Isolation, Characterization and Screening of Bacterial Isolates from Lake Magadi for Exoenzyme and Antimicrobial activity

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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 beloved dear family; my husband Edward Nderitu Karanja, son Shawn Karanja and daughter Melissa Nderitu. Thanks to all of you for the support you have and are still giving me. Without your encouragement and support this journey would be long and tough.

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LIST OF ABBREVIATIONS AND ACRONYMS

ARDRA	Amplified Ribosomal DNA Restriction Analysis
Bp	Base pairs
CMC	Carboxymethylcelullose
DNA	Deoxyribonucleic Acid
EDTA	Ethylene Diamine Tetra-Acetic Acid
G+C	Guanine and Cytosine
KMM1	Kenya Minimal Medium 1
LB	Luria Bertani
Nm	Nanometers
OD	Optical Density
PCR	Polymerase Chain Reaction
SDS	Sodium Dodecyl Sulfate

ABSTRACT

Microorganisms from soda lakes have attracted attention as a possible source of novel enzymes and metabolites for use in biotechnology and developing new applications such as medicine, food, and research reagents. Many studies on alkaliphilic bacteria; isolation, characterization and identification, have been done on Kenyan soda lakes. However, very little has been documented on Lake Magadi, a hyper saline lake with up to 30% salinity levels. This study sought to bioprospect for alkaliphilic bacteria from Lake Magadi that could produce novel bioactive compounds and document for further exploitation. 55 isolates were isolated using different media prepared with filter-sterilized water from the lake. These were characterized using cultural, biochemical and molecular approaches, and screened for production of extracellular enzymes as well as potential for production of antimicrobial compounds. The bacteria were Gram positive and Gram negative, and they grew well at pH ranging from 5 – 10, temperature range of 25 – 50 ^{o}C and sodium chloride range of 0- 30 %. The isolates produced various extracellular enzymes such as amylases, lipases, proteases, cellulases and esterases. Antimicrobial assays done to determine the isolates range of *in vitro* activity against test organisms exhibited a range of inhibitory effects. 17 isolates produced coloured pigments into the media indicating that they could produce diverse bioactive metabolites.

Analysis of partial sequences using Blast showed that 80 % of the isolates were affiliated to the genus *Bacillus* while 20 % were affiliated to members of *Gammaproteobacteria*. Isolates A5, A14 and A30 clustered with *Bacillus* at 96-97 %

similarity. A11 scored 96 %, and had several neighbors with similar percentage similarity such as *Alcaligenes faecalis* strain CL-10.3a, *Streptomyces sp*. VITSVK5, *Achromobacter sp*. DBTN3, *Bordetella* sp. VVAR and uncultured *beta proteobacterium* clone L21. A19 clustered with members of the genus *Stenotrophomonas* with a score of 95 % similarity. These could represent novel species within the Lake ecosystem. A22 had *Anoxybacillus sp*. C163a and *Kocuria sp.* M14 as the nearest neighbors in BLAST with 80 % and 81 % similarity respectively. A32 and A47 clustered with *Bacillus* at 80 % and 81 % similarity respectively while A31 clustered with *Klebsiella pneumoniae* at 87% similarity. These could represent novel genera of organisms. This study demonstrated that the extreme environment of Lake Magadi harbors novel Alkaliphilic bacteria that can produce enzymes and antimicrobial compounds.

CHAPTER ONE

1.0: INTRODUCTION

1.1 Background information

Soda lakes represent the most stable naturally occurring highly alkaline environments on earth. They commonly have pH values greater than 10 (occasionally reaching pH 12) and are characterized by the presence of large amounts of Na_2CO_3, (usually as $Na_2CO_3.10H_2O$ or Na_2CO_3, $NaHCO_3.2H_2O$) and are depleted of Mg^{2+} and Ca^{2+} because of the insolubility of carbonates under alkaline conditions (Duckworth, 1996; Grant, 2006). The rates of evaporation of soda lakes exceed any inflow. Such environments are found in arid and semi-arid areas of tropical or subtropical rain-shadow deserts of North America; in the continental interiors of Asia and areas of tectonic rifting such as the East African Rift Valley. Despite apparently hostile conditions; extremely high salinity and alkalinity levels, these caustic lakes are the most productive aquatic environments in the world, with productivity rates an order of magnitude greater than the mean rate for all aquatic environments on Earth. This is presumably because of the high ambient temperatures, high light intensities, availability of phosphates and unlimited access to CO_2 in these carbonate rich waters (Grant, 2006).

One of the most striking features of many alkaline, saline lakes is the coloration of the waters. Depending on a variety of conditions related to water chemistry, dense populations of micro-organisms may colour the lakes green, orange, purple, pink, or

red, due to the massive permanent or seasonal blooms of specific algae, cyanobacteria, eubacteria or archaebacteria (Grant *et al*., 1990).

Organisms found in these environments are described as extremophiles and are usually characterized only by one distinctive extreme such as temperature, pH or salt. However, others are multi-extremophiles, for example alkalithermophiles (Kevbrin *et al.*, 1998), which are micro-organisms that can survive high alkaline conditions and elevated temperatures (Melack and Kilham, 1974; Grant *et al*., 1990).

Microorganisms from extreme environments also possess useful physiological properties; for example, their enzymes are alkali and heat stable and therefore, suitable for exothermic industrial processes (Adams and Kelly, 1995 and Kranz *et al*., 1997). Sodium-dependent transport systems have been reported, which generate a sodium motive force via H^+/Na^+ antiport systems. Internal H^+ is exchanged with Na^+ by the cells, and Na^+ then accompanies substrates into the cells (Horikoshi, 1998). Sodium ions in the surrounding environment are essential for effective solute transport through the membranes of alkaliphilic *Bacillus* spp. Genes responsible for the alkaliphily of *Bacillus halodurans* C-125 and *Bacillus firmus* OF4 have been analyzed (Horikoshi, 1999).

Cell walls of several alkaliphilic microorganisms contain a large amount of acidic amino acids. The acidic charges on these components may act as charged membranes, reducing the pH on the cell surface between 8 and 9 (Horikoshi, 1998) thereby allowing the cell to maintain a neutral internal pH. Plasma membranes may also

maintain pH homeostasis by using the Na^+/H^+ antiporter system, the K^+/H^+ antiporter, and ATPase-driven H^+ expulsion (Horikoshi, 1999).

The East African Rift Valley contains a number of alkaline soda lakes, including Lakes Bogoria, Elmenteita, Nakuru and Magadi. They have been shown to support a dense and diverse population of aerobic, organotropic, halophilic, alkaliphilic, and alkalitolerant representatives of major bacterial phyla (Duckworth *et al.,* 1996; Jones *et al.,* 1998; Grant *et al.,* 1999 and Zavarzin *et al.,* 1999).

Between these bacterial phyla, there is cycling of carbon, sulfur, and nitrogen under aerobic and anaerobic conditions present in the lakes. A marked difference in prokaryotic communities has been observed between the strongly hypersaline, alkaline brines of Lake Magadi and Lake Natron in the Rift Valley, Owens Lake in California and some of the Wadi Natrun depression in Egypt with salt concentration approaching saturation (30 % or greater) and the more dilute waters of lakes Elmenteita, Nakuru and Bogoria with salinities on the order of 5 % w/v (Jones *et al.,* 1994 and 1998).

Despite these differences, photosynthetic primary production appears to play an important role in the soda lake environment and, presumably, supports the rest of microbial community (Grant *et al*., 1999; Jones *et al*., 1998). The less alkaline lakes are usually dominated by dense blooms of cyanobacteria while the hypersaline lakes, on occasion (i.e. after extensive rainfall causes dilution of the brines) support blooms of both cyanobacteria and alkaliphilic anoxygenic phototrophs belonging to the genera *Ectothiorhodospira* and *Halorhodospira* (Grant *et al*., 1999; Jones *et al*., 1998).

Studies on Lake Magadi have revealed various extreme alkaliphilic, moderate halophilic and benthic cyanobacteria. They were identified, by morphology to be *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). Unicellular cyanobacteria were observed to develop mostly at higher salinities, approximately 7 % and 10 % NaCl, while trichomic forms were better suited to lower salinity and alkalinity (Zavarzin *et al*., 1999). These are not only essential for the fixation of nitrogen in this environment, but they are also producers of oxygen (Melack and Kilham, 1974).

A Eukaryotic micro-algae was isolated from Lake Magadi water samples; that was identified, by morphology, as *Chlorella minutissima* (Gerasimenko *et al.*, 1999). Alkaliphilic cyanobacteria, notably *Arthrospira platensis*, and *Cyanospira rippkae*, are responsible for photosynthetic primary production in dilute lakes. There is also an unquantified contribution to primary productivity made by anoxygenic phototrophic bacteria of the genus *Ectothiorhodospira* (Jones *et al*., 1998). In hypersaline lakes cyanobacteria and anoxygenic phototrophs from the genus *Halorhodospira* and also *Rhodobaca bogoriensis* may be responsible for primary productivity (Milford *et al*., 2000).

Although the soda lakes of the Rift Valley are eutrophic, relatively shallow presumably oxygen limited, they maintain dense populations of non phototrophic

aerobic organotrophic bacteria that utilize products of photosynthesis as well as products of anaerobic destruction. Viable counts of aerobic organotrophs from a range of diluted lakes indicate 10^5 - 10^6 cfu ml $^{-1}$ (Grant *et al.,* 1990). A number of aerobic chemoorganotrophic, alkaliphilic isolates obtained from several East African soda lakes were studied in detail, though only a few of these isolates are published. The majority of Gram negative isolates were members of proteobacteria (Duckworth *et al.*, 1996 and Jones *et al*., 1998). A study on Lake Magadi, Elmenteita, Nakuru and Bogoria revealed the presence of a diverse population of aerobic sulfur oxidizing bacteria of the genera *Thioalkalimicrobium* and *Thioalkalivibrio* (Sorokin *et al*., 2001).

The anaerobic alkaline saline environment has received less attention. The predominant biological process in soda water bodies is sulfate reduction. It is responsible for not only the final steps of organic matter degradation but also for generating alkaline conditions as a result of transformation of sulfate to sulfide (Zavarzin *et al*., 1999). *Desulfonatronovibrio hydrogenovorans,* a member of proteobacteria was isolated from mud in a drainage ditch at Lake Magadi. These may play the universal role of hydrogen sink in a sulfidogenic anaerobic alkaliphilic community (Zhilina *et al*., 1997). Hydrogen acetogenesis also provides an available hydrogen sink. A representative of the homeacetogenic bacteria (strain Z-7937) was isolated from Lake Magadi samples (Zhilina and Zavarzin, 1994).

An anaerobic culture of hydrogenotrophic denitrifier, morphologically similar to *Paracoccus* was isolated from Lake Magadi (Zavarzin *et al.,* 1999). Chemoorganotrophic populations are biochemically very active, hydrolyzing many different polymers and producing sugars and amino acids. These may be used as substrates for the fermentation of simple compounds by anaerobic fermentors. Fatty acids produced by anaerobes may be consumed by other groups such as the acetogenic bacteria, including *Natroniella acetigena*, *Thermosyntropha lipolytica* (Svetlichnyi *et al*., 1996) and *Tindallia magadiensis* (Zavarzin *et al*., 1999).

Organic material degraded by anaerobic digestion produces substrates for methanogens such as *Methanosalus zhilinaeae* isolated from Lake Magadi (Zhilina and Zavarzin, 1994). The methane produced is oxidized by methane-oxidizers, methanotrophs, assigned to the *Methylobacter* genus, although a recently isolated methane oxidizer, AMO1, is most closely related to *Methylmicrobium pelagium* (Sorokin *et al*., 2000).

The sulfur cycle in these lakes utilizes sulfur and sulfate presumably generated by *Ectothiorhodospira* and *Halorhodospiria sp*., and also aerobic sulfur-oxidizers (Sorokin *et al.,* 2001). Sulfate-reducing bacteria then complete the cycle (Zhilina *et al*., 1997; Pikuta *et al.,* 1998).

The nitrogen cycle in these lakes involves the production of ammonia by fermentative anaerobes such as *Tindallia magadii* (Kevbrin *et al.,* 1998). Ammonia is utilized by methanotrophs and nitrifiers, producing nitrate. Nitrate, in turn, is utilized by the

chemoorganotrophs, creating a link between the nitrogen and carbon cycles. Much of this diversity has been discovered by traditional culturing and taxonomic procedures.

Previously, application of cultivation-independent molecular techniques for the detection of new prokaryotic (Bacteria and Archaea) diversity in Lake Magadi has been done. Samples were collected in a dry period from sediment under the trona and 14 aerobic spore forming strains were isolated (Baumgarte, 2003). These were analyzed on the basis of ARDRA and sequence analysis. Sequences were detected clustering within two major groups of established lines of bacteria: mainly the groups of Gram-positive bacteria, with low G+C content (Firmicutes) and the gamma subdivision of the Proteobacteria. Cultivation-dependent analysis of the isolates revealed a unicellular, unicyanobacterial, non-axenic culture, representing a predominant species isolated from Lake Magadi (Baumgarte, 2003). However, very little information has been documented on microbial diversity of Lake Magadi.

1.2 Statement of the problem

The demand for new antibiotics continues to grow due to the rapid spread of antibiotic-resistant pathogens causing life-threatening infections. Nature still remains the richest and the most versatile source for new antibiotics (Baltz, 2006; Koehn and Carter, 2005; Pelaez, 2006). It is estimated that as few as 0.1–1% of the organisms living in the biosphere have been cultured and characterized in a laboratory setting. The other 99% of the population may represent novel genetic diversity (Handelsman

et al. 1998). These microorganisms represent a diverse and still undiscovered reservoir of novel strains that may produce novel natural compounds (Baltz, 2007).

Microbial communities in the soda lakes have attracted attention as a possible source of novel enzymes and metabolites for use in biotechnology. Enzymes from alkaliphiles have long term stability in detergent products, energy cost saving by lowering the washing temperatures, quicker and more reliable product, reduced effluent problems during the process, and stability in the presence of detergent additives such as bleach activators, softeners, bleaches and perfumes (Horikoshi, 1999). Due to the unusual properties of these enzymes they are expected to fill the gap between biological and chemical processes and have been greatly employed in laundry detergents (Horikoshi, 1999; Bordenstein, 2008). However, very little research has been carried out on Lake Magadi hence less information is available.

1.3 Justification

The earth's biological diversity is disappearing at an ever increasing rate. The documentation of this loss has been based primarily on large organisms, such as mammals and vascular plants; however, evidence exists of an increasing decline in the diversity of the less conspicuous organisms such as bacteria, fungi, and bryophytes (Duckworth *et al.*, 1996, Zavarzin *et al*., 1999). These poorly known but speciose groups of organisms may be more vital to long term ecosystem survival than the well-known macro fauna and flora.

Extremophiles have a worldwide distribution, and are found in a wide range of habitats, including extreme environments such as deserts, areas with high salt concentrations, very low or high pH levels or ionizing radiation as well as in deep sea sediments **(**Zhilina and Zavarzin, 1994). Extensive studies on alkaliphilic bacteria; isolation, characterization and identification have been carried out in the Kenyan soda lakes.

The use of biological systems or agents to catalyze chemical transformations on industrial scale is well established and includes both free enzymes and whole cells (Faber, 1992). The enantio-selectivity of enzymes makes them suited for resolving racemic mixtures by means of kinetic resolution (Chen *et al.,* 1982; Sih and Wu, 1989).

Knowledge of the spatial and temporal variation of organisms producing the different enzymes and factors affecting enzyme activity are important to understand and optimize. For instance up to 60–70% of the organic matter in the incoming wastewater of domestic wastewater treatment plants is accounted for by proteins and lipids (Martinez *et al*., 1996). Removal of these compounds through mechanical methods is expensive and therefore enzymes provide a cheap way to remove them from these wastes. Besides this, industrially useful enzymes with novel applications, or which improve upon the activities of the ones currently being used, are frequently being sought (Marrs *et al*., 1999).

Many currently employed alkaliphiles enzymes are very useful as tools for biotechnological exploitation. Research has revealed a great diversity of bacterial extremophiles that could produce a large pool of enzymes to choose from for developing new biotechnological applications such as medicine, food, and research reagents. The stability and activity of thermophilic enzymes can be controlled by separate molecular determinants. These enzymes can be used as molecular templates to design highly stable enzymes that have high activity at high temperatures (Vieille and Zeikusl, 2001).

The alkaliphilic bacteria from extreme environment are a particular focus for the discovery of new bioactive compounds. These bacterial communities should therefore, be isolated, characterized and identified for maximum utilization in areas such as production of industrial valuable enzymes and metabolites, general genetic resources for applications like production of signals, genetic analysis, mechanisms of membrane transport, pH regulation and in the taxonomy of alkaliphilic microorganisms (Horikoshi, 1999). It can safely be assumed that the extremophilic organisms derived from the soda lake environment have a great, yet-to-be-exploited potential for a variety of biotechnological applications.

Since culture-independent studies have shown that soda lake environments harbour diverse groups of bacteria with potential to produce diverse bioactive compounds, this study carried out on Lake Magadi was geared towards use of enrichment strategies to

isolate new bacterial members, characterization and screening the isolates for bioactive compounds.

1.4 Hypothesis

Lake Magadi harbors novel alkaliphilic bacteria that have biotechnological potential in production of novel bioactive compounds.

1.5 Objectives

1.5.1 General Objective

To bioprospect for novel alkaliphilic bacteria that could produce novel bioactive compounds for use in biotechnology.

1.5.2 Specific Objectives

1. To isolate bacteria from Lake Magadi.
2. To characterize and identify the bacterial isolates using cultural, biochemical and molecular approaches.
3. To screen the bacterial isolates for production of extracellular enzymes.
4. To screen the bacterial isolates for bioactive metabolites.

CHAPTER TWO

2.0: LITERATURE REVIEW

2.1 Microbial diversity of soda lakes in the world

Basic knowledge about molecular mechanisms of alkaliphilic microbes, stem mainly from studies by Horikoshi (1999), on *Bacillus halodurans* C-125 and by Keller and Zengle, (2003)**.** Their studies on hot springs, in Yellowstone National Park (Wyoming, U.S.A), revealed large bacterial diversity in the hot spring samples with twelve new division-level lineages. The study showed that members of the bacterial domain seemed to outnumber the Archaea in this hydrothermal environment (Keller and Zengle, 2003)**.**

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

Of the 20 isolates, only 4 were Gram-negative, and Gram-positive isolates were diverse and predominant. However, the majority of the clones obtained from Baer Soda Lake were related to *Proteobacteria,* with only about 10 % of the clones affiliated with Gram-positive bacteria. 26 alkaliphilic and alkalitolerant *Bacillus* species have been identified from alkaline environments (Yumoto *et a*l., 2003; Olivera *et al*., 2005; Nogi *et al*., 2005; Ghosh *et al*., 2007; Lee *et al*., 2008; Borsodi *et al*., 2008).

Northern Egypt has a set of desert alkaline soda lakes in the Wadi Natrun area, which due to their lower surface elevation, are fed by underground water from the river Nile. They have an intensive microbial flora (Imhoff *et al.,* 1979, 1996) and are known as a source for the isolation of various mesophilic alkaliphiles. Alkaliphilic aerobic bacterium *Alkalilimnicola halodurans* was isolated from sediments of Lake Natron which was affiliated with members of the family *Ectothiorhodospiraceae* (Baumgarte, 2003).

A novel, obligately anaerobic, alkalithermophilic, chemo-organotropic bacterium was isolated from the sediment of an alkaline hot spring located on Paoha Island in Mono Lake, California, USA. This rod-shaped bacterium could also reduce Fe (III) and Se (IV) in the presence of organic matter. On the basis of physiological properties, 16S rRNA gene sequence and DNA–DNA hybridization data, the strain has been identified as *Anaerobranca californiensis* sp. Nov. (Vladimir *et al.,* 2004).

2.2 Kenyan Soda lakes and microbial biodiversity

The salinities of soda lakes found in Kenya range from approximately 5 % total salts (w/v) to 30 % and pH values from 9 to above 11.5 (Duckworth *et al.*,1996). Lake Magadi in Kenya is among the most stable highly alkaline environments on earth, with a consistent pH of 10.5 to 12 (Duckworth *et al.,* 1996; Grant, 2006). Alkaliphilic communities contain representatives of major trophic groups. Primary producers dominate the lakes (Melack and Kilham, 1974). Kenyan soda lakes have revealed a typical predominance of dense blooms of *Cyanobacteria* in less saline alkaline lakes (Mwirichia *et al.,* 2010). The predominant filamentous species are *Spirulina platensis, Spirulina maxima* and *Cyanospira (Anabaenopsis)* (Melack and Kilham, 1974; Tindall *et al.*, 1984 and Florenzano *et al.*, 1985). The unicellular species *Chorococcus spp.*, *Synechococcus sp.* or *Synechocystis* have also been found, and in some cases they may be the dominant primary producers (Grant *et al.,* 1990; Mwatha and Grant, 1993; Grant, 2006).

The Kenyan soda lakes such as Bogoria and Elmenteita are also characterized by hot springs which host both hyperthermophilic and haloalkalithermophilic microorganisms. Hyperthermophilic Bacteria and Archaea represent the organisms at the upper-temperature limits of life (Stetter and Zillig, 1985; Brock, 1986; Stetter, 1992). They grow fastest (optimally) between 80 and 105 °C and are unable to grow below 60 °C. Their adaptations towards high pH and elevated temperature draw attention not only as a source of industrially valuable enzymes but also for studying adaptive mechanisms to extreme environmental parameters.

Phototrophic eukaryotes of the diatoms belonging to the genera *Nitzchia* and *Navicula* are predominant in these ecosystems (Tindall *et al.*, 1984). Other groups represented include alkaliphilic anoxygenic phototrophic bacteria, mainly of the genera *Ectothiorhodospira* and *Halorhodospira* (Melack and Kilham, 1974). Anoxygenic phototrophic bacteria are also capable of forming visible blooms in soda lakes and members of the genera *Ectothiorhodospira* and *Halorhodospira* provide substantial contributions to primary production (Grant, 1990; Grant and Horikoshi, 1992; Grant, 2006). The genera *Ectothiorhodospira* and *Halorhodospira* are able to oxidise sulphide to sulphate, depositing extracellular elemental sulphur (Hecky and Kilham, 1973). Remarkable primary productivity supports a diverse and stable population of aerobic organotropic bacteria in East African soda lakes (Tindall *et al*., 1980).

Anaerobic groups consist of the acetogenic ammonifiers and alkaliphilic hydrogenotrophic sulphate reducers *Desulfonatronovibrio* and *Desulfonatronum,* obligately autotrophic sulphur-oxidizing bacteria, methane-oxidizing *Methylobacter alcaliphilus* and alkaliphilic *Methylomicrobium* sp. able to oxidize methane and ammonia (Zarvazin *et al.,* 1999).

Studies on the low saline lakes of the Kenyan Rift Valley such as Bogoria, Crater Lake Sonachi, Elementeita and Nakuru revealed the presence of diverse populations of aerobic sulfur oxidizing bacteria of genera *Thioalkalimicrobium* and *Thioalkolivibrio* (Sorokin *et al*., 2001). Anaerobic alkalithermophiles from Lake Bogoria include *Thermosyntropha lipolytica* (Svetlitshnyi *et al.,* 1996) and *Anaerobranca gottschalkii*

(Prowe and Antranikian, 2001). Several *Bacillus* strains such as M8 C22, M8-C11 (FJ 764771), M14-C16 (FJ 764778), M4-C7 (FJ 764769), M10-C8 (FJ 764774), M14-C6 (FJ 764777), M1-C6 (FJ 764768), M8-C14 (FJ 764772), M9-C3 (FJ 764773) and M10-C17 (FJ 764775) were isolated from Lake Elmenteita (Mwirichia, 2009 and Mwirichia *et al.,* 2009).

Studies done to assess the microbial diversity of Lake Elmenteita using a culture-independent approach revealed diverse groups of bacteria that are involved in complex metabolic interactions within the Lake's ecosystem. The main Phyla revealed included; Cyanobacteria, Firmicutes, Spirochetes, Actinobacteria, Planctomyces, Proteobacteria, Bacteroidetes, Chloroflexi and Chlorobi. Most of the clones identified were affiliated to as-yet uncultured bacteria. There was an occurrence of clones representing members of the class *Betaproteobacteria* which have not been previously reported from either the East African soda lakes (Rees *et al.,* 2004 and Mwirichia *et al.,* 2010) or the other soda lakes such as Wadi al-Natrun in Egypt (Mesbah *et al.*, 2007). The functional role of members of this phylum in the soda lake environment needs to be further investigated preferably using a culture-dependent approach (Mwirichia *et al.,* 2010).

In another study where different enrichment and isolation media were used, in an attempt to isolate novel groups of bacteria from Lake Elmenteita, phylogenetic analysis of 181 partial 16S rRNA gene sequences with excellent quality, showed that the majority of the isolates were affiliated to the class *Gammaproteobacteria* and to

the genus *Bacillus*. Isolates from the genus *Halomonas* and *Bacillus* constituted 37 and 31 % of the total sequenced isolates, respectively. Other groups recovered were related to *Marinospirillum, Idiomarina, Vibrio, Enterococcus, Alkalimonas, Alkalibacterium, Amphibacillus, Marinilactibacillus* and the Actinobacteria, *Nocardiopsis* and *Streptomyces* (Mwirichia *et al*., 2010).

Organotrophic bacteria of the phylum Actinobacteria, namely *Bogoriella caseilytica* (Groth *et al*., 1997) and *Cellulomonas bogoriensis* (Brian *et al*., 2005) have also been described from Lake Bogoria in Kenya. Others are members of the genus *Dietzia natronolimnaea* (Duckworth *et al*., 1998), the genera *Arthrobacter* and *Terrabacter* (Duckworth *et al.*, 1998) from Lake Oloiden, Kenya.

Other alkalithermophilic strains of an autotrophic, carbon dependant and nitrite oxidizing bacteria have also been isolated from Siberian and Kenyan soda lakes (Jones *et al.*, 1998). The strains isolated from diverse locations form a compact species group related to *Nitrobacter* but different from the known species (Sorokin *et al.*, 1998, Grant, 2006).

A large amount of current research involving Lake Magadi is to try to purify and culture novel forms of bacteria able to live in alkaline lakes. Two haloalkaliphilic strains *Spirochaeta alkalica* and *S. africana* have been isolated (Zhilina *et al*., 1997). In 2004, eight new strains of denitrifying bacteria were found in a lagoon with a pH of 10 (Boltianskaya *et al*., 2004). Another research in 2007 described experiments that

isolated a new genus and species of bacteria known as *Methylohalomonas lacus* (Sorokin *et al*., 2007).

2.3 Application of alkaliphiles in biotechnology

Microbial communities in the soda lakes have attracted attention as a possible source of novel enzymes and metabolites for use in biotechnology. Microbes are a preferred source of enzymes since they are cheaper to produce and their enzyme content is more predictable and controllable (Plummer and Tarentino, 1991; Aeed *et al.,* 1992; Adams and Kelly, 1995 and Kranz *et al*., 1997; Grant, 2006).

According to Duckworth *et al.*, (1996), thermoalkaliphilic bacteria are believed to have biotechnological potential such as sources of alkali-stable enzymes. Around 100 types of bacteria have previously been randomly isolated from samples of soil, water and sediments in and around the dilute soda lakes of Bogoria, Elmenteita, Nakuru and Sonachi. When the bacterial isolates were subjected to a preliminary numerical taxonomic analysis, they indicated considerable taxonomic diversity (Grant and Horikoshi, 1992; Grant, 2006).

The advances in the application of alkaliphilic-or alkalitolerant-based biomolecules during the past 20 years are due to the introduction of proteolytic enzymes classified as serine protease in the detergent industry. Since the discovery of this enzyme in the 1970s, attention has been centred on alkaliphilic enzymes such that within a few years, a large number of enzymes have been available. Industrial applications of alkaliphiles have been investigated and some enzymes have been commercialized (Denizci *et al*.,

2004; Nogi *et al*., 2005). Of the enzymes now available to industry, enzymes such as proteases, cellulases, lipases and pullulanases are by far the most widely employed and they still remain the target biomolecules. (Rainey *et al*., 1994; Takami & Horikoshi, 2000; Demirjian *et al*., 2001; Maugeri *et al*., 2001; Grant, 2006).

Proteases from extremophiles are also applied in the manufacture of leather, xylanases for use in the pulp paper industry and cyclodextrin glucanotransferase for cyclodextrin manufacture from starch, frequently used in foodstuffs, chemicals, cosmetics and pharmaceuticals (Grant *et al*., 1990, Takami & Krulwich, 2000; Gupta *et al*., 2002; Saeki *et al*., 2002; Oner *et al*., 2006). Glycosyl transferases and hydrolases from extremophiles are important because they can perform reactions at high temperatures and high contents of organic solvents. Subsequently, they have advantages over 'conventional' enzymes (Grant *et. al*., 1990; Horikoshi, 1996; Bordenstein, 2008).

Detergent enzymes account for approximately 60 % of total worldwide enzyme production. They usually have a pH range of 8 and 10.5 (Horikoshi, 1999). The main reason for selecting enzymes from alkaliphiles is their long term stability in detergent products, energy cost saving by lowering the washing temperatures, quicker and more reliable product, reduced effluent problems during the process, and stability in the presence of detergent additives such as bleach activators, softeners, bleaches and perfumes. Due to the unusual properties of these enzymes they are expected to fill the gap between biological and chemical processes and have been greatly employed in laundry detergents (Horikoshi, 1999, Bordenstein, 2008). Many currently employed

alkaliphiles enzymes are very useful as tools for biotechnological exploitation. Clearly, there are both a wide range of potential applications and many benefits to be gained from them which thus far have hardly been exploited.

Studies on alkaline enzymes have concentrated largely on those organisms which have been easily observed in the natural environment (Horikoshi, 1999). For example, large numbers of alkaliphilic *Bacillus* species have been isolated over the years, many due to the systematic work of Horikoshi and co-workers. The majority of halobacteria examined have retinal - based pigments capable of the light mediated translocation of ions across the cell membrane. Bacteriorhodopsin as a light driven proton pump and halorhodopsin as an inward chloride pump became perfect models for energy conversion, opening interesting biotechnological perspectives for the use of these molecules in different applications, including halographic techniques and information storage (Oren, 1998; Oner *et al*., 2006). It has also been documented that the carotenoid pigment of halobacteria, trap solar radiation, increasing the ambient temperature and evaporation in salterns, hastening the deposition of sea salt (Tindall, 1988; Bordenstein, 2008). It can safely be assumed that the extremophilic organisms derived from the soda lake environment have a great, yet-to-be-exploited potential for a variety of biotechnological applications.

2.4 Application of alkaliphiles in pharmaceutical industry

Micro-organisms are highly efficient in their ability to produce many kinds of bioactive compounds (Zeynep and Metin, 2001). A large number of antibiotics are

produced by various types of bacteria, such as Actinomycetes. Screening bacteria from alkaline habitats or those grown under extreme cultural conditions remains a profitable area for investigation. Some new antibiotics were produced by certain bacteria when an alkaline medium with high alkalinity (pH 9 to 10.5) was used (Sato *et al*., 1983). The alkaliphilic Actinomycete *Nocardiopsis* strain, a producer of phenazine, successfully grew at pH 10.0 in culture medium (Tsai *et al*., 1995). 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 smegmatis,* and *Candida albicans* (Tsai *et al*., 1995).

The preliminary results have encouraged further research work to identify the metabolites produced by alkaliphilic bacteria (Eltem and Ucar, 1998). The discovery of these bioactive compounds provides evidence that organisms from such environments are also capable of producing antibiotic-type compounds. Alkaliphilic producers of novel bioactive agents still await exploitation.

Since the discovery of penicillin by Alexander Fleming in 1928 and its development by Chain and Florey in the 1940s there have been tremendous developments in the medicinal use of microbial metabolites and their derivatives. These include the immunosuppressants cyclosporine A, FK506 and rapamycin (Van Middlesworth and Cannell, 1998), antihyperlipidemics lovastatin and the discovery of guggulsterone

(Urizar *et al*., 2002), as anti-diabetic drugs, hormone antagonists, anti-cancer drugs, and agricultural and pharmaceutical agents (Grabley and Thiericke, 1999).

2.5 Characterization of alkaliphiles

Duckworth *et al*., 1996, 2000) characterized alkaliphiles in their detailed work on the "Phylogenetic Diversity of Soda Lake Alkaliphiles", using several different media in enrichment and isolation, under the same specified conditions. 16S rRNA genes from a range of aerobic chemoorganotrophic, alkaliphilic Bacteria and Archaea were sequenced and subjected to phylogenetic analysis. Gram-negative alkaliphiles were found to be confined to Proteobacteria, with many isolates related to the *Halomonas / Deleya* group. Gram-positive alkaliphiles were found in both high % G + C and low % G + C divisions of the Gram-positive lineage, with many isolates being related to the *Bacillus* group, others to *Arthrobacter* spp. Alkaliphilic Archaea were relatively closely related to members of the genera *Natronococcus* and *Natronobacterium.* An anaerobic, thermophilic isolate was assigned to a new genus within the *Thermotogales.*

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

The comprehensive molecular work on the application of results from the discovery of molecular biology and biochemical studies, such as protein purification and characterization, facilitated by the cloning and expressing of genes from hyperthermophiles and mesophilic hosts revealed a great diversity of bacterial extremophiles (Vieille and Zeikusl, 2001, Helen *et al*., 2003). This represents a large pool of enzymes to choose from for developing new biotechnological applications. The stability and activity of thermophilic enzymes can be controlled by separate molecular determinants. These enzymes can be used as molecular templates to design highly stable enzymes that have high activity at high temperatures (Vieille and Zeikusl, 2001). Such an achievement could greatly enhance the range of applications for these enzymes in areas including medicine, food and research reagents.

CHAPTER THREE

3.0: MATERIALS AND METHODS

3.1 Study site

Lake Magadi is a hyper saline lake that lies in the southern part of the Kenyan Rift Valley close to the Tanzanian border, between Lake Natron in the south and fresh water Lake Naivasha to the north. It is approximately 2° S and 36° E of the Equator at an elevation of about 600 m above sea level, and lies in the lowest part of the trough in a naturally formed closed lake basin. The lake covers an area of 90 km^2 and is one of the smaller Rift Valley lakes (Behr and Röhricht, 2000). Evaporation is intense during the dry season (3500 mm per annum), the range of temperature being between 22 °C and 34 °C. The Loita Hills and the Mau Escarpment to the west shield the valley floor from rainfall resulting in an annual total of approximately 500 mm of rainfall in the two rainy seasons (Behr and Röhricht, 2000). The lake water lies below the surface and surface water is usually only found around the edges of the crystalline deposits where thermal springs feed the lake (Tindall, 1980).

There are no Permanent Rivers entering Lake Magadi basin and solutes are supplied mainly by a series of alkaline springs with temperatures as high as 86 °C. The springs are located around the perimeter of the lake. Where the salinity is low enough and the temperatures are not too high, these peripheral lagoons support a thriving colony of fish *Tilapia grahami*, which can tolerate a pH of 10.5 and temperature of 39 °C. In some saline lagoons, probably in the absence of Tilapia, mass accumulations of

microorganisms may be observed (Tindall, 1988). The crystalline trona deposits of the lake itself are coloured off-white, red/orange, or red/purple. Closer examination of the surface trona deposits show that under appropriate conditions, a visible microbial stratification occurs which resembles stromatolitic formations found in other benthic saline environments (Tindall, 1980). The lower layer of the lake is a region of degrading organic matter rendered black by sulfate reduction (Behr and Röhricht, 2000). In summary, in terms of water chemistry and mineralogy, Lake Magadi is an example of a typical alkaline saline lake at the stage of maximum evaporite productivity. It is located in the rain shadow of mountains with a large catchment area.

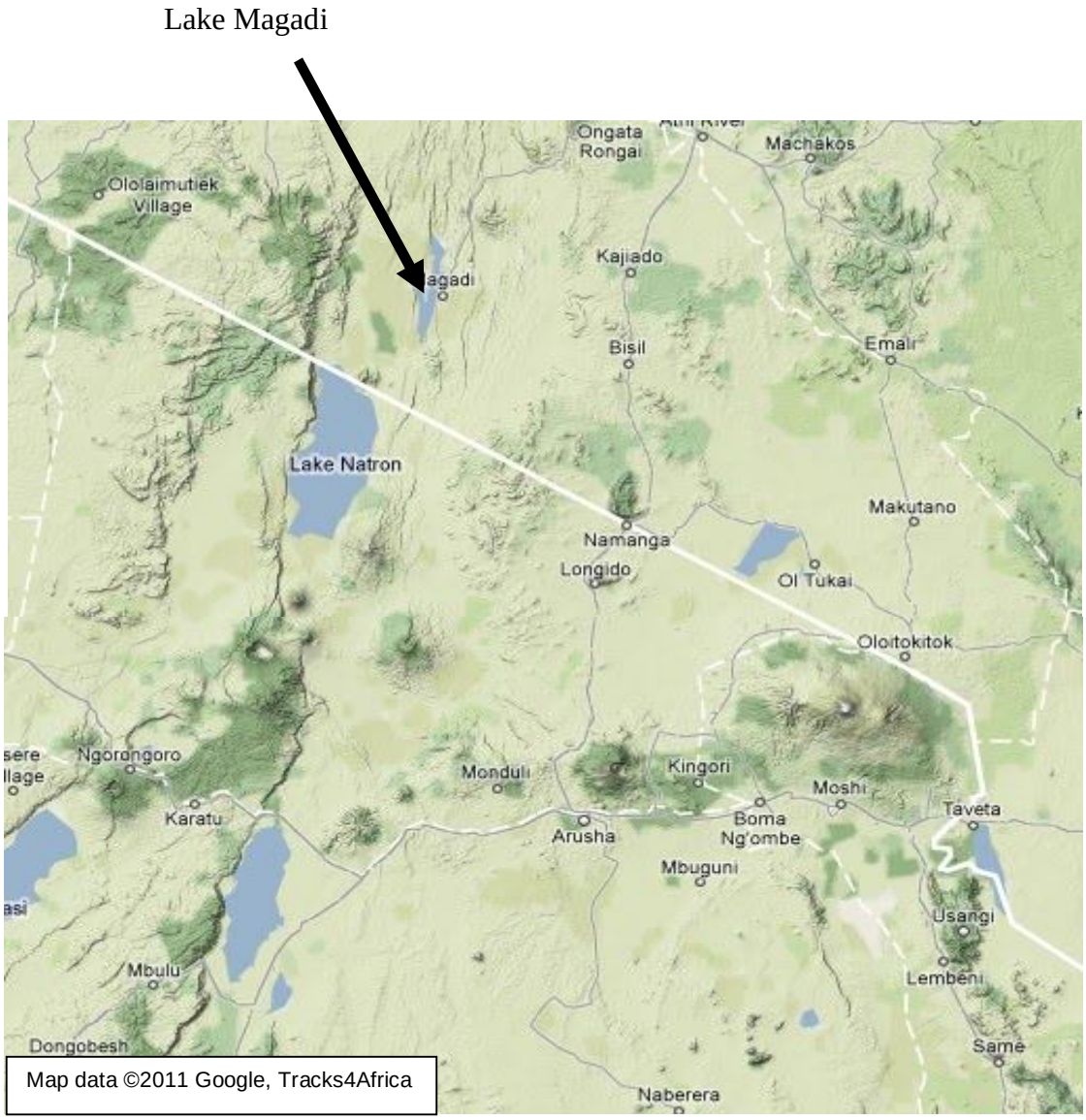

Figure 3.1: A Google map showing the location of Lake Magadi

Figure 3.2a: Sampling point station 1 (hot springs, (48 °C))

Figure 3.2b: Sampling point station 2 (39.4 °C)

3.2 Sample collection

Sediment, salt, water samples and microbial mats were collected on 10th and 11th February, 2010 in Lake Magadi at three points that differed in alkalinity levels. The samples were collected randomly at each station in sterile bottles, preserved in dry ice and transported to the laboratory at JKUAT, Kenya. Once in the laboratory, the samples were divided into two sets. One set was preserved at -80 ^{o}C while the other was used for work on the isolation, characterization and screening of the bacterial isolates.

3.3 Isolation and culturing of bacteria

100µl of the sample diluents prepared from various sample types and sterile distilled water were inoculated on Kenya Minimal Medium for aerobes (KMM1), which was based on MM5 medium (Boga *et al.*, 2003). KMM1 medium contained NaCl [1.7g], KCl [6.5 g], $MgCl_2.6H_2O$ [0.50 g], $CaCl_2.2H_2O$ [0.10 g], NH_4Cl [5.6 g], $NaSO_4$ [1.0 g], and KH_2PO_4 [1.0 g] per litre. The medium was supplemented with yeast extract

and casamino acids (each 0.1 %; Difco, Detroit, MI, USA) and autoclaved. After the medium had cooled, the following were added from sterile stock solutions; 1M Na-Phosphate buffer [40 ml; pH 7.0], SL 11 [2 ml], Se/W solution [2 ml], 7-Vitamin solution [2 ml], Folic acid [2 ml; 50 mg/l], Riboflavin [2 ml; 50 mg/l], Branched chain VFAs [2 ml; 25 mM] and Lipoic acid [2 ml; 1 mM]. Media with additional substrates glucose and gelatin, each 0.1 % w/v was prepared and solidified with agar (1.5 % w/v). The diluents were also inoculated in differential solid media supplemented with different sodium chloride concentrations (NaCl is a Na^+ source for halophiles); 0 %, 5 %, 10 %, 20 % and 30 % and pH 7, 8.5 and 10; and in natural media prepared using lake water. Cycloheximide (0.01 mg) was added to all the media to prevent fungal growth. Cultures were incubated at 30 ^oC for up to seven days to allow adequate growth for the various fast and slow growing isolates. Individual colonies which grew on the plates were re-streaked onto KMM1 media where they had grown best, while others were re-inoculated in freshly prepared LB-Kanamycin agar (Fluka). Different colonies were selected based on morphology and restreaked several times in LB-Kanamycin agar to obtain pure cultures. Microbial cultures were stocked in the isolation medium supplemented with 20 % glycerol and kept at -80 °C (Demain and Davies, 1999).

3.4 Characterization and Identification of bacterial Isolates

Colonial morphologies of the isolates were described using standard microbiological criteria, with special emphasis on pigmentation, colour, shape, size and form. These

characteristics were described for cultures grown at optimum temperature, pH, and salt concentration.

Preliminary characterization by Gram staining was done (using safranin) of each of the isolates using the method of Dussault (1955) and observed under a light microscope at ×100 (Keast *et al*., 1984). The Gram staining technique was used to categorize the isolates into Gram negative and Gram positive (Cappuccino and Sherman, 2002).

3.4.1 Physiochemical characterization of bacterial isolates

3.4.1.1 Growth at different sodium chloride concentration

The ability of isolates to grow at different sodium chloride concentrations was determined using LB Media supplemented with NaCl: 0 %, 5 %, 10 %, 15 %, 20 % and 30 % sodium chloride and 1 % sodium carbonate. The media was inoculated with each of the bacterial isolates and incubated at 30 ^{o}C, 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.

3.4.1.2 Growth at various temperatures

Bacteria, as a group of organisms, exist over a wide range of temperatures. However, individual species can only exist within a narrower spectrum of temperatures as it

normally influences the rate of chemical reactions through its action on cellular enzymes (Cappuccino and Sherman, 2002). The aim of the experiment was to determine the optimum temperature requirements for growth of the isolates. LB solid Media at pH 7.0 was prepared, sterilized and dispensed in sterile petri dishes. Each batch was inoculated with the isolates and incubated at temperatures 20, 25, 30, 35, 40, 45 and 50 ^{o}C. Growth of isolates was checked 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.

3.4.1.3 Effect of pH on growth of the isolates

Growth and survival of microorganisms is greatly influenced by the pH of the environment, and all bacteria and other microorganisms differ as to their requirements. Each species has the ability to grow within a specific pH range, which may be broad or limited, with the most rapid growth occurring within a narrow optimum range (Cappuccino and Sherman, 2002). The aim of the experiment was to determine the optimum pH requirements for the isolates. LB solid Media was prepared and pH was adjusted to 5, 7, 8.5 and 10 using 1 M HCl and 1 M NaOH. This was sterilized and dispensed in sterile Petri dishes. Each medium was inoculated with ten isolates and incubated at 30 ^{o}C. Growth of isolates was checked 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.

3.5 Screening for production of enzymes

Bacterial isolates were screened for their ability to produce extracellular enzymes i.e. amylases, proteases, xylanases, lipases and cellulases. The ability of the isolates to utilize substrates such as starch, xylan, cellulose, CMC, olive oil, and skimmed milk indicate 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 (Castro *et al.,* 1993, Cappuccino and Sherman, 2002).

3.5.1 Determination of amylolytic activity

The isolates were cultured on basal media (1 % KH_2PO_4, 0.0 1 % $MgSO_4.7H_2O$, 0.005 % $CaCl.2H_2O$, 4 % NaCl and 1 % Na_2CO_3) supplemented with 1 % starch (Merck), as the sole carbon source **(**Horikoshi, 1971**).** The medium was then inoculated by the spotting of 10 isolates per plate prior to incubation at 30 ^oC. After 48 h the plates were flooded with iodine solution ((Sigma–Aldrich) Cappuccino and Sherman, 2002). Clear halos around the colonies indicated extracellular amylase production while negative results were indicated by blue black colour all over the plate (Castro *et al.,* 1993).

3.5.2 Determination of the of xylanolytic activity

The isolates were cultured on basal media (1 % KH_2PO_4, 0.01 % $MgSO_4.7H_2O$, 0.005 % $CaCl_2.2H_2O$, 4 % NaCl and 1 % Na_2CO_3) supplemented with 1 % xylan (Fluka) as the sole carbon source, by the method described by (Lee and Lee, 1997). The medium was then inoculated with the isolates and incubated for 48 hours at 30 °C. These were flooded with 1 % Congo red dye. The dye was then replaced with NaCl (1 M) and subsequently rinsed with distilled water. The plates were observed for halos around the colonies, as indication of positive polymer degradation.

3.5.3 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 % $MgSO_4.7H_2O$, 0.00 5% $CaCl_2.2H_2O$, 4 % NaCl and 1 % Na_2CO_3) supplemented with 1 % cellulose and 1 % CMC separately. Each medium was then inoculated by spotting of 10 isolates per plate followed by incubation for 48 hours at 30 ^{o}C. The plates were then flooded with 1 % Congo red dye. The dye was then replaced with NaCl (1 M) and subsequently rinsed with distilled water. The plates were observed for halos around the colonies, as indication of positive polymer degradation.

3.5.4 Determination of lipolytic/ esterase activity

The isolates were cultured on basal media (1 % KH_2PO_4, 0.0 1% $MgSO_4.7H_2O$, 0.005 % $CaCl_2.2H_2O$, 4 % NaCl and 1 % Na_2CO_3) supplemented with 1 % olive oil

(domestic grade) as the sole carbon source. The medium was then thereafter inoculated by the spotting of 10 isolates per plate and incubated for at least 48 hours at 30 ^{o}C. The media was observed for zones of precipitation of calcium crystals around each isolate. Positive isolates for lipase/esterases production were indicated by the precipitation of calcium crystals around the colonies.

3.5.5 Determination of the proteolytic activity

For the determination of proteolytic activity, skimmed milk was used following the method of (Lee *et al*., 2005). The isolates were cultured on basal media (1 % KH_2PO_4, 0.01 % $MgSO_4.7H_2O$, 0.005 % $CaCl_2.2H_2O$, 4 % NaCl and 1 % Na_2CO_3) supplemented with 1 % skimmed milk. The medium was then inoculated by the spotting of 10 isolates per plate, incubated at 30 ^{o}C and observation for zones of clearing after 48 hours. Positive isolates for protease production exhibited a zone of proteolysis as demonstrated by clearing zones (Cappuccino and Sherman, 2002).

3.6 Screening the bacterial isolates for 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 in *vitro* plate assays. Each bacterial isolate was cultured onto antibiotic production broth media that contained the following; mannitol (5.0 g Fluka), soya bean flour (5.0 g), dry yeast (0.9 g), agar (10.0

g) and distilled water (1 L). The isolates were incubated at 30 ^{o}C 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 30°C for 24 hours.

McFarland standards of both 0.5 and 1.0 concentrations were made using barium chloride and sulphuric acid. These were used to check on the turbidity of the cells of both test organisms and the isolates under investigation. 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 Mueller Hinton agar (Fluka) seeded with the test organisms. These were incubated for 24 - 48 hours at 30 ^{o}C after which the results were recorded. The positive control consisted of commercial Kanamycin antibiotic (1 mg/ml) while negative control consisted of un-inoculated plate. Inhibition activity was evaluated visually by scoring for inhibition of growth of test bacteria on the plates (Fatope *et al.*, 2000, Fatope, 1995).

3.7 Molecular characterization of bacterial isolates

All the isolates showing positive results for any of the substrates or the production of antibiotics were selected for this analysis. Pure subcultures of the selected isolates were inoculated in 20 ml of freshly prepared LB broth and incubated for 24 hours in a shaker incubator at 30 ^{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 mycelial pellet was 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 were added 5µl of

Lysozyme (20 mg/ml) and 5 µl of RNAse A (20 mg/ml), 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) was added and contents were mixed by inverting the eppendorf several times. 10 µl of Proteinase K (20 mg/ml) was added, mixed gently and incubation at 50 ^{o}C for 1 hour. Extraction followed the phenol/chloroform method (Sambrook *et al*., 1989). Presence of DNA was checked on 1 % agarose and visualized under ultraviolet by staining with ethidium bromide. The genomic DNA was used as templates for subsequent PCR amplification.

3.8 PCR amplification of 16S rRNA genes

Total DNA from each isolate was used as a template for amplification of the 16S rRNA genes. Nearly full-length 16S rRNA gene sequences were PCR-amplified using bacterial primer pair 8F forward 5'-AG (A/G) GTTTGATCCTGGCT-3') and 1492R reverse, 5'-CGGCTACCTTGTTACGACTT-3' (Sigma) according to the position in relation to *Escherichia coli* gene sequence (Embley and Stackebrandt, 1994; Lane, 1991). Amplification was performed using Peqlab primus 96 PCR machine. Amplification was carried out in a 40µl mixture containing 5µl of PCR buffer (×10), 3µl dNTP's (2.5mM), 1µl (5 pmol) of 8F forward primer, 1µl (5pmol) of 1492R reverse primer, 0.3µl *Taq* polymerase, 1.5µl of template DNA and 28.2µl of water. The control contained all the above except the DNA template. Reaction mixtures were subjected to the following temperature cycling profiles repeated for 35 cycles: Initial activation of the enzyme at 96 ^{o}C for five minutes, denaturation at 95 ^{o}C for 30 seconds, primer annealing at 53 ^{o}C for 30 seconds, chain extension at 72 ^{o}C for 1.0

minute and a final extension at 72 ^{o}C for 10 minutes (Roux, 1995). Amplification products (5µl) were separated on a 1 % agarose gel in 1× TBE buffer and visualized under ultraviolet by staining with ethidium bromide (Sambrook *et al.*, 1989).

3.9 Purification of PCR products

The PCR products were purified using the QIAquick PCR purification Kit protocol (Qiagen, Germany) according to manufacturer's instructions.

3.10 Phylogenetic data analysis

Partial sequences were generated at the sequencing facility at ILRI, (BecA-ILRI Hub Services, SegoliP) sequencing facility using the primer 1492R. The 16S rDNA gene sequences of the selected fifty five isolates were compared to the sequences in the public databases using Basic Local Alignment Search Tool (BLAST) in the National Centre for Biotechnology Information (NCBI) website (htt://www.ncbi.nih.gov). Alignment was done using CLASTAL W 1.6 software. The 16S rDNA gene sequences with high similarity to those determined in the study were retrieved and added to the sequences from this study. Sequencing alignment was done using Mega 4 (Tamura *et al*., 2007).

Phylogenetic data was analyzed using neighbor joining method (Saitou and Nei, 1987; Tamura *et al.,* 2004) and Maximum composite Likelihood method using Mega 4 (Tamura *et al*., 2007) and Bootstrap analysis using Mega 4 (Felsenstein, 1985).

CHAPTER FOUR

4.0: RESULTS

4.1 Physical characteristics at sampling site

The physical parameters during sampling of the lake are presented below **(Table 4.1)**.

Table 4.1: Summary of sampling parameters for water samples from Lake Magadi during the sampling period

Parameter	**Average**
pH	9.8 ± 0.42
Total Dissolved Solids (TDS)	2.94 ± 4.9mg/l
Temperature (T)	45.27 ± 6.29°C
Dissolved Oxygen (DO)	7.53 ± 8.54mg/l
Conductivity (C)	8.74 ± 10.22 ms

Sampling Stations (N) = 3; pH range = 9.49 -10.28; TDS = 0.02-8.6 mg/l; T =39.4 - 51.9°C; DO =2.6 - 17.4 mg/l; C = 0.04 - 20 ms.

Sediment, salt, water, foam and microbial mat samples were collected from three sampling points and coded **(Table 4.2)**.

Table 4.2: Sample types collected from the three sampling stations of Lake Magadi and their description

Sampling station	Sample type	Code
1	Salt	S11
	Microbial mat (hot springs- 44.5 °C)	S12
	Microbial mat (Hot springs - biofilm 48 °C)	S13
	Microbial mat (Lake Surface near the hot springs)	S14
	Mud (sediment) in the lake	S15
	Mud (sediment) outside the lake)	S16
	Water	S17
2	Salt	S21
	Foam (on the water surface)	S22
	Water	S23
	Mud (sediment) outside the lake)	S24
	Mud from below water column (in the lake)	S25
3	Sediment (salt)	S31
	Water	S32

4.2 Isolation of bacteria

The inoculated plates were incubated at 30 ^{o}C and observations were made as from day 4 of growth **(Plate 4.1a-c)**.

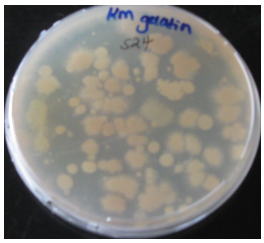

Plate 4.1a: A photograph showing a KMM+gelatin medium culture plate with different colonies before isolation of individual colonies. There is low diversity with a few colonies.

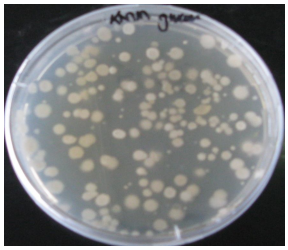

Plate 4.1b: A photograph showing a KMM+glucose medium culture plate with different colonies before isolation of individual colonies. There is low diversity.

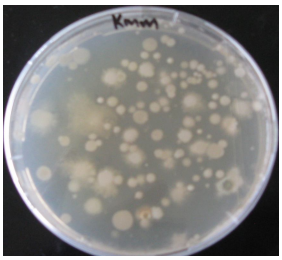

Plate 4.1c: A photograph showing a KMM1 medium culture plate with different colonies before isolation of individual colonies. There is high diversity.

A total of a hundred and thirty nine pure isolates were obtained from the three sampling stations of Lake Magadi. After testing their ability to hydrolyze various substrates, 78 isolates that could utilize most of the substrates used were selected for characterization. Among these, 55 isolates exhibited antimicrobial activity against the test organisms used and these were investigated further by molecular characterization.

4.3 Morphological characterization of isolates

4.3.1 Colony and Cell Morphology

Morphological characterization was based on classical macroscopic techniques of color, form, shape, and elevation of pure colonies. Most colonies were able to grow within 4-5 days of incubation at 30 $^{\circ}$C. The colony morphology of the isolates obtained from Lake Magadi ranged from circular, entire, flat and filamentous. They were smooth and the colour ranged from white to cream, with dark brown, reddish brown and light brown in pigmentation. 80 % of the isolates were Gram positive while 20 % were Gram negative. The cells ranged from long rods; short rods while others were filamentous **(Table 4.3)**.

Table 4.3: Morphological Characteristics of the isolates

	Colony Characterization				Cell characterization	
Strain	**Colony colour**	**Colony form**	**Colony elevation**	**Colony margin**	**Cell arrangement**	**Gram reaction**
A1	Dark brown	Irregular	Raised	Entire	Filamentous	+
A2	Brown	Spindle	Flat	Undulate	Rods	+
A3	Dark brown	Circular	Flat	Undulate	Rods	+
A4	Brownish black	Irregular	Flat	Undulate	Rods	+
A5	White	Circular	Flat	Entire	Filamentous	+
A6	Reddish brown	Spindle	Flat	Undulate	Rods	+
A7	Cream	Irregular	Flat	Undulate	Rods	+
A8	Cream	Irregular	Flat	Undulate	Rods	+
A9	Light brown	Circular	Flat	Undulate	Rods	+
A10	Brown	Spindle	Flat	Entire	Cocci	+
A11	Cream	Irregular	Flat	Undulate	Rods	+
A12	Cream	Circular	Flat	Entire	Filamentous	+
A13	Cream	Circular	Flat	Undulate	Rods	+
A14	Cream	Spindle	Flat	Undulate	Rods	+
A15	Cream	Circular	Flat	Undulate	Rods	+
A16	Cream	Circular	Flat	Undulate	Rods	+
A17	Cream	Irregular	Flat	Undulate	Rods	+
A18	Cream	Irregular	Flat	Undulate	Rods	+
A19	Cream	Irregular	Flat	Entire	Rods	-
A20	Cream	Circular	Flat	Undulate	Filamentous	+
A21	Brown	Spindle	Flat	Undulate	Rods	+
A22	Cream	Circular	Flat	Undulate	Rods	+
A23	Cream	Irregular	Flat	Undulate	Rods	-
A24	Cream	Circular	Flat	Undulate	Rods	+
A25	Cream	Circular	Flat	Undulate	Cocci	+
A26	Brown	Irregular	Flat	Undulate	Filamentous	+
A27	Cream	Irregular	Flat	Entire	Cocci	+
A28	Cream	Circular	Flat	Undulate	Cocci	+
A29	Cream	Circular	Flat	Undulate	Rods	+
A30	Cream	Irregular	Flat	Undulate	Rods	+
A31	Cream	Circular	Flat	Undulate	Rods	-
A32	Cream	Spindle	Flat	Undulate	Rods	+
A33	Cream	Irregular	Flat	Undulate	Rods	+
A34	Cream	Circular	Flat	Undulate	Rods	+
A35	Brown	Spindle	Flat	Undulate	Rods	+
A36	Cream	Irregular	Flat	Undulate	Rods	+
A37	Cream	Irregular	Flat	Undulate	Cocci	+
A38	Cream	Irregular	Flat	Undulate	Rods	+
A39	Cream	Irregular	Flat	Undulate	Rods	+
A40	Brown	Spindle	Raised	Entire	Filamentous	+
A41	Brown	Spindle	Raised	Entire	Filamentous	+
A42	Brown	Irregular	Flat	Undulate	Rods	+
A43	Cream	Irregular	Flat	Undulate	Filamentous	+
A44	Brown	Irregular	Flat	Undulate	Filamentous	+
A45	Cream	Irregular	Flat	Undulate	Rods	+
A46	Brown	Spindle	Flat	Undulate	Cocci	-
A47	Cream	Circular	Flat	Entire	Filamentous	+
A48	Cream	Irregular	Flat	Undulate	Rods	+
A49	Brown	Irregular	Flat	Undulate	Rods	-
A50	Cream	Irregular	Flat	Undulate	Filamentous	+
A51	Dark brown	Circular	Flat	Undulate	Rods	+
A52	Cream	Irregular	Flat	Undulate	Rods	+
A53	Cream	Irregular	Flat	Undulate	Rods	+
A54	Brownish black	Spindle	Flat	Undulate	Filamentous	+
A55	Cream	Irregular	Flat	Undulate	Filamentous	+

4.4 Physiochemical characterization of isolates

4.4.1 Growth at different sodium chloride concentration

The isolates showed varied tolerance to different concentrations of sodium chloride. The number of isolates that grew increased with increase in salt concentration from 0 % up to 5 % sodium chloride, where the highest growth was recorded followed by 10 % and gradually decreased towards 20 % with minimal growth at 30 % **(Table 4.4)**. Growth was scored using four levels of positive sign (+), whereby one positive (+) indicated minimal growth, two positives (+ +) indicated average growth, and three positives (+ +) indicated satisfactory growth while four positives (+ + + +) indicated excellent growth **(Plate 4.2)**.

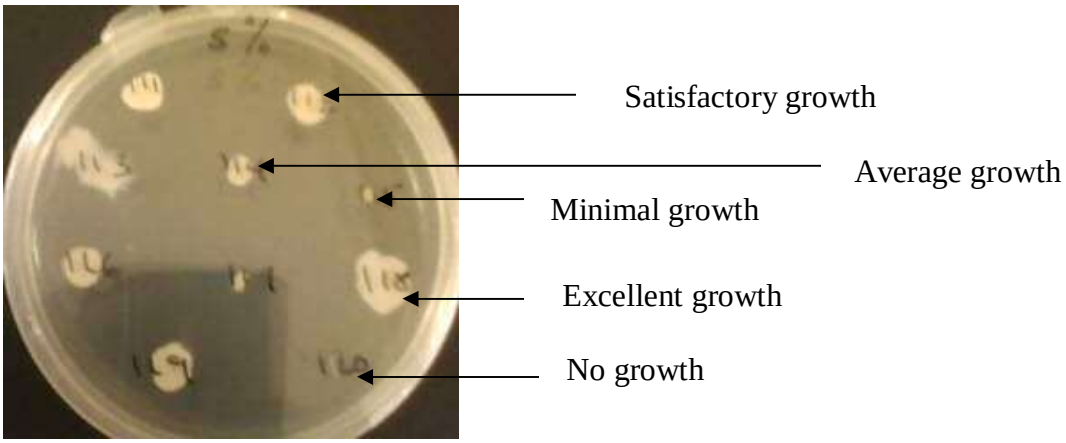

Plate 4.2: Bacterial isolates growth as scored at 5 % NaCl concentration

.**Table 4.4:** Growth of isolates from Lake Magadi at different salt concentrations

Isolates	0 %	5 %	10 %	20 %	30 %
A1	+ +	+ + + +	+ + +	+	+
A2	+ +	+ + +	+ + +	+	+
A3	+ + +	+ + +	+ + +	+	+
A4	+ + +	+ + +	+ + +	+	+
A5	+ + +	+ + +	+ + +	+	+
A6	+ + +	+ + + +	+ + +	+	+
A7	+ + +	+ + +	+ + +	+	+
A8	+ + +	+ + +	+ + +	+	+
A9	+ + +	+ + + +	+ + +	+	+
A10	+	+ + ++	+ + +	+ +	+
A11	+ + +	+ + +	+ + +	+	+
A12	+ + +	+ + + +	+ + +	+	+
A13	+ + +	+ + +	+ + +	+	+
A14	+ + +	+ + +	+ + +	+	+
A15	+ + +	+ + +	+ + +	+	+
A16	+ + +	+ + +	+ + +	+	+
A17	+ + +	+ + +	+ + +	+	+
A18	+ + +	+ + +	+ + +	+	+
A19	+ +	+ + + +	+ + +	+	+
A20	+ + +	+ + +	+ + +	+	+
A21	+ + +	+ + +	+ + +	+	+
A22	+ + +	+ + + +	+ + +	+	+
A23	+ + +	+++	+ + +	+	+
A24	+ +	+ + +	+ + +	+	+
A25	+ +	+ + + +	+ + +	+	+
A26	+ + +	+ + +	+ + +	+	+
A27	+ + +	+ + +	+ + +	+	+
A28	+ + +	+ + +	+ + +	+	+
A29	+ + +	+ + + +	+ + +	+	+
A30	+ +	+ + +	+ + +	+	+
A31	+ +	+ + +	+ + +	+	+
A32	++ +	+ + +	+ + +	+	+
A33	+ +	+ + + +	+ + +	+	+
A34	+ + +	+ + +	+ + +	+	+
A35	+ + +	+ + + +	+ + +	+	+
A36	+ +	+ + + +	+ + +	+	+
A37	+ +	+ + + +	+ + +	+	+
A38	+ +	+ + +	+ + +	+	+
A39	+ +	+ + +	+ + +	+	+
A40	+ +	+ + + +	+ + +	+	+
A41	+ + +	+ + + +	+ + +	+	+
A42	+ +	+ + +	+ + +	+	+
A43	+ + +	+ + + +	+ + +	+	+
A44	+ + +	+ + +	+ + +	+	+
A45	+ +	+ + + +	+ + +	+	+
A46	+ +	+ + +	+ + +	+	+
A47	+	+ + +	+ + +	+	+
A48	+ + +	+ + ++	+ + +	+	+
A49	+ +	+ + + +	+ + +	+ +	+
A50	+ +	+ + + +	+ + +	+	+
A51	+	+ + +	+ + +	+	+
A52	+ +	+ + +	+ + +	+	+
A53	+ + +	+ + ++	+ + +	+	+
A54	+ + +	+ + +	+ + +	+	+
A55	+ +	+ + + +	+ + +	+	+

4.4.2 Growth at different temperature

All the isolates grew at wide range of temperatures including room temperature (25 ^{o}C - 50 ^{o}C). The optimum growth of the isolates was observed between 30 – 45 ^{o}C **(Plate 4.3)**. However, good growth was observed even at 50 ^{o}C **(Table 4.5)**.

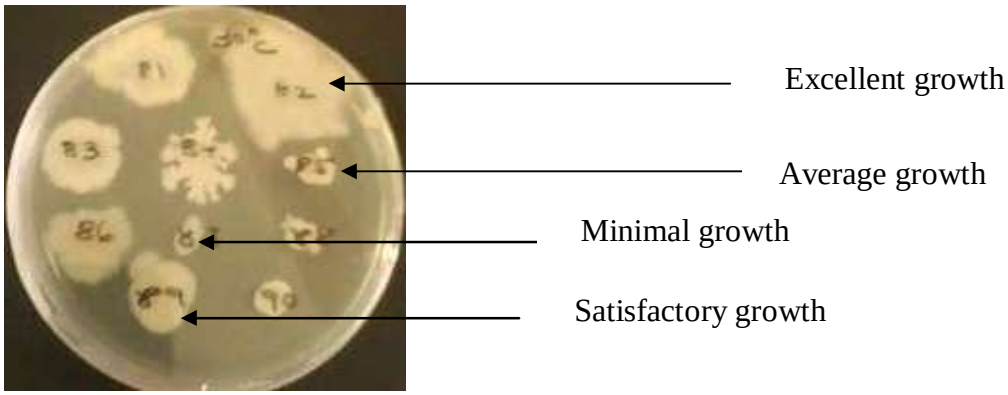

Plate 4.3: Bacterial isolates growth as scored at 30° C

Table 4.5: Growth of the isolates from Lake Magadi at different temperature levels

Isolates	25 °C	30 °C	35 °C	40 °C	45 °C	50 °C
A1	+ +	+ + +	+ + +	+ + +	+ +	+ +
A2	+ + +	+ + +	+ + +	+ + +	+ +	+ +
A3	+ + +	+ + +	+ + +	+ + +	+ +	+ +
A4	+ + +	+ + +	+ + +	+ +	+ +	+ +
A5	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
A6	+ +	+ + +	+ + +	+ + +	+ + +	+ + +
A7	+ + +	+ + +	+ +	+ +	+ +	+ +
A8	+ + +	+ + +	+ +	+ +	+ +	+ +
A9	+ +	+ + +	+ + +	+ + +	+ + +	+ + +
A10	+ + +	+ + +	+ + +	+ + +	+ + +	+ +
A11	+ + +	+ + +	+ + +	+ + +	+ +	+ +
A12	+ + +	+ + +	+ + +	+ + +	+ +	+ +
A13	+ + +	+ + +	+ + +	+ + +	+ +	+ +
A14	+ + +	+ + +	+ + +	+ + +	+ + +	+ +
A15	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
A16	+ + + +	+ + +	+ + +	+ + +	+ + +	+ + +
A17	+ +	+ + +	+ + +	+ + +	+ +	+ +
A18	+ + +	+ + +	+ +	+ +	+ +	+ +
A19	+ + +	+ + +	+ + +	+ + +	+ +	+ +
A20	+ + +	+ + +	+ + +	+ + +	+ +	+ +
A21	+ + +	+ + +	+ + + +	+ + + +	+ + + +	+ + + +
A22	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
A23	+ +	+ + +	+ + +	+ + +	+ + +	+ +
A24	+ + +	+ + +	+ + +	+ +	+ +	+ +
A25	+ + +	+ + +	+ +	+ +	+ +	+ +
A26	+ + +	+ + +	+ + + +	+ + +	+ + +	+ + +
A27	+ +	+ + +	+ + +	+ + +	+ + +	+ + +
A28	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
A29	+ +	+ + +	+ + +	+ + +	+ + +	+ + +
A30	+ + +	+ + +	+ + +	+ +	+ +	+
A31	+ + +	+ + +	+ +	+ +	+ +	+ +
A32	+ + +	+ + +	+ +	+ +	+ +	+ +
A33	+ + +	+ + +	+ +	+ +	+ +	+
A34	+ + +	+ + +	+ + + +	+ + + +	+ + + +	+ + +
A35	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
A36	+ + +	+ + +	+ + +	+ +	+ +	+ +
A37	+ + +	+ + +	+ + +	+ +	+ +	+ +
A38	+ + +	+ + +	+ +	+ + +	+ + +	+ + +
A39	+ + +	+ + +	+ +	+ + +	+ + +	+ + +
A40	+ + +	+ + +	+ +	+ +	+ +	+ +
A41	+ + +	+ + +	+ + +	+ +	+ +	+ +
A42	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
A43	+ +	+ + +	+ + +	+ +	+ +	+ +
A44	+ + +	+ + +	+ +	+ +	+ +	+ +
A45	+ + +	+ + +	+ + +	+ + +	+ +	+ +
A46	+ +	+ + +	+ + +	+ + +	+ + +	+ + +
A47	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
A48	+ +	+ + +	+ + +	+ + + +	+ + + +	+ + +
A49	+ + +	+ + +	+ + +	+ +	+ +	+
A50	+ +	+ + +	+ + +	+ + +	+ +	+ +
A51	+ + +	+ + +	+ + +	+ + +	+ +	+
A52	+ + +	+ + +	+ +	+ +	+ +	+ +
A53	+ +	+ + +	+ + +	+ + +	+ + +	+ +
A54	+ +	+ + +	+ +	+ + +	+ + +	+ + +
A55	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +

4.4.3 Growth at varied pH

Although the isolates were from an alkaline environment, they were able to grow at wide range of pH, including acidic pH 5 and neutral pH **(Plate 4.4)**. There was growth at pH 5 for most of the isolates and this was observed to increase with increase in pH. The highest overall growth was observed at pH10 **(Table 4.6)**.

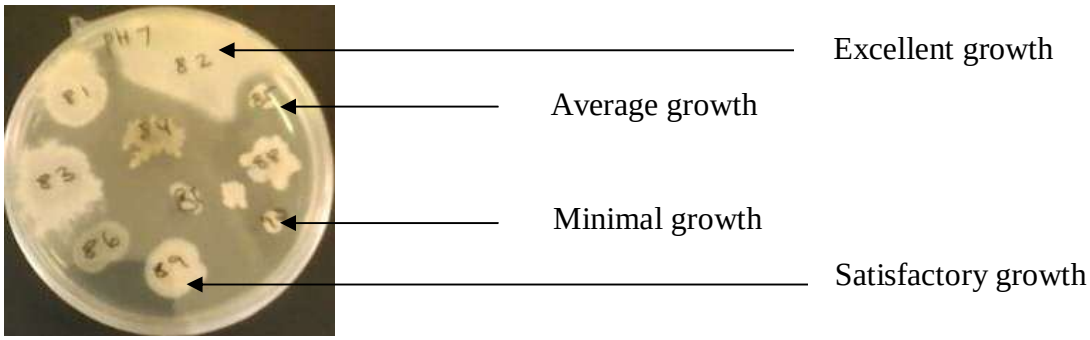

Plate 4.4: Bacterial isolates growth as scored at pH 7

Table 4.6: Growth of the isolates from Lake Magadi at varied pH

Isolates	pH 5.0	pH 7.0	pH 8.5	pH 10.0
A1	+ +	+ +	+ + +	+ + + +
A2	+ +	+ +	+ + +	+ + +
A3	+ +	+ + +	+ + + +	+ + + +
A4	+ +	+ +	+ + +	+ + +
A5	+ +	+ + +	+ + +	+ + + +
A6	+ +	+ + +	+ + +	+ + + +
A7	+ +	+ +	+ +	+ + +
A8	+ +	+ +	+ + +	+ + +
A9	+ + +	+ + +	+ + +	+ + + +
A10	+ +	+ +	+ +	+ +
A11	+ +	+ + +	+ + +	+ + + +
A12	+ +	+ + +	+ + +	+ + + +
A13	+ + +	+ + +	+ + + +	+ + + +
A14	+	+ + +	+ + +	+ + +
A15	+ +	+ + +	+ + +	+ + +
A16	+ + +	+ + +	+ + +	+ + +
A17	+ +	+ +	+ + +	+ + +
A18	+ +	+ + +	+ + +	+ + + +
A19	+ + +	+ + +	+ + +	+ + +
A20	+	+ +	+ + +	+ + +
A21	+ +	+ + +	+ + +	+ + +
A22	+ + +	+ + +	+ + +	+ + +
A23	+ +	+ + +	+ + +	+ + + +
A24	+ +	+ + +	+ + +	+ + +
A25	+ +	+ + +	+ + +	+ + +
A26	+ +	+ + +	+ + +	+ + +
A27	+ +	+ +	+ + +	+ + +
A28	+ +	+ + +	+ + +	+ + +
A29	+ +	+ + +	+ + +	+ + + +
A30	+ +	+ + +	+ + +	+ + + +
A31	+ +	+ +	+ +	+ + +
A32	+	+ +	+ + +	+ + +
A33	+ +	+ +	+ + +	+ + + +
A34	+ + +	+ + +	+ + + +	+ + + +
A35	+ +	+ +	+ + +	+ + +
A36	+ +	+ +	+ + +	+ + +
A37	+ +	+ +	+ + +	+ + +
A38	+ +	+ +	+ + +	+ + +
A39	+ +	+ + +	+ + +	+ + +
A40	+ +	+ + +	+ + +	+ + +
A41	+ +	+ + +	+ + +	+ + +
A42	+	+ +	+ + +	+ + + +
A43	+ +	+ +	+ + +	+ + + +
A44	+ +	+ + +	+ +	+ + +
A45	-	+ +	+ +	+ + +
A46	+ +	+ + +	+ + +	+ + +
A47	+ +	+ +	+ +	+ + +
A48	+ + +	+ + +	+ + +	+ + +
A49	+ +	+ +	+ + +	+ + +
A50	+	+ +	+ +	+ + +
A51	+ +	+ +	+ + +	+ + + +
A52	+ +	+ +	+ + +	+ + +
A53	+	+ +	+ + +	+ + +
A54	+ +	+ + +	+ + +	+ + + +
A55	+ +	+ + +	+ + +	+ + +

4.5 Screening the isolates for production of extracellular enzymes

All the 55 isolates selected for molecular characterization hydrolyzed starch. This was indicated by clear halos around the isolate. The isolates also exhibited a zone of proteolysis as demonstrated by clearing zones and these were positive for protease production. 16 isolates were positive for production of xylanase, 17 isolates produced Carboxymethylcellulase, 22 isolates produced cellulase and 29 isolates produced esterase and lipase **(Table 4.7)**. The **Plate 4.5a – e** represents some isolates from Lake Magadi grown on basal media and showing positive results for xylan utilization hence production of xylanase; cellulose utilization hence production of cellulase; CMC utilization hence production of hemicellulases and olive oil utilization hence the production of esterase and lipase enzymes respectively.

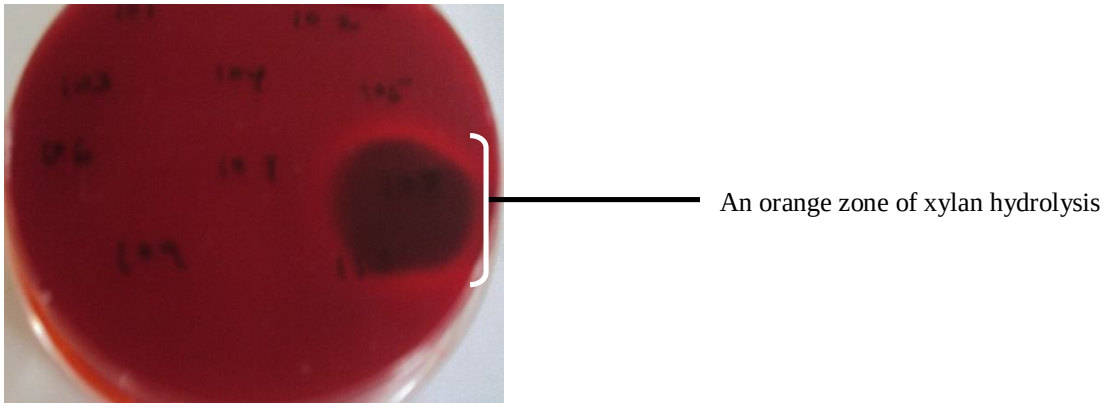

Plate 4.5a: Hydrolysis of Xylan by isolate A1.

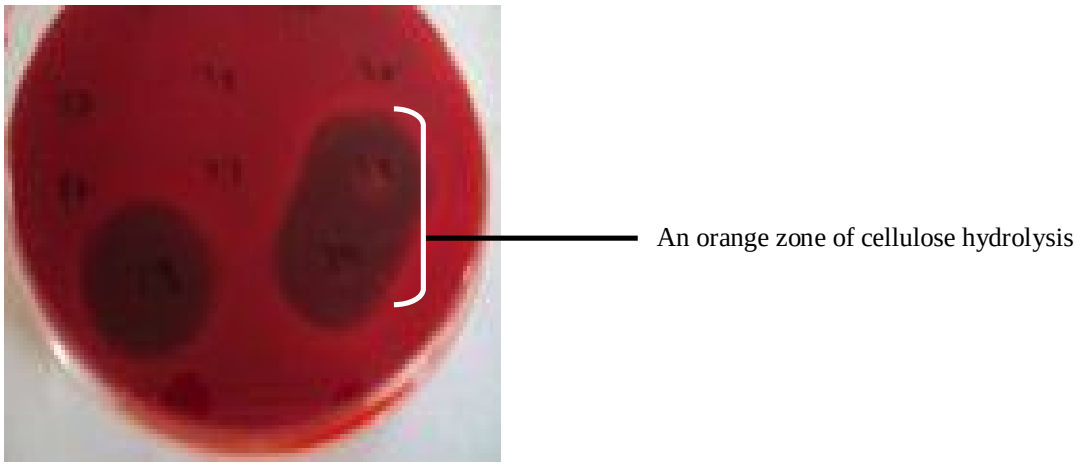

Plate 4.5b: Hydrolysis of cellulose by isolates A1, A2 and A5.

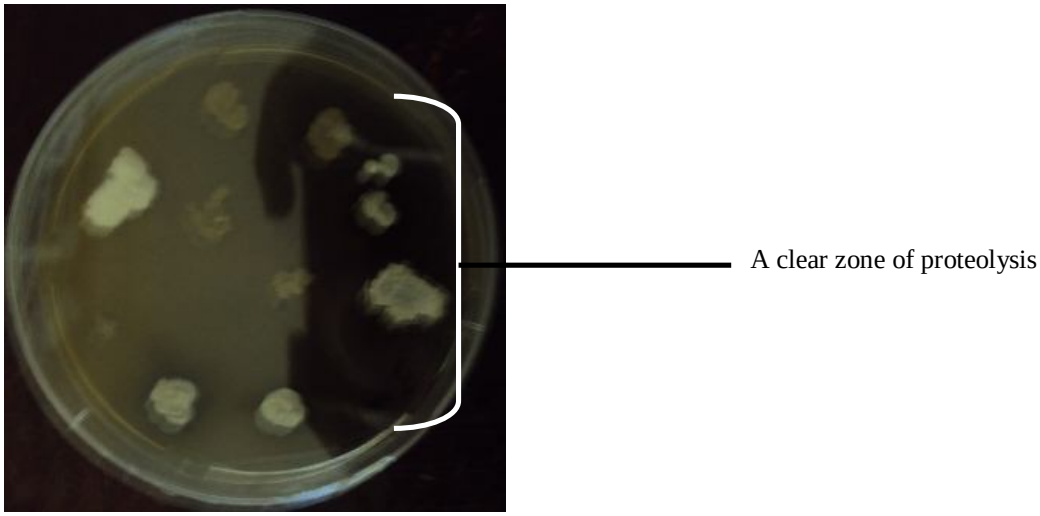

Plate 4.5c: Hydrolysis of skim milk by isolates A10 and A12.

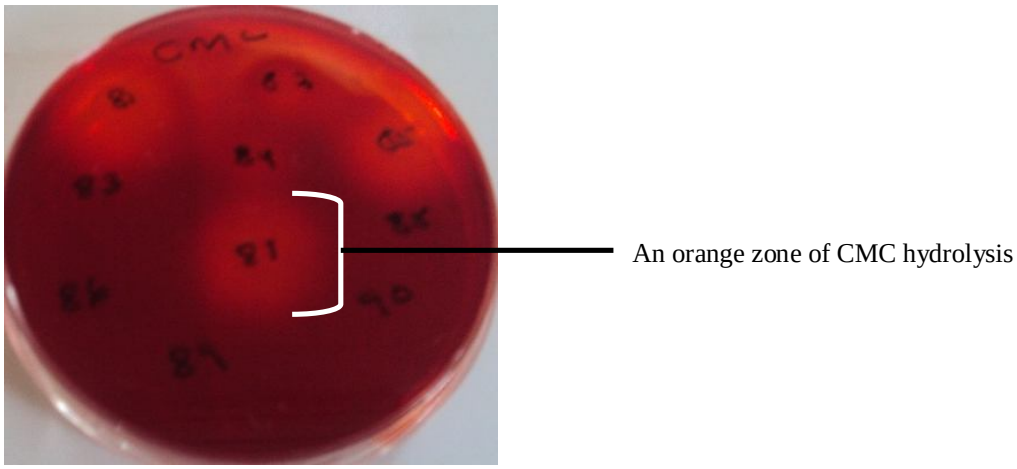

Plate 4.5d: Hydrolysis of CMC by isolates A6, A19 and A33.

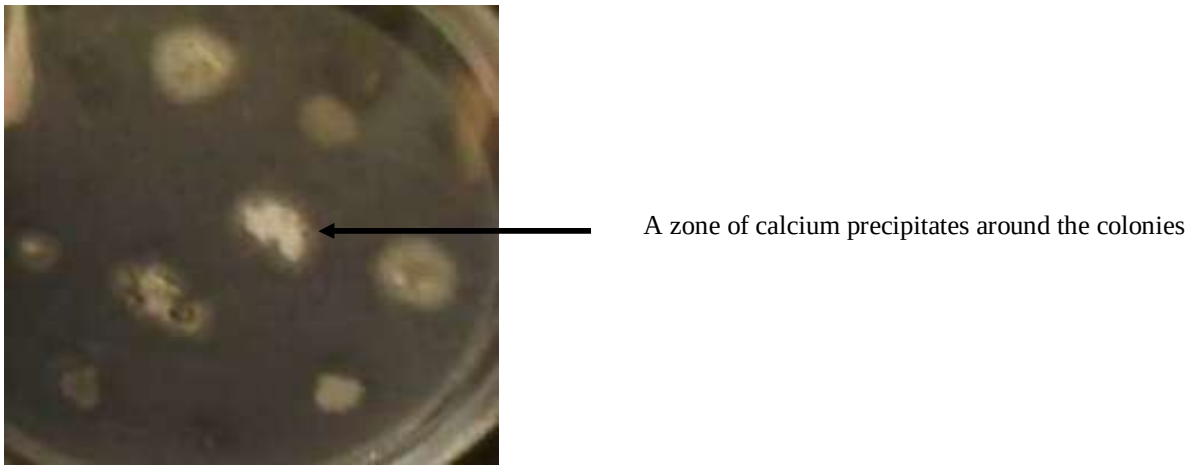

Plate 4.5e: Hydrolysis of olive oil by isolates A1, A 15, A19 and A33.

Table 4.7: Isolates from Lake Magadi that produced enzymes

Isolates	Protease	Amylase	Xylanase	CMC	Cellulase	Esterase & lipase
A1	+	+	+	+	+	+
A2	+	+	-	-	+	+
A3	+	+	-	-	-	+
A4	+	+	-	-	-	+
A5	+	+	-	-	+	-
A6	+	+	+	+	+	+
A7	+	+	-	-	-	-
A8	+	+	+	+	+	+
A9	+	+	-	-	-	-
A10	+	+	-	-	-	-
A11	+	+	-	-	-	-
A12	+	+	-	-	+	-
A13	+	+	-	-	-	-
A14	+	+	-	-	-	-
A15	+	+	-	-	-	+
A16	+	+	-	-	-	-
A17	+	+	-	-	-	-
A18	+	+	-	-	-	-
A19	+	+	+	+	+	+
A20	+	+	-	-	-	+
A21	+	+	-	-	-	+
A22	+	+	-	-	+	-
A23	+	+	-	-	-	-
A24	+	+	-	-	-	-
A25	+	+	-	-	-	-
A26	+	+	+	+	+	+
A27	+	+	-	-	-	-
A28	+	+	-	-	-	-
A29	+	+	-	-	-	+
A30	+	+	-	-	-	-
A31	+	+	-	-	-	-
A32	+	+	+	+	+	+
A33	+	+	+	+	+	+
A34	+	+	-	-	-	-
A35	+	+	-	-	-	+
A36	+	+	-	-	+	+
A37	+	+	-	-	-	-
A38	+	+	-	-	-	-
A39	+	+	-	-	-	+
A40	+	+	+	+	+	+
A41	+	+	+	+	+	+
A42	+	+	-	+	+	+
A43	+	+	+	+	-	+
A44	+	+	+	+	+	+
A45	+	+	-	-	-	-
A46	+	+	-	-	-	+
A47	+	+	+	+	+	+
A48	+	+	+	-	-	-
A49	+	+	+	+	+	+
A50	+	+	-	-	-	+
A51	+	+	+	+	+	+
A52	+	+	-	-	+	+
A53	+	+	+	+	+	+
A54	+	+	+	+	+	+
A55	+	+	-	-	-	-

4.6 Screening of isolates for antimicrobial activity.

The isolates were tested for their antagonistic activity against test bacteria and fungi, to determine the level of antagonism of each isolate against the individual test organisms. Out of the 55 isolates, 54 isolates tested positive for antibiotic activity against *Staphylococcus aureus* (NCTC 10788) **(Plate 4.6a)** with inhibition zones ranging from 15-30 mm apart from isolate A11 that showed no inhibitory effect **(Table 4.8)**. Out of the 55 isolates, 32 isolates tested positive against *Pseudomonas aeruginosa* (ATCC 27853) **(Plate 4.6b)** with inhibition diameters ranging from 5-12 mm **(Table 4.8)**. 18 isolates were active against *Bacillus subtilis* (ATCC 55732) **(Plate 4.6c)** with inhibition diameters ranging from 6-12 mm **(Table 4.8)**. 22 isolates were active against *Escherichia coli* (NCTC 10418) **(Plate 4.6d)** with inhibition diameters ranging from 6-12 mm **(Table 4.8)**. None of the isolates had antimicrobial activity against *Candida albicans* (ATCC 90028) **(Table 4.8)**. Those isolates that were not active against test organisms had no growth, hence allowing growth of test organisms on Mueller Hinton agar plate.

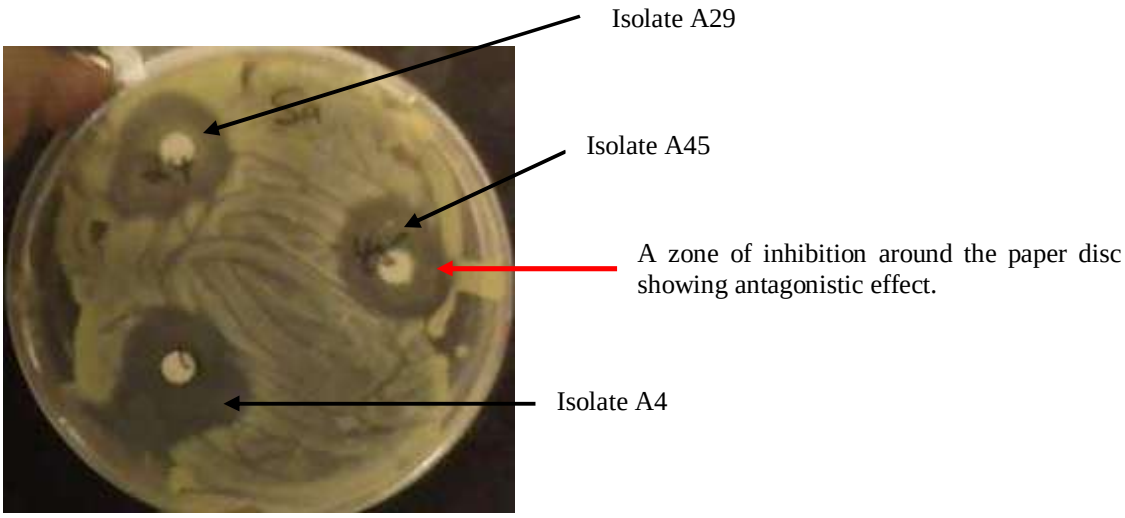

Plate 4.6a: Antagonistic activity of isolates A4, A29 and A45 against *S. aureus* (NCTC 10788).

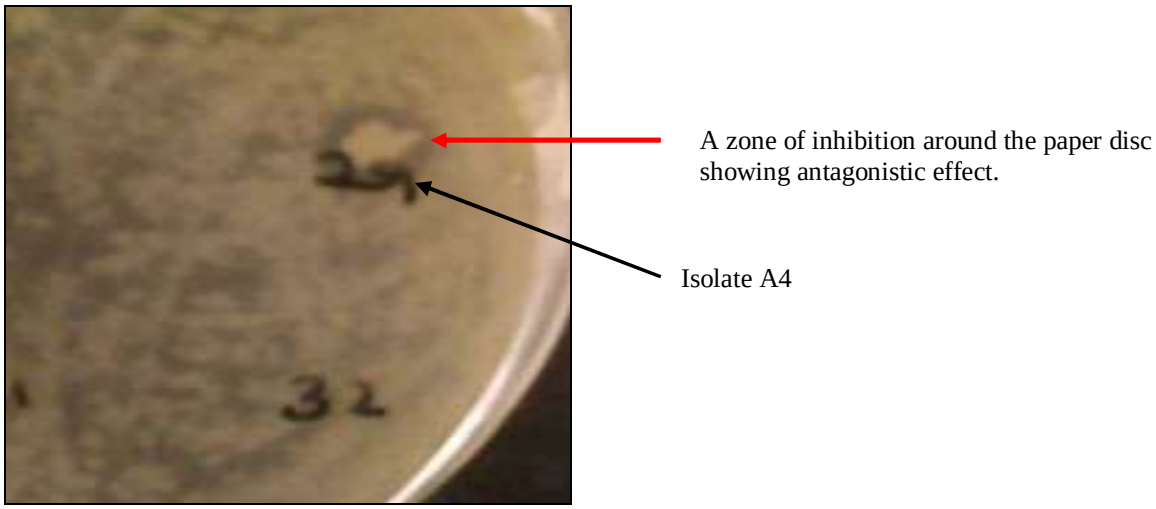

Plate 4.6b: Antagonistic activity of isolate A29 against *P. aeruginosa* (ATCC 27853)

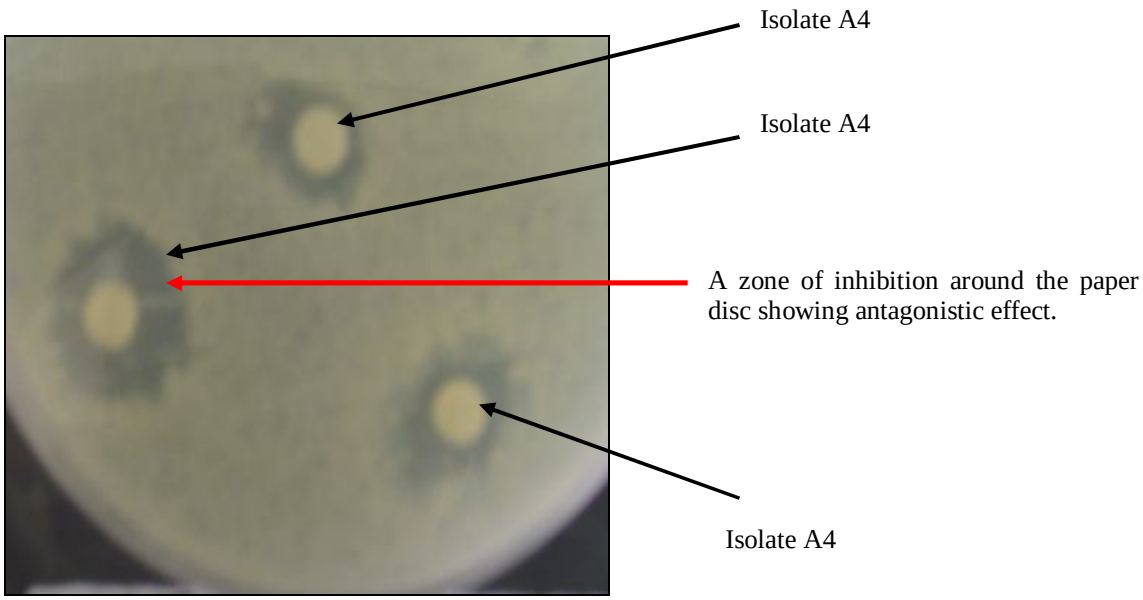

Plate 4.6c: Antagonistic activity of isolates A5, A9 and A23 against *B. subtilis* (ATCC 55732)

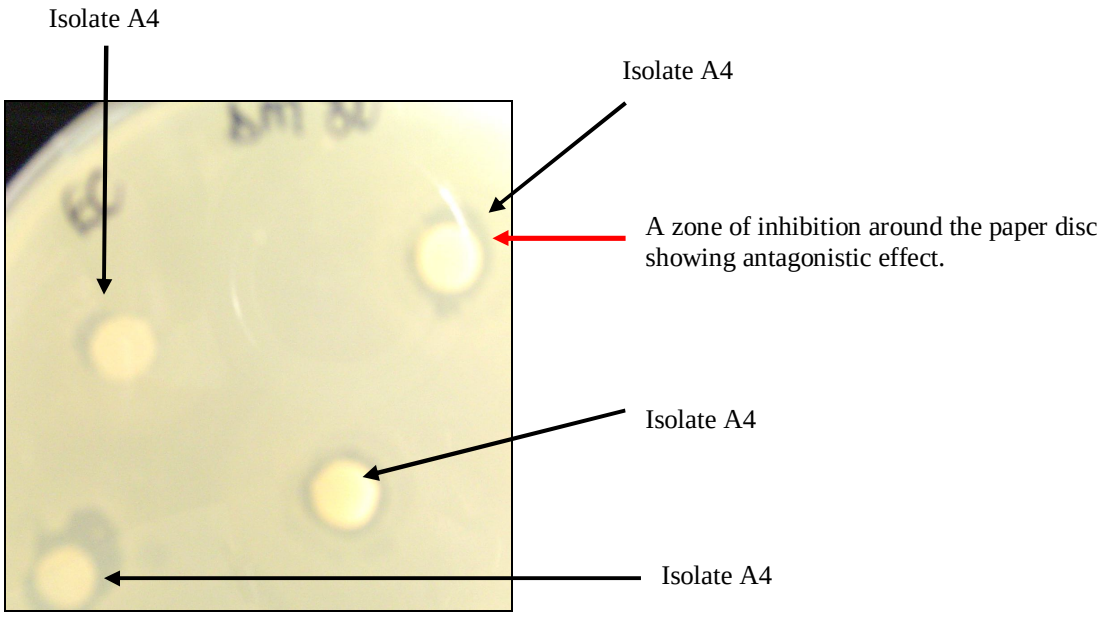

Plate 4.6d: Antagonistic activity of isolates A1, A10, A13 and A23 against *E.coli* (NCTC 10418)

Table 4.8: Screening of bacterial isolates from Lake Magadi for antimicrobial activity

Isolates	*E. coli*	*P. aeruginosa*	*B. subtilis*	*S. aureus*	*C. albicans*
A1	+	+	-	+	-
A2	+	+	-	+	-
A3	+	+	-	+	-
A4	-	+	-	+	-
A5	-	-	+	+	-
A6	+	+	+	+	-
A7	+	+	-	+	-
A8	-	+	-	+	-
A9	-	+	+	+	-
A10	+	+	+	+	-
A11	-	+	+	-	-
A12	-	+	-	+	-
A13	+	+	+	+	-
A14	+	+	-	+	-
A15	-	+	-	+	-
A16	-	-	-	+	-
A17	-	-	-	+	-
A18	-	+	-	+	-
A19	-	+	-	+	-
A20	-	-	-	+	-
A21	-	-	-	+	-
A22	-	-	-	+	-
A23	+	+	+	+	-
A24	-	-	-	+	-
A25	-	-	-	+	-
A26	+	+	+	+	-
A27	+	-	-	+	-
A28	+	-	-	+	-
A29	-	-	-	+	-
A30	-	-	-	+	-
A31	-	+	-	+	-
A32	-	-	+	+	-
A33	-	-	-	+	-
A34	+	+	-	+	-
A35	-	-	+	+	-
A36	-	+	-	+	-
A37	-	+	-	+	-
A38	-	-	-	+	-
A39	-	-	-	+	-
A40	+	+	+	+	-
A41	+	+	+	+	-
A42	+	+	+	+	-
A43	+	+	+	+	-
A44	+	+	+	+	-
A45	-	-	-	+	-
A46	-	-	-	+	-
A47	+	+	+	+	-
A48	-	-	-	+	-
A49	-	+	-	+	-
A50	+	-	-	+	-
A51	+	+	-	+	-
A52	-	-	-	+	-
A53	-	-	-	+	-
A54	+	+	+	+	-
A55	+	+	+	+	-

4.7 Molecular characterization of bacterial isolates

4.8.1 PCR amplification of 16s rRNA gene from isolates

Genomic DNA was extracted from all the selected 55 active isolates. 16S rRNA gene amplification with bacterial specific primers yielded an amplification product of approximately 1600 bp. **(Figure 4.1).**

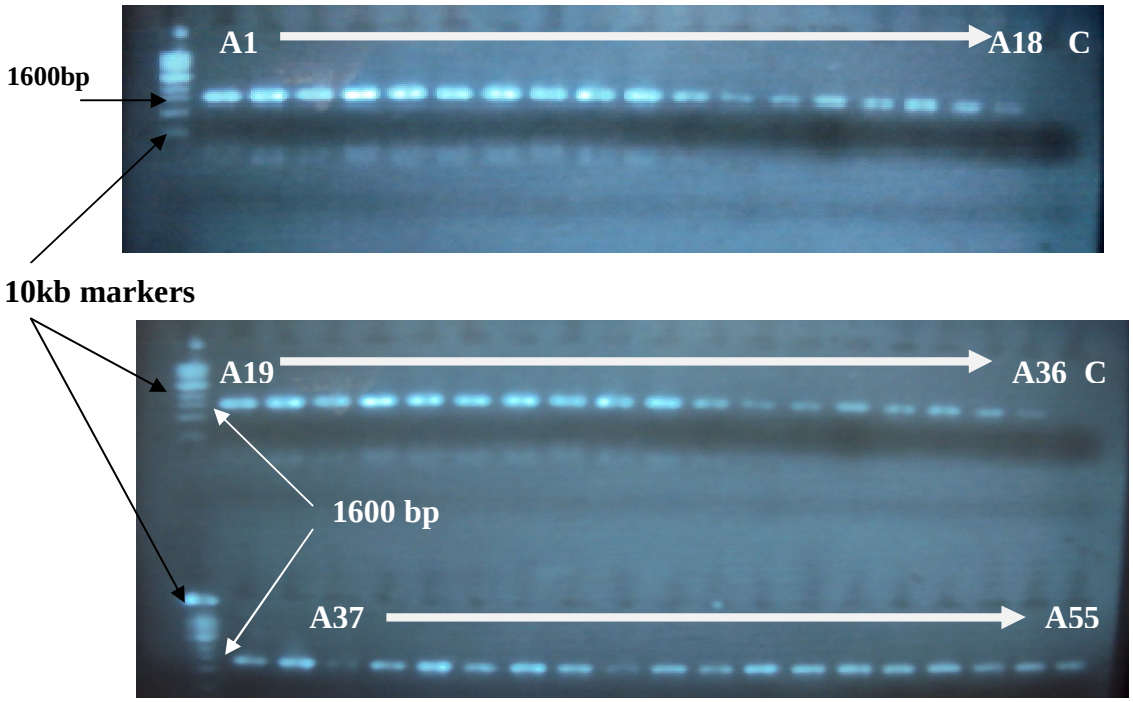

Figure 4.1: A 1 % agarose gel showing PCR amplification of 16S rRNA gene of the 55 bacterial isolates and a control visualized after ethidium bromide staining

C = Control

4.8.2 Phylogenetic analysis of sequences

BLAST analysis of the partial sequences showed that 37 isolates (80 %) were from the genus *Bacillus* within the Firmicutes in the domain bacteria **(Figure 4.2)** with similarities between 91% and 100%. Among these were; *Bacillus subtilis, Bacillus pumilus, Bacillus licheniformis and Bacillus amyloliquefaciens among others* **(Table 4.9).** Nine isolates belonged to the class *Gammaproteobacteria* in the phylum proteobacteria with similarities between 80% and 99 %. The isolates were affiliated to microorganisms belonging to the genus *Klebsiella, Rhodobacter, Stenotrophomonas, Alcaligenes, Brevibacterium, Anoxybacillus, Xenorhabdus,* and *Proteus* **(Table 4.9)**. However, isolates A5, A11, A14, A19 and A30 had sequence similarity of between 95 - 97 % and these could represent novel species. These are highlighted in yellow, while isolates A22, A31, A32 and A47 had sequence similarity of between 80-93 %. These could represent novel genera and are highlighted in red **(Table 4.9)**.

Table 4.9: Blast results of isolates from Lake Magadi and their close relatives.

Sample ID	Station	BP	Acc No.	Next neighbour in BLAST	% Identity
A1	S13	348	HQ222833	*Bacillus Subtilis* strain NB-05	100%
A2	S25	595	HM372880	*Bacillus amyloliquefaciens* strain ZDS-1	99%
A3	S22	862	AY973269	*Bacillus subtilis* strain 233	98%
A4	S15	798	GQ144705	*Bacillus subtilis* strain GSC-3	100%
A5	S13	344	GU191141	*Bacillus sp.* SDNS	96%
A6	S23	528	HQ154645	*Bacillus subtilis* strain 00856	98%
A7	S14	862	GQ407182	*Bacillus* sp. DV9-37	99%
A8	S22	840	HM849728	*Bacillus mucilaginosus* strain s-2	98%
A9	S16	836	HM579804	*Bacillus sp.* HY13(2010)	100%
A10	S23	806	HM854250	*Bacillus subtilis subsp.* inaquosorum KTH-61	99%
A11	S12	298	HQ113218	*Alcaligenes faecalis* strain CL-10.3a	96%
A12	S15	492	GQ169376	*Bacillus subtilis* subsp. spizizenii strain N22	99%
A13	S14	572	DQ520804	*Bacillaceae bacterium* NR88	99%
A14	S32	497	HM055601	*Bacillus licheniformis* strain GD3a	97%
A15	S24	806	EU231615	*Bacillus subtilis* strain TCCC17018	99%
A16	S23	807	EF062991	*Bacillus sp.* JM4	100%
A17	S24	805	EU262980	*Bacillus subtilis* strain XJPL-YB-23	99%
A18	S22	566	GQ153538	*Bacillus subtilis* strain Itb57	98%
A19	S22	452	HM161861	*Stenotrophomonas sp.* strain 2BQN19	95%
A20	S23	682	HM854243	*Bacillus tequilensis* strain KTH-48	99%
A21	S16	594	HM209756	*Bacillus methylotrophicus* strain GRM804	100%
A22	S25	265	FJ430013	*Anoxybacillus sp.* C163a	80%
A23	S24	701	DQ001154	*Rhodobacter capsulatus* strain MT1131	99%
A24	S17	650	EU379282	*Bacillus pumilus* strain 4RS-5b	99%
A25	S22	906	FJ592172	*Bacillus aerophilus* strain BH-11	99%
A26	S23	917	HM849728	*Bacillus mucilaginosus* strain s-2	99%
A27	S17	830	DQ238044	*Bacillus sp.* DCA-5	99%
A28	S15	606	HQ015739	*Staphylococcus sp.* B3RO05	98%
A29	S16	721	FJ620574	*Bacillus subtilis* strain SACs7	99%
A30	S23	441	HQ149694	*Bacillus subtilis* strain AVNM-108	96%
A31	S13	343	FN689724	*Klebsiella pneumoniae*	87%
A32	S12	437	GU125640	*Bacillus amyloliquefaciens* strain	91%
A33	S24	705	HM854255	*Brevibacterium halotolerans* strain KNA-24	99%
A34	S32	459	GU568196	*Bacillus axarquiensis* strain LNXM37	98%
A35	S14	562	HM598464	*Bacillus velezensis* strain Z-26	98%
A38	S13	516	EU257698	*Bacillus subtilis* strain F2	98%
A39	S21	692	HM480326	*Bacillus subtilis* strain WIFD5	98%
A41	S25	654	GQ303178	*Bacillus subtilis* strain AR1	97%
A46	S22	651	EU867352	*Bacillus sp.* CCGE2081	99%
A47	S21	330	GQ246653	*Bacillus sp.* M1T1B7	93%
A48	S14	330	FJ006875	*Bacillus sp.* WPCB033	99%
A49	S24	656	AB507816	*Xenorhabdus hominickii, Proteus mirabilis*	98%
A51	S14	716	GQ867223	*Bacillus sp.* BRAZ7A	99%
A54	S23	461	EU717842	*Bacillus licheniformis* strain X-14	99%
A55	S12	452	EU082292	*Bacillus sp.* LAMI 008	99%

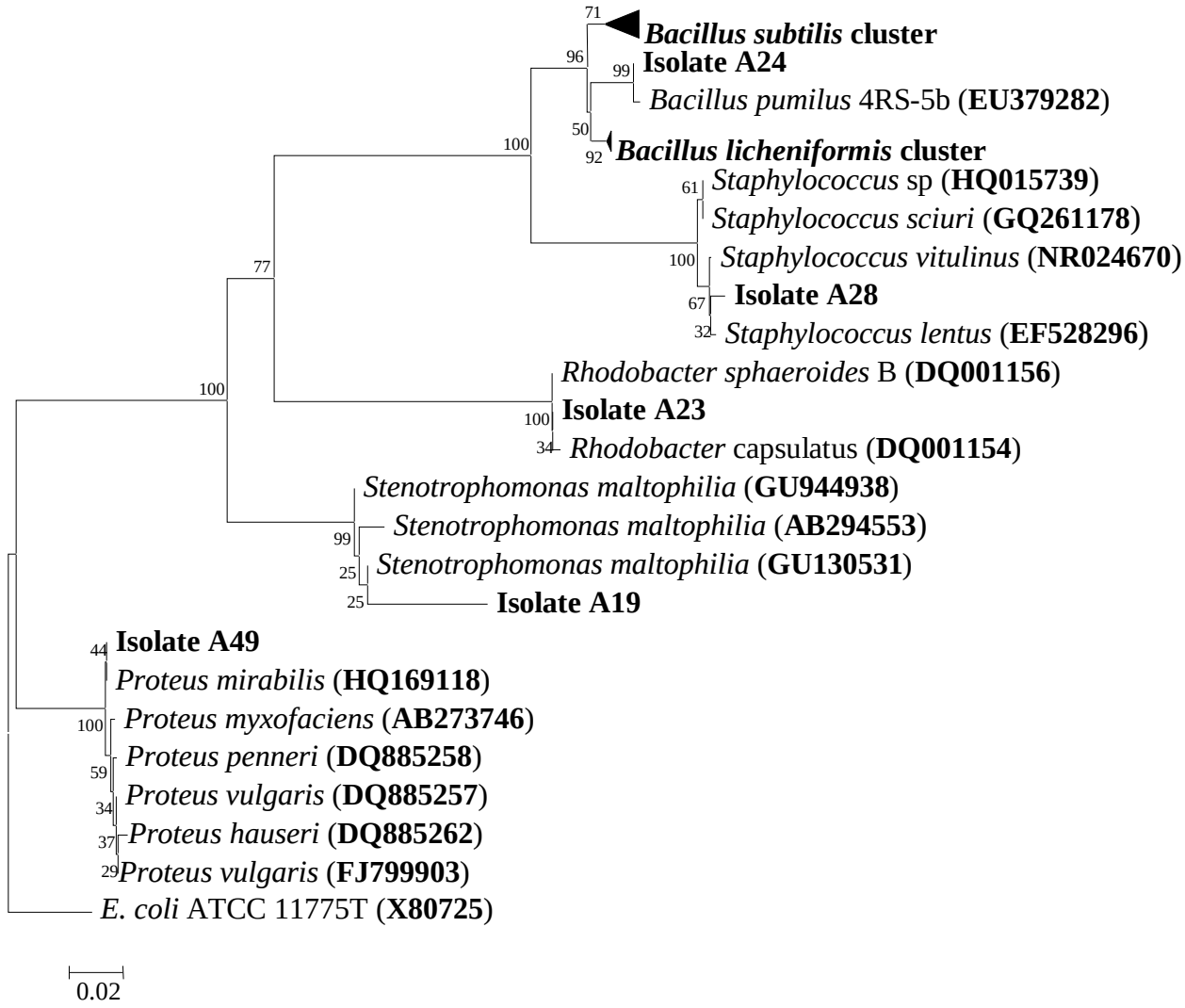

Figure 4.2: Phylogenetic affiliation of the isolates to members of firmicutes and proteobacteria. Isolates are indicated in bold. Only bootstrap values above 50 are shown.

CHAPTER FIVE

5.0: DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 DISCUSSION

The aim of this study was to isolate bacterial groups from Lake Magadi for subsequent characterization using cultural, biochemical and molecular approaches, and assess their biotechnological potential in production of extracellular enzymes and bioactive metabolites.

Physiochemical characterization of the isolates showed that the highest growth was observed at pH 8.5 and 10. However, the isolates were able to grow at pH 5 **(Table 4.6)** which suggests that they are tolerant to acidic conditions (Moreira & Siqueira, 2002). This growth at pH range of 5 to10 concurred with an earlier study by Horikoshi (1998) which showed that a low to high pH range of 5.7 to 9.0, favours growth of alkaliphiles and that a pH range 9.0 to 10 may serve as their selective optimum pH. Organisms that live at the extremes of pH are able to do so by maintaining their cytoplasm at the same pH as their mesophilic relatives, thus obviating the need for evolution of altered internal physiology. Active mechanisms to achieve this may involve secondary proton uptake mediated by membrane-associated antiporters. Passive mechanisms include negatively charged cell wall polymers in alkaliphiles, unusual bioenergetics, unusual permeability properties, positive surface charges, high internal buffer capacity, over expression of H+ export enzymes and unique transporters for acidophiles. Alkaliphiles maintain an internal pH of 7.5 - 8.5,

regardless of the environmental pH. At high pH, alkaliphilic bacteria activate a sodium-ion pump, which begins a process that lowers the internal pH by transporting hydrogen ions into the cell. Alkaliphiles' cell membranes have a special composition that protects them in Lake's highly alkaline conditions (Gilmour, 1990, Douglas, 1996, DeLong and Pace, 2001).

All the isolates grew within temperature range of 25 ^{o}C - 50 ^{o}C. The optimum growth was recorded between 30 ^{o}C - 45 ^{o}C. However, good growth was recorded even at 50 ^{o}C **(Table 4.5)**. The temperatures recorded at sampling points ranged from 39.4 ^{o}C and 51.9 ^{o}C. This growth of isolates at temperatures lower that the study site conditions indicates that the bacterial communities in Lake Magadi are adapted to a wide range of temperatures. It therefore confirms a study on bacterial strains previously isolated from Lake Magadi that clustered with neighbors that grow well at temperature range of 10 ^{o}C - 55 ^{o}C (Baumgarte, 2003).

The isolated alkaliphiles from the Lake Magadi were able to tolerate concentrations of up to 20 % with the optimum sodium chloride concentration being 5 % - 10 % **(Table 4.4)**. Growth of the isolates in culture medium at different sodium chloride levels (varying between 0 % and 30 %) indicated tolerance to salinity and an adaptability of these isolates to adverse growth conditions. This is in agreement with the earlier studies on *Bacillus okuhidensis* and *Bacillus halodurans* (Zhiyu *et al*., 2002). Bacteria can survive for millions of years in fluid inclusions of salt deposits including and evaporates by adaptation to these potentially deadly environments. To prevent loss of water from the cell, halophiles offset the high salt in the environment by accumulating

compounds such as potassium and glycine-betaine. This allows a balance of salts inside and outside of the cell preventing water from flowing outward as would be the case if lower salt levels existed within the cells (Norton and Grant, 1988; Rothschild and Mancinelli, 2001; Mancinelli and Rothschild, 2002). Studies done on isolates from Lake Magadi show that bacterial isolates grow well at sodium chloride concentrations below 5 % and as high as 30 % concentration (Baumgarte, 2003). In this study, growth of the isolates improved with increase in salt concentration from 0 % up to 5 % sodium chloride, where the highest growth was recorded. The isolates showed satisfactory growth at 10 % and gradually decreased towards 20 % with minimal growth at 30 % **(Table 4.4)**.

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

The BLAST results showed that 81 % of the isolates were from the genus *Bacillus* within the Firmicutes in the domain bacteria while others belonged to the class *Gammaproteobacteria* in the phylum Proteobacteria with similarities between 80 % and 100 %. This concurs with an earlier study in Lake Elmenteita, where phylogenetic

analysis of 181 partial 16S rRNA gene sequences with excellent quality showed that the majority of the isolates were affiliated to the class *Gammaproteobacteria* and to the genus *Bacillus* (Mwirichia *et al*., 2010). Other groups that were recovered along with this class of micro organisms in that study were related to *Marinospirillum, Idiomarina, Vibrio, Enterococcus, Alkalimonas, Alkalibacterium, Amphibacillus, Marinilactibacillus* and the actinobacteria *Nocardiopsis* and *Streptomyces* (Mwirichia *et al*., 2010).

In this study, Firmicutes belonging to the Low G + C group were more diverse and abundant than Gram-negative Proteobacteria. This concurs 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).

About 81 % of the isolates in this study were affiliated to micro organisms belonging to the genus *Bacillus.* Among these were; *Bacillus subtilis***,** *Bacillus pumilus, Bacillus licheniformis* and *Bacillus amyloliquefaciens* among others **(Table 4.9)**. *Bacillus* species are among the most commonly found aerobic, eubacterial alkaliphiles in soda lakes as well as selected environments (Guffanti *et al.*, 1980; Horikoshi and Akiba, 1982; Kruwich and Guffanti, 1983, 1986; Takami *et al*., 1999). The isolates formed three clusters of *Bacillus* on the phylogenetic tree namely; *Bacillus subtilis* (thirteen isolates), *Bacillus pumilus* (one isolate) and *Bacillus licheniformis* (six isolates).

Earlier studies grouped *Bacilli* isolated from soda lakes into two clusters of alkaliphiles and alkaline-tolerant isolates based on physiological and biochemical characteristics as well as DNA base composition, hybridization, and 16SrDNA analyses (Nielsen *et al.* 1994, 1995).

Members of the genus *Bacillus* that have been previously isolated from Lake Magadi clustered with *Bacilli* members that are alkalitolerant, obligate alkaliphilic while others grow at pH range of 5 - 10 (Baumgarte, 2003). Some of these include; *Bacillus agaradhaerens, Bacillus clarkia, Bacillus alcalophilus, Bacillus silvestris, Bacillus haloalkaliphilus and Bacillus pseudofirmus.* These were isolated from forest soil, waste water, bauxite waste, garden soil, brine/mud in Wadi Natrun, Lake Bank soil, river bank soil, horse and elephant manure, rotting wood, solar salterns and saline soils. This is an indication that many alkaliphilic or alkalitolerant members of the *Bacilli* are fairly ubiquitous (Baumgarte, 2003).

The genus *Bacillus* has many species that exist; hence the heterogeneity in physiology, ecology and genetics of the genus *Bacilli.* This genus has a vast diversity of physiological types such as degraders of most substrates from plants and animals, antibiotic producers, heterotrophics, nitrifiers, denitrifiers, nitrogen fixers, acidophiles, alkaliphiles, thermophiles and psychrophiles among others (Slepecky, 1972; Norris *et al*., 1981; Claus and Berkeley, 1986). According to the studies of Nakamura *et al*. (1984), the genus has many species and at one time, 145 species made up the genus. There are several validly published new species shown to be genetically and

phenotypically distinct from other *Bacillus* species that have not been described in the *Bergey's* Manual of Systematic Bacteriology.

The isolates A2, A3, A6, A8, A10, A12, A18, A21, A32, A38, A41 and A46 were closely related to a *Bacillus subtilis* cluster which includes; *Bacillus subtilis* strain SACs7 (FJ620574), *Bacillus subtilis* H 10 - 7 (FJ392727), *Bacillus subtilis* subsp. natto strain ZD - 6 (EF660339) and *Bacillus subtilis* strain Itb57 (GQ153538) with 94 - 100 % similarity **(Figure 4.6)**.

B. subtilis produces the proteolytic enzyme subtilisin. *B. subtilis* spores can survive the extreme heat during cooking. *B. subtilis* is responsible for causing ropiness a sticky, stringy consistency caused by bacterial production of long-chain polysaccharides in spoiled bread dough (Madigan and Martinko, 2005). Isolate A24 clustered closely with *Bacillus pumilus* strain 4RS - 5bc (EU379282) and scored 99 % similarity. *B. pumilus* is a ubiquitous Gram-positive, aerobic, rod-shaped endospore-forming bacterium that can be isolated from a wide variety of soils, plants and environmental surfaces, and even from the interior of Sonoran desert basalt (Benardini *et al.*, 2003).

The isolates A7, A13, A16, A27 and A54 clustered closely with *Bacillus licheniformis.* This group include; *Bacillus licheniformis* strain MKU 2 (DQ071561), *Bacillus licheniformis* strain SHL1 (HQ111516), *Bacillus sp.* DV9 - 37 (GQ407182) and *Bacillus sp*. enrichment culture clone (HM1237) with 99 - 100 % similarity. *Bacillus licheniformis* is a rod-shaped, Gram-positive bacterium (Veith *et al.*, 2004). It produces a variety of extracellular enzymes that are associated with the cycling of

nutrients in nature. *B. licheniformis* produces a protease enzyme that can survive at high pH levels (an optimum pH at around 9 and 10). This protease is a desired ingredient in laundry detergent due to its ability to be used in low temperatures, which prevents shrinkage and fading colors. *B. licheniformis* is used to make the antibiotic Bacitracin. Bacitracin is composed of a mixture of the cyclic polypeptides that *B. licheniformis* produces; ironically the purpose of Bacitracin is to inhibit the growth of *B. licheniformis*. Bacitracin lyses the cells of *B. licheniformis* in the presence of cadmium or zinc ions (Snoke and Cornell, 1965). Current research shows that *B. licheniformis* contributes to nutrient cycling and has antifungal activity. Novozymes Biofungicide Green Releaf contains *B. licheniformis* strain SB3086 as an active main ingredient.

Isolates A5, A14 and A30 had members of the genus *Bacillus* as their nearest neighbors in BLAST. They however scored 96-97 % similarity indicating that these could represent new species. Isolate A11 scored 96 %, and had several neighbors with similar percentage similarity such as *Alcaligenes faecalis* strain CL-10.3a, *Streptomyces sp*. VITSVK5, *Achromobacter sp*. DBTN3, *Bordetella* sp. VVAR and uncultured *beta proteobacterium* clone L21. This isolate could represent novel species and should be further researched on **(Table 4.9)**.

Isolate A28 clustered closely with members of the genus *Staphylococcus* with a score of 98 % similarity. The species includes *Staphylococcus sciuri* (GQ261178), *Staphylococcus vitulinus* (NR024670) and *Staphylococcus lentus* (EF528296).

Staphylococcus is a genus of Gram-positive bacteria that appear coccoid under the microscope and form in grape-like clusters. It produces catalase, has an appropriate cell wall structure and G + C content of DNA in a range of 30 - 40 mol %. It has ability to produce coagulase, an enzyme that causes blood clot formation (Ryan and Ray, 2004). This genus includes at least forty species, nine of these have two subspecies and one has three subspecies (Harris and Foster, 2002). Most are harmless and reside normally on the skin and mucous membranes of humans and other organisms. They are a small component of soil microbial flora though they have not been reported from soda lakes (Madigan and Martinko, 2005).

19 % of the isolates from Lake Magadi were affiliated to members of the class *Gammaproteobacteria.* The genera in this class included; *Klebsiella, Rhodobacter, Stenotrophomonas, Alcaligenes, Brevibacterium, Anoxybacillus, Xenorhabdus,* and *Proteus* **(Table 4.9)**. This concurs with earlier studies in soda Lake Elmenteita, where BLAST analysis of the partial sequences shows that 60 % isolates belonged to the class *Gammaproteobacteria.* These were affiliated to the genus *Halomonas, Marinospirillum* and *Idiomarina* species (Mwirichia *et al*., 2010). Another study by Grant (2004); forty cloned sequences were found to be similar to that of known bacterial isolates (>97 % sequence similarity), represented by the species of the genera *Brevundimonas, Comamonas, Alcaligenes, Stenotrophomonas,* and *Klebsiella*. In a cultivation-dependent analysis of microbial diversity in Lake Magadi, the third commonly encountered group of sequence types comprised three isolates that were

closely affiliated with the *Halomonas* group of the gamma subdivision of Proteobacteria (Berendes *et al.*, 1996, Baumgarte, 2003).

In this study, isolate A31 had members of various genera as the nearest neighbors in BLAST with 86 - 87 % similarity **(Table 4.9)**. Some of these include; *Klebsiella pneumoniae, Pantoea agglomerans, Enterobacter sp*. SY36, *Raoultella terrigena* strain, *Salmonella sp*. Co9936, Rainbow trout intestinal bacterium, Nitrogen-fixing bacterium CAN9B, *Citrobacter sp*., *Pantoea stewartii* strain Ast2. This isolate could represent novel genus and should be further investigated on in order to fully identify the organism. It hydrolyzed both skim milk and starch indicating that it was able to produce extracellular proteases and amylases. It had inhibition activity against Gram-positive and Gram-negative bacteria; hence it could have an ability to produce broad spectrum antimicrobial compounds.

Isolate A23 was closely related to the genus *Rhodobacter.* The species include *Rhodobacter sphaeroides* B (DQ001156) and *Rhodobacter capsulatus* (DQ001154) with 99 % similarity. *Rhodobacter sp.* is a rod-shaped, Gram-negative, purple non-sulfur photo heterotrophic bacterium belonging to the α-3 subclass of Proteobacteria. They are found in soil, in anoxic zones of waters, mud, sludge, and in organic-rich water habitats. These are metabolically diverse organisms, able to grow in a wide range of lifestyles including aerobic, anaerobic, anoxygenic, photosynthetic, fermentation as well as diazotrophic growth modes (Blankenship *et al.*, 1995). They have potential for bioremediation, such as detoxifying metal oxides. They also

produce the carbon and reductant storage molecule polyhydroxybutyrate (PHB) that can be used as bioplastic (Choudhary *et al*., 2007). Some of the applications to biotechnology include: production of indole under anoxygenic conditions (Devi *et al*., 2000; Ranjith, *et al*., 2007) and synthesize zinc sulphate nanoparticles (Bai *et al*., 2006). *R. sphaeroides* OU5 is grown on L-tryptophan as sole source of nitrogen in the absence of oxygen. The metabolite has phytohormonal activity and phytotoxicity against tumorous cell lines and also inhibitory activity of cyclooxygenase-2. *R. sphaeroides* is valuable for the extraction of carotenoids. Studies found that carotenoids having antioxidant activity and provitamin A function are able to inhibit various types of cancer and protect from cardiovascular disease and age-related muscular degeneration (Zhenxin *et al*., 2007).

Isolate A49 clustered closely with members of the genus *Proteus* with a score of 98 % similarity **(Table 4.9)**. The species include *Proteus mirabilis* and *Proteus vulgaris. Proteus mirabilis* is a Gram-negative, facultatively anaerobic, rod shaped bacterium that causes 90 % of all '*Proteus*' infections in humans. It can utilize urea and citrate; produce hydrogen sulfide gas, and forms clear films on growth media. It is motile, possesses peritrichous flagella and is known for its swarming ability. Noteworthy is the ability of this species to inhibit growth of unrelated strains resulting in a macroscopically visible line of reduced bacterial growth where two swarming strains intersect (Frénod and Emmanuel, 2006).

Proteus vulgaris is a rod-shaped, Gram-negative bacterium that inhabits the intestinal tracts of humans and animals. It can be found in soil, water and fecal matter. It is grouped with the *Enterobacteriaceae* and is an opportunistic pathogen of humans. It is known to cause urinary tract and wound infections. Like other members of *Enterobacteriaceae* present in alkaline saline environments, members of the *Proteus* genus play an important role in the remineralization of organic matter within the ecosystem. They are the major contributors in the transformation of organic carbon, sulfur, nitrogenous compounds and metals with an important role in food webs and nutrient cycling (Grant *et al*., 1990, Amarja *et al*., 2008).

There are many reports on the isolation and characterization of Gram-negative bacteria from different soda lakes. Studies on the Inner Mongolian soda lake (Ma *et al*., 2004) indicated dominance of Gram-negative bacteria of the, β, γ and δ groups, with a low percentage of Gram-positive bacteria. Similar results were also observed in the case of the hyper alkaline spring waters in Jordan (Pedersen *et al.,* 2004). *Halomonas, Stenotrophomonas, Alcaligenes* and *Paracoccus* have already been reported from different soda lakes (Duckworth *et al*., 1996; Zavarzin *et al*., 1999; Duckworth *et al*., 2000 and Ma *et al*., 2004). The "Xanthomonas group" became of increasing interest since several strains with fundamentally different phenetic characteristics compared with the known plant and human pathogenic genera were isolated from different and sometimes extreme environments and were assigned to this branch based on their 16S rDNA sequences (Finkmann *et al.*, 2000). Among these, chemolithoautotrophic Fe (II)-oxidizing strains were isolated from groundwater (Emerson and Moyer, 1997), an

alkaliphilic *Stenotrophomonas*-like strain was isolated from Lake Natron, Kenya, (Duckworth *et al.*, 1996), and *Stenotrophomonas maltophilia*-like strains were isolated from the gut and faeces of the arthropod *Folsomia candida* (Hoffmann *et al*., 1998). In a previous study on East African soda lakes, Gram-negative lipolytic isolates affiliated with members of the genera *Pseudomonas* and *Stenotrophomonas* have been identified (Jones *et al*., 1994; Duckworth *et al*., 1996; 2000 and Amarja *et al*., 2008). In Lake Elmenteita, *Pseudomonas* / *Stenotrophomonas* strains 45E3 and 97NT4 were identified (Grant, 1992).

Stenotrophomonas maltophilia is an aerobic, non-fermentative, Gram-negative bacterium. It is an uncommon bacterium and human infection is difficult to treat (Gilligan, 2003; Waters *et al*., 2007). Initially classified as *Pseudomonas maltophilia*, *S. maltophilia* was also grouped in the genus *Xanthomonas* before eventually becoming the type species of the genus *Stenotrophomonas* in 1993 (Al-Jasser, 2006). In this study, isolate A19 clustered with members of the genus *Stenotrophomonas* with a score of 95 % similarity **(Table 4.9)**. This isolate hydrolyzed all the six substrates, an indication of its ability to produce the various extracellular enzymes. It had inhibition activity against Gram-positive and Gram-negative bacteria; hence its ability to produce broad spectrum antimicrobial compounds. This isolate could represent novel species within this genus and should be investigated further.

Isolate A22 had *Anoxybacillus sp*. C163a and *Kocuria sp.* M14 as the nearest neighbors in BLAST with 80 % and 81 % similarity respectively **(Table 4.9)**. The

isolate hydrolyzed skim milk, starch and olive oil and had inhibition activity against Gram-positive test bacteria; hence its ability to produce narrow spectrum antimicrobial compounds. This isolate could represent novel genera within the lake's ecosystem.

Isolates A32 and A47 had *Bacillus* genus as the nearest neighbors in BLAST with 80 % and 81 % similarity respectively **(Table 4.9)**. These isolates hydrolyzed all the six substrates indicating their ability to produce the various extracellular enzymes. They had inhibition activity against Gram-positive and Gram-negative bacteria; hence their ability to produce broad spectrum antimicrobial compounds. They however scored very low percentage similarity, hence could represent a new genera within the lake's ecosystem.

The DNA sequences of five isolates (A5, A11, A14 A19 and A 30) showed identity of 95 % - 97 % similarity with the previously known sequences in the GenBank database **(Table 4.9)**. These could represent novel species of organisms within the lake's ecosystem. Four isolates (A22, A31, A32 and A 47) showed identity of 80 % - 93 % similarity, and could represent novel genera of organisms. 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 many biotechnologically important cultures.

Microbial communities in natural alkaline environments such as soda lakes have attracted attention as a possible source of novel enzymes and metabolites for use in biotechnology. Most of the microorganisms isolated are able to produce hydrolytic

enzymes such as lipase, amylase, cellulase, hemicellulase, esterase and proteases at alkaline pH. The stability of these enzymes at alkaline pH is attributed to their habitat (alkaline lake) and growth profile in a wide range of pH. Of the 139 isolates screened in this study, 78 were able to utilize between 1 and 6 of the substrates screened. The 55 isolates selected for molecular characterization hydrolyzed starch and skim milk. This is a characteristic that confirm their role in the decomposition of organic matter in the habitats (Crawford, 1988; Kieser *et al*., 2000). Studies have shown that during vegetative growth and subsequent sporulation, a variety of proteases are produced (Priest, 1977), indicating their role in decomposition of organic matter in nature.

Alkaliphilic micro-organisms of the genus *Bacillus* in particular, have been shown to be of considerable biotechnological importance because of their ability to produce extracellular alkaline enzymes such as protease (Horikoshi, 1971), and amylases (Boyer and Ingle, 1972) that are tolerant to high pH and high temperatures (Hamamoto and Horikoshi, 1992; Nielsen *et al*., 1994). This concurs with findings of other researchers in which peptides have been shown to be by far the most abundant nitrogenous compounds in organic matter (Sowden *et al*., 1976; Schnitzer, 1985). Enzymes produced by *B. subtilis* such as amylase are widely used as additives in laundry detergents. In addition, a strain of *B. subtilis* formerly known as *Bacillus natto* is used in the commercial production of the Japanese food natto (Nielsen *et al*., 1994).

In another study, alkaliphilic bacteria isolated from sediment samples of the alkaline Lonar Lake; *Arthrobacter ramosus* and *Bacillus alcalophilus,* exhibited high protease

activity using soya cake as a sole source of carbon and nitrogen (Kanekar *et al*., 2002). Protease activity was optimum at 1 % initial substrate concentration, at 30 °C and under shake culture condition for both organisms. The enzyme was thermostable at (65 °C), pH 12 and also active in the presence of commercial detergent (Kanekar *et al*., 2002).

Besides starch and skim milk, sixteen (16) of the studied isolates hydrolyzed xylan **(Table 4.7).** This is a characteristic shared with *Bacilli* strain C-125 (JCM9153) and *Bacillus halodurans* (Zhiyu *et al*., 2002). Seventeen (17) isolates hydrolyzed CMC, twenty two (22), cellulose and twenty nine (29), olive oil **(Table 4.7)**. Hydrolysis of the various substrates was an indication of the ability in the various isolates to produce hemicellulases, cellulases, lipases and esterases that are of industrial importance e.g. Cellulases have been introduced as laundry additives.

In this study, isolates A 2 and A 32 clustered closely with *Bacillus amyloliquefaciens* **(Table 4.9)**. *Bacillus amyloliquefaciens* is the source of a natural antibiotic barnase, enzyme amylase used in starch hydrolysis, the protease subtilisin used with detergents and the BamH1 restriction enzyme used in DNA research (Skerman *et al*., 1980). Therefore, microbial communities from Lake Magadi are a probable source of these enzymes as indicated by the enzyme activity test results of this study **(Table 4.7)**.

Microorganisms are a prolific source of secondary metabolites that have anti-bacterial, anti-fungal, anti-tumor or anti-protozoal activities making them a target for isolation in large-scale screening programs in industries. Around 23,000 bioactive secondary

metabolites produced by microorganisms have been reported and are of commercial interest (Bibb, 2005). Many of these secondary metabolites are potent antibiotics (Berdy, 2005). Members of genus *Bacillus* are capable of producing antibiotics as secondary metabolites in the late logarithmic or early stationary phase of growth of batch cultures. About 169 of these secondary metabolites have been produced and recorded. Various strains of *B. subtilis* are known to have produced 68 antibiotics while *Bacillus brevis* have produced 23 known antibiotics (Katz and Demain, 1977). *Bacillus pumilus is* known for its fungicidal activity by production of an anti-fungal compound (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 smegmatis,* and *Candida albicans* (Eltem and Ucar, 1998).

In this study, isolates were studied for the potential to produce antimicrobial compounds. The ability of individual isolates to inhibit the growth of Gram-positive and Gram-negative test organisms was tested. The isolates showed antagonistic activity that differed from one isolate to another. This could be due to the expected different modes of action and activity of the individual biochemical constituents of the respective isolates (Mao *et al.,* 2006).

Fifty four (54) of the studied isolates had inhibitory effects on *Staphylococcus aureus* with inhibition diameters ranging from 15 mm to 30 mm (**Table 4.8**). Isolate A11, a probable novel species that clustered with *Alcaligenes* had no inhibition against *Staphylococcus aureus* but it inhibited growth of *Pseudomonas aeruginosa* and *Bacillus subtilis.* This was consistent with earlier studies that have shown that most of the antibiotics are active against Gram-positive organisms, although there are exceptions (Eltem and Ucar, 1998).

Twenty two (22) of the studied isolates showed antagonistic effect against the Gram-negative *Escherichia coli,* with inhibition diameters of 6 mm to 12 mm (**Table 4.8**). Twenty (20) of these isolates clustered with members of the genus *Bacillus,* (A23) with *Rhodobacter* and (A28) with *Staphylococcus*. Thirty two (32) of the studied isolates had antagonistic effect against *Pseudomonas aeruginosa* with inhibition diameters ranging from 5 mm to 12 mm. Eighteen (18) of the studied isolates had antagonistic effect against *Bacillus subtilis* with inhibition diameters ranging from 6 mm to 12 mm (**Table 4.8**). Sixteen (16) of these isolates clustered with members of the genus *Bacillus*, *Rhodobacter* (A23) and *Alcaligenes* (A11). None of the isolates had antagonistic activity against test fungi, *Candida albicans.*

A study carried out by Foldes *et al*. (2000) reported that, the difference in microbial sensitivity may also be attributed to the experimental conditions. They also stated that, the various forms of antagonism depend on the concentration and the amounts of the active substance(s) present in each individual bacterial isolate, causing a difference in

the levels of antagonism, which could be high in one and low in another. This could also be the probable reason as to why, the bacterial antimicrobial screening activity exhibited different levels of antagonistic activity against the different test organisms.

In addition, seventeen (17) of the studied isolates produced pigments into the media that ranged from dark brown, reddish brown and light brown in colour. This probably was an indication of the isolates ability to produce diverse secondary metabolites (**Table 4.8**).

B. subtilis strain QST 713 (marketed as QST 713 or Serenade) has a natural fungicidal activity, and is employed as a biological control agent. It was popular worldwide before the introduction of consumer antibiotics as an immunostimulatory agent to aid treatment of gastrointestinal and urinary tract diseases (Shylakhovenko, 2003). It is still widely used in Western Europe and the Middle East as an alternative medicine. *B. subtilis* is also used to produce hyaluronic acid, useful in the joint-care sector in healthcare (Mazza, 1994).

In this study, Lake Magadi has been shown to harbour a wealth of diverse microorganisms with probable useful commercial properties. 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 to our understanding of diversity and provides useful information on cultures in this extreme environment.

5.2 CONCLUSIONS

- The study has demonstrated that L. Magadi harbours Alkaliphilic bacteria species. 139 isolates were obtained; 55 isolates were characterized and identified.

- The isolated bacteria grew well at pH ranging from 5 – 10, temperature range of 25 – 50 ^{o}C, NaCl range of 0- 30 % and they utilized a wide variety of sugars as carbon source. The above conditions are therefore to be adopted if the isolates are to be exploited industrially.

- The bacterial isolates hydrolyzed skim milk, starch, Xylan, Cellulose, CMC and olive oil, indicating that they could be a potential source of protease, amylase, xylanase, cellulase, hemicellulase, esterase and lipase enzymes at alkaline pH.

- Isolates showed antimicrobial activity against Gram positive and Gram negative test bacteria *(Staphylococcus aureus, Pseudomonas aeruginosa, Bacillus subtilis* and *Escherichia coli)***.** Six of the nine novel isolates had broad spectrum activity

- Molecular characterization of isolates indicates that all of them belong to domain Bacteria. 80 % of the isolates were affiliated to microorganisms belonging to the genus *Bacillus*, phylum of Firmicutes and 20 % of the isolates were affiliated to members of the class *Gammaproteobacteria*. Firmicutes

belonging to the Low G + C group are more diverse and abundant than Gram-negative Proteobacteria. Five (5) isolates showed identity of 95 - 97 % similarity with the previously known sequences in the GenBank database. These represent novel species of organisms within the lake ecosystem. Four (4) isolates showed identity of 80 -93 % similarity, representing novel genera of organisms within the lake ecosystem.

5.3 RECOMMENDATIONS

- Further research on isolation and characterization of the specific enzymes and antimicrobial compounds produced by these microorganisms is of great importance. This will help to elucidate the structures and biochemical characteristics of any novel enzymes and bioactive metabolites detected.

- Further analysis of bacteria is necessary for complete characterization and identification of more alkaliphilic strains by carrying out full sequencing.

- The novel organisms need to be further confirmed by methods such as fatty acid analyses and DNA-DNA hybridization.
- Modification of protocols to allow the isolation of more diverse genera.

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APPENDICES

Appendix 1: LB-Kanamycin Agar (1 Litre)

10 g Sodium Chloride

10 g Tryptone

5 g Yeast extract

15 g agar

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

Adjust pH to 7.5 with NaOH and autoclave

Cool to 55 ^{o}C

Add 10 ml of 10 mg/ml filter sterilized Kanamycin (antibiotic) and pour on plates.

Appendix 2: 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.

Appendix 3: 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 4: 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)

Appendix 5: Antibiotic production media

Mannitol 5.0 g

Soya bean flour 5.0 g

Dry yeast 0.9 g

Agar 10.0 g

Appendix 6: 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 7: Electrophoresis buffer Working Concentrated stock

TBE buffer 10×

Chemical	**Volume**
Tris	108 g
Boric Acid	55 g
$Na_2EDTA.2H_2O$	9.3 g

Adjust the volume to 1 liter with ddH_2O 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 8: EDTA 0.5 M pH 8.0

Dissolve 186.1 g of disodium ethylenediaminetetra-acetate (EDTA.2H_2O Sigma ED2SS mw 372.2) in 800 ml of ddH_2O. 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 9: Ethidium Bromide l0 ×

Dissolve 1.0 g of EtBr in a final volume of 100 ml ddH_2O. Wrap the bottle in aluminum foil and stir several hours to get a true solution. Store at 4 °C.

To make the 1× stock used to stain gels take 10 ml of the 10× stock and bring to a final volume of 100 ml using ddH_2O. Wrap bottle in aluminum foil and store at room temperature.

Appendix 10: Proteinase K

To 1 ml of ddH_2O add 20 mg of Proteinase K (Promega # 52066). This gives a 20mg / ml stock.

Appendix 11: SDS 10 %

Dissolve 100 g of electrophoresis-grade SDS in 800 ml ddH_2O. Heat the solution to dissolve. Bring to a final volume of 1000 ml using ddH_2O. Do not autoclave.

Appendix 12: 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 ddH_2O and autoclave.

Appendix 13: Tris 1 M p H 7.4

Dissolve 121.1 g of Tris base in 800 ml of ddH_2O and adjust the pH to 7.4 with concentrated HCL. Bring the final volume to 1000 ml with ddH_2O. Divide into 100 ml bottles and autoclave.