated by blue black colour all over the plate (Castro *et al.*, 1993).

- **Xylanases**

The isolates were cultured on basal media (1% KH<sub>2</sub>PO<sub>4</sub>, 0.01% MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.005% CaCl<sub>2</sub>·2H<sub>2</sub>O, 4% NaCl and 1% Na<sub>2</sub>CO<sub>3</sub>) supplemented with 2.5% xylan as the sole carbon source, by the method described by Lee and Lee (1997). The medium was then inoculated with the 36 isolates in duplicate for replication and observed for zones of clearing, by flooding them on plates with 1% Congo red after 48 h of incubation at 55°C. The colour around positive isolates for xylanase production changed from red to orange while the colour around the negative isolates remained red.

- **Cellulase**

The production of cellulose was determined using media that contains cellulose and carboxymethylcellulose. The isolates were cultured on basal media (1% KH<sub>2</sub>PO<sub>4</sub>, 0.01% MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.005% CaCl<sub>2</sub>·2H<sub>2</sub>O, 4% NaCl and 1% Na<sub>2</sub>CO<sub>3</sub>) supplemented with 1% cellulose and carboxymethylcellulose separately. Each medium was then inoculated with smear spots of 10 isolates per plate of the 36 isolates in duplicate for replication, for 48 h at 55°C. The plates were then flooded with 1% Congo red and observed for zones of clearing. Positive isolates for cellulose production were indicated by change of colour around the area surrounding the isolate, from red to orange while the negative isolates had the colour around the isolates remaining red.

- **Lipases**

Media with Olive oil as the substrate was used to check the production of lipase monitored by inoculating the 36 isolates on basal media (1%  $\text{KH}_2\text{PO}_4$ , 0.01%  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.005%  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 4% NaCl and 1%  $\text{Na}_2\text{CO}_3$ ) supplemented with 1% olive oil. The medium was then inoculated with smear spots of 10 isolates per plate in duplicate for replication, for at least 72 h at 55°C then observed for zones of clearing. Positive isolates for lipase production were indicated by clear zones around them while the negative isolates were indicated by lack of clearing zones.

- **Proteases**

For the determination of proteolytic activities, casein was used following the method of Lee *et al.* (2005). The isolates were cultured on basal media (1%  $\text{KH}_2\text{PO}_4$ , 0.01%  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.005%  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 4% NaCl and 1%  $\text{Na}_2\text{CO}_3$ ) supplemented with 5% casein. The medium was then inoculated with smear spots of 10 isolates per plate of the 36 isolates in duplicate for replication and incubated at 55°C then observed for zones of clearing after 48 h. Positive isolates for protease production exhibited a zone of proteolysis as demonstrated by clearing zones while the negative isolates did not have the clearing zones (Cappuccino and Sherman, 2002).

Determination of protease production was complemented with the Gelatin Liquefaction test. (3.6.3). Nutrient broth (0.8%) was supplemented with 0.4% gelatin, 1.5% agar, 4% sodium chloride and 1% sodium carbonate and autoclaved. On setting, each plate was inoculated with the 36 isolates respectively in duplicate for replication

(Cappuccino and Sherman, 2002). After incubation for eight days at 55°C, the cultures were flooded with 1M H<sub>2</sub>SO<sub>4</sub> containing 0.05 g of sodium sulphite. Cultures that liquefied gelatin were considered positive for gelatin hydrolysis and hence positive protease production while those which did not liquefy gelatin were considered to be negative for gelatin hydrolysis.

### **3.8 Molecular characterization of the isolates**

#### **3.8.1 Extraction of genomic DNA**

Each of the 36 isolates was grown in one ml of 1.3% nutrient broth supplemented with 3% sodium chloride and 1% sodium carbonate and incubated at 55°C for 24 h. Total genomic DNA of the isolates was extracted from their cells in duplicate using two lysis buffers as solution A (50 mM Tris pH 8.5, 50 mM EDTA pH 8.0 and 25% sucrose solution) and solution B (10 mM Tris pH 8.5, 5 mM EDTA pH 8.0 and 1% SDS). Bacterial cultures (1.8 mg) were added into the centrifuge tube with 500 µl of solution A, vortexed for five seconds and centrifuged for one minute at 13,000 rpm. The supernatant was discarded and the isolates resuspended in 200 µl of solution A and vortexed for five seconds. This was followed by 5 µl of 20 mg/l lysozyme treatment, gently mixed and incubated at 37°C for one hour to lyse the cell wall. Solution B (600 µl) was then added and gently mixed by inverting the tubes severally, followed by the addition of 10 µl of proteinase K (20 mg/l) and the mixture incubated at 50°C for 30 minutes. Equal volumes of phenol/chloroform were used for extraction.



The mixture was centrifuged for 15 minutes at 13,000 rpm after the total volume was divided into two halves of 400  $\mu$ l.

The entire volume of the supernatant was transferred to clean 1.5 ml tubes, and the extraction repeated. An equal volume of Chloroform/Isoamylalcohol (24:4) was then added, and the content centrifuged at 13,000 rpm for 15 minutes. The supernatant was transferred to clean 1.5 ml tubes and the step repeated to remove all the phenol. Addition of 0.1 volumes of 3M potassium acetate as well as ethanol for precipitation followed and the mixture was left at  $-80^{\circ}\text{C}$  overnight.

The extracted genomic DNA was defrosted, centrifuged at 13,000 rpm for five minutes, the ethanol discarded and the pellet air dried on the bench for 20 minutes. The pellet was then resuspended in 100  $\mu$ l of TE buffer and the DNA quality checked on 0.8% agarose gel after staining with ethidium bromide. Then the remaining volume was stored at  $-20^{\circ}\text{C}$ . The genomic DNA was used as templates for subsequent PCR amplification (Appendix B).

### **3.8.2 Polymerase chain reaction (PCR)**

Total genomic DNA was used as a template for amplification of the 16S rDNA gene. The gene encoding for the 16S rDNA was amplified by PCR using bacterial universal primer pair combination of forward primer Bac 8F (5'AGR CTT TGA TCC TGG CTC AG 3') and the reverse primer Bac1492-R (5'CGG CTA CCT TGT TAC GAC TT3'), according to the position in relation to *Escherichia coli* gene sequence (Lane,

1991; Embley and Stackebrandt, 1994). Amplification was performed using an advanced primus 96 thermal cycler. Amplification was carried out in a 40  $\mu$ l mixture and the reaction mixture consisted of 30  $\mu$ l ddH<sub>2</sub>O, 4  $\mu$ l buffer 10 $\times$ , 1  $\mu$ l dNTP's 2 mM, 0.8  $\mu$ l of 10 Mm, 27F forward primer, 0.8  $\mu$ l of 10 mM 1492R reverse primer, 2.4  $\mu$ l MgCl<sub>2</sub> 25 mM, and 0.4  $\mu$ l *Taq* polymerase 5U/ $\mu$ l which was distributed to PCR tubes and 1  $\mu$ l of template DNA added. Reaction mixtures were then subjected to the following temperature cycling profiles: Denaturation at 94°C for 45 seconds, primer annealing at 55°C for 50 seconds, extension at 72°C for one minute 30 seconds repeated for 35 cycles and a final extension at 72°C for 10 minutes (Roux, 1995). Amplification products (20  $\mu$ l) were separated on a 1% agarose gel in 1 $\times$  TBE buffer and visualized by ethidium bromide staining (Sambrook *et al.*, 1989).

### **3.8.3 Restriction fragment length polymorphism (RFLP)**

Digestion restriction was performed on the PCR amplicons using the restriction endonucleases *Hae* III and *Msp* I separately. Both digestions were incubated at 37°C for one hour in a final volume of 10  $\mu$ l that consisted of 6  $\mu$ l of the amplicon, 1  $\mu$ l of the buffer, 3.5  $\mu$ l of ddH<sub>2</sub>O, 0.1  $\mu$ l of BSA and 0.4  $\mu$ l of the restriction enzyme then the RFLP patterns were verified on 2.5% agarose gel after staining with ethidium bromide. Two representatives from each of the groups with different RFLP profile patterns were then selected, purified and sequenced.

### **3.8.4 Purification of PCR products**

The PCR products were purified using the QIAquick PCR purification Kit protocol (Qiagen, Germany) according to manufacturer's instructions. Five volumes of buffer PB (Qiagen, Germany) was added to one volume of the PCR sample and vortexed to mix. The mixture was then transferred to QIAquick spin column and then centrifuged for three minutes at 8,000 rpm. The flow-through was discarded, and the QIAquick column placed back into the same tubes. To wash the DNA, 740  $\mu$ l buffer PE was added to the QIAquick column twice and centrifuged for one minute. The flow-through was discarded and the column centrifuged again for an additional one minute at 8,000 rpm to remove residual ethanol from buffer PE. The QIAquick column was then placed in a 1.5 ml micro centrifuge and 30  $\mu$ l of buffer EB (10 mM Tris-Cl, pH 8.5) added to elute DNA. The tubes were then centrifuged for one minute, the spin column removed and DNA allowed to stand for one minute at room temperature before storing at  $-20^{\circ}\text{C}$  for application (Sambrook *et al.*, 1989).

### **3.8.5 Sequencing and phylogenetic analysis**

Purified samples for sequencing were prepared for sequencing by again running on a gel to check the quality of the PCR products and quantifying the products to determine the concentration of the purified products. The products were also labeled and sealed and then packed in ice together with the gel photograph indicating the labels, quantity loaded during electrophoresis and the method used for purification for transfer to ILRI. Partial sequences were generated at the sequencing facility at ILRI, BecA-ILRI Hub Services > SegoliP Sequencing Facility using the primer 518R. The 16S rDNA

gene sequences of the six selected isolates were compared to the sequences in the public databases using Basic Local Alignment Search Tool (BLAST) in the National Centre for Biotechnology Information (NCBI) website (<http://www.ncbi.nih.gov>). Alignment was done using CLASTAL W 1.6. The 16S rDNA gene sequences with high similarity to those determined in the study were retrieved and added to database and aligned with Mega 4 (Tamura *et al.*, 2007). The alignments were corrected manually where necessary based on conserved regions using CHROMAS LITE.

To show the evolutionary relationships of these taxa, the evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987; Tamura *et al.*, 2004). The optimal tree with the sum of branch length = 0.81476834 was drawn and the percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) shown next to the branches. Bootstrap analysis using Mega 4, was performed to attach confidence estimates for the tree topologies (Felsenstein, 1985). The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and were in the units of the number of base substitutions per site. There were a total of 420 positions in the final dataset (Tamura *et al.*, 2007).

### **3.9 Data Analysis**

Data was analyzed by ANOVA tests (Appendix D) using SPSS 12.0 and SAS 9.1 softwares and separation of mean done using Tukeys' test and graphs drawn. Phylogenetic data was analyzed using neighbor joining method (Saitou and Nei, 1987; Tamura *et al.*, 2004), Maximum composite Likelihood method using Mega 4 (Tamura *et al.*, 2007) and Bootstrap analysis using Mega 4 (Felsenstein, 1985).

## 4.0 CHAPTER FOUR

### 4.1 RESULTS

#### 4.2 Physical characteristics at the sampling site

The physical parameters at Lake Bogoria during the first and second sampling of the thermal springs are presented in (Table 4.1). These values are the raw figures of the conditions at the sampling sites of the thermal waters at Lake Bogoria during the two sampling times (seasons).

**Table 4.1:** Physical parameters at the hot springs of Lake Bogoria during the first and second sampling

Sampling / Conditions	First sampling	Second sampling
Atmospheric temperature	37°C	37°C
Hot spring water temperature	98°C	84.1°C
Stream waterway temperature	78.5°C	74.3°C
Surface elevation	989m	989m
pH	7.4	7.9
Conductivity	0.05	1
Dissolved oxygen	23mg/l	8.6mg/l
Position	00 15 19N 036 05 00E	00 15 19N 36 05 00E

Temperatures during the first sampling were higher than the second sampling. The first sampling was during the dry season, evaporation was high and the water level was low while the second sampling was during the wet season. The rains had caused

some erosion of soils from the surroundings to the lake, the temperatures were lower, the water level in the lake was higher and therefore the pH was slightly higher as well as the solubility of carbonate salts.

### **4.3 Isolation of alkalithermophiles**

A total of 36 isolates were obtained from the hot springs of Lake Bogoria, 29 from the samples obtained from the pure hot springs water and seven from microbial mats in the hot springs waterway. Out of a total of 36 isolates, nine isolates were obtained during the first sampling while 27 isolates were obtained during the second sampling from the hot springs of Lake Bogoria.

### **4.4 Morphological characterization of isolates**

Morphological characterization was based on classical macroscopic techniques of color, arrangement and shape of pure colonies. Microscopic characterization was performed using the Gram reaction and cell shape after simple staining. The 36 isolates obtained from the hot springs of Lake Bogoria formed circular, entire, and flat colony morphology. Colonies ranged from white, cream to light brown in pigmentation (Plate: 4.1). Figure 4.1 shows the morphological and microscopical characteristics of a representative isolate LB<sub>2</sub> from the hot springs of Lake Bogoria.

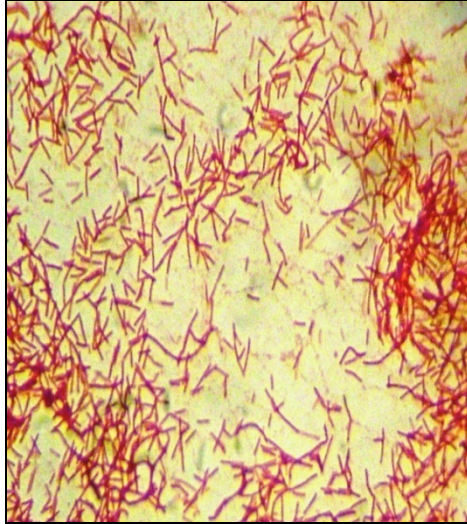
Microscopy of these isolates revealed that twenty seven of the isolates were Gram-positive rods while nine of them were gram variable, in a wide variety of arrangements such as diplobacilli, streptobacilli and single bacilli (Table 4.2).

**Table 4.2:** Morphological characteristics of the 36 alkalithermophilic isolates from the hot springs of Lake Bogoria

<b>MORPHOLOGICAL CHARACTERISTICS</b>				
<b>Strain</b>	<b>Colony colour</b>	<b>Cell shape</b>	<b>Gram reaction</b>	<b>Arrangement</b>
LB <sub>1</sub>	Cream	Rods	Gram variable	Streptobacilli
LB <sub>2</sub>	Light brown	Rods	Gram variable	Streptobacilli
LB <sub>3</sub>	Cream	Long rods	Gram variable	Streptobacilli
LB <sub>4</sub>	White	Rods	Gram positive	Single bacilli
LB <sub>5</sub>	White	Rods	Gram positive	Streptobacilli
LB <sub>6</sub>	White	Rods	Gram positive	Diplobacilli
LB <sub>7</sub>	Cream	Long rods	Gram positive	Streptobacilli
LB <sub>8</sub>	Cream	Long rods	Gram positive	Diplobacilli
LB <sub>9</sub>	Cream	Short rods	Gram positive	Streptobacilli
LB <sub>10</sub>	Cream	Long rods	Gram positive	Streptobacilli
LB <sub>11</sub>	Cream	Rods	Gram positive	Single bacilli
LB <sub>12</sub>	White	Long rods in chains	Gram positive	Diplobacilli
LB <sub>13</sub>	White	Long rods in chains	Gram positive	Diplobacilli
LB <sub>14</sub>	Cream	Long rods in chains	Gram positive	Streptobacilli
LB <sub>15</sub>	Cream	Rods	Gram positive	Diplobacilli
LB <sub>16</sub>	Light brown	Short rods	Gram positive	Streptobacilli
LB <sub>17</sub>	Cream	Long rods in chains	Gram positive	Streptobacilli
LB <sub>18</sub>	Cream	Short rods	Gram positive	Streptobacilli
LB <sub>19</sub>	Cream	Rods in chains	Gram positive	Single bacilli
LB <sub>20</sub>	Cream	Short rods	Gram positive	Single bacilli
LB <sub>21</sub>	Cream	Long rods in chains	Gram positive	Diplobacilli
LB <sub>22</sub>	Cream	Rods	Gram positive	Diplobacilli
LB <sub>23</sub>	Cream	Long rods	Gram positive	Single bacilli
LB <sub>24</sub>	Cream	Short rods	Gram positive	Single bacilli
LB <sub>25</sub>	Cream	Long rods in chains	Gram positive	Streptobacilli
LB <sub>26</sub>	White	Long rods	Gram positive	Streptobacilli
LB <sub>27</sub>	Cream	Long rods	Gram positive	Diplobacilli
LB <sub>28</sub>	Cream	Short rods	Gram positive	Streptobacilli
LB <sub>29</sub>	Cream	Long rods	Gram variable	Streptobacilli
LB <sub>30</sub>	Cream	Rods	Gram variable	Single bacilli
LB <sub>31</sub>	Cream	Rods	Gram variable	Single bacilli
LB <sub>32</sub>	Cream	Long rods	Gram variable	Streptobacilli
LB <sub>33</sub>	Cream	Rods	Gram variable	Diplobacilli
LB <sub>34</sub>	Cream	Long rods	Gram variable	Streptobacilli
LB <sub>35</sub>	Cream	Long rods	Gram positive	Streptobacilli
LB <sub>36</sub>	Cream	Long thin rods	Gram positive	Single bacilli



**LB** = Lake Bogoria



**Figure 4.1:** A photograph of a pure culture of Isolate LB<sub>2</sub> isolated from the hot springs of Lake Bogoria, on a glass slide under a light microscope ( $\times 100$ ) Showing morphological characteristics of isolate LB<sub>2</sub>



**Plate 4.1:** A photograph of a pure plate culture of Isolate LB<sub>2</sub> isolated from the hot springs of Lake Bogoria. Grown on Tryptic Soy Agar. Showing morphological characteristics of isolate LB<sub>2</sub>

## 4.5 Physiological characteristics

### 4.5.1 Growth at various salt concentrations.

All the 36 isolates resisted sodium chloride and grew. The highest growth was recorded followed by 20% then 10% followed by 7% and gradually decreasing towards 2% (Table 4.3.). All the 36 isolates required NaCl for growth. This is one of the lake conditions and 4% NaCl was used throughout the culturing, purification, stocking and testing of the isolates. Isolates LB<sub>17</sub>, LB<sub>1</sub>, LB<sub>13</sub>, LB<sub>4</sub> and LB<sub>20</sub> had the lowest growth while isolates LB<sub>26</sub>, LB<sub>30</sub>, LB<sub>16</sub>, LB<sub>33</sub> and LB<sub>30</sub> recorded the highest growth in 2%, 5%, 7%, 10% and 20% sodium chloride concentration respectively (Table 4.3).

**Table 4.3:** Growth Of alkalithermophilic isolates from the hot springs of Lake Bogoria at varied sodium chloride concentration.

Samples	2%NaCl	5%NaCl	7% NaCl	10%NaCl	20% NaCl
LB <sub>1</sub>	0.073 <sup>bc</sup>	0.015 <sup>q</sup>	0.089 <sup>kl</sup>	0.164 <sup>de</sup>	0.134 <sup>ijklmn</sup>
LB <sub>2</sub>	0.072 <sup>bc</sup>	0.106 <sup>p</sup>	0.128 <sup>efghi</sup>	0.137 <sup>l</sup>	0.119 <sup>mon</sup>
LB <sub>3</sub>	0.054 <sup>fg</sup>	0.108 <sup>op</sup>	0.115 <sup>ghij</sup>	0.118 <sup>o</sup>	0.165 <sup>defgi</sup>
LB <sub>4</sub>	0.053 <sup>fgh</sup>	0.122 <sup>ijklmn</sup>	0.0148 <sup>bcdefg</sup>	0.152 <sup>gh</sup>	0.164 <sup>efghi</sup>
LB <sub>5</sub>	0.044 <sup>kl</sup>	0.112 <sup>nop</sup>	0.101 <sup>kil</sup>	0.133 <sup>mn</sup>	0.153 <sup>ghijkl</sup>
LB <sub>6</sub>	0.053 <sup>fgh</sup>	0.108 <sup>op</sup>	0.133 <sup>defghi</sup>	0.111 <sup>p</sup>	0.154 <sup>ghijkl</sup>
LB <sub>7</sub>	0.049 <sup>ghij</sup>	0.123 <sup>ijklmn</sup>	0.109 <sup>hijk</sup>	0.136 <sup>lmn</sup>	0.248 <sup>c</sup>
LB <sub>8</sub>	0.032 <sup>no</sup>	0.115 <sup>nop</sup>	0.123 <sup>fghij</sup>	0.119 <sup>o</sup>	0.165 <sup>defgi</sup>
LB <sub>9</sub>	0.031 <sup>nop</sup>	0.134 <sup>ghij</sup>	0.137 <sup>defghi</sup>	0.162 <sup>ef</sup>	0.175 <sup>defg</sup>
LB <sub>10</sub>	0.048 <sup>ghij</sup>	0.115 <sup>nop</sup>	0.120 <sup>fghij</sup>	0.132 <sup>n</sup>	0.138 <sup>hijklmn</sup>
LB <sub>11</sub>	0.052 <sup>fghi</sup>	0.127 <sup>ijklm</sup>	0.132 <sup>defghi</sup>	0.133 <sup>n</sup>	0.172 <sup>defg</sup>
LB <sub>12</sub>	0.069 <sup>cd</sup>	0.163 <sup>bc</sup>	0.142 <sup>cdefgh</sup>	0.140 <sup>kl</sup>	0.130 <sup>ijklmn</sup>
LB <sub>13</sub>	0.025 <sup>qp</sup>	0.166 <sup>b</sup>	0.064 <sup>l</sup>	0.142 <sup>k</sup>	0.233 <sup>c</sup>
LB <sub>14</sub>	0.063 <sup>de</sup>	0.115 <sup>nop</sup>	0.183 <sup>ab</sup>	0.097 <sup>q</sup>	0.155 <sup>fghijk</sup>
LB <sub>15</sub>	0.037 <sup>mn</sup>	0.132 <sup>hijk</sup>	0.153 <sup>abcdef</sup>	0.173 <sup>c</sup>	0.179 <sup>defg</sup>
LB <sub>16</sub>	0.023 <sup>qr</sup>	0.122 <sup>klmn</sup>	0.190 <sup>a</sup>	0.163 <sup>ef</sup>	0.151 <sup>ghijklm</sup>
LB <sub>17</sub>	0.017 <sup>r</sup>	0.158 <sup>bcd</sup>	0.113 <sup>ghij</sup>	0.163 <sup>def</sup>	0.108 <sup>no</sup>
LB <sub>18</sub>	0.075 <sup>bc</sup>	0.128 <sup>ijklm</sup>	0.116 <sup>fghij</sup>	0.156 <sup>g</sup>	0.168 <sup>defg</sup>
LB <sub>19</sub>	0.046 <sup>ghijk</sup>	0.136 <sup>fghi</sup>	0.142 <sup>cdefgh</sup>	0.161 <sup>i</sup>	0.190 <sup>de</sup>
LB <sub>20</sub>	0.045 <sup>jk</sup>	0.131 <sup>hijkl</sup>	0.143 <sup>cdefgh</sup>	0.183 <sup>b</sup>	0.067 <sup>p</sup>
LB <sub>21</sub>	0.042 <sup>ijklm</sup>	0.120 <sup>mnl</sup>	0.142 <sup>cdefgh</sup>	0.155 <sup>g</sup>	0.198 <sup>d</sup>
LB <sub>22</sub>	0.064 <sup>de</sup>	0.143 <sup>efgh</sup>	0.123 <sup>fghij</sup>	0.167 <sup>d</sup>	0.117 <sup>no</sup>
LB <sub>23</sub>	0.078 <sup>b</sup>	0.119 <sup>omn</sup>	0.128 <sup>efghi</sup>	0.164 <sup>def</sup>	0.162 <sup>efghij</sup>
LB <sub>24</sub>	0.0413 <sup>klm</sup>	0.146 <sup>def</sup>	0.165 <sup>abcde</sup>	0.138 <sup>l</sup>	0.188 <sup>def</sup>
LB <sub>25</sub>	0.047 <sup>ghijk</sup>	0.117 <sup>mnop</sup>	0.154 <sup>abcdef</sup>	0.162 <sup>ef</sup>	0.156 <sup>fghijk</sup>
LB <sub>26</sub>	0.217 <sup>a</sup>	0.114 <sup>nop</sup>	0.133 <sup>defghi</sup>	0.166 <sup>de</sup>	0.158 <sup>efghij</sup>
LB <sub>27</sub>	0.054 <sup>fg</sup>	0.108 <sup>op</sup>	0.132 <sup>defghi</sup>	0.146 <sup>ij</sup>	0.172 <sup>defg</sup>
LB <sub>28</sub>	0.028 <sup>opq</sup>	0.145 <sup>efg</sup>	0.177 <sup>abc</sup>	0.166 <sup>de</sup>	0.282 <sup>b</sup>
LB <sub>29</sub>	0.046 <sup>ijk</sup>	0.150 <sup>de</sup>	0.074 <sup>kl</sup>	0.152 <sup>gh</sup>	0.116 <sup>no</sup>
LB <sub>30</sub>	0.023 <sup>qr</sup>	0.183 <sup>a</sup>	0.134 <sup>defghi</sup>	0.148 <sup>hi</sup>	0.347 <sup>a</sup>
LB <sub>31</sub>	0.018 <sup>r</sup>	0.129 <sup>ijklm</sup>	0.145 <sup>cdefgh</sup>	0.144 <sup>jk</sup>	0.171 <sup>defg</sup>
LB <sub>32</sub>	0.022 <sup>qr</sup>	0.131 <sup>hijkl</sup>	0.167 <sup>abcd</sup>	0.188 <sup>a</sup>	0.173 <sup>defg</sup>
LB <sub>33</sub>	0.044 <sup>ijkl</sup>	0.145 <sup>efg</sup>	0.133 <sup>defghi</sup>	0.191 <sup>a</sup>	0.125 <sup>klmno</sup>
LB <sub>34</sub>	0.038 <sup>lmn</sup>	0.153 <sup>cde</sup>	0.108 <sup>hijk</sup>	0.182 <sup>b</sup>	0.094 <sup>op</sup>
LB <sub>35</sub>	0.037 <sup>mn</sup>	0.123 <sup>ijklmn</sup>	0.122 <sup>fghij</sup>	0.177 <sup>c</sup>	0.1231 <sup>mno</sup>
LB <sub>36</sub>	0.042 <sup>ijklm</sup>	0.132 <sup>hijk</sup>	0.137 <sup>defghi</sup>	0.184 <sup>b</sup>	0.287 <sup>b</sup>

**Key**

i). Numbers represent means of growth of 36 isolates for each treatment at varied sodium chloride concentration.

ii). Mean separated using Tukeys' test by the same letter in the same row are not significantly different (P<0.05) from each other.

#### **4.5.2 Growth of isolates at different temperature**

All the 36 isolates grew at wide range of temperatures including room temperature (24°C- 65°C). The optimum (highest) growth of the isolates was observed at 55°C and in decreasing order followed by 40°C, then 24°C with the least general growth observed at 65°C (Table 4.4). In addition, at 24°C isolate LB<sub>6</sub> exhibited the highest growth and LB<sub>19</sub> had the lowest growth. Isolate LB<sub>12</sub> had the highest growth at temperature 40°C while there was little difference in growth for the rest of the isolates with lower growth. At 65°C, isolate LB<sub>35</sub> grew best as compared to the rest of the isolates while isolate LB<sub>33</sub> had the least growth. (Table 4.4)

**Table 4.4:** Growth of alkalithermophilic isolates from the hot springs of Lake Bogoria at varied temperature.

Samples	24°C	40°C	55°C	65°C
LB <sub>1</sub>	0.113 <sup>p</sup>	0.050 <sup>b</sup>	0.1333 <sup>o</sup>	0.181 <sup>de</sup>
LB <sub>2</sub>	0.125 <sup>no</sup>	0.030 <sup>b</sup>	0.0933 <sup>p</sup>	0.238 <sup>d</sup>
LB <sub>3</sub>	0.132 <sup>mn</sup>	0.253 <sup>b</sup>	0.3513 <sup>ef</sup>	0.470 <sup>b</sup>
LB <sub>4</sub>	0.175 <sup>g</sup>	0.196 <sup>b</sup>	0.2190 <sup>mn</sup>	0.054 <sup>hijk</sup>
LB <sub>5</sub>	0.192 <sup>f</sup>	0.375 <sup>b</sup>	0.4173 <sup>d</sup>	0.089 <sup>fghij</sup>
LB <sub>6</sub>	0.773 <sup>a</sup>	0.318 <sup>b</sup>	0.451 <sup>c</sup>	0.054 <sup>hijk</sup>
LB <sub>7</sub>	0.083 <sup>r</sup>	0.251 <sup>b</sup>	0.2703 <sup>ij</sup>	0.033 <sup>hijk</sup>
LB <sub>8</sub>	0.142 <sup>kl</sup>	0.218 <sup>b</sup>	0.2543 <sup>kl</sup>	0.141 <sup>ef</sup>
LB <sub>9</sub>	0.135 <sup>lm</sup>	0.276 <sup>b</sup>	0.3153 <sup>gh</sup>	0.198 <sup>de</sup>
LB <sub>10</sub>	0.526 <sup>b</sup>	0.298 <sup>b</sup>	0.3383 <sup>efg</sup>	0.323 <sup>c</sup>
LB <sub>11</sub>	0.523 <sup>b</sup>	0.364 <sup>b</sup>	0.3663 <sup>e</sup>	0.052 <sup>hijk</sup>
LB <sub>12</sub>	0.057 <sup>t</sup>	1.459 <sup>a</sup>	0.3693 <sup>e</sup>	0.049 <sup>hijk</sup>
LB <sub>13</sub>	0.067 <sup>s</sup>	0.162 <sup>b</sup>	0.2137 <sup>mn</sup>	0.082 <sup>fghij</sup>
LB <sub>14</sub>	0.036 <sup>u</sup>	0.079 <sup>b</sup>	0.0877 <sup>p</sup>	0.017 <sup>jk</sup>
LB <sub>15</sub>	0.074 <sup>s</sup>	0.212 <sup>b</sup>	0.2183 <sup>mn</sup>	0.082 <sup>fghij</sup>
LB <sub>16</sub>	0.165 <sup>h</sup>	0.338 <sup>b</sup>	0.414 <sup>d</sup>	0.042 <sup>hijk</sup>
LB <sub>17</sub>	0.028 <sup>v</sup>	0.757 <sup>ab</sup>	0.815 <sup>a</sup>	0.098 <sup>fgh</sup>
LB <sub>18</sub>	0.098 <sup>q</sup>	0.223 <sup>b</sup>	0.2633 <sup>ijk</sup>	0.031 <sup>hijk</sup>
LB <sub>19</sub>	0.051 <sup>t</sup>	0.251 <sup>b</sup>	0.3247 <sup>fgh</sup>	0.063 <sup>hgijk</sup>
LB <sub>20</sub>	0.086 <sup>r</sup>	0.212 <sup>b</sup>	0.22131 <sup>mn</sup>	0.048 <sup>hijk</sup>
LB <sub>21</sub>	0.043 <sup>u</sup>	0.118 <sup>b</sup>	0.2157 <sup>mn</sup>	0.0723 <sup>fghijk</sup>
LB <sub>22</sub>	0.081 <sup>r</sup>	0.164 <sup>b</sup>	0.2010 <sup>n</sup>	0.415 <sup>b</sup>
LB <sub>23</sub>	0.283 <sup>c</sup>	0.319 <sup>b</sup>	0.3570 <sup>ef</sup>	0.103 <sup>fgh</sup>
LB <sub>24</sub>	0.151 <sup>ij</sup>	0.176 <sup>b</sup>	0.3367 <sup>efg</sup>	0.022 <sup>ijk</sup>
LB <sub>25</sub>	0.167 <sup>h</sup>	0.263 <sup>b</sup>	0.2967 <sup>hi</sup>	0.091 <sup>fghi</sup>
LB <sub>26</sub>	0.201 <sup>e</sup>	0.213 <sup>b</sup>	0.2353 <sup>klm</sup>	0.074 <sup>fghijk</sup>
LB <sub>27</sub>	0.148 <sup>jk</sup>	0.287 <sup>b</sup>	0.3473 <sup>efg</sup>	0.066 <sup>hgijk</sup>
LB <sub>28</sub>	0.124 <sup>o</sup>	0.284 <sup>b</sup>	0.3587 <sup>e</sup>	0.132 <sup>efg</sup>
LB <sub>29</sub>	0.175 <sup>g</sup>	0.193 <sup>b</sup>	0.3240 <sup>fgh</sup>	0.067 <sup>hgijk</sup>
LB <sub>30</sub>	0.138 <sup>lm</sup>	0.117 <sup>b</sup>	0.3473 <sup>efg</sup>	0.0723 <sup>fghijk</sup>
LB <sub>31</sub>	0.124 <sup>o</sup>	0.418 <sup>b</sup>	0.6123 <sup>b</sup>	0.052 <sup>hijk</sup>
LB <sub>32</sub>	0.215 <sup>d</sup>	0.383 <sup>b</sup>	0.4153 <sup>d</sup>	0.018 <sup>ijk</sup>
LB <sub>33</sub>	0.156 <sup>i</sup>	0.133 <sup>b</sup>	0.1370 <sup>o</sup>	0.001 <sup>k</sup>
LB <sub>34</sub>	0.133 <sup>m</sup>	0.181 <sup>b</sup>	0.1973 <sup>n</sup>	0.053 <sup>hijk</sup>
LB <sub>35</sub>	0.113 <sup>p</sup>	0.193 <sup>b</sup>	0.1950 <sup>n</sup>	0.640 <sup>a</sup>
LB <sub>36</sub>	0.123 <sup>o</sup>	0.131 <sup>b</sup>	0.1370 <sup>o</sup>	0.131 <sup>efg</sup>

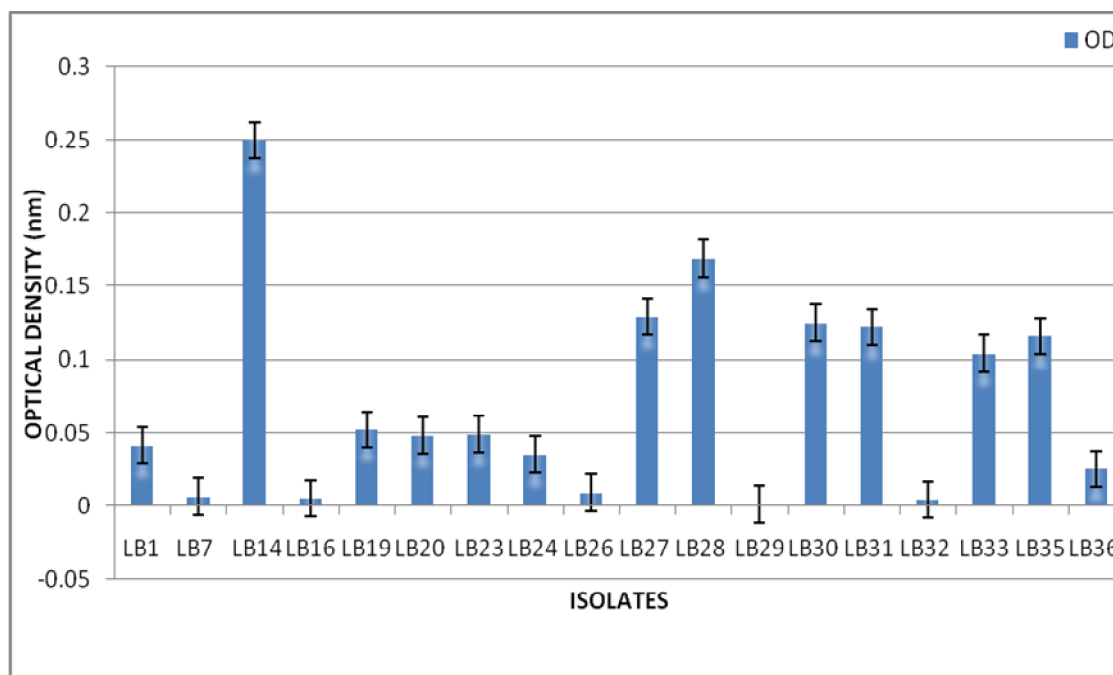
**Key**

i). Numbers represent mean of growth of 36 isolates for each treatment at varied temperature.

ii). Mean separated using Tukeys' test by the same letter in the same row are not significantly different (P<0.05) from each other.

### 4.5.3 Growth in 0.001% Lysozyme

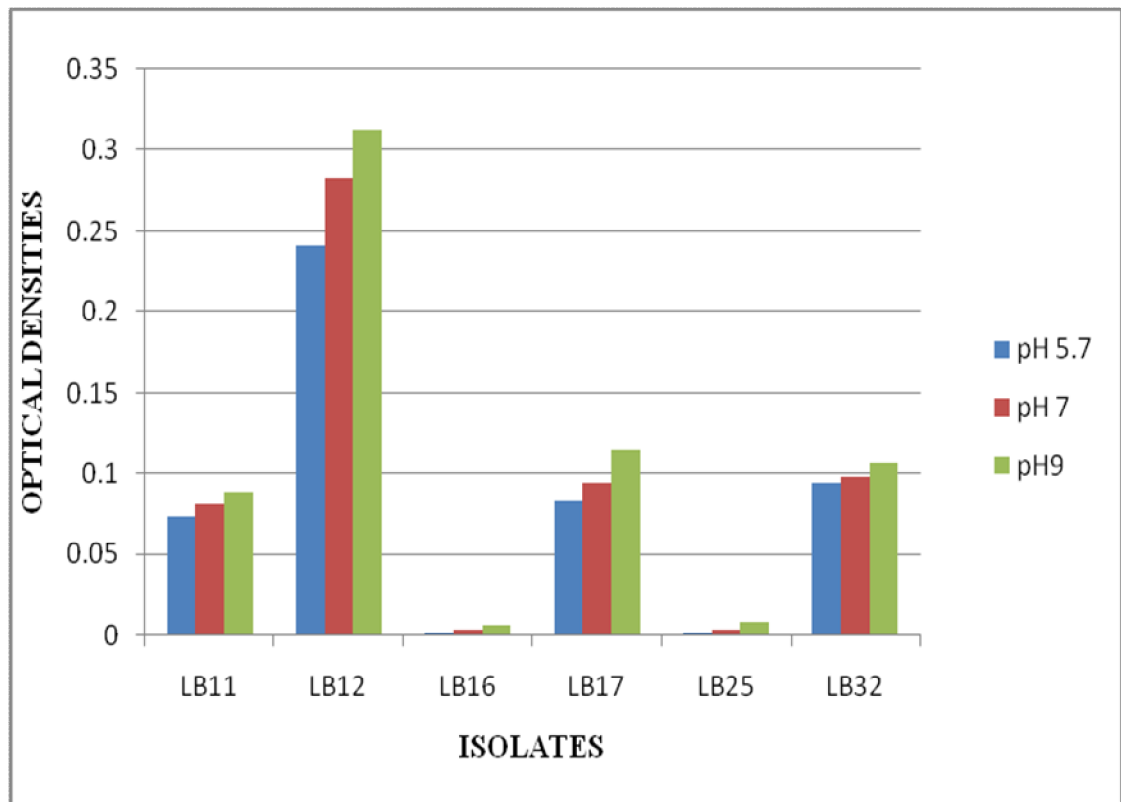
Among the alkalithermophiles isolated from the hot springs of Lake Bogoria, 18 isolates tested positive for growth in 0.001% lysozyme enzyme showing that they have the ability to resist the lipolytic activity of lysozyme to dissolve their cell wall and hence their growth could not be inhibited by the enzyme. For these isolates, isolate LB<sub>14</sub> had the highest resistance to lysozyme followed by isolate LB<sub>28</sub> then LB<sub>27</sub>, LB<sub>30</sub>, LB<sub>31</sub>, LB<sub>35</sub> and LB<sub>33</sub> among others (Figure 4.2). The isolates that showed the least resistance to the lysozyme were LB<sub>29</sub> followed by LB<sub>32</sub>, LB<sub>16</sub>, LB<sub>7</sub>, and then LB<sub>26</sub> (Figure 4.2). However, 18 other isolates could not resist the lipolytic activity of the enzyme hence did not grow.



**Figure 4.2:** Growth of the alkalithermophilic isolates that tested positive for growth in 0.001% lysozyme. ( $P < 0.05$ )

#### 4. 5.4 Effect of pH growth of the isolates

Although all the 36 isolates are alkalithermophiles, they were able to grow at wide range of pH, including acidic pH 5.7 and neutral pH. There was little growth at pH 5.7 for most of the isolates and growth was observed to increase with increase in pH from 5.7-9.0 where growth was optimum for all isolates. For the six selected isolates basing on difference in their characteristics, the highest overall growth was observed in isolate LB<sub>12</sub> while the lowest overall growth was exhibited by isolates LB<sub>16</sub> and LB<sub>25</sub>. The growth of isolates LB<sub>11</sub>, LB<sub>17</sub> and LB<sub>32</sub> was almost the same for the three different pH. (Figure 4.3)



**Figure 4.3:** Growth of the alkalithermophilic isolates selected for sequencing at varied pH

Being alkalithermophiles, the isolated micro-organisms grew well at alkaline pH. However, they could also survive in an acidic pH though with very low growth (Figure 4.3).

#### 4.6 Biochemical characterization

All the biochemical tests were carried out at 55°C unless stated otherwise and control experiments and replication were used to check the validity and precision of the results. The isolates were subjected to various tests but only the selected 10 representatives basing on the differences in the characteristics of the isolates are summarized in Table 4.5. All the isolates tested positive for starch hydrolysis; plate 4.2 and utilization of xylan; plate 4.3, decarboxylation of L-lysine ,L-ornithine except isolate 33 and L-arginine, positive for VP test; there was increase in pH after growth in all the isolates, and anaerobic growth (Table 4.5)



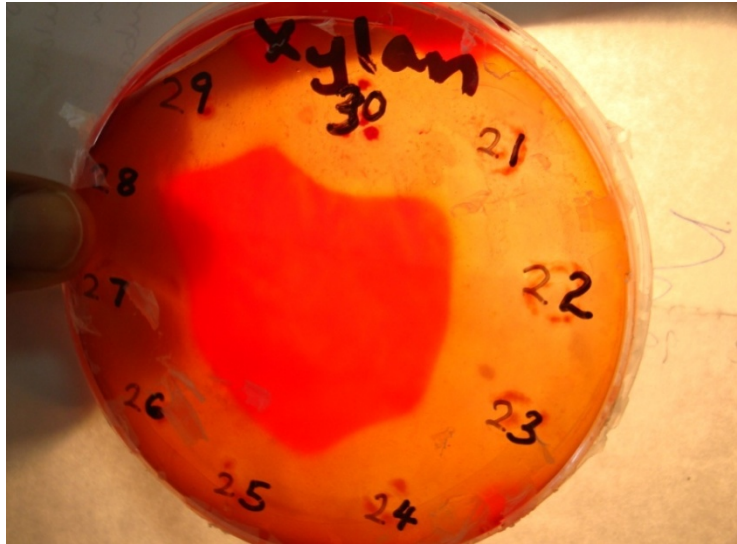
**Plate 4.2:** A photograph of pure culture isolates from the hot springs of Lake Bogoria, grown on basal media, supplemented with 1% starch, 4% NaCl and 1% Na<sub>2</sub>CO<sub>3</sub> and showing clearing zone around the isolates and a blue black color where there was no inoculation (at the center). positive results for starch hydrolysis

**Table 4.5:** Biochemical characteristics of 10 representatives selected basing on the differences in characteristics of isolates from the hot springs of Lake Bogoria.

-Negative results and + Positive results.

<b>BIOCHEMICAL TEST</b>										
ISOLATE/ TEST	LB <sub>2</sub>	LB <sub>4</sub>	LB <sub>11</sub>	LB <sub>12</sub>	LB <sub>14</sub>	LB <sub>15</sub>	LB <sub>16</sub>	LB <sub>17</sub>	LB <sub>25</sub>	LB <sub>32</sub>
MR	-	-	-	-	-	-	-	-	-	-
VP	+	+	+	+	+	+	+	+	+	+
GELATIN	-	-	-	+	-	+	+	-	-	+
MOTILITY	-	-	-	-	-	-	-	-	-	-
CITRATE	-	-	-	-	-	-	-	-	-	-
TYROSINE	-	+	-	-	-	-	-	-	+	-
NITRATE REDUCTION	-	-	-	-	-	-	-	-	-	+
ANAEROBIC GROWTH	+	+	+	+	+	+	+	+	+	+
Production of: H <sub>2</sub> S	-	-	-	-	-	-	-	-	-	-
INDOLE	-	-	-	-	-	-	-	-	-	-
Deaminase of:- PHENYLALAMIN	-	-	-	-	-	-	-	-	-	-
Growth in: LYSOSYME 0.001%	+	+	+	+	+	+	+	+	+	+
Decarboxylation of: L-ARGENINE	+	+	+	+	+	+	+	+	+	+
L- LYSIN	+	+	+	+	+	+	+	+	+	+
L- ORNITHINE	+	+	+	+	+	+	+	+	+	+
Utilization of: STARCH	+	+	+	+	+	+	+	+	+	+
XYLAN	+	+	+	+	+	+	+	+	+	+
OLIVE OIL	-	-	+	+	+	+	+	+	+	+
CMC	-	-	-	-	-	-	-	-	-	-
SKIMED MILK	-	-	+	+	-	-	-	+	-	+
TWEEN 20	-	-	-	+	-	-	-	-	-	-
TWEEN 80	-	-	-	-	-	-	+	-	-	-





**Plate 4.3:** A photograph of pure isolates from the hot springs of Lake Bogoria, grown on basal media, supplemented with 2.5% xylan, 4% NaCl and 1% Na<sub>2</sub>CO<sub>3</sub> and showing clearing zone around the isolates and a red color where there was no inoculation (at the center). Positive results for xylan utilization

All the isolates tested negative for utilization of cellulose, motility, and hydrogen sulphide production, acid production from sugars, gas production from glucose, phenylalanine deaminase, indole test and citrate test (Table 4.5). For other tests, isolates LB<sub>5</sub>, LB<sub>12</sub>, LB<sub>15</sub> and LB<sub>16</sub> tested positive for gelatin liquefaction, LB<sub>32</sub> was positive for nitrate reduction, LB<sub>4</sub> was positive for tyrosine test, LB<sub>12</sub> positive for tween 20 and LB<sub>16</sub> was positive for tween 80 utilization (Table 4.5).

#### **4.4.1 Screening of the isolates for enzyme production**

Isolates LB<sub>11</sub>, LB<sub>12</sub>, LB<sub>16</sub>, LB<sub>17</sub> and LB<sub>25</sub>, were positive for catalase production, LB<sub>11</sub>, LB<sub>12</sub>, LB<sub>17</sub> and LB<sub>32</sub> were positive for proteases as well as for gelatinase production and all the 36 isolates tested positive for production of extracellular amylase and xylanase but also tested negative for the production of cellulase enzyme (Table 4.6)

**Table 4.6:** Alkalithermophilic isolates from the hot springs of Lake Bogoria that have the potential to produce enzymes.

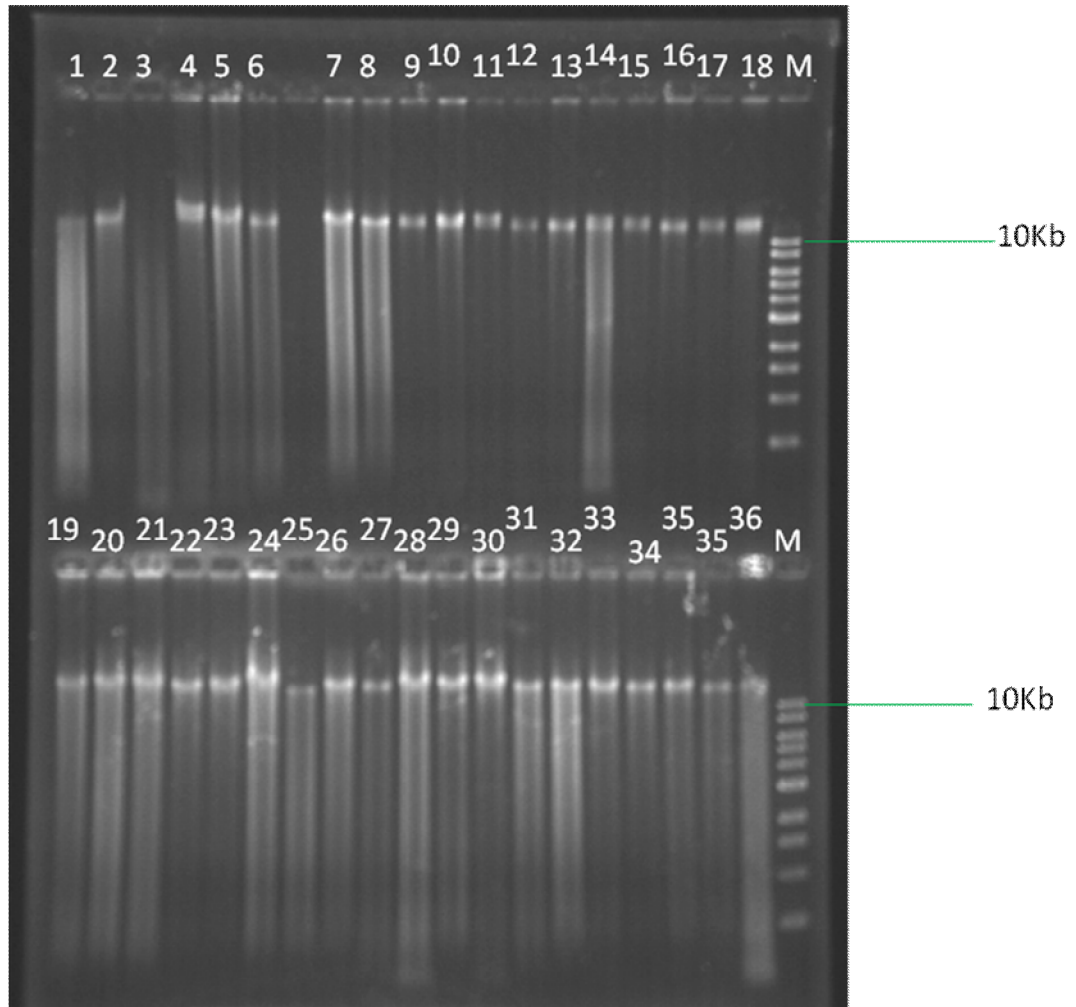
- Negative results and + Positive results.

Production of Enzyme /Isolate	LB <sub>11</sub>	LB <sub>12</sub>	LB <sub>16</sub>	LB <sub>17</sub>	LB <sub>25</sub>	LB <sub>32</sub>
Amylase	+	+	+	+	+	+
Xylanase	+	+	+	+	+	+
Cellulase	-	-	-	-	-	-
Protease	+	+	-	+	-	+
Catalase	+	+	+	+	+	-
Gelatinase	+	+	-	+	-	+

#### 4.7 Molecular characterization

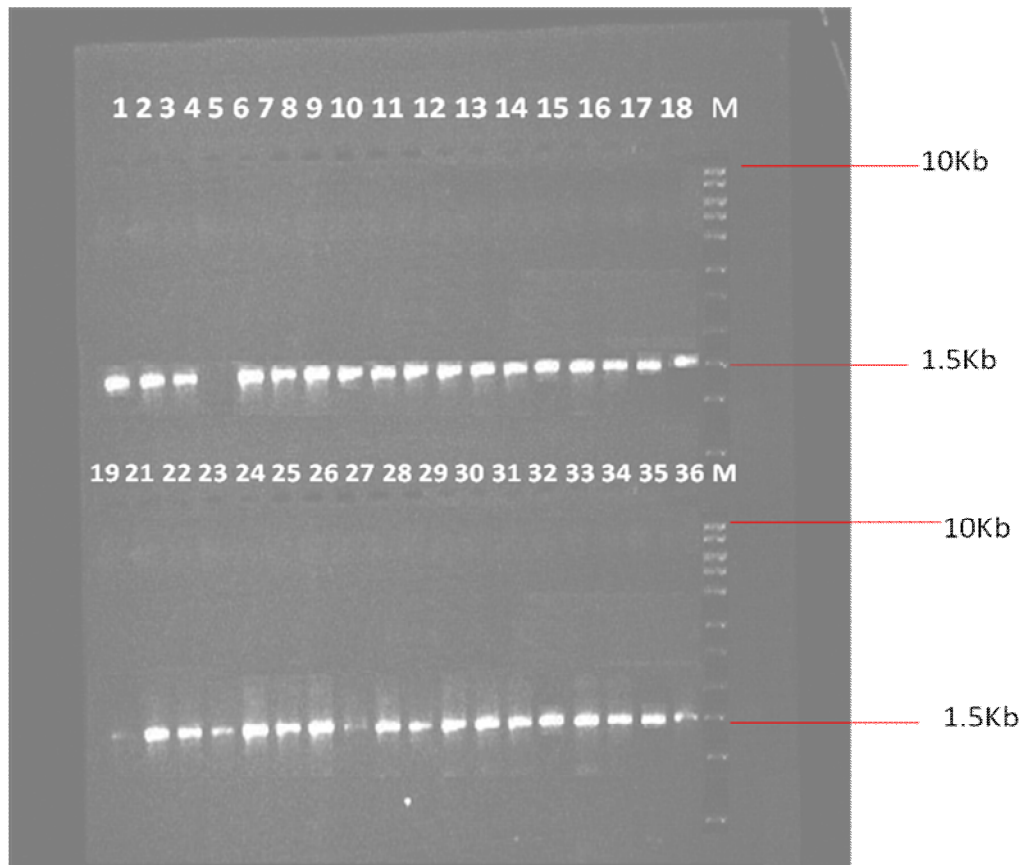
After biochemical tests, the strains were further analyzed using molecular techniques.

The 16S rDNA region was used for the molecular characterization. DNA from all the pure isolates obtained from the hot springs of Lake Bogoria was extracted using the phenol/chloroform technique. Figure 4.5 shows genomic DNA extractions of the isolated strains from the hot springs of Lake Bogoria, visualised under UV light after ethidium bromide staining and using a DNA 10Kb ladder (sigma).



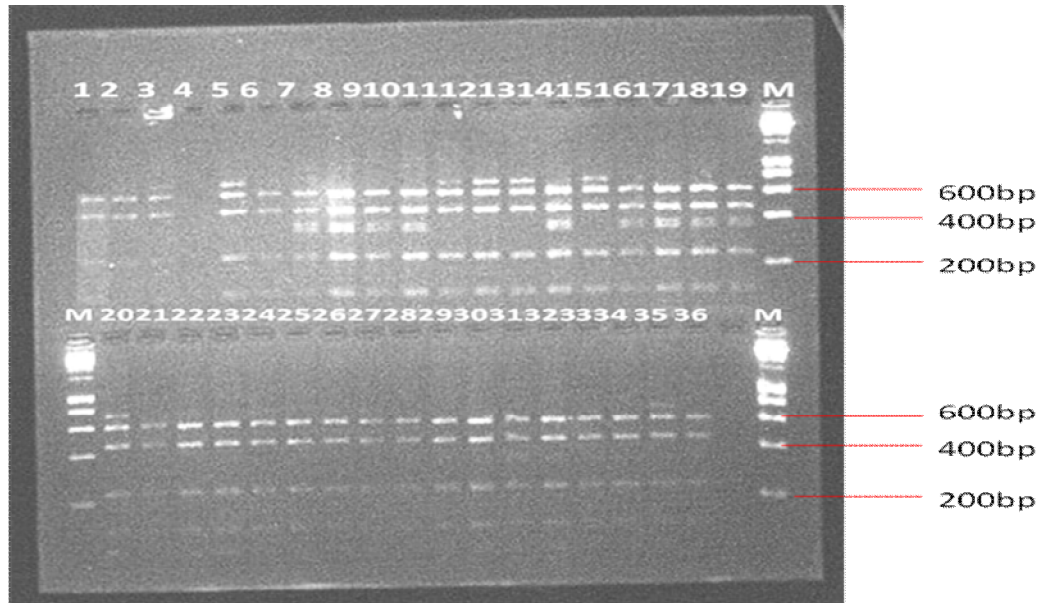
**Figure 4.4:** Gel electrophoresis photograph of DNA bands of isolates from the hot springs of Lake Bogoria, visualized by UV illumination

The amplification of 16S rDNA from the extracted genomic DNA of isolates from the hot springs of Lake Bogoria was done using universal primers Bac 8F and Bac 1492R and only one isolate; isolate LB<sub>4</sub> out of the 36 isolates was not amplified by the used protocol. The isolates were then visualised under UV light after ethidium bromide staining to check the quality of the PCR products (Figure 4.6).

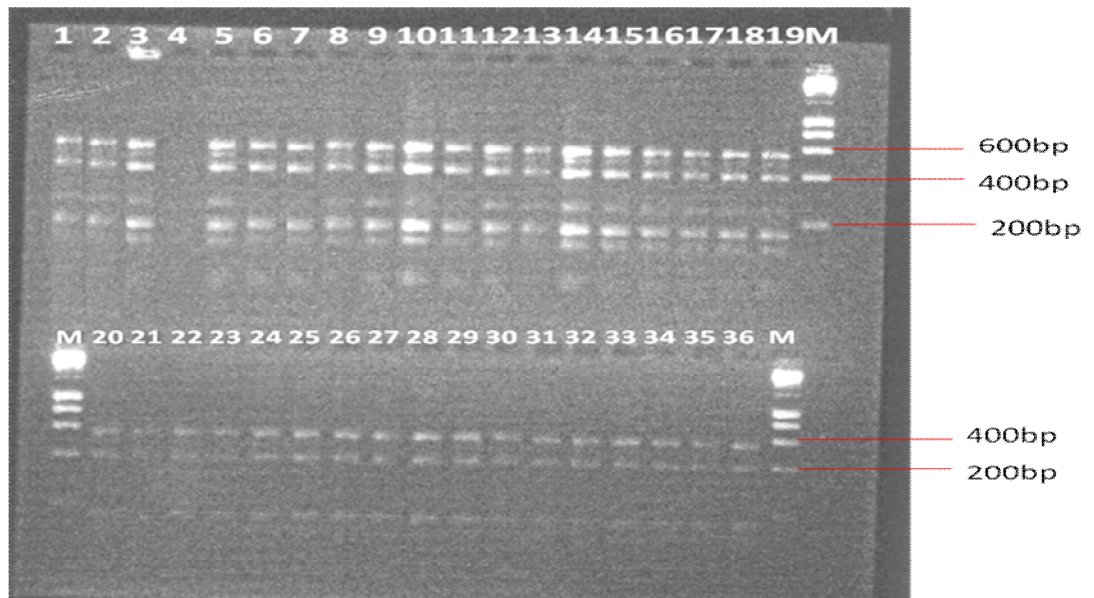


**Figure 4.5:** A photograph of amplified 16S rDNA (1.5 kb) using forward primers Bac 8F and reverse primer Bac 1492R from alkalithermophiles Isolated from the hot springs of Lake Bogoria.

PCR products were subjected to restriction endonucleases *Hae* III enzyme and were restricted into various profile patterns (Figure 4.7). The same PCR amplicons were subjected to restriction by *Msp* I enzyme which showed a lower diversity of the isolated alkalithermophiles (Figure 4.8). This led to the grouping of the 36 alkalithrmophilic isolates into three main groups, according to their RFLP profiles (Figure 4.9) and selection of the unique isolates for sequencing.

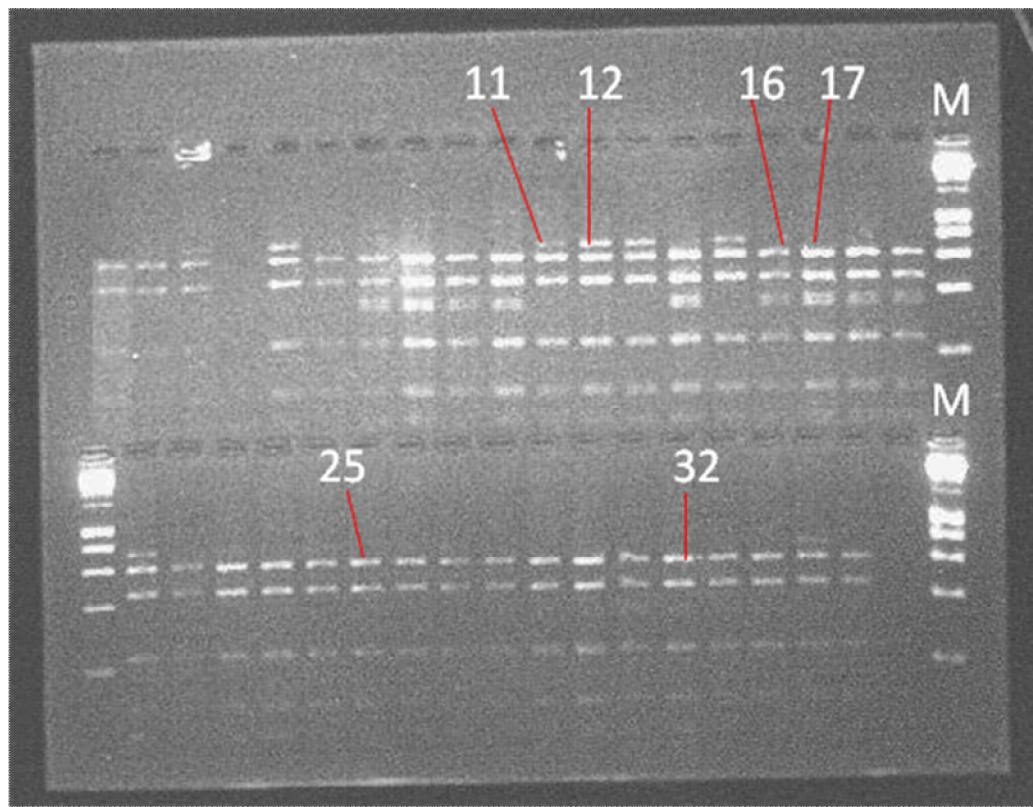


**Figure 4.6:** A photograph of restriction fragment length polymorphism by *Hae* III restriction enzyme showing RFLP profile patterns of the 36 isolates from the hot springs of Lake Bogoria in 2.5% agarose gel, Using Bioline Hyperladder 1 (10Kb) marker.



**Figure 4.7:** A photograph of restriction fragment length polymorphism by *Msp* I restriction endonuclease showing RFLP patterns of 36 alkalithermophilic strains isolated from the hot springs of Lake Bogoria in 2.5% agarose gel. Using Bioline Hyper ladder 1 (10Kb)

Two representatives with similar RFLP profile patterns from each of the three groups with different RFLP profile patterns were selected (Group one 11 and 12, Group two 16 and 17 and Group three 25 and 32). The selected isolates were then purified and sequenced (Figure 4.9).

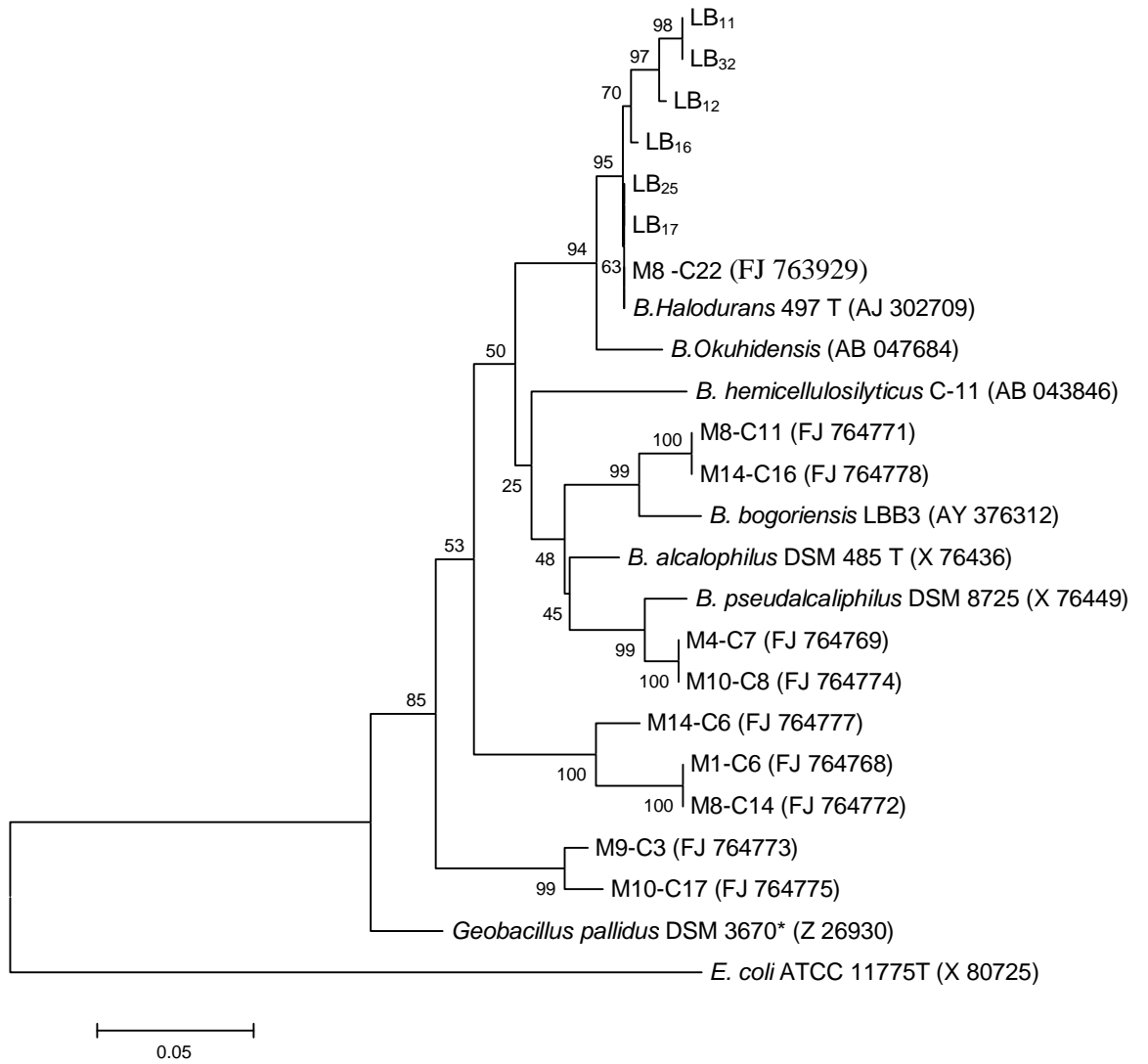


**Figure 4.8:** A photograph of RFLP profiles of 16S rDNA sequences of Alkalithermophilic isolates from the hot springs of Lake Bogoria selected for sequencing basing on their RFLP profile differences.

Sequenced PCR amplicons of the six selected isolates basing on the RFLP profile patterns of strains from the hot springs of Lake Bogoria were used in phylogenetic analysis. The BLAST search results showed that all the isolates belong to the bacterial domain and were clustered within phylum; *Firmicutes*. These isolates had sequence similarity of between 99-100% with known members of *Bacilli* sp. (Table 4.8). The phylogenetic tree constructed showed the phylogenetic position of each isolate (Figure 4.10).

**Table 4.8:** Blast search results of the sequenced isolates from the hot springs of Lake Bogoria and their close relatives.

<b>Isolate</b>	<b>Blast results (Close relatives)</b>	<b>Percentage similarity</b>
LB <sub>11</sub>	<i>Bacillus halodurans</i> DSM497T (AJ 302709)	99%
LB <sub>12</sub>	<i>Bacillus halodurans</i> DSM497T (AJ 302709)	99%
LB <sub>16</sub>	<i>Bacillus</i> sp. M8-C22. (FJ 763929)	100%
LB <sub>17</sub>	<i>Bacillus</i> sp. M8-C22. (FJ 763929)	100%
LB <sub>25</sub>	<i>Bacillus</i> sp. M8-C22 (FJ 763929)	100%
LB <sub>32</sub>	<i>Bacillus halodurans</i> DSM497T (AJ 302709)	99%



**Figure 4.9:** Neighbor-joining distance tree using 16S rDNA sequences of *Bacillus* strains isolated from the hot springs of Lake Bogoria.



## **5.0 CHAPTER FIVE:**

### **5.1 DISCUSSION**

The objectives of this research were to determine the physico-chemical characteristics of the hot springs in Lake Bogoria, isolate a large number of useful alkalithermophiles from the hot springs of Lake Bogoria, characterize and identify them using morphological, physiological, biochemical and molecular methods and then screen the isolates for useful secondary metabolites.

The hot springs of Lake Bogoria have extreme physico-chemical conditions (Table 4.1) suitable for the extremophiles such as alkalithermophiles. A total of 36 isolates were isolated from the hot springs of Lake Bogoria, 29 from the hot spring water and seven from microbial mats in the hot springs waterway. Out of a total of 36 isolates, 27 were isolated during the second sampling while nine were from the first sampling. The high number of isolates during the second sampling could have been due to the lower temperature of 84.1°C recorded which was closer to the culturing temperature of 55°C than the 98°C recorded during the first sampling (Table 4.1). Modified Horikoshi media (Horikoshi, 1971), was used in this study to increase the chances of isolation of alkalithermophiles. The media was modified by addition of 4% sodium chloride and varying the carbon source with glucose, starch, cellulose or xylose.

Cultivation of alkalithermophiles using modified Horikoshi medium with starch as a carbon source sustained the highest morphotypes as compared to the rest of the media.

However, it showed a low diversity within morphotypes recovered since most of the isolates represented the same bacterium. This could be attributed to the fact that cultivation is known to capture a small fraction of microbial diversity in a given sample. The low diversity of isolates obtained from water sampled at Lake Bogoria hot springs could have been due to the fact that very low diversity in hot environments is common. Most of them are distributed in many mesophilic environments, but others have been isolated only from one specific location (Wiegel, 1998). It remains possible that there are other strains of alkalithermophiles present in Lake Bogoria thermal water but since two extreme conditions were imposed at the same time; (high pH and elevated temperature), low diversity was achieved. The combination of two extreme conditions of physico-chemical growth parameters restricts the range at which microorganisms can proliferate more than in a single growth condition.

Most of the genera isolated from the hot spring environments included the spore forming bacteria, isolated from mesophilic environments such as *Clostridium thermoalkalophilum* (Wiegel, 1998). This is in agreement with the fact that a variety of alkaliphiles have been isolated and characterized from these locations (Jones *et al.*, 1998; Zavarzin *et al.*, 1999; Martins *et al.*, 2001) including anaerobic alkalithermophiles from Lake Bogoria: *Thermosyntropha lipolytica* (Svetlitsnyi *et al.*, 1996), *Anaerobranca gottschalkii* (Prowe and Antranikian, 2001) and some unidentified strains. Other *Bacillus* strains such as M8-C22 (FJ763929), M8-C11 (FJ 764771), M14-C16 (FJ 764778), M4-C7 (FJ 764769), M10-C8 (FJ 764774), M14-C6

(FJ 764777), M1-C6 (FJ 764768), M8-C14 (FJ 764772), M9-C3 (FJ 764773), and M10-C17 (FJ 764775) (Figure 4.10) were isolated from Lake Elementaita by Mwirichia (2009).

Kevbrin *et al.* (1998) isolated an alkaliphilic, obligately anaerobic, fermentative, asporogenous bacterium with a gram-positive cell wall structure from soda deposits in Lake Magadi, Kenya. 16S rDNA sequence analysis of this bacterium showed that it belongs phylogenetically to cluster XI of the low-G+C gram-positive bacteria. On the basis of its distinct phylogenetic position and unique physiological properties, they proposed a new genus and new species, *Tindallia magadii*, for this strain. An alkaliphilic bacterium, strain C-125 (JCM 9153), isolated from alkaliphilic soil in 1975 was identified as a member of genus *Bacillus* but later re identified as *B. halodurans* based on 16S rDNA sequence and DNA-DNA hybridization analysis (Takami *et al.*, 2000). Alkaliphilic aerobic bacterium *Alkalilimnicola halodurans* was also isolated from sediments of Lake Natron which was affiliated with members of the family Ectothiorhodospiraceae (Baumgarte, 2003)

All the 36 isolates from the hot springs of Lake Bogoria belonged to the bacterial domain which is in line with observations in hot springs in Yellowstone National Park (Wyoming, U.S.A), which revealed remarkable bacterial diversity with twelve new division-level lineages and the study showed that members of the bacterial domain seemed to outnumber the archaea in this hydrothermal environment (Keller and

Zengle, 2003). Various studies have successfully isolated *Geobacillus* species from all continents, specifically where geothermal biotopes exist (McMullan *et al.*, 2004). *Geobacillus gargensis* (Nazina *et al.*, 2004) was isolated from Garga hot spring, Transbaikal. *Geobacillus* shares alkaliphily with *Bacillus halodurans*.

The isolates showed white, cream to light brown pigments and had short to long thin rods (others in chains). Twenty seven of them were Gram positive *Bacillus* but nine isolates were gram variable (Table 4.2). Restriction Fragment Length Polymorphism (RFLP) analysis of PCR amplicons with 16S rDNA grouped the isolates into three different RFLP profile patterns (Figure 4.9). However, analysis of the sequences showed that all the three RFLP profiles belong to the genus *Bacillus* (Table 4.8), domain of bacteria, phylum firmicutes, class *Bacilli*, order *Bacillales*, family *Bacillaceae*. There are many species that exist in genus *Bacillus* hence heterogeneity and a vast diversity of physiological types such as antibiotic producers, alkalophiles, thermophiles and psychrophiles, among others (Slepecky, 1972; Norris *et al.*, 1981; Claus and Berkeley, 1986). According to the studies of Nakamura *et al.*, (1984), at one time, 145 species made up the genus and several validly published new species, distinct from other *Bacillus* species have not yet been described in the *Bergey's Manual of Systematic Bacteriology*. In addition, literature contains many experiments done using *Bacillus* isolates, that have not yet been properly identified to species such as; *Bacillus* sp. strain C-59 and *Bacillus* sp. strain N-6, both alkalophilic organisms with unusual bioenergetic properties (Kitada and Horikoshi, 1987; Kitada *et al.*, 1989)

and *Bacillus* sp. strain MGA3, a thermophilic methanol-utilizing species; mutants which are capable of producing large amounts of lysine (Guettler and Hanson, 1988; Schndel *et al.*, 1989). The generic intrageneric heterogeneity is as great as the bacterial families exist (Priest *et al.*, 1988).

Analysis of 16S rRNA molecules by oligonucleotide sequencing (Fox *et al.*, 1977; Stackebrandt and Wose, 1979) would have been an effective approach to *Bacillus* taxonomy. However, a 16S rRNA sequencing survey of Jurtshuk *et al.* (1989) defined three major *Bacillus* taxonomic cluster groups, by determining complete or partial sequences of 16S rRNA genes on 35 recognized neotype reference strains or type species using the technique of Lane *et al.*, (1985). Phylogenetic analyses using three different approaches (Sneath and Sokal, 1973; Fitch and Margoliash, 1967; Saitou and Nei, 1987) showed three major groupings of *Bacillus* spp., referred to as clusters I, II and III. The 16S rRNA bacillus cluster groups were quite different from those previously noted (Stackebrandt *et al.*, 1987).

Although *Bacillus halodurans* was not included in the clusters described by Jurtshuk *et al.* (1989) it may belong to cluster I, subgroup A of *Bacillus subtilis* due to their genetic and physiological similarity. Takami *et al.* (2000) established that *Bacillus halodurans* C-125 is quite similar to *B. subtilis* in terms of genome size, G+C content of the genomic DNA and the physiological properties used for taxonomical identification. *B. halodurans* encodes a 71% sequence identity to *B. subtilis*. The

phylogenetic placement of C-125 based on 16S rDNA sequence analysis indicates that this organism is more closely related to *B. subtilis* than to any other member of the genus *Bacillus*. Using orthologous proteins to compare *B. halodurans* and *B. subtilis* genomes from the replication origin region (*oriC*), more than 20 bases in the *B. halodurans* nucleotide sequence continuously matched those of *B. subtilis*. About 1500 genes, some of which constitute operons; genes associated with mobility and chemotaxis, protein secretion, cell division, the main glycolytic pathways, metabolism of nucleotides and nucleic acids, DNA replication, RNA modification and other functions are similar. On the other hand, the region around 112–153° in the *B. halodurans* genome corresponds to the region around 212–240° in the *B. subtilis* genome. The fact that alkalithermophilic bacteria belong to the Gram positive *Bacillus* subphylum, (Wiegel, 1998), and the common abundance of this genus in hot environments, such as thermal springs may explain why *Bacilli* were present in the hot springs of Lake Bogoria as well as the preference of the genus to extreme environment with high pH and elevated temperature.

There are earlier reports of Gram positive aerobic isolates of low G+C divisions found in soda lakes and were associated mainly with the diverse bacilli taxon especially *Bacillus alcalophilus* and *Bacillus clarkii* (Duckworth *et al.*, 1996). The isolates grew at temperature rang of 40°C-65°C with optimal growth temperature of 55°C (Table 4.4). Growth at a pH range of between 5.7 and 10 is consistent with earlier studies which showed that a low to high pH ranging from 5.7 to 9.0 (Figure 4.3), favors their

growth and a range 9.0 to 10 may serve as the optimum pH (Horikoshi, 1998). This is in agreement with the study of Takami *et al.* (2000) that *Bacillus halodurans* is an alkaliphilic bacterium that grows optimally above pH 9.5. All the isolates were able to tolerate up to 20% sodium chloride concentrations with the optimum salt concentration being 10% (Table 4.3). Adaptation to high salt concentration has been reproduced in vitro (Okazaki and Okami 1975; Okami and Okazaki, 1978).

The ability of alkalithermophiles to adapt and grow optimally at environmental and physicochemical parameters unsuitable for the growth of other micro-organism and their fast growth, makes them good models for research. They can be grown easily and in large numbers whenever required, can produce thermostable enzymes which are attractive catalysts since their temperature optima can reduce contamination in economically feasible non aseptic applications and their adaptations towards high pH and elevated temperature is useful in studying adaptive mechanisms to extreme environmental parameters (Kevbrin *et al.*, 1998).

The isolates did not form organic acids or gas from sugars. This is supported by the characteristics of *Bacillus sphaericus*, *Bacillus subtilis* and *Bacillus halodurans* which give negative results for acid or gas production from glucose (Gordon *et al.*, 1973). However, all the 36 isolated alkalithermophiles were positive for decarboxylation of L-lysine, L-arginine and L-ornithine (Table 4.5). Isolate LB<sub>12</sub> was able to decompose tween 20 consistent with the characteristics of *Bogoriella caseilytica* gen. nov., sp.

nov (Ingrid *et al.*, 1997). The inability of isolate LB<sub>11</sub> to produce gelatinase, indole or H<sub>2</sub>S is similar to the properties of *Bacillus okuhidensis* and *Bacillus halodurans* strains as confirmed by earlier studies of Zhiyu *et al.* (2002).

Out of 36 isolates, 18 resisted the lysoytic activity of lysozyme (Figure 4.2). This is unusual with gram positive bacteria and could be because some of the isolates were gram variable or the properties of their cell membrane changed because of growing at high temperature. This is supported by the studies of Gould (1983) that with respect to thermophily, thermophilic organisms can grow at high temperatures due to changes in the physical properties of their membrane structure of the gram positive cells and in the lipid composition in response to growth at high temperatures.

The hot springs of Lake Bogoria, harbor alkalithermophiles which have the potential to produce useful secondary metabolites such as enzymes. The 36 isolates produced a variety of extracellular enzymes including amylases, lipases, gelatinases, proteases and xylanases (Table 4.6). This is in agreement with earlier studies in which it was noted that alkaliphilic micro-organisms, species of the genus *Bacillus* in particular, are of considerable biotechnological importance because of their ability to produce some extracellular alkaline enzymes such as protease (Horikoshi, 1971), pectinase (Horikoshi, 1972) and amylases (Boyer and Ingle, 1972) that are resistant to high pH or high temperature conditions (Hamamoto and Horikoshi, 1992; Nielsen *et al.*, 1994).



These unusual enzymes can be used in industrial and other processes (Yamagata, 1993; Nielsen *et al.*, 1995).

All the 36 Isolates were non-cellulolytic and also xylanolytic, which is in agreement with the characteristics of *Bacillus* strain C-125 (JCM 9153) (Zhiyu *et al.*, 2002) and consistent with earlier findings in which xylosomes associated with anaerobes had been proposed and demonstrated for low G+C gram-positive bacteria strains. However, for several of the anaerobic firmicutes (such as *Thermoanaerobacter* and *Thermoanaerobacterium*), protrusions of cell wall compounds have been observed to contain extracellular starch hydrolyzing enzymes (Antranikian *et al.*, 1987).

Isolates, LB<sub>11</sub>, LB<sub>12</sub>, LB<sub>17</sub> and LB<sub>32</sub> are proteolytic, which is in agreement with the characteristics of the genus *Bacillus*. Studies have shown that during vegetative growth and subsequent sporulation, a variety of proteases are produced (Priest, 1977), indicating their role in decomposition of organic matter in nature. This is in agreement with findings of other researchers in which peptides have been shown to be by far the most abundant nitrogenous compounds in organic matter (Sowden *et al.*, 1976; Schnitzer, 1985). Isolate LB<sub>32</sub> is catalase negative, has rods that are usually non-motile, does not reduce nitrate and is phenylalanine negative. This is similar to the characteristics of *Bacillus okuhidensis* and *Bacillus halodurans* (Zhiyu *et al.*, 2002). *B. halodurans* is a rod shaped gram positive, aerobic or anaerobic bacteria. There has been considerable interest in producing large quantities of enzymes for industrial

purposes; for detergent supplementation, in the brewing industry, the food industry, leather manufacturing, bread making, and in the paper industry (Debabov, 1982). Genes that code for a variety of unique thermostable enzymes such as amylase can be cloned and overproduced in industrial hosts (Zeikus, 1979; Wiegel and Ljungndahl, 1981).

*Bacillus halodurans* 497T (AJ302709) is similar to *bacillus* MIR 32 since they have similar sequences and both utilize xylan, have high stability in alkaline environment and grow under alkaline and neutral conditions. *Bacillus halodurans* C-125 is Gram positive, Anaerobic or aerobic and has similar phylogenetic properties with *Bacillus subtilis* and non cellulolytic.

What the next line of development will be, is unclear (Horikoshi, 1999), but it may be the wider application of enzymes. Alkaline enzymes should find additional uses in various fields of industry, such as chiral-molecule synthesis, biological wood pulping, and more production of sophisticated enzyme detergents. It is expected that in the near future, further aerobic and anaerobic alkalithermophiles with exciting properties will be isolated from extreme environments using traditional and novel microbial culture techniques and molecular survey methods such as metagenomics. These studies will extend our understanding of the boundaries for conditions under which life can thrive on earth (Kevbrin *et al.*, 1998). Subsequently, this will lead to new theories of how life

could have evolved on early earth and whether it could presently or in the future exist in extraterrestrial habitats.

## **5.2 Conclusion**

The hot springs of Lake Bogoria have an average temperature of 90°C and an average pH of 9 which are extreme conditions suitable for the inhabitation of extremophiles such as alkalithermophiles.

By the use of the modified Horikoshi media (Horikoshi 1971) by varying the carbon source (cellulose, xylan, starch and glucose) and addition of 4% sodium chloride, novel alkalitolerant *Bacillus* isolates related to *B. halodurans* were isolated from the hot springs of Lake Bogoria.

The isolates obtained from the hot springs of Lake Bogoria were thermotolerant, alkalitolerant and halotolerant because they grew at wide range of temperature (24°C-65°C), pH (5.7-9.0) and sodium chloride concentration (2-20%) and therefore able to survive in extreme conditions

The alkalithermophiles isolated from the hot springs of Lake Bogoria were xylanolytic, non-cellulolytic, amylolytic, and some lipolytic and proteolytic.

### **5.3 Recommendation**

More research is required to design studies that would compare the diversity of alkalithermophiles in different seasons of the year such as the rainy and dry seasons.

Different protocols and novel microbial culture techniques should be designed so as to allow the isolation of more diverse genera.

Total characterization and identification of alkalithermophilic strains from the hot springs of Lake Bogoria, that might have escaped cultivation is essential.

Extensive research on the specific secondary metabolites released by alkalithermophiles from the hot springs of Lake Bogoria should be done.

More research in various fields of industry, such as chiral-molecule synthesis, biological wood pulping, and more production of sophisticated enzyme detergents, should be done to find additional uses for Alkali stable enzymes.

Molecular methods such as metagenomics studies and clone libraries should be used to study the useful functional genes in alkalithermophiles found in hot springs of soda lakes.

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## **APPENDICES**

### **APPENDIX A**

#### **MEDIA PREPARATION**

##### **MODIFIED HORIKOSHI MEDIA**

###### **Formula**

10.0 g glucose

5.0 g (Difco) yeast extract

5.0 g peptone

1.0 g  $K_2HPO_4$

0.2 g  $MgSO_4 \cdot 7H_2O$

10 g  $Na_2CO_3$

40 g NaCl

###### **Preparation**

Suspend the components in 700 ml of distilled water and 10 g  $Na_2CO_3$ , and 40 g NaCl in 300 ml of distilled water separately to make one litre, mix well to dissolve the medium completely. Sterilize by autoclaving at 121°C for 15 minutes. Dispense in 3 ml in sterile tubes under sterile conditions.

**Use;** For the enrichment of bacteria during culture

##### **BASAL MEDIA**

###### **Formula**

1%  $KH_2PO_4$

0.01%  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$

0.005%  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$

4%  $\text{NaCl}$

1%  $\text{Na}_2\text{CO}_3$

### **Preparation**

Suspend the components in one litre of distilled water. Stir well to dissolve the medium completely. Sterilize by autoclaving at  $121^\circ\text{C}$  for 15 minutes. Cool to  $45\text{-}50^\circ\text{C}$  and pour in 15-20 ml amounts into Petri dishes.

**Use;** To test for the production of enzymes

## DIFFERENTIAL AGAR

### **Formula**

1%  $\text{KH}_2\text{PO}_4$

0.01%  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$

0.005%  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$

0.14% Agar

4%  $\text{NaCl}$

1%  $\text{Na}_2\text{CO}_3$

### **Preparation**

Suspend the components in one litre of distilled water. Stir well to dissolve the medium completely. Sterilize by autoclaving at  $121^\circ\text{C}$  for 15 minutes. Cool to  $45\text{-}50^\circ\text{C}$  and pour in 15 – 20 ml amounts into Petri dishes

**Use;** General purpose broth media

## NUTRIENT BROTH

### **Formula**

1.3% nutrient broth

3% sodium chloride

1% sodium carbonate

### **Preparation**

Suspend the components in one litre of distilled water. Stir well to dissolve the medium completely. Dispense in 3-10 ml in tubes then Sterilize by autoclaving at 121°C for 15 minutes.

**Use;** General purpose broth media

## TRIPTIC SOY AGAR

4% Tryptic soy agar

4% sodium chloride

1% sodium carbonate

### **Preparation**

Suspend the components in one litre of distilled water. Stir well to dissolve the medium completely. Sterilize by autoclaving at 121°C for 15 minutes. Cool to 45-50 °C and pour in 15 – 20 ml amounts into Petri dishes

**Use;** For sub-culturing the isolates and as the common media after isolation

## NITRATE BROTH

0.8% Nutrient

0.1%  $\text{KNO}_4$

4%  $\text{NaCl}$

1%  $\text{Na}_2\text{CO}_3$

### **Preparation**

Suspend the components in one litre of distilled water. Stir well to dissolve the medium completely. Dispense in 3-10 ml in tubes then Sterilize by autoclaving at  $121^\circ\text{C}$  for 15 minutes.

**Use:** to test for nitrate reduction to nitrites.

## METHYL RED-VOGES-PROSKAUER (MR-VP) MEDIA

1.7% MR-VP Media

4%  $\text{NaCl}$

1%  $\text{Na}_2\text{NO}_3$

### **Preparation**

Suspend the components in one litre of distilled water. Stir well to dissolve the medium completely. Dispense in 5 ml in tubes then Sterilize by autoclaving at  $121^\circ\text{C}$  for 15 minutes and measure the pH before inoculation.

**Use:** used to determine the ability of isolates to oxidize glucose with the production and stabilization of high concentrations of acid end products.

## SULPHUR-INDOLE MORTILITY (SIM) AGAR MEDIA

### **Formula**

3.6% SIM

4% NaCl

1% Na<sub>2</sub>NO<sub>3</sub>

### **Preparation**

Suspend the components in one litre of distilled water. Stir well and bring it to boil to dissolve the medium completely. Dispense in 3-10 ml in tubes then Sterilize by autoclaving at 121°C for 15 minutes. Make slants as the media cools.

**Use;** to test for the production of tryptophanase enzyme and the ability to produce hydrogen sulfide from substrates,

## LYSOZYME BROTH

### **Formula**

1.0 g/l nutrient broth

1.0 ml Lysozyme

### **Preparation**

Suspend the nutrient broth in one litre of distilled water, Stir well to dissolve the medium completely and adjust the pH to 6.9 at 25°C. Sterilize by autoclaving at 121°C for 15 minutes. At the same time suspend Lysozyme in 100 ml of distilled water, Stir well to dissolve and filter sterilize. Add 1.0 ml of sterile Lysozyme solution to 99.0 ml of cooled nutrient broth. Aseptically distribute 2.5 ml to sterile tubes.

**Use;** To determine the resistance of the isolates to Lysozyme enzyme

## **APPENDIX B**

### **DNA EXTRACTION REAGENTS**

- Solution 1
  - 50 mM Tris pH 8.5
  - 50 mM EDTA pH 8.0
  - 25% Sucrose solution
  
- Solution 2
  - 10 mM Tris pH 8.5
  - 5 mM EDTA pH 8.0
  - 1% SDS
  
- Lysozyme 20 mg/ml
- Proteinase K 20 mg/ml
  
- Phenol
  
- Chloroform
  
- Absolute ethanol.
  
- 3M potassium acetate
  
- Isoamylalcohol
  
- TE buffer
  
- TBE buffer
  
- Ethidium bromide
  
- Agarose gel



## Electrophoresis buffer Working Concentrated stock

### TBE buffer 10×

chemical	volume
Tris	108 g
Boric Acid	55 g
Na <sub>2</sub> EDTA.2H <sub>2</sub> O	9.3 g

Adjust the volume to one liter with ddH<sub>2</sub>O and divide into 500 ml bottles

**Running conditions:** use 1× TBE as the running buffer. Load 2 μl of sequencing reactions/well making sure to low out wells with a syringe first then Run the gel at 80W for 1 h interval.

### EDTA 0.5 M pH 8.0

Dissolve 186.1 g of disodium ethylenediaminetetra-acetate (EDTA 2H<sub>2</sub>O Sigma ED2SS mw 372.2) in 800 ml of ddH<sub>2</sub>O. Stir vigorously and adjust the pH to 8.0 with NaOH pellets (EDTA will not go into solution until the pH is near 8.0, so add some of the pellets before trying to adjust the pH. Bring it to a final volume of 1000 ml. Divide into 100 ml aliquots and autoclave.

### Ethidium Bromide 10×

Dissolve 1.0 g of EtBr in a final volume of 100 ml ddH<sub>2</sub>O. Wrap the bottle in aluminum foil and stir several hours to get a true solution. Store at 4°C. To make the 1× stock used to stain gels take 10 ml of the 10× stock and bring to a final volume of 100 ml using ddH<sub>2</sub>O. Wrap bottle in aluminum foil and store at room temperature.

### **Proteinase K**

To one ml of ddH<sub>2</sub>O add 20 mg of Proteinase K (Promega # 52066). This gives a 20 mg /ml stock.

### **SDS 10%**

Dissolve 100 g of electrophoresis-grade SDS in 800 ml ddH<sub>2</sub>O. Heat the solution to dissolve. Bring to a final volume of 1000 ml using ddH<sub>2</sub>O. Do not autoclave

### **TE pH 8.0**

<b>chemical</b>	<b>volume</b>
1 M Tris pH 8.0	2 ml
0.5 M EDTA pH 8.0	400 $\mu$ l

Bring it to a final volume of 100 ml using ddH<sub>2</sub>O and Autoclave.

### **Tris 1 M pH 7.4**

Dissolve 121.1 g of Tris base in 800 ml of ddH<sub>2</sub>O and adjust the pH to 7.4 with concentrated HCl. Bring the final volume to 1000 ml with ddH<sub>2</sub>O. Divide into 100 ml bottles and autoclave.

## APPENDIX C

### SAMPLING INFORMATION SHEET

Media type (Modified Horikosh media with)	Tube number	Amount of media	Sample type	Inoculum amount	Place of inoculation (environment)
Glucose	1	20 ml	Pure hot spring water	2 ml	Field
	2	20 ml	Pure hot spring water	2 ml	Lab
	3	20 ml	Microbial mat	2 ml	Field
	4	20 ml	Microbial mat	2 ml	Lab
	5	20 ml	–	–	Field
	6	20 ml	–	–	Lab
Cellulose	1	20 ml	Pure hot spring water	2 ml	Field
	2	20 ml	Pure hot spring water	2 ml	Lab
	3	20 ml	Microbial mat	2 ml	Field
	4	20 ml	Microbial mat	2 ml	Lab
	5	20 ml	–	–	Field
	6	20 ml	–	–	Lab
Xylose	1	20 ml	Pure hot spring water	2 ml	Field
	2	20 ml	Pure hot spring water	2 ml	Lab
	3	20 ml	Microbial mat	2 ml	Field
	4	20 ml	Microbial mat	2 ml	Lab
	5	20 ml	–	–	Field
	6	20 ml	–	–	Lab
Starch	1	20 ml	Pure hot spring water	2 ml	Field
	2	20 ml	Pure hot spring water	2 ml	Lab
	3	20 ml	Microbial mat	2 ml	Field
	4	20 ml	Microbial mat	2 ml	Lab
	5	20 ml	–	–	Field
	6	20 ml	–	–	Lab

#### Key

- A total of 28 tubes were used each with 20 ml of varied media were used.
- One tube of each media was left in the lab uninoculated; unexposed controls: (-)
- One tube of each media was taken to the field uninoculated; exposed controls: (-)
- Eight tubes were inoculated in the field immediately after sampling.
- Eight other tubes were inoculated in the lab three days after sampling.
- Four varied media were used for enrichment.

## APPENDIX D

### ANOVA tables

#### I. Growth at different temperature

**Dependent Variable: 24°C**

<b>Source of variation</b>	<b>DF</b>	<b>Mean Square</b>	<b>F value</b>	<b>Pr &gt; F</b>
Sample Treatments	35	0.06691863	12746.4	<.001

**Temperature 40°c**

<b>Source of variation</b>	<b>DF</b>	<b>Mean Square</b>	<b>F value</b>	<b>Pr &gt; F</b>
Sample Treatments	35	0.17280209	1.73	0.0255

**Temperature 55°c**

<b>Source of variation</b>	<b>DF</b>	<b>Mean Square</b>	<b>F value</b>	<b>Pr &gt; F</b>
Sample Treatments	35	0.18380219	1.84	0.0352

### Temperature 65°c

<b>Source of variation</b>	<b>DF</b>	<b>Mean Square</b>	<b>F value</b>	<b>Pr &gt; F</b>
Sample Treatments	35	0.05706263	112.39	<.0001

## II. Growth at different sodium chloride concentration

### 2% NaCl

<b>Source of variation</b>	<b>DF</b>	<b>Mean Square</b>	<b>F value</b>	<b>Pr &gt; F</b>
Sample Treatments	35	0.00325140	765.03	<.0001

### 5% NaCl

<b>Source of variation</b>	<b>DF</b>	<b>Mean Square</b>	<b>F value</b>	<b>Pr &gt; F</b>
Sample Treatments	35	0.00212738	155.35	<.0001

### 7% NaCl

<b>Source of variation</b>	<b>DF</b>	<b>Mean Square</b>	<b>F value</b>	<b>Pr &gt; F</b>
Sample Treatments	35	0.00216235	16.05	<.0001

**10% NaCl**

<b>Source of variation</b>	<b>DF</b>	<b>Mean Square</b>	<b>F value</b>	<b>Pr &gt; F</b>
Sample Treatments	35	0.00149006	930.21	<.0001

**20% NaCl**

<b>Source of variation</b>	<b>DF</b>	<b>Mean Square</b>	<b>F value</b>	<b>Pr &gt; F</b>
Sample Treatments	35	0.00922280	90.94	<.0001