ISOLATION AND CHARACTERIZATION OF EXTREMOPHILIC BACTERIA FROM THE HOT SPRINGS OF LAKE MAGADI, KENYA

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Isolation and characterization of extremophilic bacteria from the hot springs of Lake Magadi, Kenya

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DECLARATION

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

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DEDICATION

This work is dedicated to my dear family; my beloved husband Japheth Kilonzo, to my Lovely sons; Israel Amani and Caleb Kibali. I appreciate the support you have accorded me during the course of my studies. Without your encouragement and support this journey would have been longer and tougher. To my loving parents James Mulango and Beatrice Mulango, who encouraged and supported me all through to this level of education. God bless you all.

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LIST OF ABBREVIATIONS AND ACRONYMS

Bp Base pairs

CMC Carboxymethylcelullose

DNA Deoxyribonucleic Acid

EDTA Ethylene Diamine Tetra-Acetic Acid

G+C Guanine and Cytosine

LB Luria Bertani

PCR Polymerase Chain Reaction

SDS Sodium Dodecyl Sulfate

BLAST Basic Local Alignment Search Tool

dNTP 2'-deoxynucleoside-5' triphosphate

EDTA Ethylene-Diamine-tetra-Acetic Acid

MEGA Molecular Evolutionary Genetic Analysis

NCBI National Centre for Biotechnology Information

PCR Polymerase Chain Reaction

rRNA Ribosomal Ribonuclaic Acid

Tris Tris-(hydroxymethyl) – aminomethane

ABSTRACT

Hot springs are aquatic environments with extremely high temperatures. They harbor a group of extremophiles called haloalkaliphilic bacteria that have developed mechanisms to thrive at high temperature, pH and salinity conditions comparable to those prevailing in various industrial processes. Research on these microbes has been motivated by their great biotechnological potential such as production of useful secondary metabolites and extremozymes with industrial application. Isolation, characterization and identification of haloalkaliphilic bacteria have been done on Kenyan soda lakes. However, more information about the hot springs of Lake Magadi, a hyper saline lake with up to 30 w/v % salinity, 12.5 pH levels and 86°C is needed. This work was aimed at isolating, characterizing and screening extremophiles from Lake Magadi hot springs for the ability to produce useful biotechnological enzymes. Collection of water, wet sediments and microbial mats was carried out from the springs in the main lake at a temperature of 45.1°C and from Little Magadi Nasikie eng'ida (temperature of 81 and 83.6°C). A total of 44 bacteria isolates were recovered using selective media prepared with filter-sterilized water from the lake. These were characterized using morphological, physiological, biochemical and molecular approaches. The isolates were also screened for production of extracellular enzymes and antimicrobial activity. Majorities (80%) of bacteria isolated were Gram positive and few (20%) were Gram negative. They grew well at pH range of 6 –14(optimum 9-12), temperature range of 30 – 60°C (optimum 40-50°C) and sodium chloride (salt)range of 0- 15 % (optimum 0-7.5%). The bacterial isolates produced various extracellular enzymes such as amylases, cellulases, proteases, lipases and esterases. Antimicrobial assays done against test organisms (bacteria and fungi) showed that the isolates had range of inhibitory effects. All isolates produced various colored pigments in the media indicating that they could be a source of diverse bioactive metabolites. Bacterial DNA was extracted using phenol-chloroform extraction method and isopropyl alcohol precipitation method. Nearly full-length 16S rRNA gene sequences were PCR amplified using forward bacterial Primer 8F and reverse bacterial primer 1492R to get 1500 bp of the PCR product. Sequencing of the 16S rDNA of the isolates was done and sequences for 7 isolates were without ambiguities. BLAST results revealed that the isolates belonged to domain bacteria, 90% of isolated microbes had an affiliation to phylum Firmicutes, class *Bacilli*, order *Bacillates*, family *Bacillaceae* and genus *Bacillus* while 10% was affiliated to phylum *Proteobacteria*, class *Betaproteobacteria*, Order *Burkholderiales*, family *Alcaligenaceae* and genus *Alcaligenes*. Isolates HS3, HS10, HS13, HS18, HS29 and HS41clustered with members of genus *Bacillus* with similarity scores ranging between 86%–98%, while isolate HS28 clustered to genus *Alcaligenes* with 96% sequence similarity. Strains from *Bacillus* family included; *Bacillus siamensis, Bacillus licheniformis, Bacillus methylotrophicus, Bacillus amyloliquefaciens, Bacillus pumilus,* and *Bacillus sonorensis* while one strain that belonged to *Alcaligenes* family was *Alcaligenes feacalis*. Based on the results, the isolates recovered were halotolerant, alkalitolerant and thermotolerant. This study demonstrates that extreme environments of the hot springs of Lake Magadi harbor

extremophilic bacteria with the potential to produce enzymes and antimicrobial compounds. Further research should focus on different protocols and novel microbial culture techniques which allow the identification of a bigger diversity of genera accompanied by extensive research on the specific secondary metabolites released by extremophiles from Lake Magadi hot springs.

CHAPTER ONE

INTRODUCTION

1.1 Background information

Soda lakes and soda deserts are the best representatives of stable naturally occurring high pH environments on Earth (Grant et al., 2006). Some of the most studied soda lakes are; Great Salt Lake (Utah, USA), the Dead Sea (Israel), Mono Lake (U.S. A), Lake Wadi An-Natrun (Egypt) and Lake Magadi (Kenya) (Jones et al., 1999; Satyanarayana et al., 2005). Soda lakes are therefore characterized by high salt concentrations and high pH, which occurs between 9.5 and 11 (Tindall 1988; Banciu & Sorokin, 2013; Vavourakis et al., 2016). The lakes are characterized by presence of large amounts of Na_2CO_3, and lack Mg^{2+} and Ca^{2+} because of the inability of carbonates to be soluble under alkaline conditions (Duckworth, 1996; Grant et al., 2006). Hypersaline environments are widely distributed on earth where they exist either as natural water bodies, such as permanent saline lakes, ephemeral salt pans and salt marshes, or as artificial solar salterns (Nyakeri et al., 2018).

Saline environments are often situated in remote geographical locations and can be divided into two broad categories: the thalassohaline environments such as the Great Salt Lake in Utah, playas, brine springs from underground, salt deposits and solar salterns that have similar salt composition to seawater in that sodium and chloride are the dominant ions (Litchfield & Gillevet, 2002) and the athalassohaline water bodies (such as the Dead sea, Lake Magadi in Kenya, Wadi Natrun in Egypt, the soda lakes of Antarctica and Big Soda Lake and Mono Lake in California) which are dominated by potassium, magnesium, or sodium (Oren, 2002; Litchfield; Gillevet, 2002). Soda lakes are commonly 9-10 times more concentrated than sea water which is generally defined as having 3.5% (w/v) dissolved salts (Satyanarayana et al., 2005; Setati, 2009). Both

natural and artificial hypersaline environments harbor remarkably high and diverse microbial cell densities (Oren, 2002).

Saline soda lakes are thought to be among some of the major productive aquatic environments on Earth (Melack, 1981; Sokorin et al., 2016), with the productivity rates in an order of magnitude greater than the mean rate for all aquatic environments on earth (Melack & Kilham, 1974; Tindal, 1986). Production yields within these haloalkaline environments stand at >10 g carbon m^{-2} day^{-1}, against the average primary production in streams and non-alkaline lakes which stands at 0.6g cm^{-2} day^{-1} (Jones, 1998). This is presumably due to availability of phosphates, unlimited access to carbon dioxide, high ambient temperatures and high light intensities (Xue et al., 2006b). Haloalkaline lakes have been found to support dense and diverse populations of aerobic, organotropic, halophilic, alkalithermophilic and alkalitolerant microbes which represent some major bacterial phyla (Zavarzin et al., 1999).

In general, any environmental condition that is perceived to be beyond the normal acceptable range is an extreme condition (Satyanarayana et al., 2005). However, a variety of microbes have been characterized by conditions such as high temperatures, pH, pressure, salt concentration, nutrient concentration, radiation, harmful heavy metals, organic solvents and toxic compounds (Satyanarayana et al., 2005). Some of these organisms not only tolerate some specific extreme conditions, but also require them for survival and growth (Bowers et al., 2009). Therefore, organisms that grow optimally at environmental and physicochemical parameters too harsh for normal life to exist are referred to as extremophiles (Michael & Barry, 1997; Satyanarayana et al., 2005).

Most of the described extremophiles are characterized and categorized by one distinct extreme parameter such as temperature (thermophiles), salinity (halophiles) or pH (alkaliphiles). However, others are polyextremophiles since they tolerate and grow under two or more extreme conditions (Kevbrin, 1998; Setati, 2009) for example alkalithermophiles. Polyextremophilic organisms can survive the combination of high

alkaline and saline conditions as well as elevated temperatures. Thus alkalithermophiles, are extremophilic bacteria which not only survive but grow optimally under conditions considered harsh and inhospitable from human point of view (Mesbah & Wiegel, 2012).

The East African Rift Valley contains a number of soda lakes that have been studied with a keen interest and these lakes include; Lake Bogoria, Lake Elmenteita, Lake Nakuru and Lake Magadi. Detailed limnological and microbiological studies have been done on these lakes over many years (Mwirichia et al., 2009; Mwirichia et al., 2010; Akhwale, 2015; Kambura, 2016). Like other extreme environments, soda lakes have become a source of research attention because of the perception that they harbor microbial life forms with valuable biotechnological applications (Zhilina et al., 2012). Additionally, they may also provide new perspectives on the true extent of microbial diversity and clues to the evolution of life on Earth (Canganella & Wiegel, 2014).

Haloalkaliphilic bacteria belonging to all major trophic groups have been described in East African soda lakes, including lakes with exceptionally high levels of heavy of metals (Muyzer, 2014). A diverse microbial array is present in the trona beds and concentrated alkaline\saline brines of lakes such as Magadi (Kenya). Lake Magadi, where salt concentration is extremely high, is the center of commercial enterprise (Seckback, 2012). Moderately, saline soda lake brines harbor more diverse microbial communities than other saline environments (Dimitriu et al., 2008). Several studies on alkalithermophilic bacteria from saline soda lakes have shown the dominant presence of *Alphaproteobacteria, Gammaproteobacteria, Firmicutes, Bacteroidetes* and *cyanobacteria* (Pagaling et al., 2009).

Studies on Lake Magadi have revealed various extreme and moderate extremophilic cyanobacteria. They were identified as *Synechocystis salina, Aphanothece stagnina, Chamaesiphon sublobosus, Rhabdoderma lineare, Synechococcus elongates, Phormidium ambiguum, Phormidium foveolarum, Phormidium retzii, Oscillatoria limnetica, Spirulina fusiformisans* and *S. laxassima* (Dubinin et al., 1995; Kambura,

2016). Unicellular cyanobacteria were observed to grow well at higher salinity levels (7 % and 10 % NaCl), than the trichomic forms which were better suited at lower salinity and alkalinity levels (Zavarzin et al., 1999; Kambura, 2016). Many of the microbes that have been recovered from the hypersaline brines of Lake Magadi are obligate alkalithermophiles (Grant & Sokorin, 2011) and represent separate alkalithermophilic lineages within pre-established taxa (Zavarzin et al., 1999).

Other studies on Lake Magadi (culture independent and dependent) also revealed a diverse population of aerobic, halophilic, alkaliphilic, haloalkaliphilic, organotropic and alkalitolerant bacterial phyla (Kambura, 2016). Though a number of extremophiles have been isolated and described from Lake Magadi, its ecology has not been fully understood or exploited, yet this is crucial for biotechnological development (Nyakeri et al., 2018). Alternative culture-independent approaches have also proven to be very useful in expanding the information of known diversity of microbial communities thriving under the extreme conditions of pH, salinity and temperature. These approaches were the first molecular methods to be used in the study of bacterial diversity in Lake Magadi (Kenya). Cloning and denaturing gradient gel electrophoresis (DGGE) of 16S rRNA gene fragments was thereafter used to detect the presence of novel prokaryotic isolates in various soda lakes in East Africa (Mesbah & Wiegel, 2007). More recently, next-generation sequencing of PCR-amplified regions of the 16S rRNA gene have also been used (Lanzén et al., 2013). The main objective of this study was to add more knowledge to the previous culture-independent and dependent studies on Lake Magadi through isolating and characterizing bacteria from the hot springs of the lake.

1.2 Statement of the problem

Kenyan soda lakes are an example of relatively simple but highly productive ecosystems that can serve as models for studying microbial diversity under extreme conditions. The soda lakes are also characterized by hot springs which host both hyperthermophilic and

haloalkalithermophilic microorganisms. Despite this, the diversity, biotechnological potential as Lake Magadi, East African Rift Valley's most saline soda lake, has not been fully understood and exploited, yet understanding and conserving soda lake biodiversity is the basis of future developments in biotechnology (Mwirichia et al., 2010).

Currently, there is rapid spread of antibiotic-resistant pathogens which cause life-threatening infections (Amárita et al., 2002). It is estimated that as low as 0.1-1% of the microorganisms which live in the biosphere have been cultured and even characterized in a laboratory setting. The other 99% of the microbial population could represent novel genetic diversity. These microorganisms represent an array of undiscovered novel microorganisms that can produce novel bioactive antimicrobial compounds.

1.3 Justification

Microorganisms in natural saline and alkaline environments are useful because they harbor useful biotechnological enzymes and metabolites. These organisms are of considerable industrial interest, particularly for the production of enzymes such as proteases for inclusion in laundry detergents, manufacture of synthetic leather and xylanases are used in paper industries. These bacterial communities should therefore be isolated, characterized and identified for maximum utilization such as the production of valuable metabolite compounds that can be used in industries. Extremophilic microorganisms derived from soda lakes have a great, unexploited potential that can be useful in biotechnological applications (Horikoshi, 2006). Thus, this study involved the isolation of new bacterial isolates, characterizing and screening the members for production of useful bioactive metabolites.

The combination of molecular analytical methods like 16S rRNA genes of the isolates with different cultivation techniques may hopefully yield a greater percentage of major dominant groups and reveal not only its community structure but also allow meaningful analysis of the natural ecology to be made (Jones, 1994; Duckworth et al., 1996). It will

also reveal previously undetected and unknown phylogenetic groups, some of which may be exclusive to the soda lake environment (Rees et al., 2003). Thus the findings of this research study will provide information that increases the understanding of the microbial diversity, ecology and potential biotechnological benefits of the microbes found at Lake Magadi, known worldwide as a major source of salt which is a food supplement.

1.4 Hypothesis

The hot springs of Lake Magadi do not harbor novel haloalkaliphilic bacteria `that have biotechnological potential

1.5 Objectives

1.5.1 General objective

To isolate and characterize extremophilic bacteria from the hot springs of Lake Magadi, Kenya.

1.5.2 Specific objectives

1. To isolate and characterize bacteria using morphological and physiochemical methods.
2. To screen the bacterial isolates for production of extracellular enzymes and antibiotics.
3. To characterize and identify the bacterial isolates using molecular methods.

CHAPTER TWO

LITERATURE REVIEW

2.1 General microbial biodiversity of soda lakes in the world.

Soda lakes are predominantly found in the arid, semi-arid and desert regions around the world (Samylina et al., 2014). Soda lakes from around the world that have been studied include; Mono Lake (Humayoun et al., 2003; Scholten et al., 2005) soda lakes in the Kenyan-Tanzanian Rift Valley (Rees et al., 2004), soda lakes in Mongolia (Sorokin et al., 2004) and Inner Mongolia in China (Ma et al., 2004), athalassohaline lakes of the Atacama desert, Chile (Demergasso et al., 2004), saline, meromictic lake Kaiike in Japan (Koizumi et al., 2004), saline Qinghai Lake, China (Dong et al., 2006) and athalassohaline Lake Chaka, China (Jiang et al., 2006). Despite these studies, the knowledge of microbial populations in hypersaline/alkaline environments are still limited when compared with microbial communities in marine or fresh water bodies.

The knowledge about molecular mechanisms of extremophilic microbes stemmed mainly from studies by Horikoshi (1999) on hot springs in Yellowstone National Park (Wyoming, U.S.A) that revealed large bacterial communities in the hot spring that had twelve novel division-level lineages. These studies revealed that members belonging to the bacterial domain appeared to outgrow the Archaea found the hydrothermal environment (Keller & Zengler, 2003; Simasi, 2009). Novel obligately anaerobic, alkalithermophilic, chemo-organotropic bacterium was identified from an alkaline hot spring located on Paoha Island in Mono Lake, California, USA. This bacterium is rod-shaped; it reduces Fe (III) and Se (IV) in presence of organic matter. The strain was identified as *Anaerobranca californiensis* sp. Nov on the basis of physiological

properties, 16SrRNA gene sequence and DNA–DNA hybridization data (Vladimir et al., 2004).

Subsequent studies of microbial diversity in the hot springs of Yellowstone National Park in (Wyoming) U.S.A, have shown that although all the hot springs were in close geographical proximity, they had similar temperatures (between 85°C and 95°C) and pH value (7.8–8.9), but differed remarkably in regard to their overall microbial diversity. This finding indicated that, in the common complexity of microbial diversity in soil, geochemical variations affect microbial biodiversity and that only studies that incorporate measurements of geochemical parameters will allow the understanding and prediction of biodiversity (Keller & Zengle 2003; Simasi, 2009).

Studies by Yanhe in 2004 on Baer Soda Lake located in the Inner Mongolia region of China, indicated that the 16S rDNA phylogenetic analysis of bacterial diversity in the alkaline Lake could be isolated and characterized using both culture dependent and molecular methods. Fifty three alkalithermophilic bacteria were isolated from the sediment samples, 20 of the isolates were subjected to 16S rRNA gene sequence analysis. The results showed some of the clones were related to extremophilic bacteria from soda lakes such as *Alkalispirillum*, *Thioalcalovibrio denitrificans*, and *Halomonas campisalis*, while others were related to known species with more than 97 % similarity from environments that are not alkaline. These isolates were affiliated to the genera *Bacillus, Amphibacillus, Gracilibacillus, Alkalibacterium, Salinicoccus, Exiguobactrium, Halomonas, Pseudomonas, Marinospirillum,* and *Cyclobacterium*. Out of the 20 bacterial isolates, 4 were Gram-negative while the rest of them were Gram-positive isolates (Borsodi et al., 2008).

Cultivatable alkalithermophilic bacteria were detected in Lonar Lake water sediment samples and showed the highest diversity within the Firmicutes phylum, followed by *Gammaproteobacteria, Actinobacteria, Alphaproteobacteria* and *Betaproteobacteria* phyla (Wani et al., 2006; Kumaresan et al., 2013). Many isolates from the lake were

found to produce biotechnologically relevant enzymes such as lipase, amylase, cellulase and caseinase at alkaline pH. Sediment based enrichments supplemented with C_1 substrates (methane and methanol) and C_2 substrates (ethanol and acetate) yielded isolates related to *Alkalimonas, Bacillus, Halomonas, Paracoccus, Pseudomonas, Methylophaga*, *Idiomarina* and *Rhodobaca* (Antony et al., 2013). Four novel heterotrophs; *Indibacter alkaliphilus, Georgenia satyanarayanai*, *Cecembia lonarensis* and *Nitritalea halakaliphila,* together with one novel methylotroph (*Methylophaga lonarensis*) have also been described from Lonar Lake (Srinivas et al., 2012).

Northern Egypt has a number of desert alkaline soda lakes in the Wadi Natrun region, these lakes are fed by underground water from the river Nile due to their lower surface elevation. They have diverse and dense microbial flora (Imboff et al., 1996) and are known as a source of various mesophilic alkalithermophiles. The Wadi An Natrun lakes are extreme in more than one aspect; high salt concentrations between 91.0 and 393.9 g/L have been reported, all the lakes have pH values between 8.5 and 11. They are populated by dense communities of halophilic alkalithermophilic microorganisms that have yielded a number of novel prokaryotic species of archaea and bacteria. These microorganisms participate in aerobic and anaerobic cycling of carbon, nitrogen, and sulfur, hence suggesting active cycling of these elements in the ecosystem. Molecular ecological studies based on identification of 16S rRNA sequences isolated directly from DNA molecular analysis of microbial diversity of the Wadi An Natrun performed are that of cellulyotic enrichment cultures and serine protease from the halo-alkaliphilic *Alkalibacillus* sp. (Soad et al., 2007; Mesbah, 2008; Mesbah 2016).

2.2 Microbial diversity of soda lakes in Kenya

Soda lakes in the Kenya's Great Rift Valley include Lake Elmenteita, Bogoria, Nakuru, Magadi and Sonachi. The salinity of lakes Bogoria, Nakuru, Elementaita and Sonachi ranges from around 5% to 9% total salts (W/V) but is more saturated in Lake Magadi. (Mwatha 1993; Lanzén et al., 2013). Studies on these lakes have shown that they harbor

novel species of archaea and bacteria. Soda lakes in the Kenyan Rift valley harbor a high archeal diversity which is affiliated to the genera; *Halovivax, Natronococcus, Halobiforma, Halalkalicoccus* and *Halorubrum* (Mwirichia et al., 2010).

In an attempt to isolate novel groups of bacteria from Lake Elmenteita, different media with filter-sterilized water from the lake was used. Majority of the isolated bacteria were affiliated to the genus *Bacillus* while the others belonged to genus *Gammaproteobacteria. Bacillus* strains isolated from the lake were M8-C11 (FJ 764771), M14-C16 (FJ 764778), M4-C7 (FJ 764769), M10-C8 (FJ 764774), M14-C6 (FJ 764777), M8-C22 (FJ 763929), M1-C6 (FJ 764768), M8-C14 (FJ 764772), M10-C17 (FJ 764775) and M9-C3 (FJ 764773) (Mwirichia et al., 2009). Other groups of bacteria recovered were closely related to *Idiomarina, Alkalimonas, Marinospirillum, Vibrio, Enterococcus, Marinilactibacillus, Alkalibacterium, Amphibacillus, Streptomyces* and *the actinobacteria Nocardiopsis.* Novel taxa were identified which had not been isolated previously from the soda environment (Mwirichia et al., 2010). Further, a culture-independent approach was also used to study the bacterial diversity of Lake Elementaita. The results of the studies indicated the presence of 37 orders in the Domain bacteria; Cyanobacteria and members of the phylum Firmicutes were the most represented showing that 93.1% of the sequenced clones had similarity values below 98% to both cultured and uncultured bacteria (Mwirichia et al., 2011).

In Lake Magadi, representatives of all the main trophic groups involved in the active cycling of nutrients (carbon, sulfur and nitrogen) under anaerobic and aerobic conditions have been discovered (Grant & Sokorin, 2011). The basis of primary production within Lake Magadi is however unclear, since the trona beds are often dominated by organotropic archaea (Xue et al., 2006). In a study to isolate alkaliphilic bacteria from Lake Magadi, (Kambura, 2016) used different types of media prepared with filter-sterilized water from the lake for isolating bacteria from Lake Magadi. Analysis of the partial sequences of 16S rRNA genes showed 80% of the isolates were closely related to the genus *Bacillus* while 20% were close relatives to members of

Gammaproteobacteria. Culture-independent 16SrRNA-based studies indicate that the previously uncultured fraction comprises numerous unknown bacteria and entire novel phylogenetic groups. A variety of 16S rRNA gene sequences related to putative novel Archaea (Euryarchaeota) have been retrieved from the alkaline water body (Grant et al., 1999; Kambura et al., 2016).

Haloalkaliphilic Archaea related to *Halorubrum* spp, *Natrialba*, *Natromonas* and *Natronolimnobius* were also isolated from the saltern Lake Magadi (Grant & Sorokin 2011). Metagenomic studies of the lake have showed archeal diversity of species such as *Halorubrum vacuolatum, Natronococcus occultus* and *Natrialba magadii*. Previous studies on Lake Magadi showed that the highest percentage of the isolated clones belonged to the uncultured members of Domain Archaea, order *Halobacteriales* (Mwirichia et al., 2010b; Nyakeri 2018). Biochemically reactive isolates producing a variety of extracellular hydrolytic enzymes such as lipases, proteinases and cellulases have been isolated from the halo alkaline lake. Many of the cultivable population of bacteria have been assigned to existing taxonomic groups, although most fall into new generic groupings. Studies based on 16S rRNA gene of soda lakes have revealed novel taxa yet to be cultured (Grant et al., 2004; Kambura et al., 2016).

2.3 Adaptations of extremophiles

The ability of extremophiles to survive extreme conditions shows that they must have special adaptive mechanisms for survival (Mesbah & Wiegel, 2008). Alkaline, salt and temperature stressors have significant interplay with one another. First, there is an overlap between alkaline stress and salt stress. Halophiles commonly have higher intracellular Na^+ contents than their non-halophilic counterparts. As pH rises, however, Na^+ cytotoxicity greatly increases (Padan & Krulwich, 2000). The toxicity of Na^+ is also determined by the status of cytoplasmic K^+ concentration; the higher the K^+ concentration in the cytoplasm, the more tolerant the cell becomes to intracellular Na^+.

Temperature stress overlaps with that of alkaline stress; high temperatures increase cell membrane permeability to H^+ (Konings & Driessen, 2002; Mesbah; Wiegel, 2008), which can be harmful to alkaliphiles that must keep their cytoplasmic pH 1-2.5 units lower than that of external pH. Temperature stress also overlaps with salt stress; high temperature alters membrane permeability to Na^+, though to a much lesser extent than membrane permeability to H^+. This counteracts the efficacy of systems functioning to keep cytoplasmic Na^+ concentrations low and these are essential for survival as intracellular Na^+ is cytotoxic (Padan et al., 2005).

Halophiles have been reported to have Sodium-dependent transport systems that generate 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 & Simasi, 2009).The chemiosmotic theory states that, the proton motive force in the cells is generated by the electron transport chain or by excreted H^+ derived from ATP metabolism by ATPase. H^+ is then reincorporated into the cells with co- transport of various substrates (Horikoshi, 1999). Sodium ions in the environment around are of much essence for the effective solute transportation through the membrane of alkaliphilic *Bacillus* spp. Alkaliphiles have clearly gained large amounts of genetic information by evolutionary processes and exhibit ability in their genes to cope with particular environments. Genes that are responsible for alkalinity of *Bacillus halodurans* C-125 and *Bacillus firmus* OF4 have been analyzed. Thus their genes are now a valuable source of information waiting to be exploited by biotechnologists (Horikoshi, 1999 & Horikoshi, 2006).

In psychrophilic and mesophilic bacteria and archaea, the proton permeability of the cytoplasmic membrane is kept constant by increasing the lipid acyl chain length, degree of saturation and ratio of iso- and anteiso-branched fatty acids as the temperature increases (Vossenberg et al., 1999a). However, alkalithermophilic bacteria and archaea growing at temperatures greater than 50^{o}C encounter increased membrane proton permeability because they are no longer able to compensate by adjusting the lipid

composition. The membrane proton permeability of the facultatively aerobic bacterium *Geobacillus stearothermophilus*, anaerobic *Thermotoga maritima* and the aerobic archaeon *Sulfolobus acidocaldarius* increases exponentially with temperature. The sodium ion permeability is several orders of magnitude lower than the proton permeability, thus many alkalithermophiles have Na+ coupled bioenergetics (Koning et al., 2002).

2.4 Molecular characterization of alkaliphiles

Studies by Baumgarte (2003) showed that the total DNA extraction, amplification of 16S rDNA gene, screening of clone library and sequence determination of cloned 16S rRNA genes has enabled the detection and recognition of various unknown bacterial sequence types of sediment samples taken from extreme environment of Lake Magadi. This provided more knowledge about the prokaryotic composition of the soda environment. In another study on the Lake, phylogenetic analyses of the partial 16S rDNA sequence of the isolates clustered the isolates into three genera namely *Bacillus, Closridium* (Firmicutes) and *Halomonas* (*Proteobacteria*). *Bacillus* relatives were the majority at 54% followed by *Clostridium* at 38% and *Halomonas* at a mere 8%, showing that the gram positive Firmicutes were more diverse than Gram-negative *Proteobacteria* (Nyakeri et al, 2018).

This concurs with a cultivation dependent study on Lake Magadi in which the sequences clustered in two main groups of already established lineages of bacteria that are the low G + C Gram-positive bacteria (*Bacillus* and relatives) and the gamma subdivision of the *Proteobacteria* (Baumgarte, 2003). Studies by Kambura on Lake Magadi in 2012 were done by analyzing partial sequences of 16S rRNA genes and showed that 80% of the isolates closely clustered to genus *Bacillus* and 20% closely clustered to members of *Gammaproteobacteria.*

Novel taxa were identified from Lake Elmenteita which had never been isolated before from the soda lake environment (Mwirichia et al., 2010a). These were bacteria affiliated to class *Gammaproteobacteria* and genus *Bacillus.* A culture-independent method was also used to carry out studies on the above bacterial communities. The results indicated that there was a presence of 37 orders in domain bacteria. Cyanobacteria and members of the phylum *Firmicutes* were the most represented with 93.1% of the sequenced clones having similarity values below 98% to both the cultured and uncultured bacteria (Mwirichia et al., 2011).

Results of a study by Mesbah (2008) indicate that a diverse range of prokaryotes are present in three of the largest lakes of the Wadi An Natrun. A high proportion of the sequences retrieved (42% of bacterial sequences, 53% archaeal sequences) had less than 90% 16S rRNA sequence identity to any sequences deposited in the RDP or Gen Bank, representing a microbial array different from what has currently been described, even in other hypersaline environments. These could represent potential novel phylogenetic groups.

2.5 Biotechnological and industrial potential of extremophiles

There is significant biotechnological interest in microorganisms and enzymes from alkaline environments. Several studies have focused on isolating and characterizing novel enzyme producing microbes from high pH, salt concentration and temperature environments (Kambura, 2015). Soda lakes are important sources of microbial enzymes that can function at high pH and temperature (Sorokin & Berben, 2014). According to Duckworth studies in 1996, alkalithermophilic bacteria have biotechnological potential of producing alkali-thermostable enzymes. The discovery of thermostable enzymes which in nature allow thermophiles to survive under high temperatures (Gomes & Steiner, 2004; Canganella & Wiegel, 2011) and which are amenable to many industrial process requirements have sparked an interest in the study of the biotechnological potential of other extreme environments. The remarkable structural and chemical

adaptations that ensure the survival of extremophiles in hostile environments gives rise to unique extremozymes (enzymes obtained from extremophiles) (Gomes & Steiner, 2004).

Extremophiles in particular, have been cultured and screened for molecules of industrial interest that include extremoenzymes, compatible solutes, biopolymers (Bartlett et al., 1993; Buchalo et al., 2000). Alkaline proteases, lipases, amylases, chitinases and caseinases have been reported in a wide range of bacteria isolated from soda lake environments, such as Rift valley soda lakes, Lonar Lake and Mono Lake (LeCleir et al., 2007). Several researchers have screened bacteria from different hypersaline environments through direct plating on agar media amended with substrates specific for extracellular enzymes such as lipases, proteases, amylases, cellulases and xylanases (Castro et al., 1993; Setati, 2009). A variety of bacteria that secrete these enzymes have been isolated and characterized (Castro et al., 1993; Sánchez-Porro et al., 2003; Rohban et al., 2009).

There are various examples of extremozymes that are in commercial use and they include alkaline proteases, cellulases and lipases (Jones, 2004). Through screening methods, industrial cellulases have been obtained from Gram-positive Kenyan Soda Lake isolates and have used in textile and laundry processes (Sheridan, 2004). Alkaline cellulases and lipases are used to manufacture detergents (Jones, 2004). Puradax, a cellulase used for rooting out difficult stains and reducing the spills on fabrics (Aygan & Arikan, 2008), is a critical ingredient in the manufacture of Tide Alternative Bleach detergent, whose origin is from a microbe cultured from Lake Bogoria, Kenya (Lacey, 2006). Another cellulase enzyme used to make soft, stone washed and bleached jeans was isolated from a different microbe cultured from Lake Bogoria (Lacey, 2006; Aygan & Arikan, 2008). Alkaline cellulases and lipases are also used in the manufacture of food ingredients and pitch control in the pulp and paper industry (Mattiasson et al., 2004).

Proteases from haloalkaliphiles are used in the manufacture of leather, xylanases are used in paper industry and cyclodextrin glucanotransferase is used for cyclodextrin manufacture. Starch is used in manufacture of foodstuffs, cosmetics and pharmaceuticals (Saeki et al, 2002; Oren et al, 2006; Kambura, 2011). Glycosyl transferases and hydrolases from extremophiles are of much importance because they have ability to perform reactions at high temperatures and high contents of organic solvents (Grant et al., 1990; Horikoshi, 1996; Bordenstein, 2008).

Haloalkaliphilic microorganisms use several different types of organic solutes such as osmolytes. Ectoine is one of the osmolytes accumulated intracellularly by soda lake microorganisms such as methylotrophs (Antony et al., 2013) and sulfur-oxidizing bacteria (Sorokin & Muyzer, 2010). Ectoine has many biotechnologically relevant applications, especially in molecular biology, cosmetics and therapeutics (Kumaresan et al., 2013). Thus haloalkaliphiles are thought to have significant economic potential because their enzymes are already used in detergent compositions, leather tanning and other industries. Therefore extremophiles are foreseen to find applications in the food, waste treatment and textile industries.

Another example of an extremophilic material used in biotechnology is bacteriorhodopsin an integral membrane protein. This retinal based pigment found in the halophilic archaeon *Halobacterium salinarum,* is part of a unique photosynthetic apparatus which functions as a light–driven proton pump. It is fuelled by solar energy (500-650 nm) and assists in the translocation of information and materials across cell membranes. It is a perfect model for energy conversion and its biotechnological use lies in optical information recording, spatial light modulation and holography (Haupts et al., 1999; Margesin & Schinner, 2001). The carotenoid pigment of halobacteria is used to trap solar radiation hence increasing the ambient temperature and evaporation in salterns thus hastening the deposition of sea salt (Tindall, 1988 & Bordenstein, 2008).

It can therefore be assumed that extremophilic microbes from soda lake environments have a great potential for a variety of biotechnological applications. The search for novel genes, enzymes and other biomolecules from soda lake environments can be expedited through the application of metagenomic strategies and culture dependent methods (Sorokin & Berben, 2014). If these approaches can further be complemented with improved high-throughput screening methods and the latest advances in sequencing technology, it may lead to discovery of many commercially important enzymes and biomolecules from saline and alkaline lake ecosystems.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study site

Lake Magadi is a hyper saline, alkaline lake situated in the southern part of Kenyan Rift Valley. The Lake lies in the southern part of the Gregory Rift Valley which is close to the Tanzanian border, between Lake Natron towards the south and freshwater Lake Naivasha towards the north. Lake Magadi is approximately 2°S of the equator (1.8667° S, 36.2667° E) at an elevation of 600 m above sea level covering an area of 90 km^2 (Behr & Röhricht, 2000). Its salinity may go up to 30% w/v which results in an almost solid deposit of sodium chloride and sodium carbonate, the latter existing as sodium sesquicarbonate or trona. The climatic zone where Lake Magadi lies receives erratic rainfall below 800 mm per year, with substantial annual variation. Most of the rain falls between December and May followed by a long dry season with daily temperatures frequently above 40°C.

The springs of Lake Magadi are located around the perimeter of the lake. The salinity of the Lake is low enough and its temperatures are not very high, peripheral lagoons support a thriving fish colony of *Tilapia grahami*, which tolerates a pH of 10.5 and thrives in temperature of 39 °C (Tindall, 1988). Some saline lagoons have, mass accumulations of microorganisms (Tindall, 1988). No permanent rivers enter Lake Magadi basin thus its solutes are supplied majorly by several alkaline/saline hot springs with their temperatures being as high as 86°C. The hot springs have been found to host both hyperthermophilic and haloalkalithermophilic bacteria.

Samples analyzed in this study were collected from 3 hot springs: one hot spring within the main Lake Magadi (02º 00′ 3.7″S 36º 14′ 32″ E) at 45.1ºC and pH 9.8; and two hot springs within Little Magadi “Nasikie eng’ida”(01º 43′ 28″S 36º 16′ 21″E), and

(01º 43′ 56″ S 36º 17′11″ E) at elevations of 611m and 616m, temperatures of 81 ºC and 83.6 ºC and pH range of 9.2 and 9.4 respectively.

Figure 3.1: Map showing location Lake Magadi

3.2 Measurements of physicochemical parameters

A Garmin e Trex 20, a hand-held GPS receiver with multi-constellation ability assuring an absolute positioning accuracy of 3 meters, was used to sample the points. The GPS was set up to use the local datum approved by the Survey of Kenya, which is Arc 1960.To accurately show the sampled hot springs to scale, maps were constructed in ArcGIS10.3 using the GPS coordinates captured from the lake during fieldwork (Kambura et al., 2016).

The pH, electrical conductivity (EC), temperature and total dissolved solids (TDS) of the sampling sites were determined *in situ*. The pH for each sampling point was measured with a portable pH-meter (Oakton pH, Eutech Instruments Pty. Ltd) and confirmed with indicator strips (Merck, range 5–10).

Temperature, Electrical Conductivity (EC), Total Dissolved Solids (TDS) and dissolved oxygen (DO) were measured using Electrical Chemical Analyzer(Jenway-3405) during sampling (Kambura et al., 2016).

Water analysis of samples from the hot springs of Lake Magadi and Little Magadi was done in order to identify and quantify the chemical components and properties of the hot spring waters, the samples were subjected to water chemistry analyses for pH, major cation, anions and trace elements. Water analysis of samples from Lake Magadi and Little Magadi was carried out at Crop Nutrition Laboratory Services (CNLS), Coopers, Nairobi. Cations such as Ca, Mg, K, Na, Mn, Fe, Cu, Mo, B, Zn and S were analyzed using Atomic. Absorption Spectrometry (AAS) while anion analysis was carried out using Mass spectrometry (Kambura et al., 2016).

3.3 Sample collection

All samples were collected randomly in triplicates from each hot spring. Water samples were collected using sterile 500 ml plastic containers that had been cleaned with 20 % sodium hypochlorite and UV-sterilized for one hour as described (Kambura et al., 2016). The upper 5 mm developing on the hot spring water margins of each microbial mat was collected and put into sterile 500 ml plastic jars, wet sediments were collected by scooping them using a sterilized hand shovel and putting them into sterile 50 ml Falcon tubes while water samples were collected right in the bubbling hot springs using sterilized 500 ml plastic containers. The collected samples were put on dry ice in cool boxes and transported to the Institute of Biotechnology Research laboratory at Jomo Kenyatta University of Agriculture and Technology (JKUAT), Kenya. Once in the laboratory the samples were preserved at -80.

3.4 Enrichment and isolation

Isolation of bacterial cultures was performed by the serial dilution technique using International Streptomyces project medium starch casein agar (SCA) and malt yeast extract agar (MYE) (Elliah et al., 2015). The media containing filtered lake water was supplemented with cycloheximide (0.01 mg) to inhibit fungal contamination and had the pH adjusted to 9.4. Filtered lake water was used to replicate the lake conditions, mainly salinity and pH (9.4). Fresh microbial mat was mixed and crushed with spatula on a piece of sterilized paper. Three samples of 0.5 grams of microbial mat were weighed on a sterile aluminum foil and transferred immediately to three universal bottles containing 10ml lake water (Peter, 2002).These preparations were mixed by vortexing at approximately 150 rpm for 1 minute. The microbial mat suspension was then serial diluted by transferring 1ml of the suspension to 9 ml test tube of sterile lake water. The dilutions were: 10^0, 10^{-1}, 10^{-2} and 10^{-3}. Aliquots of 200 µl from different dilutions were transferred to petri dishes containing the prepared media and spread on the surface with a sterile glass rod, for the plate count experiments. This was followed by incubation at 45^o C for 24 to72 hours allow adequate growth for the various fast and slow growing isolates. Growth was monitored until colonies started to form. Sub culturing was done in the same media and incubated at the same temperature, by picking different colonies and restreaking several times till pure cultures were obtained. Pure isolates were all transferred to LB media (common media), supplemented with 5% sodium chloride for further physiochemical, biochemical and molecular analysis. The obtained colonies were afterwards characterized based on morphological characteristics such as colour, shape, size and form. Well isolated and differentiated colonies were transferred to 20% glycerol stocks and preserved at -20^0C (Demain & Davies, 1999).

3.5 Characterization and identification of bacterial isolates

Colony morphology of the isolates obtained after sub culturing were described by use of standard microbiological criteria, especially emphasizing on pigmentation, color, shape, size and form. Physiochemical characterization was done by growing the isolates at different temperature, pH and salt concentrations. Preliminary characterization by Gram staining was done for each isolate using safranin as described by (Dussault, 1955; Sandle, 2004) and then observed under a light microscope ×100 (Keast et al., 1984; Kurt, 2016).This staining technique was used to put the isolates into two categories; Gram positive and gram negative (Cappuccino & Sherman, 2014).

3.6 Physiochemical characterization

3.6.1 Growth at different sodium chloride concentration

The ability of the isolates to grow on media fortified with various sodium chloride (NaCl) concentrations was determined using LB solid media supplemented with NaCl: 0%, 2.5%, 5%, 7.5 %, 10%, 12.5%, 15% and 17.5%; supplemented with 1% sodium carbonate. The media was incubated at 45°C (because of their thermophilic nature) and then checked for growth after 48 hours by observing the extent of growth. The level of growth was scored using four levels of positive sign, where by one positive (+) indicated minimal growth, two positives (+ +) indicated average growth, and three positives (+ + +) indicated satisfactory growth while four positives (+ + + +) indicated excellent growth.

3.6.2 Growth at various temperatures

The aim of this experiment was done to determine the optimum temperature requirements for the growth of the bacterial isolates. LB solid media at pH 7.0 was prepared, sterilized and dispensed into sterile Petri dishes. Each batch was

inoculated with the isolates and incubated at temperatures 30, 35, 40, 45, 50, 55, 60 and 65°C. The extent of growth of the isolates was observed after 48 hours of incubation. The level of growth was scored using four levels of positive sign, where by one positive (+) indicated minimal growth, two positives (+ +) indicated average growth, and three positives (+ + +) indicated satisfactory growth while four positives (+ + + +) indicated excellent growth.

3.6.3 Effect of pH on growth of the isolates

The aim of this study was to determine the optimum pH requirements for the isolates. LB solid Media was prepared and pH adjusted to 6, 7, 8, 9, 10,11,12,13 and 14 using 1M HCL and 1M NaOH. This was sterilized and dispensed into sterile Petri dishes. Each medium was inoculated with bacterial isolates and incubated at 45°C. The extent of growth of the isolates was observed after 48 hours of incubation. The level of growth was scored using four levels of positive sign, where by one positive (+) indicated minimal growth, two positives (+ +) indicated average growth, and three positives (+ + +) indicated satisfactory growth while four positives (+ + + +) indicated excellent growth.

3.7 Screening for production of enzymes

Bacterial isolates were screened for their ability to produce various extracellular enzymes i.e. xylanases, lipases amylases, esterases, proteases, pectinases and cellulases. The ability of the bacterial isolates to utilize substrates such as starch, xylan, cellulose, carboxymethylcellulose (cmc), olive oil, pectinase and skimmed milk indicated the ability to produce the respective enzymes. Positive results were indicated by the potential of the respective isolates to produce enzymes that would utilize these substrates while the negative tests were indicated by the presence of the substrate after growth of the isolates meaning that the substrate was not utilized (Castro et al., 1993; Cappuccino & Sherman, 2014).

3.7.1 Determination of amylolytic activity

The bacterial isolates were cultured on basal media (1% KH_2PO_4, 0.01% $MgSO_4.7H_2O$, 0.005% $CaCl.2H_2O$, 4%NaCl and 1% Na_2CO_3) supplemented with 1% starch as the sole carbon source. The medium was then inoculated by spotting 4 isolates per plate and incubating at 45°C. After 48 hours the plates were flooded with iodine solution to check for the presence of amylase (Cappuccino & Sherman, 2014). Clear halos around the bacterial colonies indicated that they produce extracellular amylase (Rheims et al., 1998), while negative results were indicated by blue black color all over the plate (Castro et al., 1993).

3.7.2 Determination of the xylanolytic activity

The isolates were cultured on basal media (1% KH_2PO4, 0.01% $MgSO_4.7H_2O$, 0.005% $CaCl.2H_2O$, 4%NaCl and 1% Na_2CO_3) and then supplemented with 1% xylan (Fluka) as the sole source of carbon. The medium was inoculated with the bacterial isolates and incubated for 48 hours at 45°C. The isolates were flooded with Congo red dye for 15 minutes. The dye was then poured off and plates rinsed with NaCl (1M). Subsequently, the plates were rinsed with distilled water and observed for halos around the colonies to check for the presence of xylanase enzyme which is an indication of positive polymer degradation. The colour around positive isolates for xylanase production changed from red to orange while the colour around the negative isolates remained red.

3.7.3 Determination of the cellulolytic and hemicellulolytic activity

The production of cellulose was determined using media that contained cellulose (Fluka) and carboxymethylcelullose (CMC) - Serva, Heidelberg. The isolates were cultured on basal media (1% KH_2PO_4, 0.01% $MgSO_4.7H_2O$, 0.005% $CaCl.2H_2O$, 4%NaCl and 1% Na_2CO_3) supplemented with 1% cellulose and 1%

carboxymethylcelullose (CMC) separately. Each medium prepared was then inoculated by spotting of 4 isolates per plate followed by incubation for 48 hours at 45°C. The plates were then flooded with 1% Congo red dye for 15 minutes. The dye was then rinsed with NaCl (1M) and subsequently rinsed with distilled water. The plates were then observed for halos around the colonies as an indication of positive polymer degradation. The plates that did not show halos indicated negative results.

3.7.4 Determination of lipolytic/esterase activity

The bacterial isolates were cultured on basal media (1% KH_2PO_4, 0.01% $MgSO_4.7H_2O$, 0.005% $CaCl.2H_2O$, 4%NaCl and 1% Na_2CO_3) supplemented with 1% olive oil as the sole carbon source. The media was thereafter inoculated by the spotting of 4 bacterial isolates per plate and incubated for at least 48 hours at 45°C. The media was later observed for zones of calcium crystals precipitation around each isolate. Positive isolates for lipase/esterases production were indicated by the precipitation of calcium crystals around the colonies while for negative isolates there was no calcium precipitation around the colonies.

3.7.5 Determination of the proteolytic activity

Protease enzymes are majorly derived from microbes. Microbial protease is produced in wide range of microorganisms under controlled cultivation conditions (Uyar, 2011). The isolates were cultured on basal media (1% KH_2PO_4, 0.01% $MgSO_4.7H_2O$, 0.005% $CaCl.2H_2O$, 4% NaCl and 1% Na_2CO_3) supplemented with 1% skimmed milk (Lee et al, 2005). The medium was then inoculated by the spotting of 4 isolates per plate and incubated at 45°C. The isolates were observed for clearing zones after a period of 48 hours. Positive isolates for protease production exhibited a zone of proteolysis as demonstrated by clearing zones

(Cappuccino & Sherman, 2014). Isolates that were negative for proteolysis did not have clearing zones.

3.8 Screening the bacterial isolates for the production of antimicrobial compounds

A cell based screening strategy was employed to screen the isolates for antimicrobial activity. The ability of individual isolates to inhibit the growth of test organisms; *Pseudomonas aeruginosa* (ATCC 27853), *Bacillus subtilis* (ATCC 55732), *Escherichia coli* (NCTC 10418), *Candida albicans* (ATCC 90028) and *Staphylococcus aureus* (NCTC 10788) was tested using *in vitro* plate assays. Each bacterial isolate was cultured onto nutrient broth media and incubated at 37 ℃ for five days, in a shaker incubator (200rpm) to allow sufficient air circulation, hence preventing any fermentation that could lead to acid production within the media. The test organisms were also cultured in nutrient broth and incubated at 45℃ for 24 hours. Paper discs were prepared and impregnated with 10 µl of the cell free broth of each isolate. The impregnated paper discs were allowed to dry under a fume chamber and then placed on nutrient agar (Fluka) seeded with the test organisms. These were incubated for 24 - 48 hours at 37 ℃ after which the results were recorded. Inhibition activity was evaluated visually by measuring the zone inhibition of growth of test bacteria on the plates (Fatope, 2000)

3.9 Molecular characterization of pure bacterial colonies.

3.9.1 DNA extraction

Pure cultures of the 44 isolates that are well labeled were inoculated in 20 ml of freshly prepared nutrient broth and incubated for 24 hours in a shaker incubator at 45^{o}C and 200 rpm. The cultures were transferred into 1.5 ml of eppendorf tubes,

centrifuged at 13000 rpm for five minutes and the supernatant was discarded. The bacterial cells were re-suspended in 200µl of solution A (50 mM Tris pH 8.5, 50 mM EDTA pH 8.0 and 25 % sucrose solution). To this 5µl of 35 Lysozyme (20 mg/ml) and 5 µl of RNAse (20 mg/ml) was added, gently mixed and incubated at 37 ^{o} C for one hour. Following incubation, 600 µl of solution B (10 mM Tris pH 8.5, 5 mM EDTA pH 8.0 and 1 % SDS) were added and contents mixed by inverting the eppendorf several times. 10µl of Proteinase K (20 mg/ml) was added, mixed gently and incubated at 50^{o}C for 1 hour. Bacterial DNA was extracted using phenolic-chloroform extraction and isopropyl alcohol precipitation method (Sambrook et al., 1989). The DNA pellet was then resuspended in 40 µl TE and semi quantified on a 1% agarose gel in 1xTAE buffer. Visualization was done under UV by staining with ethidium bromide (Sambrook et al., 1989). The genomic DNA obtained was stored at -20℃ (Magarvey et al., 2004 ; Mwirichia et al., 2010b).

3.9.2 PCR amplification

Nearly full-length 16S rRNA gene sequences were PCR amplified using forward bacterial Primer pair 8F 5'-AG (A/G) GTTTGATCCTGGCT-3') and reverse bacterial primer 1492R 5'CGGCTACCTTGTTACGACTT-3' to get 1500 bp of the PCR product. The PCR was carried out in a 50 µl reaction volume containing 0.25 µl of high fidelity Taq, 1.25 µl (10pmol) of 8F forward primer, 1.25 µl (10 pmol) of 1492R reverse primer, 0.5 µl (0.5ng) of template DNA, 0.5 ul of dNTPs mix (2.5mM), 4.0, 10x PCR buffer and 36.25 µl of PCR water. The PCR cycling consisted of an initial activation of the enzyme at 94^{o}C for 5 minutes followed by 35 cycles of a denaturation step at 94^{o}C for 45 seconds, primer annealing step at 55^{o}C for 60 seconds, chain elongation step at 72^{o}C for 2 minutes and a final extension at 72^{o}C for 5 minutes . PCR products were visualized under ultra violet light of ethidium bromide stained 1.5% agarose gel. The PCR products were then purified using the QIA quick PCR purification Kit (Qiagen,) following the

manufacturers' protocol. The purified products were sequenced using the ABI prism big dye terminator at Macrogen.

3.9.3 Phylogenetic data analysis

The 16S rRNA gene sequences of the bacteria isolates were viewed and edited using Chromas Lite software (www.technelysium.com.au). The sequences were aligned using CLUSTAL W 1.6 software and compared to sequences in the public databases using the Basic Local Alignment Search Tool (Blastn) search program on the National Center for Biotechnology Information (NCBI) Website (http://www.ncbi.nlm.nih.gov/) to find closely related bacterial 16S rRNA gene sequences. Phylogenetic analyses of the sequences was conducted using MEGA version 7 (Tamura et al., 2007) and their evolutionary history inferred using Neighbor Joining method (Saitou & Nei., 1987). Jukes and Cantor (1969) method was used to calculate the evolutionary distances, infer and construct the phylogeny tree. Evolutionary distances obtained were computed using the Maximum Composite Likelihood method (Robertson et al., 2009). Bootstrap consensus tree inferred from 1,000 replicates (Felsenstein., 1985) was taken to represent the evolutionary history of the taxa already analyzed.

CHAPTER FOUR

RESULTS

4.1 Physical characteristics at sampling site

The physical and chemical conditions at Lake Magadi during the sampling period of the lake are presented below in (**Table 4.1**). The three hot springs of Lake Magadi and Little Magadi were selected based on their different temperature and pH levels. These values are the raw figures of the conditions at the sampling sites. Temperatures ranged from a low of 45.1 ºC to a high of 83.6 ºC while pH ranged from 9.2 to 9.8.

Table 4.1: Physical and chemical parameters of sampling sites of Lake Magadi

Parameter	Hot spring 1	Hot spring 2	Hot spring 3
Latitude °S	02° 00′ 3.7″	01° 43′ 56″	01° 43′ 28″
Longitude °E	36° 14′ 32″	36° 17′ 11″	36° 16′ 21″
Elevation	603	616	611
Total Dissolved Solids (TDS)	1	1	1
pH	9.8	9.2	9.4
Temperature (T)	45.1	81	83.6
Dissolved Oxygen (DO)	12.4	0.71	0.04
Conductivity (C)	0.03	1	1
Sodium (ppm)	17700	17300	11300
Chlorides (ppm)	4000	4640	4220
Bicarbonates (ppm)	14200	17500	17100
Silicon (ppm)	28.1	51.1	23.8
Ammonium (ppm)	0.94	2.66	2.57
Calcium (ppm)	0.62	0.53	0.33
Magnesium (ppm)	<0.02	<0.026	<0.02
Potassium (ppm)	287	458	225
Phosphorus (ppm)	6.31	4.17	2.72
Nitrate N (ppm)	0.53	0.67	0.67
Nitrates (ppm)	2.35	2.98	2.97
Sulphur (ppm)	129	107	58.9
Sulphates (ppm)	387	322	176
Iron (ppm)	<0.01	0.012	0.014
Manganese (ppm)	0.016	0.012	<0.01
Zinc (ppm)	<0.01	<0.01	<0.01
Boron (ppm)	9.3	15.5	8.06
Copper (ppm)	0.043	<0.01	<0.01

(Adapted from Kambura et al., 2016)

4.2 Isolation of bacteria

The inoculated plates were incubated at a temperature of 45°C and observations were made after 36 hours of growth. After sub culturing 3 times, a total of 44 pure bacterial isolates were identified from microbial mats of the hot springs of Lake Magadi. These isolates were all able to grow at 50°C and between pH 9-12 and were selected for biochemical characterization and these were then investigated further by molecular characterization.

HS- Hot spring

4.3 Morphological characterization of isolates

4.3.1 Colony and Cell Morphology

Morphological characterization of the bacterial isolates was based on classical macroscopic techniques of shape, form, color, and elevation of the pure colonies. Microscopic characterization was performed using the Gram reaction and cell shape after simple staining. The colony shapes of the 44 isolates obtained from the hot springs of Lake Magadi ranged from circular, entire, flat and filamentous. The colonies of the bacteria were smooth and their color ranged from cream to light cream, brown and light brown in pigmentation. 80% of the total isolates were Gram positive while the remaining 20% were negative. Some of the cells were long rods others were short rods and the others were coccus. **Plate 4.2** shows the microscopical characteristics of isolates from the hot springs of Lake Magadi.

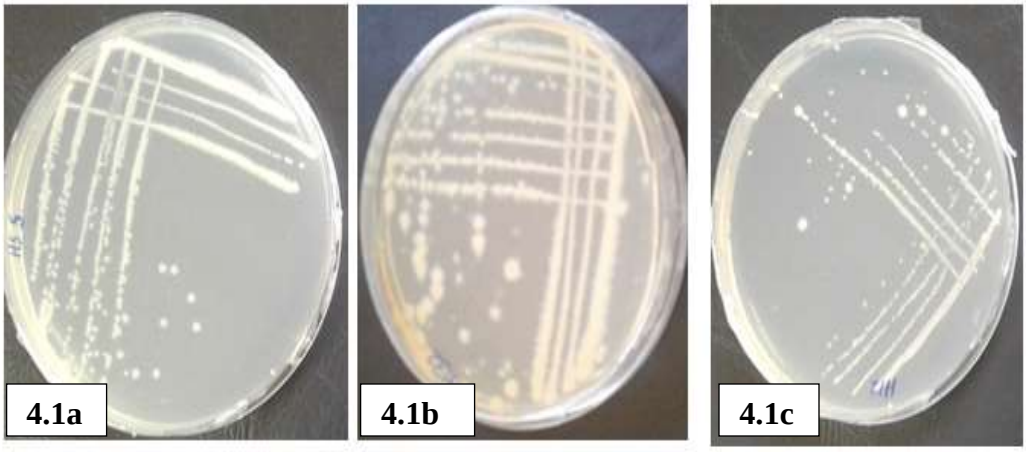

Plate 4.1a-c: A pure plate cultures of isolates HS5, HS32 and HS5 grown on nutrient agar.

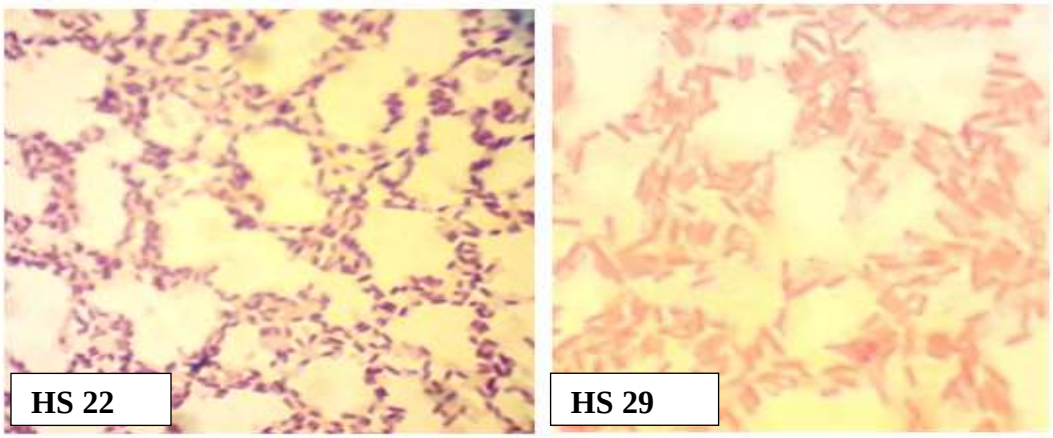

Plate 4.2 Gram positive stain reaction of isolate HS 22 & Gram negative stain reaction of isolate HS 29

Table 4.2: Morphological characteristics of the isolates from Lake Magadi hot springs

Strain	Colony color	Colony form	Colony elevation	Colony margin	Cell arrangement	Gram reaction
HS 1	Brown	Circular	Raised	Undulate	Cocci	+
HS 2	White	Circular	Raised	Filamentous	Rods	+
HS 3	Cream	Irregular	Flat	Undulate	Rods	+
HS 4	Brown	Irregular	Raised	Undulate	Rods	+
HS 5	White	Circular	Raised	Undulate	Rods	+
HS 6	Brown	Irregular	Raised	Filamentous	Rods	-
HS 7	Cream	Irregular	Raised	Entire	Cocci	+
HS 8	Cream	Circular	Flat	Entire	Rods	+
HS 9	Brown	Irregular	Raised	Undulate	Rods	+
HS 10	Light cream	Irregular	Raised	Undulate	Rods	+
HS 11	Light cream	Irregular	Flat	Undulate	Cocci	-
HS 12	Light cream	Circular	Raised	Serrated	Rods	+
HS 13	Light cream	Irregular	Raised	Undulate	Rods	+
HS 14	Cream	Irregular	Raised	Undulate	Rods	+
HS 15	Brown	Irregular	Raised	Undulate	Rods	- +
HS 16	Light cream	Irregular	Raised	Filamentous	Rods	+
HS 17	Light cream	Circular	Flat	Serrated	Rods	+
HS 18	Light cream	Circular	Flat	Entire	Rods	+
HS 19	Brown	Irregular	Raised	Undulate	Rods	+
HS 20	Cream	Circular	Raised	Entire	Rods	+
HS 21	Cream	Irregular	Flat	Undulate	Rods	+
HS 22	Cream	Irregular	Raised	Serrated	Rods	+
HS 23	Cream	Irregular	Flat	Entire	Rods	+
HS 24	Light cream	Filamentous	Raised	Filamentous	Rods	+
HS 25	Cream	Circular	Raised	Entire	Rods	+
HS 26	Cream	Circular	Raised	Entire	Rods	+
HS 27	Cream	Irregular	Flat	Undulate	Cocci	+
HS 28	Cream	Circular	Raised	Undulate	Rods	+
HS 29	Cream	Circular	Raised	Undulate	Rods	+
HS 30	Brown	Irregular	Raised	Undulate	Rods	+
HS 31	Light cream	Irregular	Raised	Undulate	Rods	+
HS 32	Cream	Irregular	Raised	Filamentous	Rods	+
HS 33	Cream	Irregular	Flat	Undulate	Rods	+
HS 34	Cream	Irregular	Raised	Undulate	Rods	+
HS 35	Light cream	Circular	Raised	Entire	Cocci	-
HS 36	Cream	Irregular	Raised	Filamentous	Rods	+
HS 37	Light cream	Circular	Raised	Serrated	Rods	+
HS 38	Light brown	Circular	Raised	Serrated	Rods	+
HS 39	Cream	Irregular	Raised	Undulate	Rods	-
HS 40	Brown	Irregular	Raised	Filamentous	Rods	+
HS 41	Light brown	Irregular	Raised	Undulate	Rods	+
HS 42	Brown	Circular	Raised	Entire	Rods	+
HS 43	Light brown	Circular	Raised	Undulate	Rods	+
HS 44	Cream	Irregular	Raised	Undulate	Rods	+

4.4 Physiochemical characterization of isolates

4.4.1 Growth at different sodium chloride concentration

The bacterial isolates showed tolerance to different concentrations of sodium chloride (Nacl). Isolates grew with increase in salt concentration from 0% up to 7.5% with the maximum growth being recorded at 7.5%. Growth of isolates started to gradually decrease from 10% up to 17.5% with minimal growth being recorded at 17.5% **(Table 4.3)**. In physiochemical characterization, growth of the isolates was measured in diameter and scored using four levels of positive sign (+), whereby one positive (+) 0-10mm indicated minimal growth, two positives (++) 10-20mm indicated average growth, and three Positives (+++) 20-30mm indicated satisfactory growth while four positives (+ + + +) 30-40mm indicated excellent growth **(Plate 4.3)**.

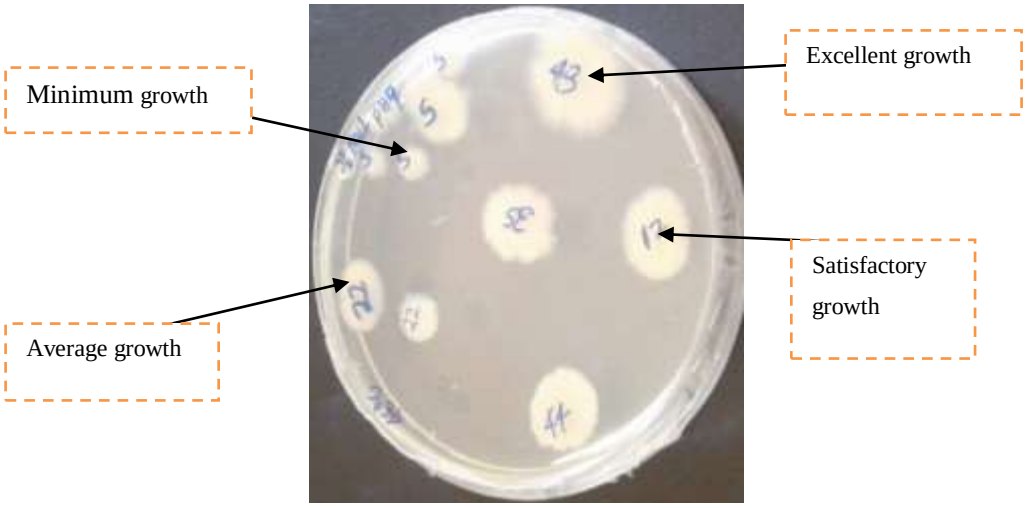

Plate 4.3: Bacterial isolates growth as scored at 5 % NaCl concentration

Table 4.3: Growth of Isolates from Lake Magadi hot springs at Different Sodium Chloride (NaCl) Concentrations

Isolates	0%	2.5%	5%	7.5%	10%	12.5%	15%	17.5
HS 1	+++	++++	++++	+++	-	-	-	-
HS 2	+++	++++	++++	++++	++	+	-	-
HS 3	+++	++++	++++	+++	-	-	-	-
HS 4	+++	++++	++++	++++	++	+	+	-
HS 5	+++	+++	++++	++++	++	-	-	-
HS 6	++	+++	++++	++++	+	+	-	-
HS 7	+++	+++	+++	+++	-	-	-	-
HS 8	+++	++++	++++	+++	-	-	-	-
HS 9	+++	++++	++++	++++	-	-	-	-
HS 10	+++	+++	+++	+++	+	-	-	-
HS 11	+++	+++	+++	+++	++	+	+	-
HS 12	+++	+++	+++	++++	+	+	+	-
HS 13	+++	+++	++++	++++	++	++	+	-
HS 14	+++	++++	++++	+++	+	+	+	-
HS 15	+++	++++	++++	++++	+	-	-	-
HS 16	+++	++++	++++	+++	-	-	-	-
HS 17	+++	+++	+++	+++	+	+	-	-
HS 18	+++	+++	+++	++++	++	++	++	-
HS 19	+++	+++	+++	++++	++	+	+	-
HS 20	+++	+++	++++	+++	+	+	-	-
HS 21	+++	+++	+++	+++	-	-	-	-
HS 22	+++	+++	+++	+++	+	+	-	-
HS 23	+++	+++	++++	++++	++	+	+	-
HS 24	+++	++++	++++	+++	+	+	-	-
HS 25	+++	+++	+++	+++	+	+	-	-
HS 26	+++	+++	+++	+++	-	-	-	-
HS 27	+++		+++	++	+	+	+	-
HS 28	+++	+++	+++	+++	-	-	-	-
HS 29	+++	+++	+++	+++	+	+	-	-
HS 30	+++	++++	++++	++++	++	+	-	-
HS 31	+++	+++	+++	+++	+	+	+	-
HS 32	+++	+++	+++	++++	++	+	-	-
HS 33	+++	++++	++++	++++	+++	+	+	-
HS 34	+++	++++++	+++	+++	+	-	-	-
HS 35	+++	+++	+++	++++	++	+	+	-
HS 36	+++	+++	+++	+++	-	-	-	-
HS 37	+++	+++	+++	+++	+	+	-	-
HS 38	+++	+++	+++	+++	+	-	-	-
HS 39	+++	+++	+++	+++	+	+	-	-
HS 40	+++	+++	+++	++++	+	+	+	-
HS 41	+++	+++	+++	+++	+	+	+	-
HS 42	+++	++++	+++	+++	+++	+	+	-
HS 43	+++	+++	++++	+++	+	+	-	-
HS 44	+++	+++	+++	+++	+	+	-	-

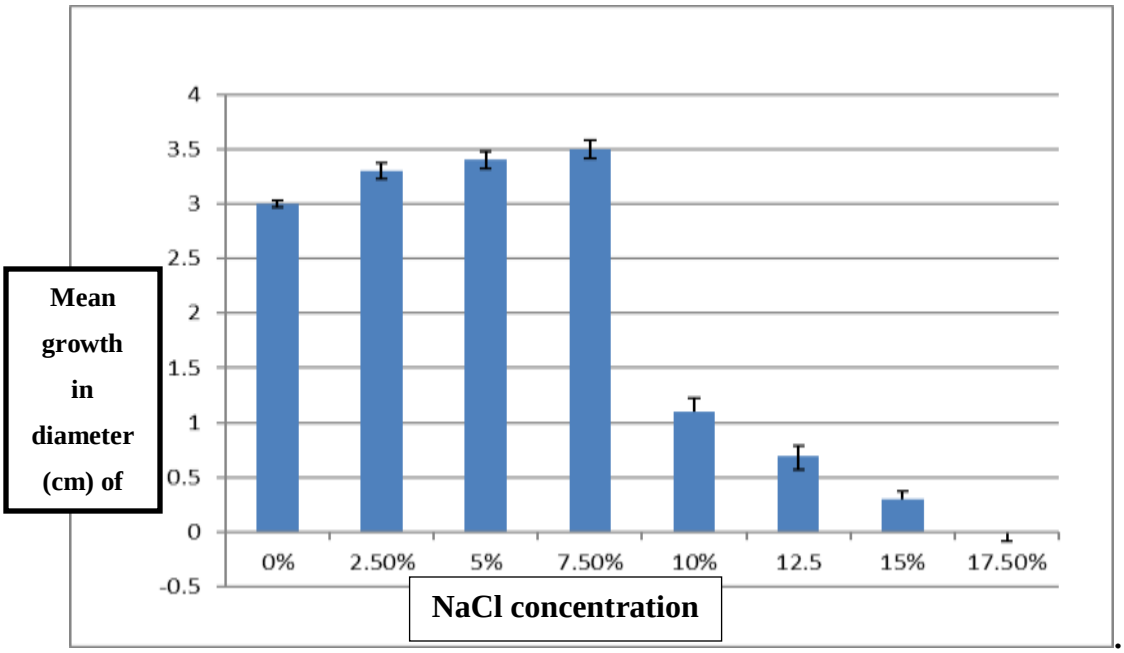

Figure 4.1 Growth of Isolates from Lake Magadi hot springs at Different Sodium Chloride (NaCl) Concentrations different Sodium Chloride (NaCl) concentrations

4.4.2 Growth at different temperature

All the isolates grew at different ranges of temperatures between 30-60°C. At 30-35°C the isolates showed slow growth. The optimum growth of the isolates was observed between 40–50°C **(Plate 4.4).** Minimum growth was observed at temperature 55°C and 60°C **(Table 4.4)**.

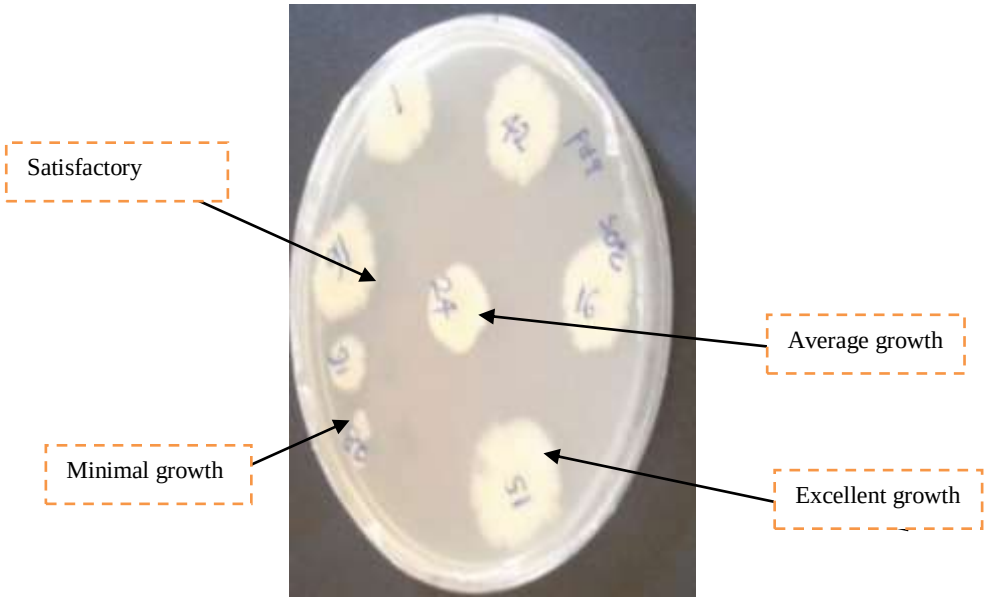

Plate 4.4: Bacterial isolates growth as scored at 50° C

Table 4.4: Growth of isolates from Lake Magadi hot springs at different temperatures

Isolates	30°C	35°C	40°C	45°C	50°C	55°C	60°C	65°C
HS 1	+++	++++	+++	+++	+++	+++	---	-
HS 2	++	+++	+++	+++	+++	+++	+++	-
HS 3	+++	++++	++++	++++	+++	---	---	-
HS 4	+++	+++	+++	+++	++++	+++	+	-
HS 5	+++	+++	+++	+++	+++	+++	+	-
HS 6	++	++++	++++	++++	++++	+++	---	-
HS 7	+++	++++	++++	++++	++++	+++	---	-
HS 8	++	+++	+++	+++	++	++	---	-
HS 9	+++	+++	+++	+++	+++	+++	+++	-
HS 10	++	+++	+++	+++	+++	+++	---	-
HS 11	++	+++	+++	+++	+++	+++	---	-
HS 12	+++	+++	+++	+++	+++	+	---	-
HS 13	++	+++	+++	+++	+++	+++	---	-
HS 14	+++	++++	++++	++++	++++	+++	++	-
HS 15	+++	+++	++++	++++	++++	+++	---	-
HS 16	+++	++++	++++	++++	+++	+++	---	-
HS 17	+++	++++	++++	+++	+++	---	---	-
HS 18	++	+++	++++	++++	+++	+++	---	-
HS 19	++	+++	+++	+++	+++	+++	+++	-
HS 20	++	+++	+++	+++	++++	+++	---	-
HS 21	+++	+++	+++	+++	+++	+++	---	-
HS 22	++	+++	+++	+++	+++	---	---	-
HS 23	+++	+++	+++	+++	+++	++	---	-
HS 24	++	+++	+++	+++	++	---	----	-
HS 25	++	+++	+++	++++	++++	++	---	-
HS 26	+++	+++	+++	+++	+++	+++	---	-
HS 27	+++	+++	+++	++++	++++	+++	---	-
HS 28	+++	+++	++++	+++	+++	++	---	-
HS 29	++	+++	+++	+++	+++	++	---	-
HS 30	+++	++++	+++	+++	+++	+++	++	-
HS 31	++	++	+++	+++	+++	+++	+	-
HS 32	+++	+++	+++	+++	+++	+++	---	-
HS 33	+++	+++	+++	++++	++++	+++	++	-
HS 34	+++	+++	++++	+++	+++	+++	+++	-
HS 35	+++	+++	+++	++++	+++	+++	++	-
HS 36	+++	+++	+++	+++	+++	---	---	-
HS 37	+++	+++	+++	+++	+++	+++	---	-
HS 38	+++	+++	+++	+++	+++	++	---	-
HS 39	++	+++	+++	++++	+++	+++	---	-
HS 40	+++	+++	+++	+++	++++	+++	---	-
HS 41	+++	+++	+++	+++	+++	+++	+	-
HS 42	+++	+++	+++	+++	++++	+++	---	-
HS 43	+++	++++	++++	++++	++++	++	---	-
HS 44	+++	+++	+++	+++	+++	+++	---	-

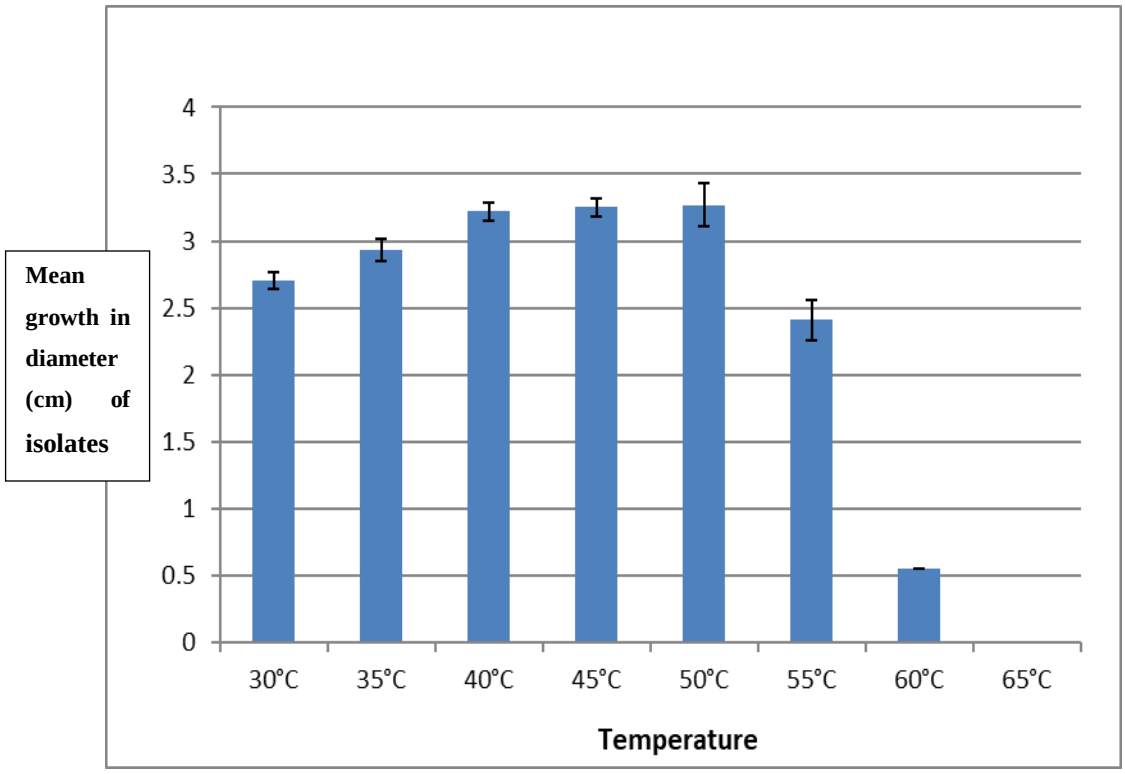

Figure 4.2: Mean growth of isolates from Lake Magadi hot springs at different

4

.4.3 Growth at varied pH

Bacterial isolates were able to grow at a broad range of pH ranging from pH 6-14. Minimum growth was observed pH 6 while optimum growth was observed between pH 7-12 **(Table 4.5)** while average growth was observed at pH 13 and 14 **(Plate 4.5)**.

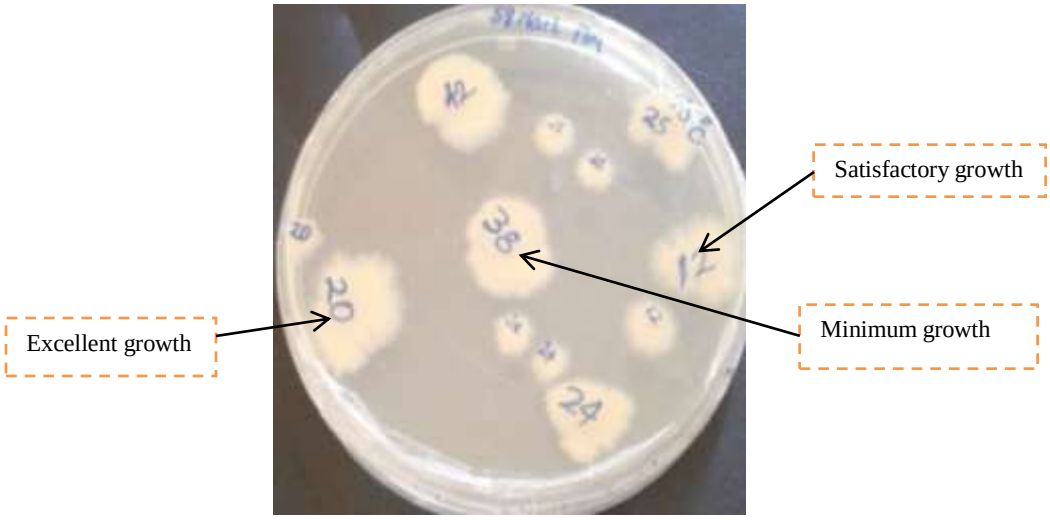

Plate 4.5: Bacterial isolates growth as scored at pH 9

Table 4.5: Growth of isolates from Lake Magadi hot springs at varied pH

Isolates	PH 5	PH 6	PH 7	PH 8	PH 9	PH 10	PH 11	PH 12	PH 13	PH 14
HS 1	---	---	+++	+++	++++	++++	++++	++++	+++	+++
HS 2	---	+	+++	+++	+++	+++	++++	+++	+++	+++
HS 3	---	---	+++	+++	+++	+++	+++	++++	+++	+++
HS 4	---	---	+++	++++	++++	++++	++++	+++	+++	+++
HS 5	---	---	+++	++++	++++	++++	++++	++++	+++	+++
HS 6	---	---	+++	++++	++++	++++	++++	+++	+++	+++
HS 7	---	---	+++	++++	++++	++++	++++	++++	+++	+++
HS 8	---	---	+++	++++	++++	+++	+++	+++	+++	+++
HS 9	---	+	+++	+++	++++	++++	++++	+++	+++	+++
HS 10	---	---	+++	+++	++++	++++	++++	++++	+++	+++
HS 11	---	---	+++	++++	++++	++++	++++	+++	+++	+++
HS 12	---	---	+++	+++	+++	+++	+++	+++	+++	+++
HS 13	---	---	+++	++++	++++	++++	++++	+++	+++	+++
HS 14	---	---	+++	+++	++++	++++	++++	+++	+++	+++
HS 15	---	---	++++	++++	+++	+++	+++	+++	+++	+++
HS 16	---	---	+++	+++	++++	++++	++++	+++	+++	+++
HS 17	---	---	+++	++++	+++	+++	+++	+++	+++	+++
HS 18	---	---	+++	+++	++++	+++	+++	+++	+++	+++
HS 19	---	+	+++	+++	+++	+++	++++	+++	---	---
HS20	---	---	+++	++++	++++	++++	++++	+++	+++	+++
HS21	---	---	+++	++++	++++	++++	++++	++++	+++	+++
HS 22	---	---	+++	++++	+++	+++	+++	+++	+++	+++
HS 23	---	---	+++	+++	+++	+++	++++	+++	+++	+++
HS 24	---	---	+++	++++	++++	++++	++++	+++	+++	+++
HS 25	---	---	+++	++++	++++	++++	++++	+++	+++	+++
HS 26	---	---	+++	++++	+++	+++	+++	+++	+++	---
HS 27	---	---	+++	++++	++++	++++	++++	+++	+++	+++
HS 28	---	---	++++	+++	+++	+++	+++	+++	+++	+++
HS 29	----	---	+++	+++	+++	+++	+++	+++	+++	+++
HS 30	---	---	+++	+++	++++	+++	+++	+++	+++	+++
HS 31	---	---	+++	+++	++++	++++	++++	+++	+++	+++
HS 32	---	+	+++	+++	++++	++++	++++	+++	+++	+++
HS 33	---	---	+++	++++	++++	+++	++++	+++	+++	+++
HS 34	---	---	+++	++++	+++	+++	+++	+++	+++	+++
HS 35	---	---	+++	++++	++++	+++	+++	+++	+++	+++
HS 36	---	---	+++	+++	+++	+++	++++	+++	+++	---
HS 37	---	+	+++	++++	+++	+++	+++	+++	+++	+++
HS 38	---	---	++++	++++	++++	+++	+++	+++	+++	+++
HS 39	---	---	+++	+++	+++	+++	++++	++++	+++	+++
HS 40	---	---	+++	++++	++++	++++	++++	+++	+++	+++
HS 41	---	+	+++	++++	++++	++++	++++	+++	+++	+++
HS 42	---	+	+++	+++	+++	+++	++++	+++	+++	+++

HS 43	---	+	+++	++++	++++	++++	++++	+++	+++	+++
HS 44	---	---	+++	++++	+++	+++	+++	+++	+++	+++

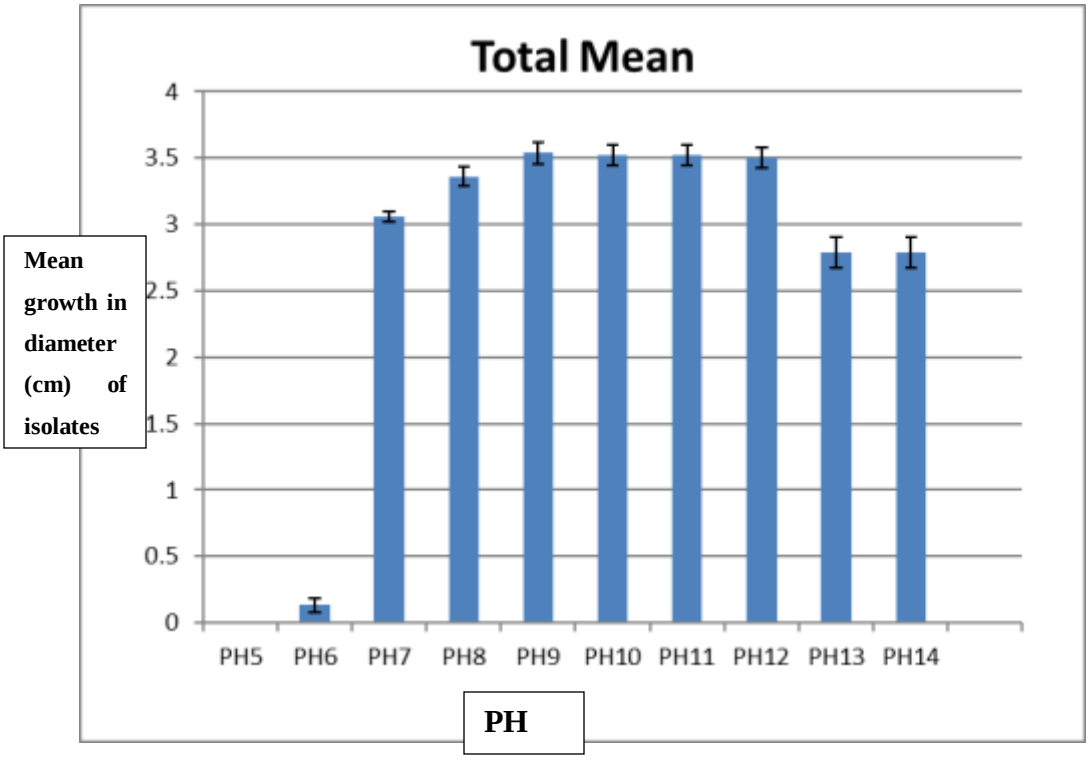

Figure 4.3: Mean growth of isolates from Lake Magadi hot springs at different pH levels levels range

4.5 Screening the isolates for production of extracellular enzymes

Bacterial isolates were screened to check for their ability to produce important extracellular enzymes on basal media supplemented with respective substrates. Enzymes tested included: cellulase, amylase, xylanase, protease, lipase, pectinase, esterase and carboxymethylcellulase. All the 44 isolates hydrolyzed starch; this was indicated by presence of clear halos around the isolate after flooding the plates with 0.6% KI solution. 40 isolates exhibited a zone of proteolysis as indicated by clearing zones around the isolate and this indicated ability of protease production. 28 isolates produced Carboxymethylcellulase, 19 isolates produced esterase and lipase, 17 isolates produced cellulase, 8 isolates showed the ability to produce of xylanase and 1 produced pectinase **(Table 4.6)**. **Plates 4.6a-e** represent some of the bacterial isolates from Lake Magadi grown on basal media showing positive results for starch utilization hence production of amylase; xylan utilization hence production of xylanase; CMC utilization hence production of hemicellulases; Skim milk utilization hence production of proteases and tween 20 utilization hence had ability to produce esterase enzymes respectively.

Plate 4.6a

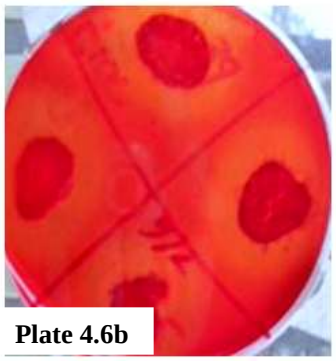

Plate 4.6b

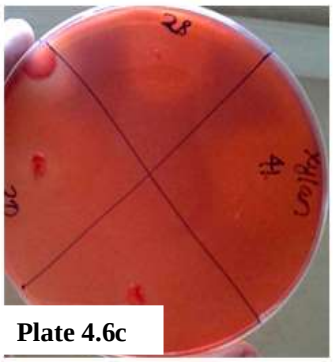

Plate 4.6c

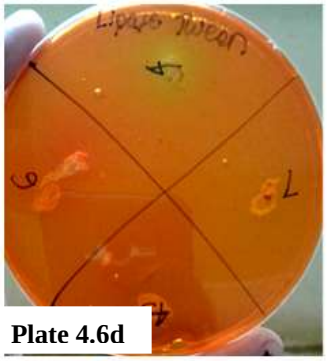

Plate 4.6d

Plate 4.6e

Plate 4.6a-e: Enzyme activity of bacterial species isolated from Lake Magadi hot springs. (a) Amylase activity (b) Cellulase activity (c) Xylanase activity (d) Esterase activity (e) Protease activity

Table 4.6: Ability of the isolates from Lake Magadi hot springs to produce extracellular enzymes

Isolates	Amylase	Protease	Cellulase	CMC	Pectinase	Lipase	Esterase	Xylanase
HS 1	+	+	+	+	-	-	+	-
HS 2	+	+	+	-	-	-	+	+
HS 3	+	+	+	-	-	-	-	-
HS 4	+	+	-	-	-	-	+	-
HS 5	+	+	-	-	-	-	+	-
HS 6	+	+	-	+	-	-	-	-
HS 7	+	+	-	+	-	-	-	-
HS 8	+	+	-	+	-	-	-	-
HS 9	+	+	-	-	-	-	+	-
HS 10	+	+	+	+	-	-	+	-
HS 11	+	+	+	+	-	-	-	-
HS 12	+	+	-	+	-	-	+	+
HS 13	+	-	-	+	-	-	-	-
HS 14	+	+	+	-	-	-	-	-
HS 15	+	+	+	+	-	-	+	-
HS 16	+	+	-	+	-	-	+	-
HS 17	+	+	-	+	-	-	+	-
HS 18	+	-	-	-	+	-	-	-
HS 19	+	+	-	+	-	-	+	-
HS 20	+	+	-	+	-	-	+	-
HS 21	+	+	-	+	-	-	-	-
HS 22	+	+	+	+	-	-	-	-
HS 23	+	+	+	-	-	+	-	-
HS 24	+	+	-	-	-	+	-	+
HS 25	+	+	-	+	-	-	+	-
HS 26	+	+	-	+	-	-	-	-
HS 27	+	+	-	-	-	-	+	-
HS 28	+	-	+	+	-	-	-	+
HS 29	+	+	-	+	-	-	-	-
HS 30	+	+	+	+	-	-	+	+
HS 31	+	+	-	-	-	-	-	-
HS 32	+	+	+	-	-	-	-	+
HS 33	+	+	+	-	-	-	-	-
HS 34	+	+	-	+	-	-	-	-
HS 35	+	+	-	+	-	-	-	-
HS 36	+	+	+	+	-	-	-	-
HS 37	+	+	+	-	-	-	-	+
HS 38	+	+	-	+	-	-	-	-
HS 39	+	+	-	+	-	-	+	-
HS 40	+	+	-	+	-	-	+	-
HS 41	+	+	+	+	-	-	-	+
HS 42	+	+	-	+	-	-	-	-
HS 43	+	-	-	-	-	-	-	-
HS 44	+	+	-	-	-	-	-	-

4.6 Screening of isolates for antimicrobial activity.

The isolates were tested to check their antagonistic activity against test bacteria and fungi and determine the level of antimicrobial activity of each bacterial isolate against the test organisms. Out of the 44 isolates, 37 isolates tested positive for antibiotic activity against *Candida albicans* (ATCC 90028) with inhibition zones ranging from 16-19 mm **(Table 4.7)**. 26 isolates tested positive against *Escherichia coli* (NCTC 10418) **(Plate 4.7a)** and their inhibition zones ranged from 8-38mm **(Table 4.7)**. 8 isolates were active against *Bacillus subtilis* (ATCC 55732) **(Plate 4.7b)** with inhibition diameters ranging from 10-15 mm **(Table 4.7)**. 6 isolates were active against *Staphylococcus aureus* (NCTC 10788) with inhibition diameters ranging from 9-17 mm **(Table 4.7)**. 7 of the isolates had antimicrobial activity against *Aspergilus Flavus* **(Plate 4.7c)** with the inhibition zone ranging from 6-10mm **(Table 4.7)**. Those isolates that were not active against test organisms had no growth, hence allowing growth of test organism on the nutrient agar plate.

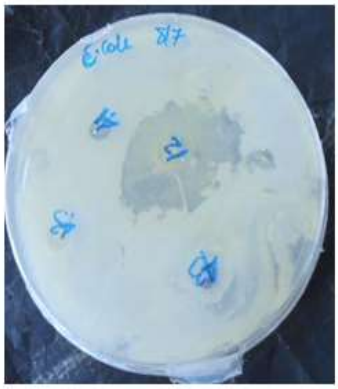

Plate 4.7a

Plate 4.7b

Plate 4.7c

Plate 4.7a-c: (a) Antagonistic activity of isolate 12 against *Escherichia coli* (b), Antagonistic activity of isolate 44 against *Bacillus Subtilis* and (c) Antagonistic activity of isolate 32 against *Aspergilus Flavus.*

Table 4.7: Screening of bacterial isolates from Lake Magadi hot springs for antimicrobial activity

Isolates	*C .albicans*	*S. aureus*	*E. coli*	*P. aeruginosa*	*B. subtilis*	*A. flavus*
HS 1	+	-	+	+	+	+
HS 2	+	-	+	+	-	-
HS 3	-	-	-	+	+	-
HS 4	+	-	+	+	-	+
HS 5	+	-	-	+	+	-
HS 6	-	+	+	-	+	-
HS 7	+	-	+	+	-	-
HS 8	+	-	-	-	-	-
HS 9	+	-	+	-	-	-
HS 10	+	-	+	+	-	-
HS 11	-	-	+	-	-	-
HS 12	-	-	+	-	-	-
HS 13	-	-	-	-	-	-
HS 14	+	-	+	-	+	-
HS 15	+	+	-	-	-	-
HS 16	+	-	-	-	-	-
HS 17	+	-	+	-	+	-
HS 18	-	+	+	+	-	-
HS 19	+	-	-	-	-	-
HS20	-	-	+	+	-	-
HS21	+	-	-	-	-	-
HS 22	+	-	+	-	-	-
HS 23	+	-	-	-	-	+
HS 24	+	-	-	-	-	-
HS 25	+	-	-	-	+	-
HS 26	+	-	-	-	-	-
HS 27	+	-	+	-	-	+
HS 28	+	+	+	+	-	-
HS 29	+	-	+	-	-	-
HS 30	+	+	+	-	-	-
HS 31	+	-	-	+	-	+
HS 32	+	-	-	+	-	+
HS 33	+	-	+	-	-	-
HS 34	+	-	+	-	-	-
HS 35	+	-	+	-	-	-
HS 36	+	-	+	-	-	-
HS 37	+	-	-	+	-	-
HS 38	+	-	+	-	-	-
HS 39	+	-	-	+	-	-
HS 40	+	-	+	+	-	+
HS 41	+	-	-	-	-	-
HS 42	+	+	+	-	-	-
HS 43	+	-	-	-	-	-
HS 44	+	-	+	-	+	-

4.7 Molecular characterization

4.7.1 PCR amplification of 16s rRNA genes from isolates

The amplification of the 16S rRNA region of the genomic DNA was done on 44 isolates using universal bacterial primers bac 8F and bac 1492R (Lane, 1991; Embley and Stackebrandt, 1994) and yielded an amplicon product of approximately 1500 bp. The amplicons were afterwards stained with ethidium bromide and visualised under UV light on 1.5 % agarose gel **(Figure 4.4)**

Figure 4.4: Profiles of PCR products of amplification of 16SrRNA gene of 32 isolates. 1.5% agarose gel and 1.5kb ladder

4.7.2 Phylogenetic analysis of sequences

BLAST analysis of the partial sequences showed that six bacterial isolates came from genus *Bacillus* which are Firmicutes with sequence percentage similarity of between 86% to 98%. Among these were; *Bacillus siamensis*, *Bacillus licheniformis, Bacillus Methylotrophicus, Bacillus amyloliquefaciens, Bacillus Pumilus* and *Bacillus sonorensis*

(**Table 4.8**). One isolate was affiliated to the genus *Alcaligenes* and belonged to *Gammaproteobacteria* class within phylum proteobacteria with the sequence similarity being 96%. The isolate was *Alcaligenes feacalis* (**Table 4.8**). Isolates HS 10 and HS 28 had sequence similarity of between 96% to 98 % respectively, these could represent novel species. Isolates HS 3, HS 13, HS 18, HS 29 and HS 41 had sequence similarity of between 86 to 90% and could represent novel genera (**Table 4.8**).

Table 4.8: BLAST search results of the sequenced isolates from Lake Magadi hot springs

Sample ID	Next neighbor in BLAST	% Identity	Accession No.
HS 3	*Bacillus siamensis*	86%	NR 117274.1
HS 10	*Bacillus Lichenformis*	98%	NR 118996.1
HS 13	*Bacillus Methylotrophicus*	90%	NR 116240.1
HS 18	*Bacillus Amyloliquefaciens*	86%	NR 137421.1
HS 28	*Alcaligenes feacalis*	96%	NR 113606.1
HS 29	*Bacillus Pumilus*	87%	NR 116191.1
HS 41	*Bacillus sonorensis*	87%	NR 113993.1

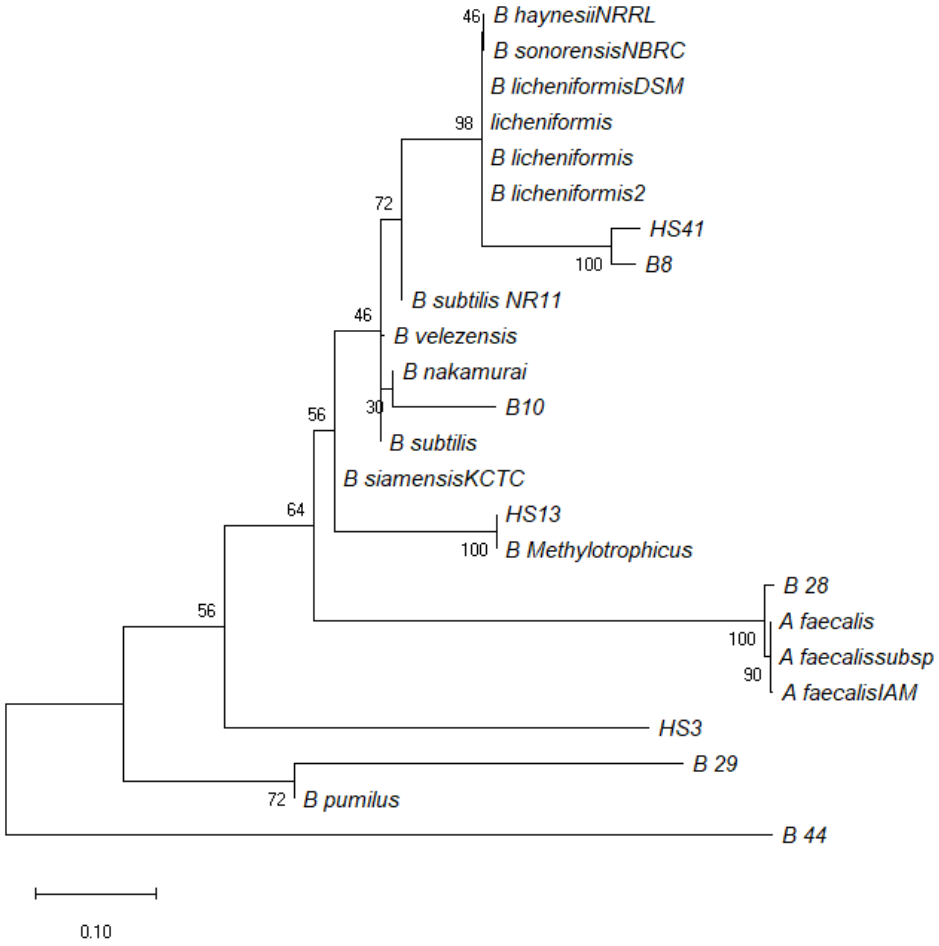

Figure 4.5: Phylogenetic tree of selected Lake Magadi isolates based on partial sequence of 16SrRNA gene

CHAPTER FIVE

GENERAL DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 Discussion

Several authors have carried out studies and characterized the microbiological inhabitants of the East African Rift Valley lakes (Melack, 1981; Vavourakis et al., 2016). Hypersaline alkaline ecosystems such as soda lakes are one of the unique ecological niches considered to harbor a physiologically highly diverse group of microorganisms, adapted to survive under at least dual extreme environmental conditions (Singh et al., 2009). The hot springs of Lake Magadi and Little Magadi are environments that experience multiple extremophilic features. Enzymes, compatible solutes and exopolysaccharides amongst all other metabolites derived from microorganisms native to these environments may be bioaugumented to drive processes with such development requirements as their natural habitat and are likely to offer more versatile bioactive compounds than the existing pool obtained from mesophiles (Ibrahim, 2013)

The isolates subjected to phenotypic, chemotaxonomic and phylogenetic analysis showed considerable phylogenetic diversity (Duckworth et al., 1996; Mwirichia et al., 2010a; 2010b). The purpose of this study was to isolate useful haloalkaliphilic bacteria from Lake Magadi hot springs, characterize and identify them using morphological, physiological, biochemical and molecular methods, then screen the isolates for production of secondary metabolites that are of biotechnological importance. A total of 44 isolates were isolated from microbial mats of the hot springs of Lake Magadi. The microbial mats had been collected from springs in the Little Magadi “Nasikie eng’ida” at temperature 83.6 °C. Different media used for isolation of extremophilic bacteria were; Malt yeast agar (MYE), starch casein agar (SCA) and differential agar. The media were

made using filtered lake water from Lake Magadi and then adjusted to pH 9.4. Preparation of the isolation media was done using Lake Magadi water, to preserve lake conditions of pH, salinity and mineral conditions as per the method of Mwirichia et al., (2010b). Though both Gram positive and Gram negative isolates were recovered in this study, the Gram positives were the majority (**Table 4.2**).

Isolates from Lake Magadi were also able to withstand salt concentrations of up to 15% (w/v) with the optimum growth being observed between 2.5% and 7.5% sodium chloride concentration **(Table 4.3).** It was observed that in this study bacteria grew well with increase of salt concentration from 0 up to 7.5% sodium chloride with the highest growth being recorded at 7.5%. Ability of the isolated microbes to grow at ≥ 5% sodium chloride concentration confirms that they are true halophiles (Mesbah et al., 2007). Studies that have been done on bacteria from Lake Magadi show that microbes grow well at sodium chloride concentrations as below as 5% and as high as 30% concentration (Baumgarte, 2003; Nyakeri, 2018). Previous studies have also indicated that, bacteria from saline lakes can live for many of years in salt deposits, thus they are adapted to these extreme 'deadly' environments (Mancinelli and Rothschild, 2002).

The biophysical property of the membrane lipids of bacteria and archaea are crucial to their sustenance in a wide variety of physical environment. Comparative studies have implicated the membrane phospholipids and glycolipids of halophiles as an important adaptation to salinity (Kushwaha et al., 1982). In general, the ability of the microbes isolated in this study to grow fast and optimally at the environmental and physicochemical parameters of high pH, elevated temperature and salinity unsuitable for the growth of other microbes makes Lake Magadi micro-organisms good models for research on adaptive mechanisms of bacteria to extreme and harsh environmental parameters.

All bacteria isolated grew within the temperature range of 30°C to 60°C. The optimum growth temperature was recorded between 40°C and 50°C. However, good growth was

also recorded at temperature 55°C **(Table 4.4)**. The temperatures that were recorded at the three sampling points ranged from 45.1ºC and 83.6ºC.Therefore this study confirms a study that was previously done on bacterial strains isolated from Lake Magadi and closely clustered with neighbors that thrive well at a temperature range of 10°C to 55°C (Baumgarte, 2003; Kambura 2012; Nyakeri 2018).

Physiochemical characterization of bacterial isolates indicated that the highest growth occurred between pH 9 to 12 (**Table 4.5**). However, there were a few isolates that grew at pH 6 this suggests that the organisms are also tolerant to slight acidic conditions (Moreira & Siqueira, 2002). Growth at pH range of 6 to10 coincides with earlier studies by Horikoshi (1998) which indicated that a pH range from low to high (5.7 to 9.0) allowed growth of alkaliphilic bacteria and that a pH range of 9.0 to 10 could be the optimum pH for growth of these microbes. Microorganisms that live at extreme pH do so by maintaining their cytoplasm at the same pH as that of mesophiles and thus obviate the need to develop an altered internal physiology (Kambura et al., 2012). pH in the neutral spectrum is achieved by several mechanisms. The acidic polymers of the cell walls function as a negatively charged matrix which reduces the pH level at the surface of the cell. Alternately, the plasma membrane use the Na^+/H^+-antiport mechanism and the ATPase driven hydrogen ion (H^+) release to maintain at least neutrality by lowering the internal pH (or below neutral cytoplasmic acidification) of the intracellular milieu (Gilmour, 1990 & DeLong, 2001).

Hydrolysis of the various substrates by the bacterial isolates was an indication of ability of the various bacterial isolates to produce enzyme; amylase, hemicellulases, lipases, cellulases, proteases and esterases that are of industrial importance e.g. cellulases have been used as laundry additives. The ability of isolates to produce enzymes concurs with earlier studies where it was noted that alkaliphilic micro-organisms of the genus *Bacillus* specifically, possess biotechnological importance because they produce useful extracellular enzymes such as protease (Horikoshi, 1971), amylases (Boyer and Ingle, 1972) and pectinase (Horikoshi, 1972) that are resistant to high pH or high temperature

conditions (Nielsen et al., 1994).

Enzymes produced by *B. subtilis* such as amylase have been widely used as special additives to laundry detergents. In addition, a strain of *B. subtilis* formerly was used in production of Japanese food natto (Nielsen et al., 1994). In other studies, alkaliphilic bacteria were isolated from sediment samples of Lonar Lake; *Arthrobacter ramosus* and *Bacillus alcalophilus*. These microbes exhibited high protease activity and thus are used in the manufacture of soya cake as a sole source of carbon and nitrogen (Kanekar et al., 2002). The enzyme was thermostable at (65 °C), pH 12 and also active in the presence of commercial detergent (Kanekar et al., 2002).

The discovery of thermostable enzymes, which in nature allow thermophiles to survive under high temperatures (Gomes & Steiner, 2004; Canganella & Wiegel, 2011) are amenable to many industrial process requirements and have sparked an interest in the study of the biotechnological potential of other extreme environments. Research interest has further been driven by the pressing demand for biocatalysts to drive industrial processes in a more economical and eco-friendly manner and the general need for more sustainable and versatile resources (Whitman et al., 1998). The remarkable structural and chemical adaptations that ensure the survival of extremophiles in hostile environments gives rise to unique extremozymes (enzymes obtained from extremophiles), compatible solutes and metabolites (Gomes & Steiner, 2004). Many of these compounds are been exploited for biotechnological applications.

In this study, isolates were also studied for their potential to produce antibiotics. The isolates showed antagonistic activities that differed from one isolate to another. This was because of the different modes of action and activity of individual biochemical constituents of the isolates (Mao et al., 2006). This could be as a result of differing of the modes of activity and action of each biochemical constituent of the isolates that were represented. Majority of the secondary metabolites that are produced by bacteria are potential antibodies and this makes a target for isolation by industries involved in large

scale screening. The microbes have become a commercial interest because they produce secondary metabolites that are anti-fungal, anti-bacterial or anti-protozoal (Berdy, 2005).

Members of genus *Bacillus* have the ability to produce antibiotics as secondary metabolites in their late logarithmic or early stationary phase of growth. Various strains of *B. subtilis* have been able to produce 68 antibiotics while *Bacillus brevis* have produced 23 known antibiotics (Katz and Demain, 1977). *Bacillus pumilus* is well known for its fungicidal activity and produces anti-fungal compounds (Bottone and Peluso, 2002). In a research study, microorganisms isolated from the alkaline saline Lake Acigol in Turkey were screened for their activity against other micro-organisms. The preliminary results indicated that alkaline-saline lake isolates exhibited antimicrobial activity against *Bacillus subtilis, Staphylococcus aureus, Micrococcus luteus, Mycobacterium megmatis,* and *Candida albicans* (Eltem and Ucar, 1998). All isolates in this study produced a variety of pigments into the media that ranged from dark brown, reddish brown and light brown in colour. This was a clear indication that the isolates have ability to produce diverse secondary metabolites.

The blastn results showed that majority of the isolates were from the class *Bacilli* in the phylum Firmicutes while one isolate belonged to the class *Gammaproteobacteria* in the phylum *Proteobacteria* with sequence similarities of between 86% to 98%. These results concur with earlier studies in Lake Magadi; where phylogenetic analysis of the 16S rRNA gene sequences with excellent quality showed that majority of the isolates were affiliated to the class *Bacilli* and class *Gammaproteobacteria* (Kambura 2012; Nyakeri 2018). In this study, Firmicutes belonging to the low G + C group were abundant than Gram-negative *Proteobacteria*. This is in agreement with a study where, in a cultivation-dependent analysis of microbial diversity in Lake Magadi, sequences were observed to cluster within two major groups of the established lineages of bacteria: mainly the low G + C Gram-positive bacteria (*Bacillus* and relatives) and the gamma subdivision of the *Proteobacteria* (Baumgarte, 2003).

Six isolates in this study were microorganisms affiliated to genus *Bacillus* while one isolate belonged to genus *Alcaligenes*. These were; *Bacillus Sonorensis, Bacillus Amyloliquefaciens, Bacillus Methylotrophicus, Bacillus Siamensis, Bacillus Pumilus, Bacillus licheniformis* and *Alcaligenes feacalis* **(Table 4.8)**. *Bacillus* species is one of the most commonly found aerobic, alkaliphiles in soda lakes environments (Takami et al, 1999). Members of genus *Bacillus* that were previously isolated from Lake Magadi clustered with *Bacilli* members that are alkalitolerant and alkaliphilic (Baumgarte, 2003; Kambura, 2012; Nyakeri, 2018.). Genus *Bacillus* has got many species; hence the heterogeneity in physiology, ecology and genetics of the genus. This genus has a big diversity of its physiological types such as degraders of substrates from plants and animals, nitrifiers, heterotrophics, acidophiles, antibiotic producers, thermophile nitrogen fixers, alkaliphiles and denitrifiers among others (Slepecky, 1972; Norris et al., 1981; Berkeley et al., 1986).

HS 3 closely clustered with *Bacillus siamensis* (NR 117274.1) and had an estimated G+C content of 51.55 mol%. The bacteria hydrolyzed starch, proteins and cellulose substrates indicating the ability to produce the amylase, protease and cellulase extracellular enzymes (**Table 4.6)**. It also had inhibition activity against Gram-positive and Gram-negative bacteria thus their ability to produce broad array of antimicrobial compounds. *B. siamensis* produces probiotics used in aquaculture however it scored a low percentage similarity, thus could represent new genera in the Lake Magadi ecosystem.

HS 10 closely clustered with *Bacillus Lichenformis* (NR 118996.1) with a similarity of 98% and had a G+C content of 52.2 mol %. *Bacillus licheniformis* is a Gram-positive rod-shaped bacterium (Veith et al., 2004). *Bacillus licheniformis* produces extracellular enzymes that are associated with nutrient cycling and possess antifungal activity. Novozymes Biofungicide Green Releaf contains *B. licheniformis* strain SB3086 as the main active ingredient (Simasi et al., 2009). *Bacillus licheniformis* also produces a useful protease enzyme that can survive in high pH levels i.e. pH 9 and 10. This protease

enzyme is used as an ingredient in laundry detergents. *B. licheniformis* also produces bacitracin which is an antibiotic made up of a mixture of cyclic polypeptides. Ironically, bacitracin lyses the cells of *Bacillus licheniformis* when cadmium or zinc ions are present (Takami & Krulwich, 2000) thus inhibiting the growth of the bacteria. This isolate had the ability to produce amylases, proteases, cellulases, hemicellulases and esterases. It also showed antagonistic activity against both gram negative and gram positive bacteria.

HS 13 showed the ability to produce amylases and hemicellulases; the bacteria however did not show any antagonistic activity against gram negative and positive bacteria. The isolate was closely related to *Bacillus Methylotrophicus* with 90% similarity to the strain and had a G+C content of 54.18 mol%. *Bacillus Methylotrophicus* is a gram positive bacterium; aerobic, motile, rod shaped and forms endospores (Binghua et al., 2016). *B. Methylotrophicus* is used as plant growth-promoting rhizobacteria since it stimulates plant growth and development via emission of volatiles.

HS 18 clustered with *Bacillus Amyloliquefaciens* with 86% sequence similarity and had a G+C content of 53.34 mol%. *Bacillus Amyloliquefaciens* strain which is gram positive, aerobic, rod-shaped and motile is known for its ability to degrade extracellular proteins and is also a source of BamH1 restriction enzyme. It produces the enzyme subtilisin, which is used in industries to make laundry detergents (Benardini et al., 2003). This isolate had ability to hydrolyze starch and pectin. It had antimicrobial activity against both Gram positive and negative bacteria hence showing ability to produce a wide variety of antibiotics.

Isolate HS 28 scored 96 % sequence similarity with *Alcaligenes faecalis* strain NR 113606.1 and *Alcaligenes feacalis* subs. The isolate was able to hydrolyze both starch and skim milk indicating its ability to produce amylases and proteases. The bacteria also showed antagonistic activity against Gram positive and negative bacteria; hence it could have an ability to produce a variety of antimicrobial compounds. Several of the isolates

from Lake Magadi have been affiliated to members of Gammaproteobacteria class. Some of the genera in this class include; *Rhodobacter, Alcaligenes, Stenotrophomonas, Klebsiella, Xenorhabdus, Brevibacterium, Proteus* and *Anoxybacillus* (Kambura et al., 2012). Blast analysis of partial sequences of isolates from soda Lake Elmenteita, showed that 60% of the isolates belonged to the class *Gammaproteobacteria*. These were closely related to genus *Idiomarina, Halomonas* and *Marinospirillum* species (Mwirichia et al., 2010). *Alcaligenes faecalis* produces an extracellular Serine Protease that is used in agriculture as a nematicide (Zheng et al., 2016).

Isolate HS 29 had 87% sequence similarity to *Bacillus pumilus* (NR 116191.1) with a G+C content of 49.03 mol%. The isolate was able to produce amylases, proteases and hemicelluloses. It also had antibiotic activity against gram positive and negative bacteria. *Bacillus pumilus* strain is rod-shaped, an aerobic and endospore-forming bacterium that has been previously isolated from a variety of plants, soils and environmental surfaces of Sonoran desert basalt (Benardini et al., 2003). *Bacillus Pumilus* produces a lipopeptide antibiotic called iturin A which is used as a bio pesticide with the potential of replacing chemical pesticides for control of pathogens (Kaushal et al., 2017).

HS 41 showed sequence similarity to *Bacillus sonorensis* (accession no.NR 113993.1) with a G+C content of 50.53 mol%. *Bacillus Sonorensis* is named after the Sonoran Desert, where the organism was collected from. It is Gram positive, motile, catalase-positive and facultative anaerobic bacteria (Nakamura, 2001). *Bacillus sonorensis* is a plant growth promoting *Rhizobacterium* that produces indole acetic acid, hydrogen cyanide, siderophore and chitinase that helps in improving growth, nutrition and yield of crops (Fhilagar et al., 2018). The bacterial isolate had the ability to produce amylases, proteases, cellulases, hemicellulases and xylanases. The bacteria could also have an ability to produce a variety of compounds because it had inhibition activity against both gram negative and gram positive bacteria.

The DNA sequences of isolates (HS 10 and HS 28) showed identity of 96% - 98% similarity with the already identified sequences in the Gen Bank database (**Table 4.8**). These isolates could represent novel species of microorganisms the lake's ecosystem. Isolates (HS 3, HS 13, HS 18, HS 29 and HS 41) showed identity of 86% - 90% similarity and could represent novel genera of microorganisms. These need to be further confirmed by methods such as fatty acid analyses and DNA-DNA hybridization. This study not only describes bacterial diversity of Lake Magadi ecosystem, but also indicates biotechnologically important cultures. In this study, Lake Magadi was found to harbor a diversity of microorganisms of commercial importance. To understand the roles and structures of these microbial communities, sequence data only is not enough (Borsodi et al., 2005). Thus, the culture-dependent approach used in the present study contributes greatly to our understanding of diversity of Lake Magadi and provides resourceful information on cultures from extreme environments.

5.2 Conclusion

This study has demonstrated that Lake Magadi hot springs harbor haloalkaliphilic bacteria species. The isolates obtained from the hot springs were alkalitolerant and halotolerant because they grew at wide range of pH (6-14) and sodium chloride concentration (0-17.5%) and therefore able to survive in extreme conditions. The above conditions can therefore be adopted if the isolates are to be exploited commercially.

The bacterial isolates were able to hydrolyze starch, Xylan, olive oil, Cellulose, skim milk and CMC indicating that they could be a potential source of amylase, xylanase, lipase, esterase cellulase, proteases and hemicellulase enzymes at alkaline pH. Thus the bacteria found in Lake Magadi hot springs have the potential to yield a wide variety of biotechnologically interesting enzymes. The isolates also showed antimicrobial and antifungal activity against Gram negative and Gram positive test bacteria; *Staphylococcus aureus, Pseudomonas aeruginosa, Bacillus subtilis, Escherichia coli* and *Aspergilus Flavus***.**

Molecular characterization of isolates indicates that all of the isolates belong to domain bacteria. Six isolates were affiliated to microorganisms belonging to the genus *Bacillus*, phylum Firmicutes and one isolate was affiliated to members of the class *Gammaproteobacteria* phylum Proteobacteria. Firmicutes belonging to the low G + C group are more diverse and abundant than Gram negative Proteobacteria. The low sequence similarity percentage values of some isolates to their nearest relatives in the blast shows that Lake Magadi might be a hot spot of many, as yet uncultured bacteria.

5.3 Recommendations

From this study, the following recommendations can be deduced;

- Different protocols and novel microbial culture techniques should be designed so as to allow the identification of a bigger diversity of genera.
- Extensive research on the specific secondary metabolites released by extremophiles from Lake Magadi hot springs 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 metagenomic, metatranscriptomics studies and clone libraries should be used to study the useful functional genes in extremophiles found in hot springs of soda lakes.

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LIST OF APPENDICES

Appendix I: LB Broth (1 Litre)

10 g Sodium Chloride

10 g Tryptone

5 g Yeast extract

Add de-ionized water to a final volume of 1 Litre.

Adjust pH to 7.5 with NaOH and autoclave.

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 at121°C for 15 minutes.

Use; General purpose broth media.

Appendix II: Nutrient broth (1 Litre)

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 at121°C for 15 minutes.

Use; General purpose broth media

Appendix III: Malt yeast extract

1% Malt extract

1% Glycerol

1% Glucose

1% Peptone

1% yeast extract

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 at121°C for 15 minutes.

Use; Isolation media

AppendixIV: Starch casein agar

0.5% Glycerol

0.5% Starch

0.5% Sodium proprionate

0.1% KNO_3

0.1% Asparagine

0.3% Casein

0.5% K_2HPO_4

0.5% $FeSO_4$

0.5% Vitamin B

Suspend the components in one liter of distilled water. Stir well to dissolve the medium completely. Dispense in 3-10 ml in tubes then Sterilize by autoclaving at121°C for 15 minutes.

Use; Isolation media

AppendixV: Basal Media

1 % KH_2PO_4

0.01 % $MgSO_4.7H_2O$

0.005 % $CaCl_2.2H_2O$

4 % NaCl

1 % Na_2CO_3

Supplemented with 2.5 % xylan, 1 % cellulose, 1 % carboxymethylcelullose, 1 % starch, 1 % skim milk or 1 % olive oil.

Appendix VI: Differential Agar

1 % KH_2PO_4 (Potassium dihydrogen Phosphate)

0.01 % $MgSO_4.7H_2O$ (Magnesium Sulphate)

0.005 % $CaCl_2.2H_2O$ (Calcium Chloride)

0.14 % Agar

4 % NaCl (Sodium Chloride)

1 % Na_2CO_3 (Sodium Carbonate)

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Appendix VII: 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
- RNase A 20 mg/ml
- Proteinase K 20 mg/ml
- Phenol
- Chloroform
- Absolute ethanol.
- 3 M NaCl
- Isopropanol

Appendix VIII: Electrophoresis buffer Working Concentrated stock

- TBE buffer 10×
- Chemical Volume
- Tris 108 g
- Boric Acid 55 g
- Na2 EDTA.2H2O 9.3 g

Adjust the volume to 1 liter with ddH2O and divide into 500ml bottles Running conditions: use 1× TBE as the running buffer. Pre run the gel at 40W for 30 minutes. Load 2µl of sequencing reactions/well making sure to low out wells with a syringe first then Run the gel at 60W for 1.5-2h interval.

Appendix IX: EDTA 0.5 M pH 8.0

- Dissolve 186.1 g of disodium ethylenediaminetetra-acetate (EDTA.2H2O Sigma ED2SS mw 372.2) in 800 ml of ddH2O.
- 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.

Appendix X: Ethidium Bromide l0 ×

- Dissolve 1.0 g of EtBr in a final volume of 100 ml ddH2O.
- 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 ddH2O.
- Wrap bottle in aluminum foil and store at room temperature.

Appendix XI: Proteinase K

To 1 ml of ddH2O add 20 mg of Proteinase K (Promega # 52066). This gives a 20mg /ml stock.

Appendix XII: SDS 10 %

Dissolve 100 g of electrophoresis-grade SDS in 800 ml ddH2O. Heat the solution to dissolve. Bring to a final volume of 1000 ml using ddH2O. Do not autoclave.

Appendix XIII: TE pH 7.4 or pH 8.0

Chemical Volume

1M Tris pH 8.0 2 ml

0.5 M EDTA pH 8.0 400 µl

Bring it to a final volume of 100 ml using ddH2O and autoclave.

Appendix XIV: Tris 1 M p H 7.4

Dissolve 121.1 g of Tris base in 800 ml of ddH2O and adjust the pH to 7.4 with concentrated HCL.

Bring the final volume to 1000 ml with ddH2O.

Divide into 100 ml bottles and autoclave.

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Isolation and characterization of haloalkaliphilic bacteria from the hot springs of Lake Magadi

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