ISOLATION AND CHARACTERIZATION OF EXTREME HALOALKALIPHILIC BACTERIA AND ARCHAEA FROM LAKE MAGADI, KENYA.

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Isolation and Characterization of extreme haloalkaliphilic Bacteria and Archaea from Lake Magadi, Kenya.

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DECLARATION

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

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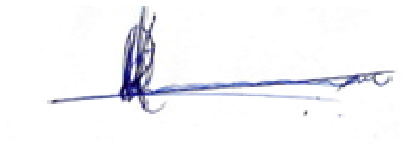

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DEDICATION

This work is dedicated to my beloved wife Susan, who despite the loneliness occasioned by my absence, not only trusted and believed in me and gave me the impetus to go on. To my Lovely children Bless Evan Misiga, Praise Moraa and Gift Favour, for whom I do endeavour to live and strive. To my loving parents Emmanuel and Bilha, who encouraged and supported me all through to this level of education. To uncle Edward for hosting me and being patient with me even when no progress was forthcoming. Above all to God, the creator of all beings, who provided the strength, health and favor to enable me see this output.

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LIST OF ABBREVIATIONS

Bp	Base pairs
DNA	Deoxyribonucleic Acid
rDNA	ribosomal Deoxyribonucleic Acid
PCR	Polymerase Chain Reaction
EDTA	Ethylene diamine tetra-acetic acid
SDS	Sodium dodecyl Sulphate
TBE	Tris Boric acid Ethylene diamine tetra-acetic acid
CMC	Carboxymethylcellulose
NaCl	Sodium Chloride Salt
TE	Tris Ethylene diamine tetra-acetic acid
JCICSB	Judicial Commission of the International Committee on Systematic Bacteriology
EtBr	Ethidium Bromide
M	Molarity
mm	Milimetre
dNTP's	Deoxynucleotide Triphosphates
ddH_2O	Double distilled water
BSA	Bovine Serum Albumin
RFLP	Restriction Fragment Length Polymorphism
G+C	Guanine and Cytosine

ABSTRACT

A variety of microbes inhabit extreme environments normally unsuitable for life such as soda lakes. Soda lake microbes have attracted attention as a possible source of novel enzymes and metabolites for use in biotechnology and are much sought for possible biotechnological use. This is because of their ability to survive and function under severe conditions comparable to those prevailing in various industrial processes. Halophiles in particular, have been cultured and screened for molecules of industrial interest that include extremoenzymes, compatible solutes and biopolymers from different hypersaline environments, including the Kenyan soda lakes. However, very little has been documented on Lake Magadi, which is not only hypersaline but also alkaline (with up to 30% salinity and 12.5 pH levels). This work aimed at isolating, characterizing and screening extremophiles from Lake Magadi for the ability to produce useful biotechnological enzymes. Isolation was done using media of two different categories (Mineral rich and Carbon rich media). Characterization of the isolates was done using cultural, biochemical and molecular approaches while their screening for the ability to produce extracellular enzymes was done by plating them on basal media supplemented with the respective substrate.

A total of 37 isolates were recovered from Lake Magadi of which 34 were Gram positive, one was Gram negative and two were Gram variable. The majority grew well at pH ranging from 6.0 – 11.0, (optimum 9.0-10.0), temperature range of 20 –45 ^{o}C (optimum of 30-35 ^{o}C) and salinity range of 5- 30 %, optimum growth was noted at 10-15%. 15 isolates produced various extracellular enzymes such as amylases, lipases and proteases.

Blast analysis of the partial sequences revealed that the study recovered bacteria affiliated to genera Bacillus, Clostridium and Halomonas from this lake, with relative abundances of 54%, 38% and 8% respectively. Isolates G-15, G-2, G-11, G-18, S-10, S-12, D-9, C-13, M6-1, C-3, X-6, S-3, M8-14, C-4, D-2, D-6, M8-12, S-11, M8-11 and C-1 clustered with members of genera Bacillus with different similarity scores ranging between 98–100%. Isolates S-2, M8-15, G-8, G-4, X-2, M6-7, S-7, G-20, C-6, C-5, G-14, G-12 and X-3 clustered with members of genus Clostridium with similarity values ranging between 80% and 99% while only isolate X-5 clustered with genus Halomonas with a similarity score of 94%. The similarity values of isolates C-6, G-12 and X-5 (94% - 97%) and those of isolates X-3 (88%), G-14 (80%), C-1 (78%), C-5 (84%) and G-20 (85%) to their closest relatives show that these could represent new species and novel genera repectively within the lake ecosystem. This study therefore reports that extreme haloalkaliphilic bacteria constitute a significant part of the microbiota that inhabit Lake Magadi, which have the ability to produce biotechnologically useful enzymes, and recommends more studies to widen the diversity of cultured microbes, use of more media types and different protocols that may increase the range of enzymes produced, and further characterization of the enzymes.

CHAPTER ONE:

1.0 INTRODUCTION

1.1 Extremophilic micro-organisms

A variety of microbes inhabit extreme environments, characterised by conditions such as high or low temperatures, pH, pressure, salt concentration, nutrient concentration, water and conditions having high levels of radiation, harmful heavy metals, organic solvents and toxic compounds (Satyanarayana *et al.,* 2005), which are too harsh for normal life to exist. In general, any environmental condition that is perceived to be beyond the normal acceptable range is an extreme condition (Satyanarayana *et al.,* 2005). A variety of organisms, however, not only tolerate specific extreme condition(s), but usually require these for survival and growth (Bowers *et al*., 2009). Organisms that are well adapted to, and grow optimally at environmental and physicochemical parameters too harsh for normal life to exist are referred to as extremophiles (Michael and Barry, 1997, Satyanarayana *et al.*, 2005). Most extremophiles are found in the microbial world and the range of environmental extremes tolerated by microbes is much broader than other life forms (Satyanarayana *et al.,* 2005).

Most of the described extremophiles are characterized only by one distinctive extreme such as temperature (thermophiles), salinity (halophiles) or pH (alkaliphiles). However, others are multi-extremophiles or polyextremophiles, if they tolerate and and grow under two or more extreme conditions (Kevbrin *et al.,* 1998; Bowers, *et al*., 2009; Setati, 2009), for example haloalkaliphiles and alkalithermophiles.

1.2 Halophilic habitats

Hypersaline environments are widely distributed on the earth's continent where they exist either as natural water bodies, such as permanent saline lakes, ephemeral salt pans and salt marshes, or as artificial solar salterns made up of a series of interconnecting evaporation and crystallizer ponds, constructed for the production of salt, potash and soda (Setati, 2009). These environments can be divided into two broad categories: the thalassohaline environments such as the Great Salt Lake in Utah, playas, brine springs from underground, salt deposits and solar salterns that have similar salt composition to seawater in that sodium and chloride are the dominant ions (Litchfield and Gillevet, 2002), and the athalassoshaline water bodies such as the Dead sea, Lake Magadi in Kenya, Wadi Natrun in Egypt, the soda lakes of Antarctica and Big Soda Lake and Mono Lake in California which are dominated by potassium, magnesium, or sodium (Oren, 2002; Litchfield and Gillevet, 2002). The most widely studied of these ecosystems are the Great Salt Lake (Utah, USA), the Dead Sea (Israël), the alkaline brines of Wadi Natrun (Egypt) and Lake Magadi (Kenya) (Post,1977; Oren, 1993; 2002; Satyanarayana *et al.,* 2005).

These water bodies are commonly 9-10 times more concentrated than sea water which is generally defined as having 3.5% (w/v) dissolved salts (Satyanarayana *et al.,* 2005; Setati, 2009). Despite this extreme salinity, both natural and artificial hypersaline environments harbour remarkably high and diverse microbial cell densities (Oren, 2000). Microorganisms that thrive in these environments have been broadly classified into halophiles and

halotolerants (Bacteria that are able to grow at moderate salt concentrations, even though they grow best in the absence of NaCl) (Bowers *et al*., 2009; Setati, 2009).

1.3 Halophilic microorganisms

These are microorganisms which grow optimally at Na^+ concentrations greater than 0.2 M (Oren, 2006). Halophiles not only require salt for their viability but also need a certain concentration of salt for their optimum growth (Post, 1977; Mouné *et al.*, 2003). They have been isolated from various saline environments such as salt lakes and soda lakes for example the Dead Sea, the Great Salt Lake and Lake Magadi (Mesbah, 2009; Oren and Guverich, 1993), solar thalassohaline salterns (originating from marine waters), subsurface salt formations (Mourne *et al.*, 2003; Mesbah *et al.*, 2007, 2009; Kim *et al*., 2007; Hedi *et al*., 2009) and fermented fish sauces (Pakdeeto *et al*., 2007).

Halophiles are categorized as slight, moderate or extreme, by the extent of their halotolerance (Ventosa *et al*., 1998). Microorganisms that grow optimally at Na^+ concentrations of 1.7 M, or the equivalent of 10% (w/v) NaCl, and greater are considered extreme halophiles (Bowers *et al*., 2009). The high concentrations of salinity makes these areas extreme environments in which relatively few organisms have been able to adapt to and occupy (Burns *et al*, 2004).

Extreme halophiles are found in all the three domains of life namely, Archaea, Eucarya and Bacteria (Bowers *et al*., 2009). Haloarchaea that belong to domain *Archaea*, phylum *Euryachaeota* and family *Halobacteriaceae* make up the majority of the prokaryotes in these environments (Asker and Ohta, 2002, Geoscience, 2002). Within the domain Eucarya,

halophiles are scarce, and the only Eucaryal micro-organism of importance in the high salt environments is the green algae *Dunaliela salina* which is cultivated for beta carotene (Oren, 2002).

Extreme halophilic bacteria, make about 25% of the prokaryotic population and are relatively equally distributed between aerobic and anaerobic species (Bowers *et al*., 2009). They belong to different phylogenetic groups such as the order *Actinomycetales* from the class *Actinobacteria* and phylum *Actinobacteria*; the order *Sphingobacteriales* from the phylum *Bacteroidetes*; the orders *Bacillales, Halanaerobiales* and *Natranaeriobiales* from the class *Bacillus* and phylum *Firmicutes*; the orders *Rhizobiales* and *Rhodospirillales* from the subphylum *α-Proteobacteria*; and the orders *Chromatiales*, *Oceanospirillales* and *Pseudomonadales* from the subphylum *γ-Proteobacteria* (Oren, 2002). Representatives of classes *Bacillus*, *Clostridia*, *Flavobacteria,* the *β*-, and *δ-Proteobacteria* and genera *Salicola, Pontibacillus, Halomonas, Marinococcus,* and *Halobacillus*, have also been documented (Oren, 2002; Hedi *et al*., 2009). The extreme bacterial halophiles exhibit various physiological and nutritional properties (Liaw and Mah, 1992; Sorokin *et al*., 2006; Mesbah *et al*., 2007) and most are unable to survive outside their high-salt native environment. Indeed, many cells are so fragile that when placed in distilled water they immediately lyse from the change in osmotic conditions (Ventosa *et al*., 1998).

In the East African soda lakes, halophilic bacteria are represented mostly by S*alinibacter rubber* and genera *Halomonas, Pseudomonas, Sternotrophomonas, Aeromonas, Vibrio, Alteromonas, Thioalkalimicrobium* and *Thioalkalivibrio* among others (Zavarzin *et al*., 1999, Talaaro and Talaaro, 2002). These organisms are referred to as haloalkaliphiles because of the alkalinity and salinity of their habitat (Bowers *et al.,* 2009).

1.4 Haloalkaliphiles

Haloalkaliphiles require both an alkaline pH (≥pH 9) and salinity (up to 33% [wt/vol] NaCl) (Horikoshi, 1999). These conditions are prevalent in the extreme soda lakes of the East African Rift Valley such as Lake Magadi and Lake Natron, and the western soda lakes of the United States of America (Jones *et al.*, 1998; Horikoshi, 1999; Jones and Grant, 2000; Zhilina *et al*., 2001; Baumgarte, 2003).

Only slightly over sixty species of extreme halophic bacteria were validly published or validated in the *International Journal of Systematic Bacteriology/Systematic and Evolutionary Microbiology* as at 2009 (Bowers *et al*., 2009). Approximately forty-five percent of the extremely halophilic species have published [Na^+] optima equal to or greater than 2.0 M but less than 3.4 M, and only thirteen microorganisms (approximately 25%) have published [Na^+] optima equal to or greater than 3.4 M (equivalent to approximately 20% (w/v) NaCl). Additionally, approximately thirty percent of the species tolerate [Na^+] 5.0 M or greater (equivalent to approximately 29% w/v NaCl) (Bowers *et al.*, 2009).

Among these microorganisms, only three--*Halorhodospira halochloris*, *Halanaerobium lacusrosei* and the unpublished *Natranaerobius 'grantii'* have been described which grow in the presence of saturated NaCl (*i.e*., 5.5 to 6.5 M, since the saturation point is dependent upon media composition, growth pH and temperature) (Imhoff and Truper, 1977, Cayol *et al*., 1994, Bowers *et al*., 2008). *Halorhodospira halochloris* (basonym *Ectothiorhodospira*) (Imhoff and Truper, 1977), has one of the highest [Na^+] optima at 4.62 M (Imhoff and Suling, 1990), *Halomonas taeanensis* is capable of growing over the unusually wide range of 0-5.13 M Na^+ (Lee *et al*., 2005) and *Natranaerobius 'grantii'* tolerates saturated NaCl concentrations in its growth medium at elevated temperature and alkaline pH (Bowers *et al*., 2008). Clearly, as the [Na^+] increases the number of known microorganisms with the adaptive mechanisms that enable them to thrive under these conditions decreases (Bowers *et al*., 2009).

1.5 Adaptations of extreme halophiles and haloalkaliphiles

These microorganisms have developed various biochemical strategies that enable them to adapt to these extreme conditions. Most halophilic and all halotolerant organisms expend energy to remove salt from their cytoplasm to avoid protein aggregation ('salting out') (Santos and Da Costa, 2002). They employ two differing strategies to prevent dessication through osmotic movement of water out of their cytoplasm which work by increasing the internal osmolarity of the cell. The first method is restricted to the moderately halophilic bacterial Order *Halanerobiale*s, the extremely halophilic archaeal Family *Halobacteriaceae* and the extremely halophilic bacterium *Salinibacter ruber* (Zhilina *et al*., 2001; Das Sarma

and Das Sarma, 2006), involves selective influx of potassium (K^+) ions into the cytoplasm which makes the entire intracellular machinery (enzymes, structural proteins, etc.), to be adapted to the high salt levels (Santos and Da Costa, 2002). The presence of this adaptation in three distinct evolutionary lineages suggests a convergent evolution of this strategy, it being unlikely to be an ancient characteristic retained in only scattered groups or through massive lateral gene transfer (Santos and Da Costa, 2002). The second, employed by the majority of bacteria, some archaea, yeasts, algae and fungi, involves the accumulation of low-molecular weight water-soluble organic compounds commonly referred to as compatible solutes, synthesised or accumulated from the environment (Santos and Da Costa, 2002; Vargas *et al*., 2008; Bursy *et al*., 2008), to counteract the deleterious effect of high salinity on cell physiology and loss of cell water (Louis and Galinski, 1997; Cánovas *et al*., 1998; Bursy *et al*., 2008). The most common compatible solutes are neutral or zwitterionic and include amino acids, sugars, polyols, bacteriorhodopsins, exopolysaccharides, hydrolases, biosurfactants, betaines and ectoines, as well as derivatives of some of these compounds (Santos and Da Costa, 2002; Bursy *et al*., 2008). Both gram positive and gram negative bacteria are known to accumulate ectoines as the predominant class of osmolytes (Bursy *et al*., 2008) with the other class of osmolytes such as glycine and betaine being accumulated only in small amounts (Louis and Galinski, 1997). Luckily for man, the synthesised compatible solutes are of biotechnological industrial interest (Kastritis *et al*., 2007).

1.6 Biotechnological applications of halophiles and haloalkaliphiles.

Extensive and intensive research efforts on extremophiles during the last decade has majorly been driven by the potential biotechnological applications associated with these microbes and their products (Santanaryanama *et al.,* 2005). The potential has been increasing exponentially with the isolation of new microbial strains, identification of novel compounds and pathways, and the molecular and biochemical characterization of cellular components (Santanaryanama *et al*., 2005).

Microbial communities in natural soda lakes have many novel and unique properties much sought in industries (Rodriguez-Valera, 1992; Margesin and Schinner, 2001). These microbes are specialist candidates for biotechnological applications because of their ability to survive and function under severe conditions comparable to those prevailing in various industrial processes (Marcella *et al.,* 2006), which normally cause the precipitation or denaturation of most other proteins (Horikoshi and Grant, 1998; Baumgarte *et al.,* 1999; Baumgarte, 2003). This ability has attracted their attention as potential sources of industrially potent enzymes (Horikoshi, 1999; Margesin and Schinner, 2001, Sánchez-Porro *et al*., 2003; Govender *et al*., 2009; Rohban *et al*., 2009).

Halophiles in particular, are being cultured and screened for molecules of industrial interest that include extremoenzymes, compatible solutes, biopolymers (Chi and Bartlett, 1993; Buchalo, *et al*., 2000). Several researchers have screened halophilic bacteria from different hypersaline environments through direct plating on agar media amended with substrates specific for extracellular hydrolytic enzymes of interest such as amylases, proteases, lipases,

DNases, pullulanases and xylanases (Castro *et al*., 1993; Setati, 2009) and quite a variety of bacteria that secrete these enzymes have been isolated and characterized (Castro *et al*., 1993; Sánchez-Porro *et al*., 2003; Rohban *et al*., 2009). The belief that many halophilic enzymes are polyextremophilic, remain optimally active, stable and able to catalyze reactions under harsh conditions typical of many industrial processes (Tokunaga *et al*., 2008; Mesbah *et al*., 2009; Moreno *et al*., 2009), has made these enzymes attractive for various biotechnological applications (Mevarech *et al*., 2000; Setati, 2009).

There are various examples of extremozymes that are now in use commercially which include alkaline proteases, cellulases and lipases (Jones, 2004). Alkaline cellulases and lipases are used to manufacture detergents (Jones, 2004). Puradax, a cellulase used "for rooting out difficult stains and reducing the spills on fabrics" (Aygan and Arikan, 2008), is a critical ingredient in the manufacture of Tide Alternative Bleach detergent, whose origin is from a microbe cultured from Lake Bogoria, Kenya (Lacey, 2006). Another cellulase enzyme used to make soft, stone washed and bleached jeans was isolated from a different microbe cultured from Lake Bogoria (Lacey, 2006; Aygan and Arikan, 2008). Alkaline cellulases and lipases are also used in the manufacture of food ingredients and pitch control in the pulp and paper industry (Mattiasson *et al*., 2004). Cyclodextrin glucanotransferase is used in the manufacture of cyclodextrin used in foodstuffs, chemicals, cosmetics and pharmaceutics industry, from starch (Grant *et al*., 1990; Horikoshi, 1996; Jones, 2004; Narang and Satyaranarayana, 2001). Alkaline and thermostable xylanases are useful in pre-

bleaching of pulps in order to reduce chlorine requirement in pulp bleaching (Archana and Satyanarayana, 1999, Sharma, *et al*., 2000).

Halophiles have been used in the bioremediation of oil contaminated hypersaline brine, soils and aquifers (Rodriguez-Valera, 1992; Gauthier *et al.*, 1992; Nazina *et al*., 2004). *Chromohalobacter beijerinckii*, often found in salted beans preserved in brine and in salted herring, is utilised in the fermentation of salty foods such as soy sauce, Chinese beans, salted cod and fish sauces (Nazina *et al*., 2004). *Dunaniella salini* and *Dunaliella bardawii*, both members of the Eukaryota domain, are cultivated for *beta carotene*, a widely used antioxidant and food colouring agent (Baumgarte, 2003; Satyaranarayana, 2005).

More industrially useful, polyextremophilic enzymes with novel applications, or which improve upon the activities of the ones currently being used are still being sought for the production of novel biotechnological products from the extreme environments of temperature, pH, or salinity (Aguilar *et al*., 1998; Marrs *et al*., 1999; Setati, 2009).

1.7 Statement of the problem

Natural soda lakes are highly productive ecosystems that provide significant opportunities for biotechnology. Despite this, the diversity, biotechnological potential and trophic relations of the inhabitants of these hypersaline ecosystems such as Lake Magadi, East African Rift Valley's most saline soda lake, is not yet fully understood and exploited, yet understanding and conserving our biodiversity is the basis of future developments in biotechnology (Mwirichia *et al.,* 2010a).

1.8 Justification of study

Natural hypersaline environments which form a significant part of the world's landscape and ecology are on the increase due to natural global changes and manmade uses like irrigation and salt harvesting. They are not only spread out throughout the world, but also account for as much water volume in the world's bodies as the fresh water bodies (Geoscience, 2002). The study of the microbial ecology of these environments will make us understand their microbial biodiversity and conservation needs, which is the basis of future developments in biotechnology (Mwirichia *et al.,* 2010a).

Hypersaline lakes and soda lakes harbour the majority of halophilic micro organisms whose diversity is low, because high salinity represents an extreme environment that relatively few organisms have been able to adapt to and occupy (Burns *et al*., 2004). Their low total diversity and wordwide occurrence make them ideal candidates for ecological studies and provides a greater degree of international comparability (Burns *et al*., 2004).

Currently, only a few of the major and dominant groups of the population of the hypersaline environment have been cultured (Burns *et al.*, 2004). Therefore its microbial diversity, community structure and natural ecology are guessed at using insignificant environmental isolates. The combination of molecular analytical methods like 16S rRNA genes of isolates with newer cultivation techniques will hopefully yield a greater percentage of major and dominant groups, reveal not only its community structure but also allow meaningful analysis of the natural ecology to be made (Jones *et al*., 1994; Duckworth *et al.*, 1996). It would also reveal previously undetected and unknown phylogenetic groups, some of which may be exclusive to the soda lake environment (Rees *et al*., 2003).

Haloalkaliphiles have great potential for microbiology and biotechnological exploitation, and they should be isolated, characterized and identified not only for taxonomy but also for maximum industrial utilization (Horikoshi, 1999). The findings of this study provide information that increases the understanding of the microbial diversity, ecology and potential biotechnological benefits of the microbes found at Lake Magadi, known world wide as a major source of salt for food.

1.9 Hypothesis

Lake Magadi in Kenya does not harbour a diversity of extreme haloalkaliphilic microorganisms.

1.10 Objectives

1.10.1 General objective

To isolate, characterize and identify haloalkaliphilic microorganisms from Lake Magadi in Kenya.

1.10.2 Specific objectives

1. To isolate haloalkaliphilic Bacteria and Archaea from Lake Magadi in Kenya.
2. To characterize and identify isolates using morphological, biochemical and molecular techniques.
3. To screen the isolates for the ability to produce biotechnologically useful enzymes.

CHAPTER TWO:

2.0 LITERATURE REVIEW

2.1 Soda lake environments

Soda lakes are highly alkaline and somewhat saline aquatic environments (Grant, 1992), formed in low Ca^{2+}, high Na^{+} arid and semi-arid tropical or semi-tropical zones where evaporation rates exceed the rate of water inflow (Duckworth *et al.*, 2000). Although they have a worldwide distribution (Abdelnasser *et al*., 2007), they are mainly confined to (sub)tropical latitudes in continental interiors or rain-shadow zones (Grant, 1992; Grant *et al.*, 1999). They are characterised by large amounts of Na_2CO_3 (usually as Na_2C0_3. $10H_20$ or $Na_2CO_3.NaHCO_3.2H_2O$) (Grant and Tindall, 1990), low levels of Mg^{2}+ and Ca^{2+} (Grant *et al.*, 1999), and concomitant increase in Cl^{-} (usually as sodium chloride), which is responsible for their salinity (Grant, 1992).

The Kenyan-Tanzanian Rift Valley contains a number of these soda lakes whose development is a consequence of geological and topographical factors (Baumgare, 2003). These lakes are characterised by a pH of around 8.5–10.5 for the dilute lakes and pH values of 12 and above in the concentrated lakes at saturation point for Na_2CO_3 and NaCl, (Cavicchioli and Thomas, 2003; Grant, 2006). Their total salts vary from about 5% w/v in the northern lakes (Bogoria, Nakuru, Elmenteita, Sonachi) to saturation (35%) in parts of the Magadi-Natron basin in the south (Jones and Grant, 1999a). Though their total salinities vary considerably according to the seasonal weather conditions, their pH, however, remains more or less constant regardless of season (Baumgarte, 2003). These

conditions make these lakes the most alkaline and saline naturally occurring sites on Earth (Grant *et al.,* 1999). The hostile nature of these conditions, their remoteness from the main centers of human activity and inaccessibility have resulted in most of them being little studied (Vargas *et al.,* 2004; Wani *et al.,* 2006; Grant, 2006).

2.2 Microbial biodiversity and productivity of Soda lakes

Despite the apparently hostile extreme caustic and saline conditions, these environments are in fact extremely productive (Zarvarzin *et al.,* 1999; Satyaranarayana, 2005). They feature a considerable microbial diversity of dense microbial communities (Grant and Tindall, 1986), Jones *et al*., 1994). The colony forming units of cyanobacteria in these lakes has been recorded at levels of 10^5- 10^6 cfu/ml^{-1} (Duckworth *et al.,* 2000). Primary production rates in excess of >10 gC $m^{-2}day^{-1}$ (Melack and Kilham, 1974; Grant *et al*., 1990) have been recorded (the mean primary productivity of streams and lakes of the world is about 0.6 g cm^{-2} day^{-1}). These lakes are therefore among the most productive aquatic environments in terms of biomass in the world (Jones and Grant, 1999a; Baumgarte, 2003). Their high productivity rates is attributed to the presence of dense blooms of cyanobacteria, (Duckworth *et al.,* 2000), relatively high ambient temperatures, high light intensities, high nutrient levels for example phosphates, the unlimited access to CO_2 via the the HCO_3^- /CO_3^{2-}/CO_2 equilibrium and lack of predation (Grant *et al.,* 1990).

Despite the high productivity and cell densities (Oren, 2002), low taxonomic biodiversity and therefore a relatively simple ecological structure has been observed in the most widely studied soda lake ecosystems (Oren, 2001), most probably due to the high salt

concentrations measured in these environments. The low diversity however, makes these ecosystems convenient model systems for microbial ecology studies (Oren, 2006).

Unlike other types of lakes, the microbial biomass of soda lakes is dominated by prokaryotes, (Grant *et al.,* 1999), consisting of all the major trophic groups of bacteria and archaea which are involved in primary production, cycling of carbon, sulfur, and nitrogen under aerobic and anaerobic conditions present in the lakes and few representatives of algae (Grant *et al.,* 1999). The trophic groups responsible for the recycling of nitrogen, carbon and sulfur in these lakes have been identified and they show obvious parallels with other aquatic systems (Jones *et al*., 1998).

The colour of soda lakes which ranges from green due to cyanobacteria, to red or orange, due to other organisms for example archaea, is dependent on their water chemistry (Grant *et al*., 1990). Lake Magadi is an example of a red soda lake (see figure 1) that harbours a number of haloalkaliphilic archaea such as *Natronobacterium gregoryi*, *Natrialba magadii*, *Halorubrum vacuolatum* and *Natronococcus occultus* and some halophic bacteria (Mwatha and Grant, 1993; Oren, 2006).

Figure 1: Red pigmentation due to haloalkaliphilic archaea natronobacteria on Trona crusts at Lake Magadi, courtesy of the Internet Encyclopedia of Science, 2003).

The East African soda lakes support a dense and diverse population of aerobic cyanobacteria, anoxygenic phototrophic bacteria (Grant and Tindall, 1986), non-phototrophic bacteria (Baumgarte, 2003), aerobic organotrophic bacteria and anaerobes of various physiologies (Grant *et al*., 1990). Anaerobic groups consist of the acetogenic ammonifiers and the hydrogenotrophic sulphate reducers *Desulfonatronovibrio* and *Desulfonatronum*, obligately autotrophic sulphur oxidising bacteria, methane oxidizing *Methylobacter alcaliphilus* and *Methylomicrobium sp* able to oxidize methane and ammonia (Zarvazin *et al*., 1999). With the addition of Proteobacteria, Natronoarchaebacteria, Spirochaetes and Thermatoga species, the soda lake community is represented in all the major functional trophic groups (Grant *et al*., 1990).

However, the diversity of the prokaryotic mirobial community found in the strongly hypersaline soda lakes of the rift valley, like lake Magadi, seems to be remarkably different from that of the dilute lakes (Jones *et al*., 1998; Jones, 2004). Production for instance in the less alkaline lakes is usually dominated by vast blooms of cyanobacteria, while in the hypersaline soda lakes, primary producers consist of blooms of cyanobacteria and alkaliphilic anoxygenic phototrophs especially those belonging to the genera *Ectothiorhodospira* and *Halorhodospira* (Jones *et al*., 1998, Jones, 2004).

It has generally been assumed that hypersaline environments with salt concentration close to saturation are dominated by the halophilic members of the domain Archaea, especially the red halophilic Archaea of the family *Halobacteriaceae*, also known as halobacteria (Oren and Guverich, 1993; Anto'n *et al*., 2002; Benloch *et al.*, 1995, 2002), and bacteria were thought to be insignificant in this kind of environment (Oren, 2002). However research done using the technique of fluorescent in situ hybridization (FISH) which directly characterises communities using their 16S rRNA sequence has led to the discovery of many extremely halophilic bacteria co-existing with the halophilic archaeal community at the highest salt concentrations (Anto'n *et al*., 2002; Martinez-Canovas *et al*., 2004). For instance, Anto'n and company found bacteria in large numbers of around 3×10^6/ml, in a marine saltern pond with a salinity of 37% in Spain (Anto'n *et al*., 1999; 2000). Martinez-Murcia, (1995) found a specific type of bacteria adapted to life at the highest salt concentrations. The bacterial restriction fragment patterns obtained from these crystallizer ponds were very different from those retrieved from lower salinity ponds. PCR studies done

by Benloch *et al*. (1999), at saltern crystallizers in Spain yielded sequences distantly related to Rhodothermus marinus (*Cytophaga/Flavobacterium/Bacteroides* phylum) (Anto'n, *et al*., 2000). Similar sequences have also been recovered from salterns in the south of France (Moune *et al*., 2000; Casamayor *et al*, 2002). These studies show that heterotrophic bacteria are as salt-dependent and salt-tolerant as the most halophilic Archaea (Oren, 2002) and therefore calls for the revision of the perception that haloarchaea are the only ecologically relevant prokaryotes in hypersaline aquatic environments (Anto'n *et al*., 2000; Ventosa, 2006).

2.3 The role of haloalkaliphiles in Soda lakes

Soda lake microbes which consists of all the major trophic groups of bacteria and archaea are thought to be significant in the ecological biochemistry of soda lakes in that they are involved in various important roles such as primary production, cycling of carbon, sulfur, nitrogen, transformation and degradation of waste and organic pollutants in saline wastewaters (Milford *et al*., 2000).

Cyanobacteria, notably *Arthrospira platensis*, and *Cyanospira rippkae* and anoxygenic phototrophic bacteria of the genus *Ectothiorhodospira* (Jones *et al*., 1998), *Halorhodospira* and *Rhodobaca bogoriensis* (Milford *et al*., 2000), may be responsible for primary productivity in the hypersaline soda lakes of the rift valley (Jones *et al*., 1998), which not only supports the rest of the microbial community but also presumably drives all the biological processes in these environment (Jones *et al*., 1998; Grant *et al*., 1999).

The nitrogen cycle in these lakes involves the production of ammonia by fermentative anaerobes such as *Tindallia magadii* and nitrification by methanotrophs and nitrifiers (Prosser, 1989; Kevbrin *et al*., 1998), in a two stage reaction (Bock and Koops, 1992; Koops and Moller, 1992). The methanotrophs first convert ammonia to nitrites which is then subsequently transformed to nitrate by the nitrifiers (Arthur *et al*., 1987). Cultured ammonia-oxidizing bacteria have been identified to belong to the gamma *(Nitrosococcus oceanus* and *N. halophilus*) and beta subclasses of *Proteobacteri*a, which has members of genera *Nitrosomonas, Nitrosospira*, *Nitrosovibrio,* and *Nitrosolobus*, as well as *Nitrosococcus mobilis* (Woese *et al.,* 1984; Utaker *et al.,* 1995). Cultivated nitrite-oxidizing bacteria have been assigned to four recognized genera: *Nitrobacter* (α-*Proteobacteria)*, *Nitrospina* (delta *Proteobacteria*, *Nitrococcus* (gamma *Proteobacteria)* (Teske *et al*., 1994), and genus *Nitrospira* that encompasses species *Nitrospira moscoviensis* and *N. marina (*Ehrich *et al*., 1995).

Halomonads capable of reducing nitrates to nitrites further reduction of the nitrite, and of anaerobic growth with and without nitrate consititute a major part of the soda lake community (Jones *et al*., 1998). These soda lake halomonads are assumed to play a major role in the nitrogen cycle of the lake (Jones and Grant 1999b) and their unique ability to reduce nitrates to nitrites sets them apart from other halomonads (*Halomonas sensu strictu*), (Jones *et al*., 1998).

The sulfur cycle is carried out by *Ectothiorhodospira* and *Halorhodospiria species w*hich produce sulfur and sulfate (the link between the carbon and sulfur cycles), aerobic sulfur oxidizing bacteria which belong to two groups namely the *Halomonas deleya* and those assigned to the novel genus *Thioalcalovibrio* (Sorokin *et al*., 2001) and sulfate-reducing bacteria; *Desulphonatronovibrio hydrogenovorans* and *Desulphonatrum lacustre*, which have been isolated from Siberian soda lakes (Zhilina *et al*., 1997; Pikuta *et al*., 1998).

Soda lake microbes are biochemically very active, and are able to utilize various organic compunds including sugars, hydrocarbons and complex polysaccharides (Hedi *et al.,* 2009). For instance, halophiles able to hydrolyze many different polymers and produce sugars and amino acids have been isolated from El-Djerid salt lake in Sebkha, Tunisia (Hedi *et al*., 2009). The sugars are used as substrates for the fermentation of simple compounds, such as fatty acids, by anaerobic fermentors (Hedi *et al.,* 2009), which in turn, may be consumed by other groups such as the acetogenic bacteria such as *Natroniella acetigena*, *Thermosyntropha lipolytica* and *Tindallia magadiensis* (Svetlichnyi *et al.*, 1996; Zavarzin *et al*., 1999).

Gauthier *et al*., (1992) isolated a halotolerant, *Marinobacter hydrocarbonoclasticus,* capable of degrading a variety of aliphatic and aromatic hydrocarbons. Nazina *et al.* (2004) has also isolated a benzene degrading halophile. These halophiles can be used in the remediation of brine contaminated soil and aquifers (Nazina *et al.,* 2004). A recombinant strain of *Deinococcus* has been created, capable of degrading organopollutants in radioactive mixed-waste environment (Setati, 2009). The recombinant remained tolerant to

the solvent effects of toluene and trichloroethylene at levels higher than those of many radioactive waste sites (Setati, 2009), suggesting potential use of genetically engineered extremophilic microbes in bioremediation of waste sites contaminated with a variety of organopollutants plus radionuclides and heavy metals (Cavicchioli and Thomas, 2003).

Soda lake microbes are therefore significant in the ecology and biogeochemistry of soda lakes (Li *et al.*, 1998). However the full extent of the soda lake microbial diversity, ecological structure and roles played by individual organisms is yet to be wholly revealed (Jones and Grant, 1999b).

CHAPTER THREE:

3.0 MATERIALS AND METHODS

3.1 Study site: Lake Magadi

Lake Magadi (1°43'-2'00'S and 36°13-36°18'E), covering an area of approximately 100 square kilometers is a hypersaline alkaline lake found in the southern part of the graben of the East African Gregory Rift Valley, close to the Kenya-Tanzanian border (see figure 2). The lake lies in the rain shadow of mountains at an altitude of 600 metres above sea level (Baumgarte, 2003). With an average annual rainfall of only about 500mm for the two rainy seasons, salinity of up to 35 % w/v or greater, pH range of 9.0-12.5, and a temperature range of between 22^0 C-34^0 C, the lake is an "alkaline saline pan" (Baumgarte *et al.*, 1999; Baumgarte, 2003). The lake consists for the most part, of an almost solid deposit of NaCl and Na_2CO_3 due to the intense evaporation (3500mm per annum), that occurs during the dry season (Baumgarte *et al.*, 1999), and active deposition of carbonate minerals notably trona ($NaHCO_3.Na_2CO_3.12\ H_2O$), which is mined on site as a source of soda ash for glass manufacture (Grant, 2006). There are very low levels of Mg^{2+} and Ca^{2+} because these cations are insoluble as carbonate minerals under alkaline conditions, especially at pH 11.5–12.0 (Grant and Tindall, 1990).

Figure 2: A Google map showing the location of Lake Magadi, courtesy of Map data ©2011 Google, Tracks 4 Africa.

3.2 Sampling

Water, soda crust and sediment samples were collected from two different sites of Lake Magadi, selected randomly, (see figures 3 and 4) using a water sampler and shovel respectively. They were placed in sterile plastic bags, labelled clearly. The bags were closed

tightly, stored in ice cooled boxes and transported to Jomo Kenyatta University of Agriculture and Technology, (JKUAT). At each sampling site varoius physical parameters such as temperature, pH,conductivity,total dissolved solutes and dissolved oxygen were noted (Mwirichia *et al*., 2010a).

Figure 3: Sampling point station 1, a hot spring

Figure4: Sampling point station 2, a salt marsh.

3.3 Enrichment and Isolation

Isolation and cultivation was performed on modified alkaline media, according to Duckworth *et al*. (1996), Xu *et al.* (2006) and Phil *et al*. (2005) using different media types, divided into two broad categories namely, the Carbon rich media (I-IV) and the mineral rich media (V-VII) (see appendix A). The media was modified by use of different kinds of carbon sources and Cycloheximidine was added to each media to exclude the growth of fungi and prepared using Lake Magadi water to replicate the natural lake conditions.

Water, sediment and soda crust samples from each sampling point were mixed in a beaker to form one representative inoculum. Enrichment was done, by inoculating 1 ml of sterile broth media with 0.1 ml of inoculum. The enrichment cultures were grown overnight at 37°C before being transferred to the media on agar plates using the spread plate method. The agar plates were incubated at 37°C and growth monitored until colonies started to form. Sub culturing was done in the same media and incubated at the same temperature, by picking different colonies and restreaking several times till pure cultures were obtained. Pure isolates were all transferred to nutrient agar (common media), supplemented with 15% sodium chloride for further biochemical and molecular analysis. Microbial cultures were stored at −80°C in the isolation medium supplemented with 40% glycerol.

3.4 Characterization and Identification of Isolates

3.4.1 Morphological characterization

Preliminary characterization of the isolates involved the examination of colonies of cultures grown at optimum temperature, pH, and salt concentration (Asker and Ohta, 1999), for morphological and cultural features such as colour and pigmentation of safranin-stained bacterial isolates under the dissecting and compound microscope using slide procedure of Hopwood (1960). This was supplemented by the classical gram staining of the individual isolates in duplicate using the Dussault (1955) modification, followed by observing under a light microscope at ×100 (Keast *et al.*, 1984) and finally confirming the results by the 3% KOH lysis test (Gregersen, 1978; Halebian *et al.*, 1981).

3.4.2 Physiological characterization

3.4.2.1 Salt tolerance test

The isolates were tested for their ability to grow at different sodium chloride concentrations by culturing them separately in 0.28% nutrient agar (diluted ten times), supplemented with varying concentrations of salt, namely; 5%, 7%, 10%, 12%, 15%, 20%, 25%, 30%, and 35%, in duplicates for replication. Growth on the agar medium was estimated as per Tindall *et al*. (1980) after incubating the plates at 37^{o}C. Two uninoculated plates with the same media served as control. The concentration of salt that allowed for the highest and lowest growth (recorded in terms of number of days taken to form colonies), was noted (Korn-Wendisch and Kutzner, 1992). Growth was scored using three levels of positive signs, where by one positive indicated minimal growth (took more than 5 days), two positives indicated average growth (took 3-4 days), three positives indicated satisfactory growth (took 2 or less days).

3.4.2.2 Temperature tolerance test

The isolates were tested for their ability to grow at different temperatures by culturing them in 0.28% nutrient agar (diluted ten times), supplemented with 15% sodium chloride and separately and in duplicates for replication. Growth was estimated on the agar medium as per Tindall *et al*. (1980) method, after incubating the plates at 15, 20, 25, 30, 35, 40, 45 and 50°C. Two uninoculated plates with the same media served as control. The temperature that allowed for the highest and lowest growth (recorded in terms of number of days taken to form colonies, was noted (Korn-Wendisch and Kutzner, 1992).

Growth was scored using three levels of positive signs, where by one positive indicated minimal growth (took more than 5 days), two positives indicated average growth (took 3-4 days), three positives indicated satisfactory growth (took 2 or less days).

3.4.2.3 Growth at different pH values

The isolates were tested for their ability to grow at different pH values by culturing them separately in 0.28% nutrient agar (diluted ten times), supplemented with 15% sodium chloride and adjusted to pH values of 5.5, 6.0, 6.5, 7.5, 8.0, 9.0, 10.0 and 11.0, in duplicates for replication. Growth on the agar medium was estimated as per Tindall *et al*. (1980), after incubating the plates at 37 ^{0}C. Two uninoculated plates with the same media served as control. The pH that allowed for the highest and lowest growth was noted (Korn-Wendisch and Kutzner, 1992). Growth was scored using three levels of positive signs, where by one positive indicated minimal growth (took more than 5 days), two positives indicated average growth (took 3-4 days), three positives indicated satisfactory growth (took 2 or less days).

3.5 Screening for enzyme production

3.5.1 Cellulase production

The isolates were tested for cellulase enzyme production by culturing them separately in basal medium containing per litre of filtered Lake Magadi water K_2HPO_4 (1.0g), $MgSO_4.7H_2O$ (0.1g), $CaCl_2.7H_2O$ (0.05g) and Agar (14.0g), supplemented with 5g of cellulose and carboxymethyl cellulose respectively. Each medium was then inoculated and incubated for 48 hours. Each plate was flooded with1% congo red dye and observed for a zone of clearing around the colony. Positive isolates for cellulase production were indicated

by clear zone around the area surrounding the isolate while the negative isolates had the colour around the isolates remaining red (Cappuccino and Sherman, 2002).

3.5.2 Xylanase production

The isolates were tested for xylanase production by culturing them separately in basal medium containing per litre of filtered Lake Magadi water: K_2HPO_4 (1.0g), $MgSO_4.7H_2O$ (0.1g), $CaCl_2.7H_2O$ (0.05g) and Agar (14.0g) supplemented with 1.25g of xylan as the sole carbon source as described by Lee and Lee (1997). Each plate of the media was then inoculated and incubated at 37^o C for 48 hours. Each plate was then flooded with1% Congo red dye and observed for a clear zone around the colony which indicated a positive result (Rheims *et al*., 1998). The colour around positive isolates for xylanase production changed from red to orange while the colour around the negative isolates remained red.

3.5.3 Amylase production

The isolates were tested for amylase production by culturing them separately in basal medium containing per litre of filtered Lake Magadi water: K_2HPO_4 (1.0g), $MgSO_4.7H_2O$ (0.1g), $CaCl_2.7H_2O$ (0.05g) and Agar (14.0g) supplemented with 5.0g of starch as the sole carbon source (Castro *et al*., 1993; Lee and Lee, 1997). Each plate of the media was then inoculated and incubated at 37^o C for 48 hours. Each plate was then flooded with1% Lugol's iodine dye (Cappuccino and Sherman, 2002) and observed for a change of colour and a clear zone around the colony which indicates a positive result (Rheims *et al*., 1998). The presence of blue black color around the colonies indicated presence of starch and therefore negative test for production of amylase, while absence of blue black color and a

clear halo zone around the colonies indicated a positive test for amyalse production (Cappuccino and Sherman, 2002).

3.5.4 Lipase production

The isolates were tested for lipase enzyme production by culturing them separately in basal medium containing per litre of filtered Lake Magadi water: K_2HPO_4 (1.0g), $MgSO_4.7H_2O$ (0.1g), $CaCl_2.7H_2O$ (0.05g) and Agar (14.0g), supplemented with 5ml of olive oil (Cappuccino and Sherman, 2002). Each inoculated plate was incubated at 37^o C for 48 hours and observed for the formation of a precipitate around the colony, which indicates a positive result while the converse indicated a negative result (Cappuccino and Sherman, 2002).

3.5.5 Protease production

Isolates ability to produce protease enzyme was done using basal media containing K_2HPO_4 (1.0g), $MgSO_4.7H_2O$ (0.1g), $CaCl_2.7H_2O$ (0.05g) and Agar (14.0g) per litre of lake Magadi water, supplemented with 1% skim milk as sole carbon source (Lee *et al.*, 2005). Each plate was then inoculated and incubated at 37^o C then observed for zones of clearing after 48h of incubation. Protease secreting isolates exhibited a zone of proteolysis as demonstrated by a clear zone surrounding the bacterial colony while the negative isolates did not have the clearing zones (Cappuccino and Sherman, 2002). Protease production was suppplemented with the gelatin liquefaction test in which nutrient broth (0.8%) was supplemented with 0.4% gelatin, 1.5% agar, per liter of lake Magadi water and autoclaved. On settling, each plate was inoculated with an isolate, in duplicate for replication

(Cappuccino and Sherman, 2002), and incubated for 5 days at 37°C, followed by flooding with 1M H_2SO_4 containing 0.05 sodium sulphite. Cultures that liquefied gelatin were considered positive for gelatin hydrolysis and hence positive protease production while those which did not liquefy gelatin were considered to be negative for gelatin hydrolysis.

3.5.6 Esterase production

The media used was a modification of Sierra (1957) and contained per litre of Lake Magadi water: peptone 10.0 g, $CaCl_2.2H_2O$ 0.1 g, agar 20.0 g, supplemented with previously sterilized 1% (v/v) Tween 80). Each plate was then inoculated and incubated at 37 ^oC then observed for the formation of precipitates around the colonies after 5 days of incubation which indicates the production of esterase enzyme.

3.6 Biochemical characterization

3.6.1 Catalase test

Catalase test detects the activity of enzyme catalase, present in most cytochrome-containing aerobic bacteria. These microbes produce hydrogen peroxide during the aerobic breakdown of sugars. Catalase decomposes hydrogen peroxide to water and oxygen.

Catalase test was done by scooping a colony of a 24-hour culture, placing it on a glass slide and adding a drop of 3% hydrogen Peroxide solution. A positive reaction was indicated by the formation of bubbles, while the absence of air bubbles indicated a negative catalase test (Cappuccino and Sherman, 2002).

3.6.2 Oxidase test

This tests the ability of a microbe to oxidize certain aromatic amines like p-aminodimethylaniline to form coloured end products. Oxidase positive bacteria possess cytochrome oxidase or indophenol oxidase (an iron containing haemoprotein), which are components of the electron transport chain of specific organisms (Kovac, 1956). These enzymes catalyze the transport of electrons from donor compounds (NADH) to electron acceptors (usually oxygen). The oxidase reagent, (N, N, N', N'-tetra-methyl-p-phenylenediamine dihydrochloride) acts as an artificial electron acceptor for the enzyme oxidase. When its oxidised in the presence of alpha naphthol, the reagent forms the coloured compound indophenol blue.

To test for the activity of this enzyme, some 24- hour old bacterial cultures were scrapped onto plastic N,N-dimethyl-1,4-phenylene diamine and alpha naphthol impregnated oxidase strips. The strips were left on the clean bench and observed after 1 minute. The development of a deep blue color at the position of the wiped colony after the 1 minute indicated a positive result for oxidase test and lack of this blue colour indicated a negative test (Cappuccino and Sherman, 2002).

3.6.3 Nitrate reduction

This tests the production of enzyme nitrate reductase which reduces nitrates that the cell uses as a final hydrogen acceptor during anaerobic respiration, to nitrites or free nitrogen gas and water. The nitrite combines with the test reagents (sulphanilic acid and alpha-naphthylamine) to form a diazo red dye.

Nitrate reduction was tested by inoculating substrates into Nitrate broth medium supplemented with 1% $KN0_3$ and incubating them for 72 hours at 37°C (Mwatha and Grant , 1993). After incubation, drops of sulphanilic acid and alpha-naphthylamine were added. Nitrate reduction was detected by withdrawing 0.5-ml samples and adding 0.2ml naphthylamine and 0.2ml sulfanilic acid reagent to each tube, as described by Smibert and Krieg (1981). Positive reactions produced an immediate cherry red colour while negative reactions remained yellow. The results were confirmed by the addition of zinc powder, in which two uninnoculated tubes were used as controls (Korn-Wendisch and Kutzner, 1992). Formation of a red colour indicated negative results while no colour change indicated that reduction was beyond nitrite (to ammonia or molecular nitrogen) (Harold, 2002).

3.6.4 Gelatin liquefaction/hydrolysis

Gelatin liquefaction detects the breakdown of gelatin to polypeptides and aminoacids by enzyme gelatinase (Harold, 2002). Gelatin protein is produced by hydrolysis of a component of the connective tissues and tendons of animals known as collagen. Gelatin is a solid at room temperature but above 25^oC it liquefies.

When gelatinase hydrolyses this protein into amino acids, it remains liquefied even at the low temperatures of an ice bath (Cappuccino and Sherman, 2002).

The bacterial isolates were inoculated onto nutrient broth supplemented with 12% gelatin and and 1.5 % agar, to demonstrate hydrolytic activity of gelatinase. One uninnoculated tube was used as control for each isolate (Cappuccino and Sherman, 2002). After

incubation, cultures that remained liquefied when placed in refrigerator at 4 ^{0}C for 30 minutes were considered positive for gelatin hydrolysis.

3.6.5 Amino acid utilization

These tests are used to detect the ability of isolates to produce enzyme decarboxylase that has the ability to decarboxylate specific amino acids into amines which are alkaline in nature, by removing the carboxyl group. The amines are indicated by change of colour of the medium from red to pink, because the media becomes more alkaline (Cappuccino and Sherman, 2002).

3.6.5.1 L-Arginine

The isolates were tested for utilization of L-Arginine by growth in a 3ml media containing 1.0% L-Arginine, 0.1% peptone, 0.03% $K_2H_2PO_4$, 0.3% Agar, and 5ml of 0.2% phenol red indicator per litre of Lake magadi water and inoculated for three days (Nazina *et al*., 2001). One uninoculated tube with the same medium served as control for each isolate. Positive isolates for $_{L}$- Argenine decarboxylase were indicated by colour change from red to pink while the negative ones remained red (Cappuccino and Sherman, 2002).

3.6.5.2 L-Lysine

The isolates were tested for utilization of L-lysine by growth in a 3ml media containing 1.0% L-lysine, 0.1% peptone, 0.03% $K_2H_2PO_4$, 0.3% Agar, and 5ml of 0.2% phenol red indicator per litre of Lake magadi water and inoculated for three days (Nazina *et al*., 2001). One uninoculated tube with the same medium served as control for each isolate. Positive isolates for $_{L}$- lysine decarboxylase were indicated by colour change from red to pink

(Rheims *et al*., (1998), while the negative ones remained red (Cappuccino and Sherman, 2002).

3.6.5.3 L-Ornithine

The isolates were tested for utilization of L-Arginine by growth in a 3ml media containing 1.0% L- Ornithine, 0.1% peptone, 0.03% $K_2H_2PO_4$, 0.3% Agar, and 5ml of 0.2% phenol red indicator per litre of Lake magadi water and inoculated for three days (Nazina *et al*., 2001). One uninoculated tube with the same medium served as control for each isolate. Positive isolates for $_L$- Ornithine decarboxylase were indicated by colour change from red to pink while the negative ones remained red (Cappuccino and Sherman, 2002).

3.6.6 Methyl Red-Voges- Proskauer (MR-VP) test

MR-VP is used to identify mixed acid fermenting bacteria.The test detects the ability of the isolates to oxidize glucose by detecting the production of sufficient acid end products (Harold, 2002). MR test detects mixed acids, which are the characteristic end products of a particular fermentation pathway that make the medium more acidic ($pH \leq 4.5$). This is detected when an indicator is added (Cappuccino and Sherman, 2002).

The VP test is used to identify bacteria that produce non-acidic or neutral end products from the organic acid products of the glucose fermentation. It specifically detects an intermediate product of the fermentation pathway that yields 2,3-butandiol known as acetoin, by the addition of Barrit's reagent(4% KOH and 5% alpha naphtol in 95% ethanol).

MR-VP broth was inoculated with each of the isolates, in duplicates, shaken and then incubated at 37°C for 72 hours after which, drops of Methyl red indicator methyl red test or

Barrit's reagent for VP test respectively, was added to aliquots of each culture. Positive reactions were those that turned red while negative reactions turned pale yellow for MR test, while for the VP test, positive tests produced a deep rose coloration (Cappuccino and Sherman, 2002).

3.6.7 Indole production and Hydrogen sulfide production tests

The test identifies isolates with the ability to produce the enzymes tryptophanase that removes the amino group from tryptophan to form indole, pyruvic acid and ammonia, and cysteine desulfurase, that produces pyruvate, ammonia and hydrogen sulfide from sulfur containing amino acids. Indole reacts with Kovacs reagent (*p*-dimethylamino-benzaldehyde) to form a deep red colour (Harold, 2002), while the iron in the medium reacts with hydrogen sulfide to produce a black precipitate (Kovac, 1956; Cappuccino and Sherman, 2002).

The isolates were were inoculated in Sulfur-Indole Motility (SIM) agar media by stabbing method in duplicate for replication, then incubated at 37 ^{o}C for 48h. Two uninnoculated tubes were used as controls. Kovac's reagent was the added to each of the 48h culture according to the protocol of (Harold, 2002). The presence of a cherry red layer in the media indicated positive result for indole production while negative results were indicated by colour remaining brown (Cappuccino and Sherman, 2002). The presence of a black coloration in the media after incubation indicated lack of hydrogen sulfide in the media (Cappuccino and Sherman, 2002). Lack of motility was detected by the confinement of the bacteria along the line of inoculation.

3.6.8 Citrate utilization

This determines the ability of a microbe to use citrate as the sole source of carbon (Harold, 2002). Citrate utilization is indicated by growth accompanied by an alkaline pH (Cappuccino and Sherman, 2002).

Simmon's citrate agar slants containing the pH indicator bromothymol blue were inoculated by streaking wih the isolates in duplicates and incubated at 37 ^{o}C for 72 hours. One uninoculated tube with the same media served as the control. Positive test were indicated by growth of the bacteria accompanied by colour change in the medium from olive green to prussian or deep blue (Cappuccino and Sherman, 2002).

3.6.9 Urease test

This test is used to determine the ability of a micro organism to produce the enzyme urease that splits urea, into CO_2 and ammonia (Harold, 2002). Ammonia makes the medium alkaline.

The isolates were inoculated in urea broth media containing phenol red indicator followed by Incubation at 37 ^{o}C. Colour change was monitored for 4 days. Positive reaction was indicated by presence of a deep pink colour, while negative tests remained yellow (Cappuccino and Sherman, 2002).

3.7 Molecular characterization

3.7.1 DNA extraction

Genomic DNA was extracted from bacterial cells grown aerobically in nutrient broth according to the protocol of Broderick *et al.* (2004). 1ml of culture cells were harvested in 1.5ml eppendorf tubes by centrifuging (13000g, 5min). 0.6 ml of BSS buffer was added to resuspend the cells followed by incubation at 30^{o}C for 30 minutes. 60 µl of 10% SDS and 3 µl of 50 mg/ml proteinase K were then added, mixed thoroughly and incubated at 55^{o}C for 2 hours. 100 µl of 5 M NaCl was added followed by thorough mixing. 80 µl of CTAB/NaCl mix was added to remove cellwall debris, denatured proteins and polysaccharides. The mixture was incubated at 65^{o}C for 30 minutes, after which an equal volume of chloroform isoamyl alcohol (24:1) was added, mixed and centrifuged for 5 minutes, to remove the CTAB complexes. The aqueous layer was then carefully transfered to a new tube and the step repeated before adding an equal volume of phenol/chloroform isoamyl alcohol (25:24:1) mix, followed by spinning at 15000 r.p.m for 5 minutes. The aqueous layer was transfered to a new tube, where 0.7 times volume of cold isopropanol was added, followed by overnight incubation at -20 ^{o}C, centrifugation at 15 000 r.p.m for 10 minutes and subsequent careful removal of isopropanol. 1 ml of 70% ethanol was then added followed by mixing by inversion and centrifugation at 15 000 r.p.m for 5 minutes, after which the ethanol was removed carefully and discarded. This procedure was repeated twice, accompanied by quick spinning, pippeting and complete air drying on the bench at room temperature. The DNA pellet was then resuspended in 40 µl TE and semi quantified on a

1% agarose gel in 1xTAE buffer. Visualization was done under UV by staining with ethidium bromide (Sambrook *et al.,* 1989) after which it was kept at -20^{0}C for future use (Magarvey *et al.,* 2004; Mwirichia *et al.,* 2010b).

3.7.2 Polymerase Chain Reaction

Amplification of the nearly full-length 16S rRNA gene sequences using bacterial primer pair 8F forward 5'-AG (A/G) GTTTGATCCTGGCT-3') and 1492R reverse, 5'-CGGCTACCTTGTTACGACTT-3'(Sigma) (Lane, 1991; Embley and Stackebrandt, 1994), was performed using a model PTC-100 thermal cycler (MJ research inc., USA) after a gradient PCR had been done to determine the optimum annealing temperature. The PCR mix consisted of 40 µl mixture containing 0.25 µl of genescript Taq, 1.0 µl (5-pmol) of 27F forward primer, 1.0 µl (5-pmol) of 1492R reverse primer, 1 µl of template DNA, 2.5 ul of dNTPs mix (2.5mM), 4.0 PCR 10x buffer (genescript) and 30.25 µl of PCR water. The PCR cycling consisted of an initial activation of the enzyme at 94^{o}C for 5 minutes followed by 35 cycles of a denaturation step at 94^{o}C for 45 seconds, primer annealing step at 53^{o}C for 60 seconds, chain elongation step at 72^{o}C for 2 minutes and a final extension at 72^{o}C for 5 minutes (Embley, 1991; Roux, 1995). The presence of amplified products and their concentration were determined by separating 7 µl of product on 1% agarose gels in 1X TAE buffer and visualizing them by ethidium bromide staining (Sambrook *et al*., 1989).

3.7.3 Purification of PCR products

The PCR products obtained were purified using the QIAquick PCR purification Kit protocol (Qiagen) and sent for sequencing.

3.7.4 Phylogenetic data analysis

Sequences were generated at the ILRI, BecA-ILRI Hub Services > SegoliP sequencing facility, using the universal bacterial primer bac 8f and bac 1492r after the samples had been prepared according to the facility's instructions. The sequences were edited by eliminating all gaps (Complete deletion option), using the CHROMAS PRO software 1.5 version. The edited 16S rDNA gene sequences were then 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 CLUSTAL W 1.6 software (Altschul *et al*., 1997), and where necessary, manually corrected based on the conserved regions. Phylogenetic analysis (evolutionary history) was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). Evolutionary distances used to infer and construct the phylogenetic tree were calculated using the Jukes and Cantor (1969) method while Felsenstein, (1985) bootstrapping method was used to determine the accuracy of the tree constructed. These evolutionary analyses were conducted in Mega 5 (Tamura *et al*., 2011).

CHAPTER FOUR:

4.0 RESULTS

4.1 Physical characteristics at sampling site

The physical parameters at the sampling stations of the lake are presented in Table 1. These values are the raw figures of the conditions at the sampling sites of Lake Magadi during the sampling.

Table 1: Physical parameters of the sampled sites of Lake Magadi during sampling

Parameter	Station 1	Station 2	Station 3
Lake Temperature	44.5^0C	39.4^0C	51.9^0C
Surrounding temp.	38.0^0C	38.0^0C	38.0^0 C
pH	9-10	9.63	10.28
Conductivity (EC)	0.04 ms	20.0 ms	6.18ms
TDS	0.02mg/l	0.2mg/l	8.6mg/l

4.2 Isolation

After several subcultures, a total of 37 pure isolates were obtained from Lake Magadi. The number of isolates obtained per media is given in table 2 below:

Table 2: Number of isolates obtained from each media.

Media	**No of isolates obtained**
Glucose	9
Starch	6
Cellulose	6
Xylose	5
DSC-97	3
M6	3
M8	5

4.3 Morphological characterization

Gram staining showed that 34 isolates were gram positive (Figure 6), 2 were gram variable and only X-5 was gram negative (Figure 5). The isolates' colony morphology ranged from short rod (27 isolates), large rods (9 isolates) and coccus (1 isolate) shapes (see table 3).

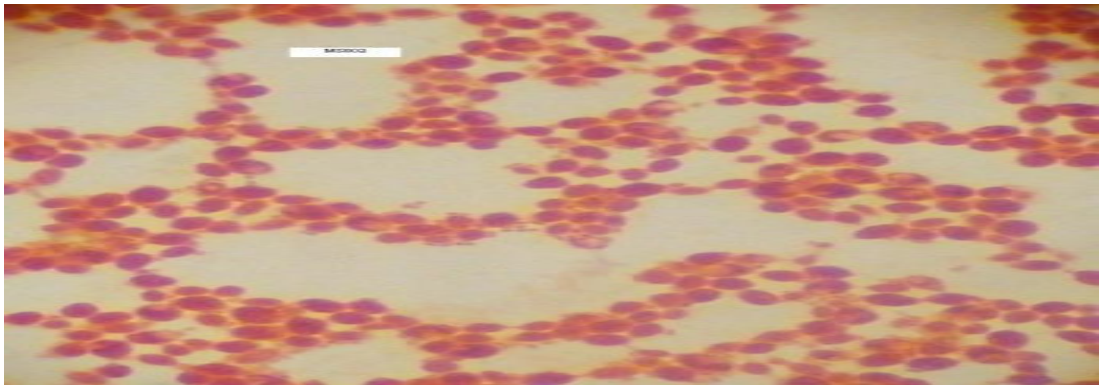


Figure 5: Photo showing the negative Gram stain reaction of isolate X-5.

Table 3: Morphological characteristics of the isolates.

Isolate	Colony color	Colony shape	Gram reaction
C1	Red-pink	Large rods	Gram positive
C3	White	Short rods	Gram positive
C4	White	Large rods	Gram positive
C5	Cream	Short rods	Gram positive
C6	Cream	Short rods	Gram positive
C13	White	Short rods	Gram positive
D2	White	Large rods	Gram positive
D6	White	Large rods	Gram positive
D9	White	Short rods	Gram positive
M6-1	White	Short rods	Gram positive
M6-7	Cream	Short rods	Gram positive
M6-10	Cream	Short rods	Gram positive
M8-1	White	Large rods	Gram positive
M8-5	White	Large rods	Gram positive
M8-12	White	Large rods	Gram positive
M8-14	White	Large rods	Gram positive
M8-15	White	Short rods	Gram positive
S2	Cream	Short rods	Gram positive
S3	Cream	Large rods	Gram positive
S7	Cream	Short rods	Gram positive
S10	Red-pink	Short rods	Gram positive
S11	Cream	Short rods	Gram positive
S12	Cream	Short rods	Gram positive
X1	Cream	Short rods	Gram positive
X2	Cream	Short rods	Gram positive
X3	Cream	Short rods	Gram positive
X5	Cream	Coccus	Gram negative
X6	Cream	Short rods	Gram positive
G2	White	Short rods	Gram variable
G4	Cream	Short rods	Gram positive
G8	Cream	Short rods	Gram positive
G11	White	Short rods	Gram positive
G12	Cream	Short rods	Gram positive
G14	Cream	Short rods	Gram variable
G15	White	Short rods	Gram positive
G18	Red-pink	Short rods	Gram positive
G20	Cream	Short rods	Gram positive

C1 G15

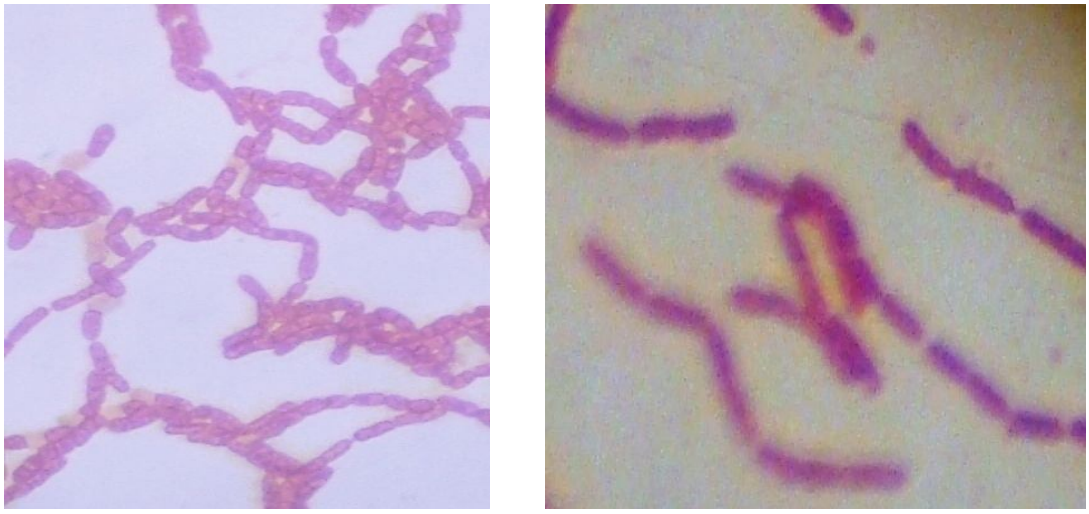


Figure 6: Photo showing the positive Gram stain reaction of isolates C1 and G15 respectively.

4.4 Physiological characterization

4.4.1 Salinity tolerance

All the 37 isolates grew in sodium chloride. Their growth was observed to improve with an increase in salt concentration from 5% up to 15% where the highest growth was recorded for the majority, followed by 20%, after which growth progressively and gradually decreased towards 25% and 30%. Most of the isolates grew poorly at sodium chloride concentration of 5%, showing that they are true halophiles. Since all the 37 isolates optimum NaCl for growth ranged between 12% and 15%, 15% NaCl was used throughout the characterization process.

Table 4: Tolerance to different salinity levels by the isolates.

Isolate	5%	7%	10%	12%	15%	20%	25%	30%	35%
S2	+	++	+++	+++	+++	++	+	+	-
S3	++	++	+++	+++	+++	++	++	+	-
S7	+	++	+++	+++	+++	++	+	+	-
S10	++	++	+++	+++	+++	++	++	+	-
S11	+	++	+++	+++	+++	++	++	+	-
S12	+	++	+++	+++	+++	++	++	+	-
X1	+	++	+++	+++	+++	++	+	+	-
X2	++	++	+++	+++	+++	+++	++	+	-
X3	-	++	+++	+++	+++	++	+	+	-
X5	+	++	+++	+++	+++	++	++	+	-
X6	+	++	+++	+++	+++	+++	+	+	-
M8-1	-	+	++	+++	+++	+++	++	+	-
M8-5	+	++	+++	+++	+++	++	++	+	-
M8-12	+	++	+++	+++	+++	++	++	+	-
M8-14	+	++	+++	+++	+++	++	+	+	-
M8-15	+	++	+++	+++	+++	+++	+	+	-
G2	+	++	+++	+++	+++	++	++	+	-
G4	+	++	+++	+++	+++	++	+	+	-
G8	+	++	+++	+++	+++	++	++	+	-
G11	+	++	++	+++	+++	+++	++	+	-
G12	+	++	++	+++	+++	+++	++	+	-
G14	+	++	++	+++	+++	+++	++	+	-
G15	+	+++	+++	+++	+++	+++	++	++	-
G18	+	++	+++	+++	+++	+++	+	+	-
G20	+	++	+++	+++	+++	+++	++	++	-
C1	+	+++	+++	+++	+++	++	+	+	-
C3	+	++	+++	+++	+++	++	++	+	-
C4	+	++	+++	+++	+++	+++	++	+	-
C5	+	++	+++	+++	+++	+++	++	+	-
C6	+	++	+++	+++	+++	++	++	+	-
C13	+	++	+++	+++	+++	++	+	+	-
D2	+	++	+++	+++	+++	++	++	+	-
D6	++	++	+++	+++	+++	++	+	+	-
D9	++	+++	+++	+++	+++	++	++	+	-
M6-1	+	++	+++	+++	+++	++	+	+	-
M6-7	++	+++	+++	+++	+++	++	++	+	-
M6-10	+	++	+++	+++	+++	++	++	+	-

4.4.2 Temperature tolerance

The isolates' temperature tolerance was tested at 15, 20, 25, 30, 35, 40 and 45^0C. None of the isolates grew at 15^oCand 45^oC. The optimum temperature for growth of the bacterial cultures was found to range between 30^oC and 35^oC.

Table 5: Tolerance to different temperature levels by the isolates.

Isolate	15 ^{o}C	20 ^{o}C	25 ^{o}C	30 ^{o}C	35 ^{o}C	40 ^{o}C	45 ^{o}C	50 ^{o}C
S2	-	++	+++	+++	+++	++	+	-
S3	-	+	++	+++	++	+	-	-
S7	-	+	+++	+++	++	+	+	-
S10	-	+	+++	+++	+++	++	+	-
S11	-	+	+++	+++	+++	++	+	-
S12	-	++	+++	+++	++	++	+	-
X1	-	++	++	+++	+++	++	+	-
X2	-	++	+++	+++	+++	+	+	-
X3	-	+	+++	+++	++	++	+	-
X5	-	+	+++	+++	+++	++	+	-
X6	-	++	+++	+++	+++	+	+	-
M8-1	-	+	+++	+++	++	+	+	-
M8-5	-	+	+++	+++	++	++	+	-
M8-12	-	+	+++	+++	++	++	-	-
M8-14	-	+	+++	+++	+++	+	-	-
M8-15	+	++	+++	+++	++	+	+	-
G2	+	+	+++	+++	+++	++	+	-
G4	-	+	++	+++	++	++	+	-
G8	-	+	+++	+++	+++	++	+	-
G11	-	+	+++	+++	++	++	-	-
G12	-	+	+++	+++	+++	++	+	+
G14	-	+	++	+++	+++	+++	++	-
G15	-	+	+++	+++	+++	++	+	-
G18	-	++	+++	+++	++	+	+	-
G20	+	+	++	+++	+++	++	+	-
C1	-	++	+++	+++	+++	++	+	-
C3	-	+	+++	+++	+++	++	++	-
C4	-	++	+++	+++	++	++	+	-
C5	-	+	+++	+++	+++	++	+	-
C6	-	++	+++	+++	++	++	+	-
C13	-	++	+++	+++	++	+	+	-
D2	-	+	+++	+++	++	++	+	-
D6	-	+	++	++	+++	++	+	-
D9	-	-	+++	+++	+++	+-	+	-
M6-1	-	+	++	+++	++	+	+	-
M6-7	-	+	+++	+++	+++	++	+	-
M6-10	-	+	++	+++	++	+	+	-

4.4.3 pH tolerance

The results showed that none of the isolates was capable of growing at the acidic pH values. Majority of the isolates grew well at pH 8.0 - 10.0, with an optimum pH value of 9.0. Growth declined as the pH increased beyond 10.5, as reflected in table 6 below.

Table 6: Tolerance to different pH levels by the isolates

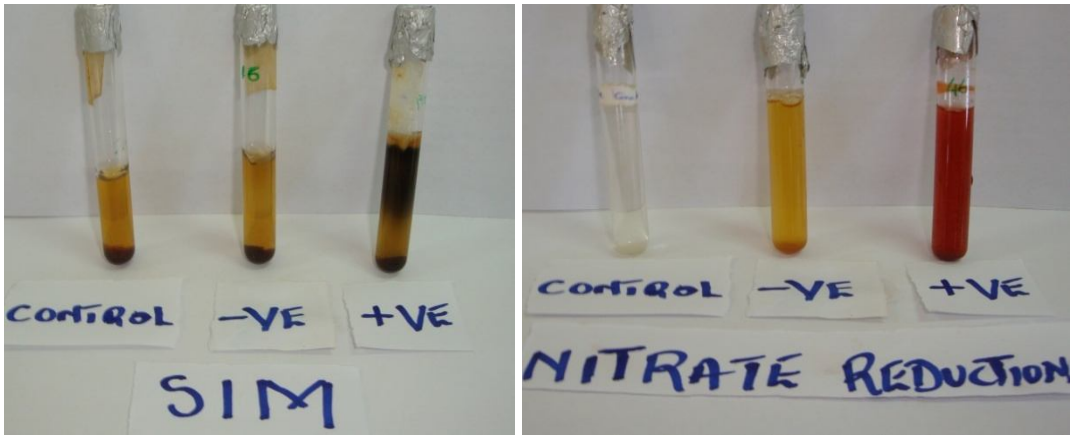
Isolate	5.5	6.0	6.5	7.5	8.0	9.0	10.0	11.0
S2	-	++	++	++	+++	+++	+++	+
S3	-	++	++	++	+++	+++	+++	+
S7	-	+	+	+	+++	+++	+	+
S10	-	+	+	++	+++	+++	+	+
S11	-	+	+	+++	+++	+++	++	++
S12	-	+	+	++	+++	+++	+++	+
X1	-	+	+	+++	++++	+++	++	+
X2	-	-	+	++	+++	+++	++	++
X3	-	+	+	++	+++	+++	++	+
X5	-	-	+	+	+++	+++	+	+
X6	-	+	+	++	+++	++	+++	++
M8-1	-	+	+	++	+++	++	++	++
M8-12	-	+	+	++	+++	+++	++	+
M8-5	-	-	+	+	+++	+++	+	+
M8-14	-	+	+	++	+++	+++	++	+
M8-15	-	+	+	++	+++	+++	+++	++
G2	-	+	+	++	+++	+++	+++	+
G4	-	+	+	++	+++	+++	++	+
G8	-	+	+	++	+++	+++	++	+
G11	-	++	++	++	++	+++	++	+
G12	-	+	+	++	++	++	+++	++
G14	-	+	+	++	++	+++	++	+
G15	-	++	++	++	++	+++	++	+
G18	-	+	+	++	++	+++	++	+
G20	-	+	+	++	++	++	+++	+
C1	-	+	+	++	+++	+++	+++	+
C3	-	+	+	++	++	+++	++	+
C4	-	+	++	++	++	+++	+++	+
C5	-	+	+	++	++	+++	++	+
C6	-	+	+	++	+++	+++	+	+
C13	-	+	+	++	+++	+++	+	+
D2	+	+	++	+++	+++	+++	+	+
D6	-	+	++	++	+++	++	+++	+
D9	+	+	++	++	+++	+++	+	+
M6-1	-	+	++	++	+++	+++	++	+
M6-7	-	+	++	+++	+++	+++	++	+
M6-10	-	+	+	++	+++	+++	++	+

Key: **+** growth took ≥ 5 days**; ++** growth took 3-4 days and **+++** growth took ≤ 2 days

4.5 Biochemical tests of the isolates

The results of the varoius biochemical tests are summarized in the table 7.

A B



C D

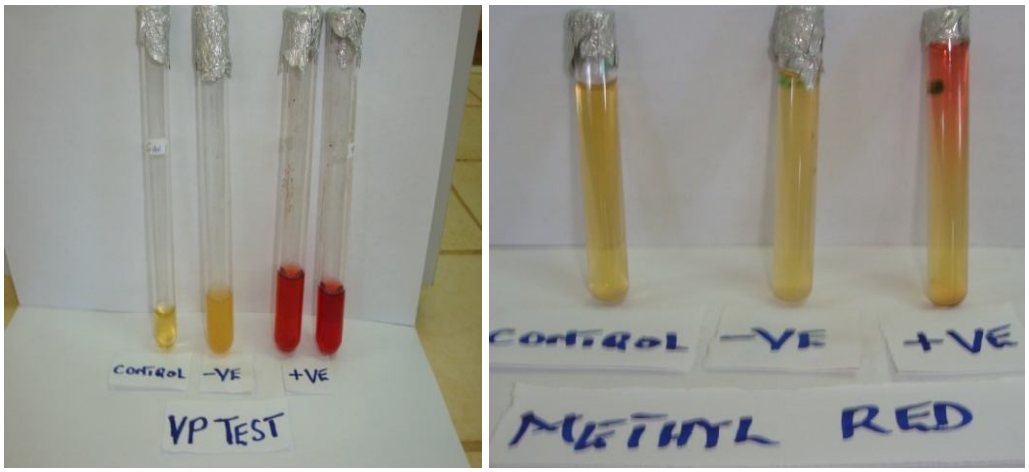


Figure 7: Biochemical tests carried out on the isolates; Sulfur-Indole test (A), Nitrate reduction test (B), Voges-Proskauer test (C) and Methyl red test (D).

Table 7: Biochemical characteristics of the isolates.

Isolates	Biochemical Tests												
	Arg	Ly	Orn	Gel	Nitrate	MR	VP	Cat	Oxid	Urea	Cit	H_2S	Ind
M6-1	+	-	+	-	-	-	-	+	+	+	-	-	-
M6-7	+	-	+	-	+	-	-	+	+	+	+	+	-
M6-10	+	-	-	-	-	-	-	+	+	-	+	+	-
C-1	-	-	+	+	-	-	-	+	+	-	-	-	-
C-3	+	-	+	-	-	-	-	+	+	-	-	+	-
C-4	-	-	+	-	-	-	-	+	+	+	-	+	-
C-5	-	-	+	-	-	-	-	+	+	+	-	-	-
C-6	-	-	+	-	-	-	-	+	+	-	-	+	-
C-13	+	-	+	-	-	-	-	+	+	+	-	-	-
X-1	-	+	-	+	-	+	-	+	+	-	-	-	-
X-2	+	+	-	+	-	+	-	-	-	+	-	-	+
X-3	+	+	-	-	-	-	-	+	+	+	-	-	-
X-5	+	-	+	-	-	-	-	+	+	-	-	-	+
X-6	+	-	-	-	-	-	-	+	+	-	-	-	-
S-2	-	-	+	-	-	-	-	+	+	+	-	+	+
S-3	-	+	-	-	-	-	-	+	+	+	-	-	-
S-7	+	+	-	+	-	+	-	-	-	-	-	-	+
S-10	-	+	+	-	+	-	-	+	+	+	-	+	+
S-11	+	+	-	-	-	-	-	+	+	+	-	-	+
S-12	-	+	-	+	-	-	-	-	+	-	-	-	-
D-2	+	-	-	-	-	-	-	+	+	+	-	+	-
D-6	-	+	+	+	-	-	-	+	+	+	-	-	-
D-9	+	+	-	+	+	+	-	+	+	-	+	-	-
G-2	+	+	+	-	-	+	-	+	+	-	-	+	-
G-4	+	-	+	+	+	+	+	+	+	+	+	+	+
G-8	+	-	-	-	-	-	-	+	+	-	+	+	-
G-11	-	-	+	-	-	+	-	+	+	-	-	+	+
G-12	-	-	-	-	-	-	-	+	+	-	-	+	-
G-14	-	+	-	-	-	-	-	+	+	-	-	+	+
G-15	-	-	+	+	-	-	-	+	+	-	-	-	-
G-18	+	-	-	-	-	+	-	+	-	+	-	+	-
G-20	+	-	+	-	-	-	-	+	+	+	-	-	-
M8-1	-	-	-	-	-	-	-	+	+	-	-	-	-
M8-5	+	-	-	-	-	-	-	+	+	-	+	+	-
M8-12	+	+	-	-	-	-	-	+	+	+	-	-	+
M8-14	+	-	+	-	-	-	-	+	-	-	+	+	+
M8-15	-	-	+	-	-	-	-	+	+	-	+	-	+

4.6 Enzyme production.

The isolates were screened for their ability to produce extracellular enymes using basal media, supplemented with the respective substrates. Enzymes tested included: cellulase, amylase, xylanase, protease, lipase,and carboxymethylcellulase. None of the isolates produced cellulase, carboxymethylcellulase, esterase and xylanase enzymes. 11 isolates produced amylase, 7 produced protease, 7 produced lipase enzymes. Some isolates produced more than one type of enzyme. No isolate yielded a postive result when screening for antimicrobial activity was done. The results for enzyme production are tabulated in table 8.

S11 C1

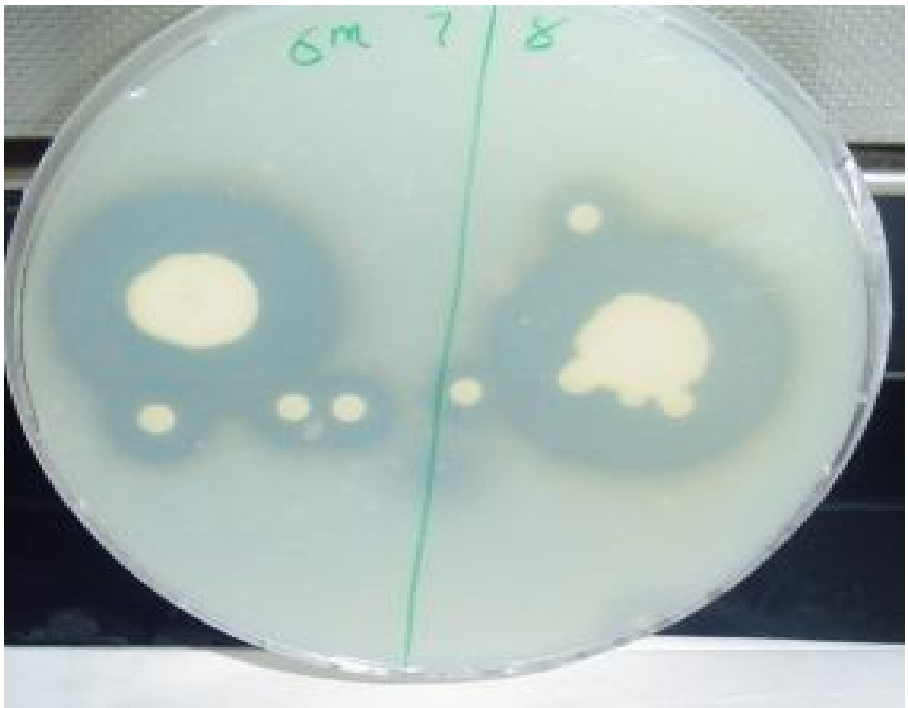


Plate 1: A photograph showing proteolytic activity on skim milk by isolate S11 and C1 respectively.

Table 8: Enzyme producing isolates.

Isolates	Amylase	Protease	Lipase	Cellulase	Methyl-cellulase	Xylanase	Esterase
G15	-	+	-	-	-	-	-
G18	-	-	+	-	-	-	-
G2	-	-	-	-	-	-	-
G4	-	-	-	-	-	-	-
G8	-	-	-	-	-	-	-
G11	-	-	-	-	-	-	-
G12	-	-	-	-	-	-	-
G14	-	-	-	-	-	-	-
G15	-	-	-	-	-	-	-
C1	+	+	-	-	-	-	-
C3	+	-	+	-	-	-	-
C4	+	-	-	-	-	-	-
C5	-	-	-	-	-	-	-
C6	-	-	-	-	-	-	-
C13	+	-	-	-	-	-	-
D2	+	-	-	-	-	-	-
D6	+	+	-	-	-	-	-
D9	-	+	-	-	-	-	-
M6-10	+	+	+	-	-	-	-
M6-1	-	-	-	-	-	-	-
M6-7	-	-	-	-	-	-	-
M8-5	-	-	-	-	-	-	-
M8-14	-	-	-	-	-	-	-
M8-15	-	-	-	-	-	-	-
M8-1	+	-	-	-	-	-	-
M8-12	+	-	+	-	-	-	-
S10	+	-	+	-	-	-	-
S11	+	+	+	-	-	-	-
S12	-	+	+	-	-	-	-
S2	-	-	-	-	-	-	-
S3	-	-	-	-	-	-	-
S7	-	-	-	-	-	-	-
X1	-	-	-	-	-	-	-
X2	-	-	-	-	-	-	-
X3	-	-	-	-	-	-	-
X5	-	-	-	-	-	-	-
X6	-	-	-	-	-	-	-

4.7 Molecular characterization

Genomic DNA from all the isolates was successfully extracted using the phenol/chloroform/CTAB modification of Broderick *et al*., (2004), method. The 16S rDNA region was then used for the molecular characterization of the isolates.

4.7.1 PCR amplification of 16s rDNA genes from isolates

The amplification of the 16S rDNA region of the genomic DNA was done using universal bacterial primers bac 8F and bac 1492R (Embley and Stackebrandt, 1994; Lane, 1991). The amplicons were then stained with ethidium bromide and visualised under UV light on 1 % agarose gel (Figure 6).

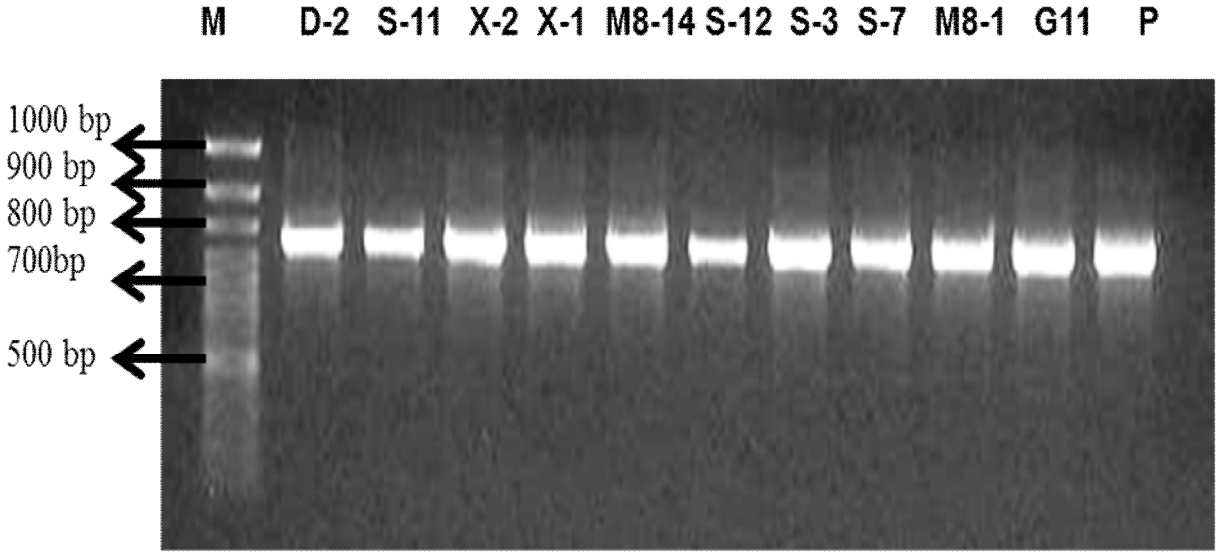


Figure 8: A photograph of PCR amplified 16S rDNA products of 10 isolates using Universal primers bac 8F and bac 1492-R, run in 1 % (W/V) agarose gel. M-Marker and P-positive control.

4.7.2 Phylogenetic analysis of sequences

Table 9: The blasted isolates, nearest neighbors in the data bank and their % relatedness.

Isolate	Length	Nearest relatives	Accession number	% similarity
C4	664 bp	*Bacillus sp*. W8B-43	HR238689	99%
C1	862 bp	*B. subtilis strain Bs 20*	HQ267754	78%
C3	659 bp	*Bacillus* sp. DB110 (2010)	HM566824	98%
C5	571 bp	*C.sporogenes strain McClung2004*	NR029231	84%
C6	873 bp	*Clostridium sp.* SA-4	AY695835	95%
D2	775 bp	*B. cereus* strain PM-2	FJ598436	99%
D6	672 bp	*B. cereus* strain OCOR1DBT	JF264468	99%
D9	560 bp	*B. licheniformis* strain C18	HQ336641	99%
G2	540 bp	*B. licheniformis* strain C18	HQ336641	99%
S10	606 bp	*B. licheniformis* strain PRL1	EF062991	99%
G4	581 bp	*Closridium sp.* N56	AB600535	99%
G8	581 bp	*Clostridium sp.* N56	AB600535	99%
G11	691 bp	*Bacillus sp.* JM4	EF062991	99%
G12	863 bp	*C. botulinum strain H04402 065*	Dq196620	97%
G15	596 bp	*B. licheniformis* strain GLU113	FN678352	100%
G18	571 bp	*B. licheniformis* strain APS2MSU	HQ637792	99%
G20	764 bp	*C. sporogenes*	AB595130	85%
G14	1021 bp	*C. sporogenes*	NR 029231	80%
X1	731 bp	*Clostridium sp*. SA-4	AY695835	99%
X2	765 bp	*Clostridium sp*. N56	AB600535	99%
X3	786 bp	*C. botulinum strain 5564*	HQ328066	88%
X5	521 bp	*H. campisalis strain H10.1.1*	GU228478	94%
X6	847 bp	*B. licheniformis strain Crk21*	GQ503331	97%
S2	529 bp	*Clostridium sp.* N56	AB600535	99%
S3	714 bp	*Bacillus sp.* OC-6	AY669167	99%
S7	712 bp	*Clostridium sp*. N56	AB600535	99%
S12	716bp	*B. licheniformis* strain C18	HQ336641	99%
S11	772 bp	*B. cereus* strain SL3G	HQ283476	99%
M6-7	686 bp	*Clostridium sp*. N56	AB600535	99%
M8-1	692 bp	*B. cereus* strain OCORIDBT	FJ264468	99%
M8-12	558 bp	*B. cereus* EBT1	JF50734	99%
M8-14	721 bp	*B. cereus* strain OCORIDBT	FJ264468	99%
M8-15	674 bp	*Clostridium sp.* N56	AB600535	99%
M6-1	562 bp	*B. licheniformis* strain APS2MSU	HQ143636	99%
C13	598 bp	*B. licheniformis* strain BPRIST039	JF700489	99%
M6-10	942 bp	*Bacillus licheniformis* strain MEL09	FJ715927	98%

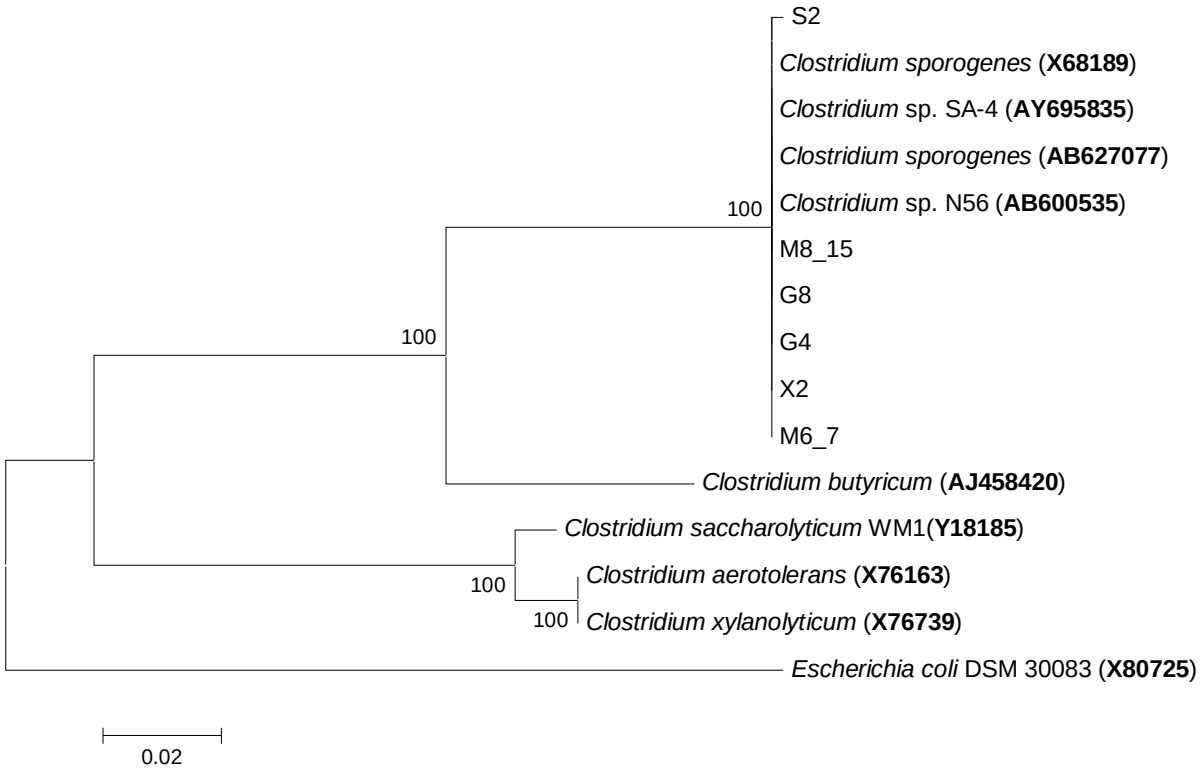


Figure 9: Evolutionary relationships of taxa in the genus *Clostridium*

The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The evolutionary distances were computed using the Jukes-Cantor method (Jukes and Cantor, 1969). There were a total of 519 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura *et al*., 2011). Only representative sequences are shown in the tree.

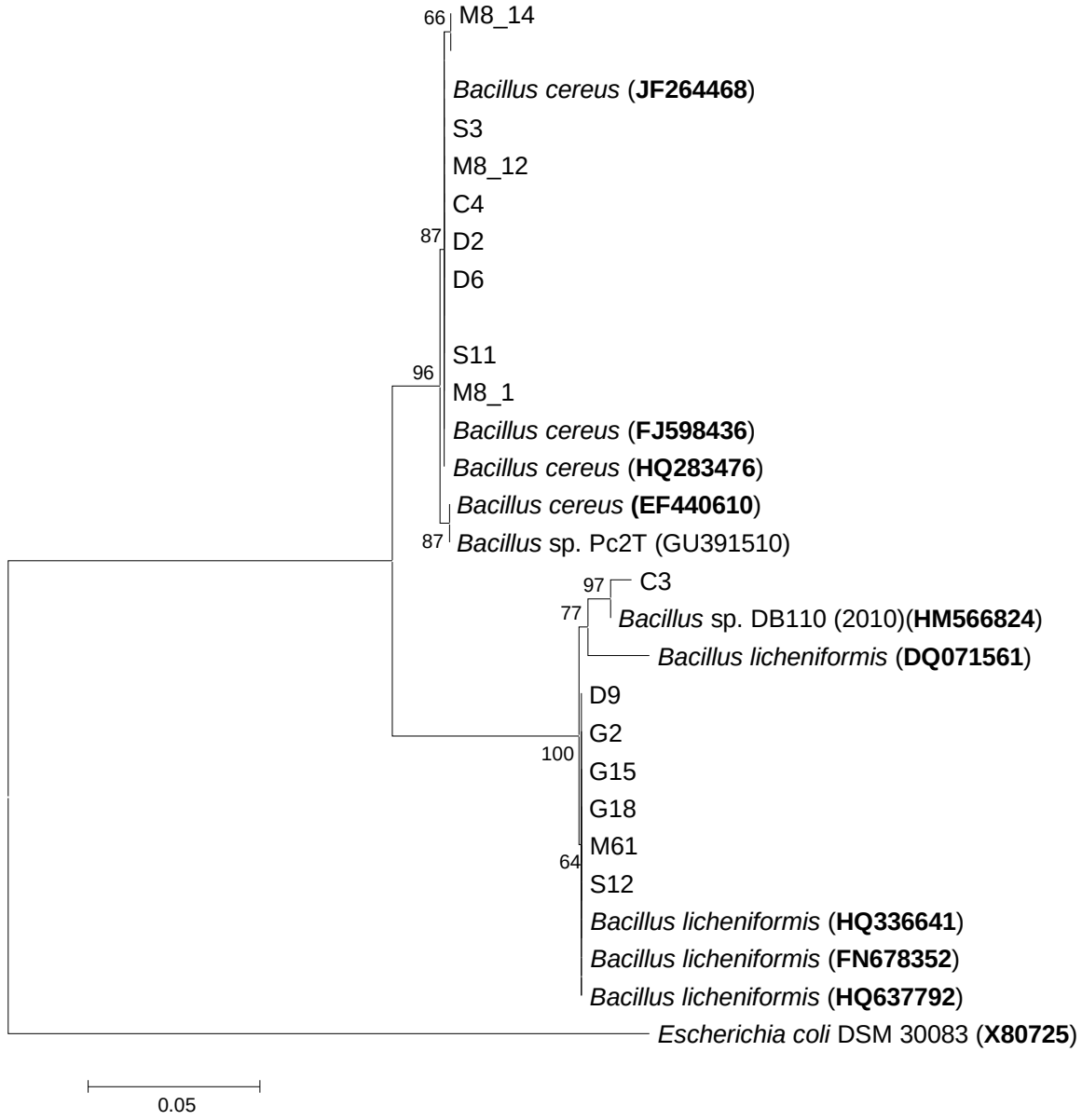


Figure 10: Evolutionary relationships of taxa in the Genus *Bacillus*.

The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The

evolutionary distances were computed using the Jukes-Cantor method (Jukes and Cantor, 1969). There were a total of 519 positions in the final datase. Evolutionary analyses were conducted in MEGA5 (Tamura *et al.*, 2011). Only representative sequences are shown in the tree.

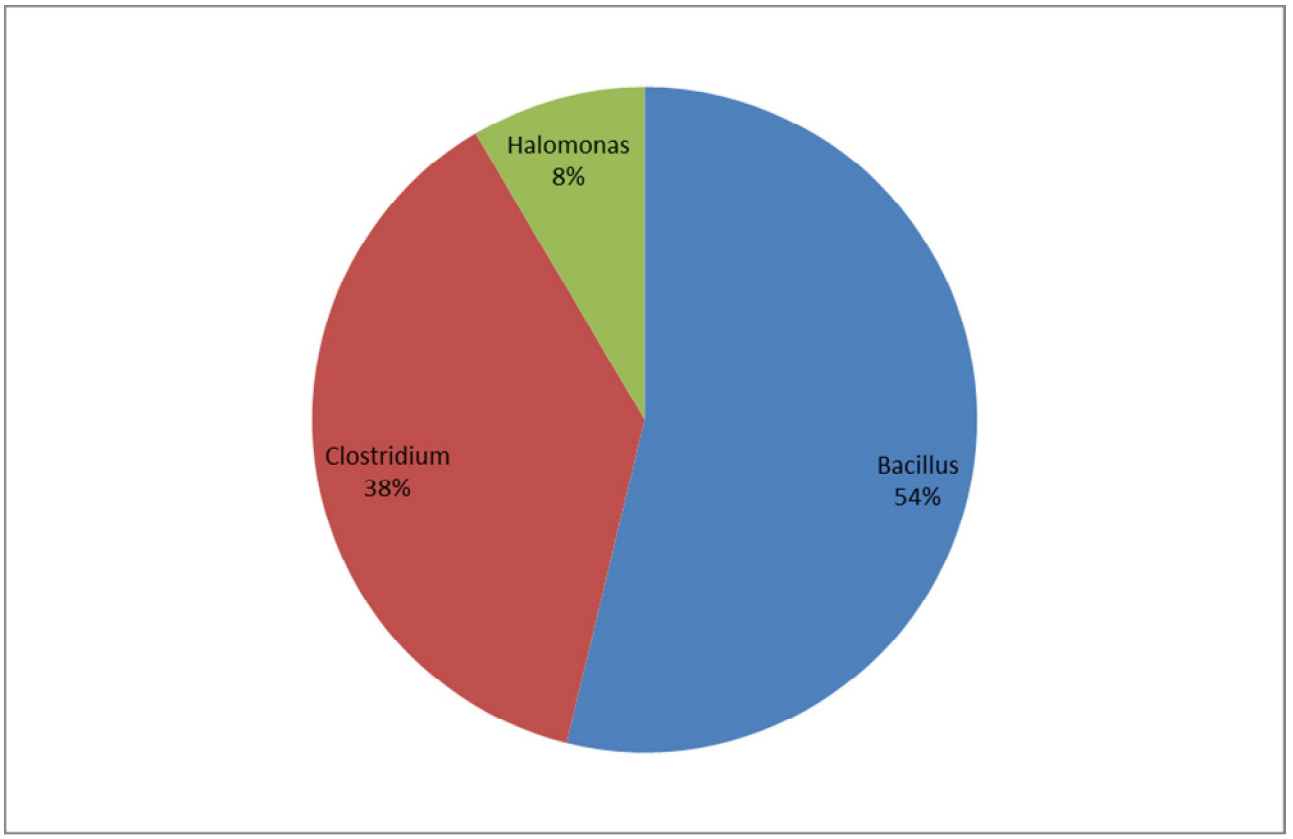


Figure 11: Pie chart showing the percentage composition of the isolates per genera.

CHAPTER FIVE:

5.0 DISCUSSION

Several authors have carried out studies and characterized the microbiological inhabitants of theEast African Rift Valley lakes (Melack, 1981). The isolates subjected to phenotypic, chemotaxonomic and phylogenetic analysis showed considerable phylogenetic diversity (Duckworth *et al.*, 1996; 2000 Jones *et al*., 1994, Mwirichia *et al.,* 2010a; 2010b).

The present study aimed at assessing the microbial diversity of Lake Magadi using a culture- dependent approach. Its objectives included the isolation, characterization and identification of the lakes' microbes using cultural, biochemical and molecular methods, and investigation of the ability of these microbes to produce enzymes of biotechnological importance. The results presented here confirm that there is a considerable phylogenetic diversity in the soda lakes.

5.1 Culturing and Isolation

A wide range of media types were used for isolation and culturing. Media 1-4 (Duckworth *et al.,* 1996) were rich in carbon while Media 5-7 (Xu *et al.,* 2006; Phil *et al.,* 2005), were rich in mineral composition. The use of a wide range of media, modified with a variety of carbon sources was used to reflect and promote the belief this would widen the range of haloalkaliphilic organisms cultured, including novel microbes not detected in soda lakes before (Mwirichia *et al*., 2010a) and promote the making of useful enzymes (Duckworth *et al.*, 1996; Mwirichia *et al.,* (2010b).

Preparation of all the isolation media was done using Lake Magadi water, to preserve lake conditions of pH, salinity and mineral conditions as per the method of Mwirichia *et al.* (2010b).

The highest total bacterial number was found in the modified carbon rich media (M1-IV), originally described by Duckworth *et al*. (1996), while a low number of isolates was detected in the mineral rich media. The carbon rich media also seemed to favour the growth of *Halomonas* and *Bacillus.*

Wagner *et al.* (1994) and Mwirichia *et al*., (2010a), using the same media in different studies, both recovered members of the genera *Bacillus* from soda lakes. Wagner (1994), equipped that media rich in organic carbon such as glucose, seems to favour the growth of the fast growing microbes, which include *Bacillus* and *Halomonas,* at the expense of other groups (Wagner *et al.*, 1994). The abundance of *Bacillus* over the other groups is probably because they produce endospores that enable them to survive from one alkaline episode until the next (Grant *et al.*, 1999).

Among the carbon rich media, the glucose containing media had the highest number of isolates. The xylose based media had the lowest number of morphotypes but is the one that realized the highest diversity, as it is the only media that yielded representatives of all the three recovered genera. In the current study representative of genus *Halomonas* were recovered from the xylose containing media only whereas in the study of Mwiricha *et al*. (2010a), the *Halomonas* relative was recovered from the glucose modification of the Duckworth) *et al.* (1996) media and media 5 (DSC-97).

Media 5 to 7, generally rich in minerals and low in organic carbon content, were prepared to target the isolation of *Halobacteria*. Though the initial morphological characterization in isolates S-10, G-18 and C-1 revealed the red and pink colony colours characteristic of *halobacteri*a, sequencing and blasting revealed that these were all related to genus *Bacillus*. Generally carbon rich media had more morphotypes (26 versus 11) and diversity when compared to the mineral rich media. For instance, M-5 isolates were all related to genus *Bacillus*, M-6 and M-8 had only one isolate each related to genus *Clostridium.*

The media composition did not seem to have an effect on the type of group recovered as the results indicate that members of genera *Bacillus* were recovered from all the 7 media types while relatives of genera *Clostridium* were recovered from six media types.

5.2 Morphological characterization of the isolates

Though both Gram positive and Gram negative isolates were recovered in this study, the Gram positives were the majority (34) **(**Table 3**)**. This is in agreement with the studies of Duckworth *et al*. (1996) and Grant *et al*. (1999), who found out that in soda lakes, the low G+C Gram positive bacteria are more abundant than the aerobic Gram-negative bacteria. Family *Halomonadaceae* probably constitute the single most important Gram-negative group in the soda lake environment while majority of the Gram positives are associated with the more diverse *Bacillus* spectrum (Wiegel, 1999).

The gram staining results were confirmed by blast results that identified the gram positive isolates as being related to genus *Bacillus* and *Clostridium*. Only one isolate was affiliated

to the the Gram negative genus of *Halomonas*, results that are in tandem with those of Wiegel, (1999).

5.3 Physiological characterization of isolates

5.3.1 Growth at different salinity levels

When cultured at different salinity [NaCl] levels, all the isolates grew within salinity range of 5% - 30%. Only isolates M8-1 and X-3 failed to grow at 5% NaCl. The optimum [NaCl] for the isolates growth ranged between 10%- 15%. Actually isolates G2, G4, G8, G11, G12, G14 G15, G-20, S-2, S-3, S-7, S-11, S-12, C-3 and C-6 had an optimum [NaCl] of 12%, isolates C-1, S-10 and G-18 had their optimum growth at 15% while the rest had an optimum growth at [NaCl] of 10% and 12% (Table 4). The ability of these isolates to grow at 5% [NaCl] and above shows that they are true halophiles and not halotolerants (Mesbah *et al*., 2007). Though the isolates grew at sodium chloride concentrations above optimum, the rate of growth was slow especially at [NaCl] of 25% and above in that it took longer for the colonies to form. This can be attributed to the numerous negative effects of higher salinity such as dehydration, inhibition of proteolytic enzymes, increased sensitivity to CO_2 and removal of O_2 from the habitat (Oren, 2002). The non pigmented isolates generally took lesser time (average of 2-3 days) to grow while their pigmented counterparts took an average of 5-7 days. Given that all the isolates grew at salinities above a [NaCl] of 20%, they all can be considered extreme halophiles (Ollivier *et al*., 1998; Duckworth *et al.,* 2000; Joshi *et al.,* 2007).

The hitherto uncultured extremely halophilic bacteria isolated by Anto'n *et al.* (2000) from multi solar saltern ponds in Spain showed high abundance and growth at salinity levels ranging from 30 to 37% and an optimum salinity for growth of between 20 and 25% total salts, typical of an extreme halophile. Haloalkaliphilic *Clostridium* and *Bacillus* species cultivated from the hypersaline East African soda lakes were able to grow well in 25-30% w/v NaCl with a minimum requirement for at least 12-15% w/v NaCl respectively (Jones *et al*., 1994; Jones and Grant, 1999a).

The *International Journal of Systematic Bacteriology/Systematic and Evolutionary Microbiology* of 2009 validly published over 60 species which are extremely halophilic. Of these species, approximately thirty percent have [Na^+] optima of less than 2.0 M (equivalent to approximately 12% w/v NaCl), nineteen of which are published at 1.7 M (equivalent to approximately 10% w/v NaCl) and approximately forty-five percent of the extremely halophilic species have published [Na^+] optima equal to or greater than 2.0 M but less than 3.4 M (Bowers *et al.*, 2009).

These results indicate that Bacteria constitute a significant and important part of the microbiota that inhabit NaCl-saturated waters previously thought to be the exclusive preserve for Archaea. Bacteria therefore seem to have also developed wide ecological competence, and are as widespread as Archaea (Anto'n *et al.*, 2000).

The ability of the microbes isolated in this study to grow fast and optimally at the environmental and physicochemical parameters of high pH, elevated temperature and salinity unsuitable for the growth of other microorganisms, makes them good models for

research, such as that of the adaptive mechanisms to extreme environmental parameters (Kevbrin *et al.*, 1998). They can also be grown easily and in large numbers whenever required to produce stable enzymes courtesy of their temperature optima which reduces chances of contamination in economically feasible non aseptic applications (Kevbrin *et al.*, 1998).

5.3.2 Growth at different temperatures

All the isolates grew at temperatures less than 45 °C and above 20 ^{o}C. Only isolates G-15 and M8-15 grew at temperatures of 15 ^{o}C. The optimum temperature for most of the isolates ranged between 30 ^{o}C and 35 ^{o}C (Table 5). This optimum temperature seems to concur with the findings of early studies carried out in Lake Magadi. Duckworth *et al.* (2000) for instance isolated a halomonad (*Halomonas magadii* sp. nov.,) that grew well between 25°C and 40 ^{o}C with an optimum temperature of 37 ^{o}C. Bolyanskaya *et al.* (2004) have also described another halomonad from an alkaline Lake in the USA capable of growing within a wide temperature range of between 10 $^{\circ}$C and 55 $^{\circ}$ C and an optimum of 36-40 $^{\circ}$C. An alkaline protease producing bacteria isolated by Abdelnasser *et al.* (2007) from Lake Wadi el-Natrun, a soda lake in Egypyt was observed to grow at temperature ranges of 25 $^{\circ}$ C and 55 $^{\circ}$C.

These bacteria grew at temperatures lower than those recorded at sampling points (which ranged from 39.4 ^{o}C and 51.9 ^{o}C). This is indicative of the adaptation of the bacterial communities in Lake Magadi to a wide range of temperatures and agrees with Baumgarte

(2003), who isolated bacterial strains from Lake Magadi which clustered with neighbors that grow well at temperature range of 10 ^{o}C - 55 ^{o}C.

5.3.3 Growth at different pH levels

All the isolates grew at pH ranges of between 6.0 -11.0 (Table 6). Only isolates D-2and D-9 were able to grow at pH level of 5.5. These shows that they all are tolerant to acidity (oren, 2006). Isolates M6-1, M8-1, M8-12, M8-15, C-1, C-3, C-5, C-6, D-2, D-6, D-9, S-2, S-10, S-11, S-12, G-2, G-11, G-15, G-18 and X-6 grew optimally at pH level of 9.0 and 10.0 while the rest of the isolates had an optimum growth at pH range of 8.0 and 10.0. Though they all grew at pH 11.0, they took a longer period for the colonies to appear than at the lower pH levels. Some of the documented pH ranges over which haloalkaliphiles and alkaliphiles grow include: 9.0 to 10 (Horikoshi; 1999), 10-10.5 (Grant *et al*., 1999), 7.0 - 11.0 (Duckworth *et al*., 2000) and 8 to 11.0 (Abdelnasser *et al.*, 2007). Zhiyu *et al.* (2002) isolated two *Bacillus* species, *Bacillus okuhidensis* and *Bacillus halodurans* that had their optimum growth at the same pH range. *Halomonas magadii* described by Duckworth *et al.*, (2000), had an optimum growth at a pH of 9.5, *Halomonas campisalis* isolated from an alkali lake in the USA had an optimum of 8.8-9.5 (Bolyanskaya *et al.*, 2004), and the one isolated from the alkaline Lonar lake in India by Joshi *et al*. (2007) had an optimum growth at pH 9. The observed pH values are therefore consistent with these earlier findings, showing that alkalinity favours the growth of haloalkaliphiles and alkaliphiles. A pH range of 9.0 to 10 may serve as their selective optimum pH (Horokoshi, 1999).

5.4 Biochemical characterization of the isolates

Isolates M6-1, M8-1, M8-12, M8-14, C-1, C-3, C-4, C-13, D-2, D-6, D-9, S-3, S-10, S-11, S-12, G-2, G-11, G-15, G-18 and X-6 all related to genus *Bacillus,* were gelatinase positive, catalase positive and produced spores. Some were citrate positive, motile and had ability to reduce nitrates (Table 8). The reduction of nitrates indicates their ability to produce enzyme nitrate reductase which reduces nitrates to nitrites or free nitrogen gas and water (Harold, 2002), that the cell uses as a final hydrogen acceptor during anaerobic respiration.

Genus *Bacillus* has a vast diversity of physiological types such as degraders of most substrates from plants and animals, nitrifiers, denitrifiers, and nitrogen fixers among others (Jones and Grant, 1999a). The fact that the majority of these *Bacilli* were isolated from media with complex carbohydrates, their abundance in a soda lake known to be rich in organic materials, and their ability to produce an asernal of extracellular hydrolytic enzymes supports the view that they may have a role in the breakdown of complex biopolymers, and subsequent mineralization of the resident organic matter in Lake Magadi (Jones and Grant, 1999a).

Isolate X-5 was oxidase positive, catalase positive; produced H_2S, l- Lysine, l- arginine, l-alanine and l ornithine positive, citrate positive and able to reduce nitrates to nitrites also. The isolate did not produce indole acid, was casein negative and was not able to hydrolyse gelatin. The ability of this isolate to reduce nitrates and its ability to utilize and grow on several substrates including the complex polysaccharides indicates that members of these groups might be involved in nutrient recycling and in the nitrogen cycle of the lake.

Halomonads capable of reducing nitrate to nitrite, further reduction of the nitrite and anaerobic growth with and without nitrate form a major part of the soda lake microbial community. Halomonads have been isolated from the littoral sediments of Lake Magadi, Lake Nakuru, and Lake Bogoria by Duckworth *et al*. (1996; 2000). For example, Zhilina *et al*. (1998) isolated pure cultures of eight strains of denitrifiers which were capable of reducing nitrates at pH 10 from the soda-depositing Lake Magadi of Kenya. The presence of nitrate-reducing microorganisms in a system is a prerequisite for a nitrogen cycle to be completed (Jones and Grant, 1999a). *Halomonas* therefore, are thought to play an important and substantial role in the nitrogen cycle of these lakes (Jones and Grant, 1999a; Jones *et al*., 1998). Isolate X-5 could therefore be involved in the nitrogen cycle.

Studies carried out on soda-containing lakes have revealed that they are rich in nitrate and organic substrates (Sorokin *et al.* 1998; 2000; Jones and Grant, 1999b). The high nutrient content is attributed to the activities of nitrogen-fixing bacteria and other nitrifiers (Sorokin *et al*., 2000) which include bacteria such as *Thioalkalivibrio denitrificans* and the representatives of the genus *Halomonas* (Duckworth *et al.*, 2000; Sorokin *et al*., 2001).

C*lostridia* are widely distributed in nature, and courtesy of their saprophytic lifestyle, they play a major role in the degradation of organic material in the soil and other natural environments (McGraw-Hill, 2002). The secretion of extracellular enzymes by many species enables them to utilize a wide variety of complex natural substrates found in the environment (Doyle, 2007).

5.5 Screening for enzyme production

The screening of the isolates for enzyme production showed an ability to produce various enzymes among them amylases, proteases and lipases. Potease activity was detected in isolates S-11, S-12, D-6, D-9, G-15, M6-10 and C-1 all of which are related to species of *Bacillus* (Table 7). Earlier studies have also isolated and characterized several *Bacillus* species that produced halophilic proteases (Kumar and Satyanarayana, 2004; Setyorini *et al.,* 2006; Shivanand and Jayaraman, 2009). For example, Horikoshi (1971) reported the production of an extracellular alkaline serine protease from alkaliphilic *Bacillus* sp. strain 221, whose optimum pH was 11.5 and retained 75% of its activity at pH 13.0. Aunstrup *et al.* (1972) reported two *Bacillus* strains, AB42 and PB12, which also produced an alkaline protease that exhibited a broad pH range (pH 9.0 to 12.0). Takami *et al.* (1989) isolated a new alkaline protease from alkaliphilic *Bacillus* sp. strain AH-101, that was most active towards casein at pH 12 to 13. Kobayashi *et al.* (1995) isolated and purified an alkaline protease from alkaliphilic *Bacillus* sp. strain KSM-K16 that was suitable for use in detergents. Abdelnasser *et al.* (2007) isolated Bacillus *halodurans* that secreted proteolytic enzymes, from Lake Wadi El Natrun.

Microbial proteases are one of the most extensively studied widely applied enzymes in industrial processes (Abdelnasser *et al.,* 2007). They are commonly used as additives in laundry detergents, food processing, pharmaceuticals, leather and diagnostic reagents, waste management as well as silver recovery from X- ray films (Karbalaei-Heidari *et al.,* 2009; Amoozegar *et al.,* 2007). Some proteases such as those from *Nesterenkonia* species have

been reported to display unique substrate specificities which might open up new application opportunities (Bakhtiar *et al.*, 2005).

Lipase activity was exhibited by isolates X-5 (a halomonad), X-6, S-10, G-18 and C-3 all of which correspond to genus *Bacillus*. Several good lipase producers from soda lakes belonging to the genus *Bacillus* such as *B. Halodurans, B. alcalophilus and Bacillus licheniformis* and *Halomonas desiderata* have been reported (Joshi *et al.*, 2007; Vargas *et al*., 2004) but not many studies have shown the production of lipolytic enzymes by members genus *halomonas* so far (Vargas *et al*., 2004). Wang *et al.* (1995) isolated a thermophilic lipase-producing *Bacillus sp*., from a hot-spring area of Yellowstone National Park that grew optimally at 60 to 65°C and in the pH range from 6 to 9.

Lipases are important as components of many washing detergents, used in paper, pulp, pharmaceutical, food, leather, chemical and waste treatment industries (Rees *et al*., 2003).

Amylase production was evidenced in isolates C-3, C-4, C-13, D-6, M8-1, M8-12, S-10, S-11, M6-10, C-1 (all related to genus *Bacillus*) and X-5 (*Halomonas*).The alkaline amylase has been produced in alkaliphilic *Bacillus* sp. strain A-40-2 (ATCC 21592) (Horikoshi, 1971). These enzyme was most active at pH 10.0 to 10.5 and retained 50% of its activity between pH 9.0 and 11.5. Kim *et al.* (1995), reported that the alkaliphilic *Bacillus* sp. strain GM8901 produced five alkaline amylases in a culture broth. McTigue *et al.* (1995) studied alkaline amylases of three alkaliphilic *Bacillus* strains namely: *Bacillus halodurans* A-59 (ATCC 21591), *Bacillus* sp. strain NCIB 11203, and *Bacillus* sp. strain IMD370. They all produced alkaline α-amylases with maxima activity at pH 10.0. Kelly *et al*. (1995) found

that the alkaline amylase of *Bacillus* sp. strain IMD370 could hydrolyze raw starch. Igarashi *et al.* (1998) isolated a novel liquefying α-amylase (LAMY) from cultures of an alkaliphilic *Bacillus* isolate, strain KSM-1378, that had a pH optimum of 8.0 to 8.5 and displayed maximum activity at 55°C. Halophilic amylases have also been produced from *Halomonas meridiana* (Coronado *et al.,* 2000).

Amylases are a class of hydrolases which catalyse the degradation of starch polymers to produce dextrins and different gluco-oligosaccharides (Gupta *et al*., 2003). They are widely employed in different biotechnological applications including the food industry where they are used extensively in the bread baking industry to improve the volume of dough, colour and crumb softness, in detergents to promote stain removal and in the paper and pulp industry for the modification of starches for coated paper (Gupta *et al*., 2003). The industrial hydrolysis of starch is commonly perfomed in the presence of high salt and low water activity and therefore the stability and ability of halophilic amylases to function optimally at extremes of pH, NaCl and elevated temperatures makes them attractive candidates for processes such as the production of syrups, treatment of saline water and waste water solutions containing starch residues in (Margesin and Schinner, 2001).

The isolation of enzyme producing bacteria from a soda lake, through direct plating on an agar media amended with substrates specific for enzymes of interest has previously been done (Govender *et al*., 2009). These studies discovered proteolytic, cellulolytic, saccharolytic and lipolytic microbes from soda lakes (Zavarzin *et al.,* 1999; Jones *et al.,* 1998; Duckworth *et al.,* 1996; Zhilina and Zavarzin, 1994). The studies isolated and

characterized a wide variety of bacteria that secrete extracellular hydrolytic enzymes such as amylases, proteases, lipases, DNases, pullulanases and xylanases (Sánchez-Porro *et al*., 2003; Rohban *et al*., 2009; Govender *et al*., 2009). In such studies just as in the current one, greater hydrolytic activity is commonly observed amongst Gram-positive halophilic bacteria than Gram-negative bacteria. Whereas most of the enzyme producing gram-positive bacteria belongs to the *Bacillus* group, enzyme producing gram-negative bacteria commonly comprise species of *Salinivibrio*, *Chromohalobacter* and *Halomonas* (Sánchez-Porro *et al*., 2003; Rohban *et al*., 2009).

Fewer enzymes were secreted than targeted in this study. This might be due to short time of incubation used that might not have allowed the microbes to reach the phase of growth when they secrete most enzymes like the celluses, xylanases.

5.6 Phylogeny and identification of isolates

Though a total of 37 isolates were sequenced, only 21 could be placed into the phylogenetic trees (Figures 10 and 11) because these are the ones that had sequences of more than 320 base pairs and identities that allowed sensible alignment according to the criteria of Rees *et al*. (2003). However the 21 isolates were representative of all the genera.

The phylogenetic analyses of the partial 16S rDNA sequence of the isolates clustered the isolates into three genera namely *Bacillus, Closridium* (Firmicutes) *and Halomonas* (Proteobacteria). Bacillus relatives were the majority at 54% followed by Clostridium at 38% and Halomonas at a mere 8%, showing that the gram positive Firmicutes were more diverse and abundant than Gram-negative Proteobacteria. This concurs with a cultivation-

dependent study on Lake Magadi in which 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).

Their identification with *Bacillus, Clostridium* and *Halomonas* groups, whose members are found in the database entries of organisms already known to be present in the Rift Valley soda lake environment, which include bacteria belonging to the genera *Bacillus, Closridium, Halomonas Micrococcus, Pseudomonas, Streptomyces* (Mwirichia *et al.,* 2010a; Rees *et al.,* 2003) is an important finding.

About 54 % of the isolates in this study were affiliated to microorganisms belonging to the genus *Bacillus.* Among these were; *Bacillus subtilis, Bacillus licheniformis* and *Bacillus cereu*s (Table 9). The isolates formed three clusters of *Bacillus* on the phylogenetic tree namely; *Bacillus subtilis* (one isolate), *Bacillus cereus* (six isolates) and *Bacillus licheniformis* (8 isolates). *Bacillus* species are among the most commonly found aerobic, eubacterial alkaliphiles in soda lakes (Horikoshi and Akiba, 1982; Takami *et al.,* 1989).

Twenty nine (29) isolates had a sequence similarity of 97% and above to their closest relatives in the BLAST, which shows that these may belong to the same species as the relatives while 7 of the isolates had a sequence similarity of less than 97% to their closest relatives in the BLAST, which shows that they may represent novel groups from the lake (Mwirichia *et al.,* 2010b).

Isolate G-15 had a 100% similarity to *Bacillus licheniformis*, isolates M6-1, G-2, G-18, C-13, D-9, S-10, G-11,and S-12 all had a 99% sequence similarity to *Bacillus licheniformis* while C-3, X-6 and M6-10 all had a 98% similarity to *Bacillus licheniformis.* This shows that all these isolates are members of the species *Bacillus licheniformis* according to Ludwig *et al*. (1998).

Bacillus licheniformis is a rod-shaped, Gram-positive bacterium that the produces a variety of extracellular enzymes associated with the cycling of nutrients in nature (Veith *et al*., 2004). *B. licheniformis* produces a protease enzyme thathas an optimum pH at around 9 and 10. This protease is utilized in laundry detergents because of its ability to be used in low temperatures, where it prevents shrinkage and fading of colors. *B. licheniformis* is used to make the antibiotic Bacitracin, which is composed of a mixture of cyclic polypeptides produced by B. *licheniformis*, whose role ironically 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). Studies done by Echigo *et al*. (2005) and by Wani *et al*. (2006) show that *B. licheniformis* contributes to nutrient cycling and has antifungal activity.

Isolates S-3, C-4, M8-12, S-11, D-2, D-6, M8-1 and M8-14 all showed relatedness to members of *Bacillus cereu*s with similarity values of 97% and above, showing that they possibly belong to this species (Ludwig *et al*., 1998). Members of genus *Bacillus* are heterotrophs and have been previously isolated from soda lakes (Jones and Grant, 1999b). They are all aerobic, gram positive, short or large rods, endospore formers, catalase and

oxidase positive and form colonies which are raised and with rhizoid or filamentous margins (Vargas *et al.*, 2004). Their dominance among the isolates may be due to their having dormant stages called endospores, that survive drying in marginal areas around the lakes (Grant *et al.*, 1999).

Isolate C-1 clustered distantly with *B. Subtilis* with a similarity value of 78%. *B. subtilis* produces the proteolytic enzyme subtilisin and its spores can survive 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). It is commonly found inhabiting in non saline environments and the similarity between isolate C-1 and *B. subtilis* shows that many strains with similar 16S rDNA sequences are widespread in nature and can thrive under very different physiological conditions (Echigo *et al*., 2005; Wani *et al.*, 2006).

X-5 is a halomonad in terms of phylogenetic placement and its general phenotypic properties. However, the 94% similarity to the 16s rDNA of *Halomonas campisalis* shows that it may belong to another strain of this species (or may represent a new species of the genus *Halomonas*). The isolate had the following morphological and biochemical properties: oxidase positive, catalase positive, circular, low convex and opaque coloured colonies, produce H_2S, l- Lysine, l- arginine, l-alanine and citrate positive and able to reduce nitrates to nitrites. The isolate did not produce indole acid, was casein negative and was not able to hydrolyse gelatin. Its physiological properties that include growing at temperature of 25-40 ^{o}C with an optimum of 35 ^{o}C, salinity range of between 5%-25% salts

and pH range of between 7-0 and 11.0 with the optimum being 9.5-10 agree with the findings of Duckworth *et al.* (2000). Halomonads have been isolated from the littoral sediments of Lake Magadi, Lake Nakuru, and Lake Bogoria and are known to constitute a major group in alkaline and saline environments characterized by ionic stress of one type or other (Duckworth *et al*., 1996; 2000, Baumgarte, 2003).

Isolates X-3 and G-12 and were all related to *Clostridium botulinum* with a percentage relatedness of 88% and 84% respectively. Since they all have sequence similarities of less than 96% to their closest relatives in the blast, the isolates represent novel groups from the lake. The low similarity values of X-3 and G-12 shows that these two may belong to new genera (Ludwig *et al.*, 1998).

Clostridium botulinum is a rod-shaped microorganism which though an anaerobe, meaning that oxygen is poisonous to its cells, tolerates traces of oxygen due to the enzyme called superoxide dismutase (SOD) which is an important antioxidant defense in nearly all cells exposed to oxygen (Doyle, 2007).

Isolates G-8, G-4, G-14 M8-15, S-2, S-7, M6-7, X-2, C-5, C-6 and G-20 all clustered with members of *Clostridium sporogenes* albeit with different sequence similarities. Isolates G-8, G-4, M8-15, S-2, S-7, M6-7 and X-2 all had a similarity value of 99% to members of *Clostridium sporogenes* while C-5, C-6 and G-20 had similarity values of 84%, 95% and 85% respectively. G-8, G-4, M8-15, S-2, S-7, M6-7 and X-2, are all members of species *Clostridium sporogenes* (Ludwig *et al*., 1998).Whereas C-6 may belong to a different species of genus *Clostridium*, the similarity values of C-5 and G-20 to their nearest

neighbors indicate that they may belong to new genera (Ludwig *et al*., 1998). *Clostridium sporogenes* represents strains of *Clostridium botulinum* that do not produce a botulin toxin (J C I C S B, 1999).

Genus C*lostridium* is not unique to the alkaline and soda environments. They have been detected in these habitats by both culture independent and culture dependant methods. For example, *Clostridium* species were cultured by Wiegel (1998) from alkaliphilic environments. Cook *et al.* (1996) reported the isolation and characterisation of *Clostridium paradoxum*, a novel alkali-thermophile. *Natronicola histidinovoran*s (Zhilina *et al.*, 1998), and the non spore forming bacterium, *Tindalia magadiensis* (Kevbrin *et al.*, 1998) found to be related to members of group X1 of the Clostridium (Collins *et al*., 1994), including *Clostridium felsineum, C. formicoaceticum* and *C. halophilum*, were derived from Lake Magadi. Group X1 and V1 saccharolytic alkaliphilic and haloalkaliphilic *Clostridia* strains have been isolated from Lakes Elmenteita, Bogoria, and Magadi by Jones *et al*. (1998) and Zhilina *et al*. (2001), respectively. Those isolated from Lake Magadi are most thought to be facultative anaerobes (Baumgarte, 2003).

The *Clostridia* are relatively large, Gram-positive, rod-shaped bacteria. All species form endospores and though most are anaerobes and therefore have a strictly fermentative mode of metabolism (Collins *et al*., 1994; Wipat and Colins, 1999), a few grow minimally in air at atmospheric pressure (Doyle, 2007). They exhibit varying degrees of intolerance to oxygen. Some are sensitive to O_2 concentrations as low as 0.5% but most species can tolerate concentrations of 3-5% (McGraw-Hill, 2002), due to the enzyme called superoxide

dismutase (SOD) which is an important antioxidant defense in nearly all cells exposed to oxygen (Doyle, 2007). The sensitivity of *Clostridia* to O_2 restricts them to habitats that contain large amounts of organic matter (Collins *et al.*,1994), which provide optimal conditions for their growth and survival (McGraw-Hill, 2002).

It is noteworthy that only two of the amplicons sequenced had 100% identity to sequences in the database. In fact some of the amplicon sequences, (X-3, G-12 and G-20), have very low identitities (88% and below) to their respective relatives in blast. Whereas amplicons with sequence identities of over 98% to a known organism may represent the same species and those that share an identity between 88% and 98% are usually considered part of the same genus (Stackebrandt and Goebel, 1994), the above isolates' rDNA could represent new genera that may be specific to this soda lake. Lake Magadi could therefore be harboring microorganisms that are still awaiting isolation (Mwiricha *et al.*, 2010b).

Though Archaea are supposedly dominant in such hypersaline environments (Oren, 2001), none was obtained in the present study. The lack of archaeal diversity may be because only a few species of the class *Halobacteria* can grow above pH 10 (David and Richard, 2001), the low organic matter content (2.14% volatile solids) in most soda lakes which is non conducive to the methanotrophic way of life favored by many Archaea and the high sulfur content of soda lakes which might have favored the growth of chemosynthetic Bacteria (Wani *et al.*, 2006). There might also be the possibility that colonies of other halophiles such as Archaea, unfortunately were not picked from agar plates during the purification,

they lost the ability to form colonies on the particular agar plates used during repeated transfers in the purification procedures, or that they simply did not form colonies because of unsuitableness of the composition of agar plates to them. The period of incubation might also have been too short to allow colonies of archea to form.

A lower diversity was obtained from this lake than anticipated. This can be attributed to the fact that two extreme conditions (salinity and alkalinity) were being investigated at the same time. The combination of two extreme conditions of physico-chemical growth parameters restricts the range at which microorganisms can proliferate more than in a single growth condition (Grant *et al.,* 1999). Such environments have a relatively restricted range of alkaliphilic inhabitants, usually *Bacillus* or related species that survive from one alkaline episode until the next by producing endospores (Grant *et al.,* 1999). The low diversity and simple ecological structure of these habitats is due to high salt concentrations that makes the number and metabolic diversity of the known microorganisms adapted to these salt concentrations rather limited (Oren, 2006). It is also possible that colonies of other halophilic bacteria other than *Bacilli, Clostridia and Halomonas,* unfortunately were not picked from agar plates during the purification.

This study indicates that the isolated microbes are plausible members of the soda lake community, demonstrates the existence of a rich, diverse and complex prokaryotic community some of which produce biotechnologically useful enzymes and that the culture dependant method is still the method of choice for unraveling both the diversity and the roles played by the individual microorganisms (Borsodi *et al.*, 2005).

CHAPTER SIX:

6.0 CONCLUSION

Lake Magadi, an extreme haloalkaline lake in the rift valley of East Africa harbours a number of extreme haloalkaliphilic bacteria. The low similarity percentage values of some isolates to their nearest reltives in the blast shows that Lake Magadi might be a hot spot of many, as yet uncultured bacteria.

Though Lake Magadi harbours both Gram positive and Gram negative Bacteria, Gram positive bacteria, especially those affiliated to genus *Bacillus* are more abundant than the gram negatives.

The bacteria found in Lake Magadi have the potential to yield a wide variety of biotechnologically interesting enzymes such as amylases, proteases and lipases and they exhibit a wide range of biochemical properties.

Modification of media that has been used to isolate microbes from alkaline areas, for example Duckworth *et al.,* 1996), can also be used to isolate microorganisms from alkaline and saline habitats and media used for the isolation of members of the domain *Archaea* for example the media of Phil *et al*. (2005) and Xu *et al.* (2006), can also be used to isolate members of the domain Bacteria.

The culture method still is and still remains the method of choice for studying both diversity and the ecological structure of a habitat such as Lake Magadi.

RECOMMENDATIONS

- More research is required to design studies that would compare the diversity of haloalkaliphiles in different seasons of the year such as the rainy and dry seasons of the year.
- Further analysis of bacteria is necessary for total characterization and identification of more haloalkaliphiles strains from Lake Magadi.
- Different protocols should be designed so as to allow the isolation of more diverse genera.
- More studies of this type should be carried out to target more industrially useful microbes.

REFERENCES

Abdelnasser, S., Nefisa, S., EI-Shayeb, M. A. and, Mabrouk, S. (2007). Isolation and Identification of Alkaline Protease Producing Alkaliphilic Bacteria from an Egyptian Soda Lake. *Journal of Applied Sciences Research*, **3** (11): 1363-1368.

Altschul, S.F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research,* **25**: 3389–3402.

Amoozegar, M. A., Fatemi, Z. A., Karbalaei-Heidari, H. R. and Razavi, M. R. (2007). Production of an extracellular alkaline metalloprotease from a newly isolated, moderately halophile, *Salinivibrio* sp. strain AF-2004. *Microbiological Research,* **162:** 369-377.

Anto'n, J., Llobet-Brossa, E., Rodriguez-Valera, F., and Amann, R. **(1999).** Fluorescence in situ hybridization analysis of the prokaryotic community inhabiting crystallizer ponds. *Environmental Microbiology*, **1:** 517-523.

Anto'n, J., Oren A., Benlloch, S., Rodríguez-Valera, F., Amann, R. and Roselló-Mora, R. (2002). *Salinibacter ruber* gen. nov., sp. nov., a novel, extremely halophilic member of the*Bacteria* from saltern crystallizer ponds**.** *International Journal of Systematic and Evolutionary Microbiology*, **52:** 485-491.

Anto'n, J., Rossello-Mora, R., Rodriguez-Valera, F., and Amann, R. **(2000)**. Extremely halophilic bacteria in crystallizer ponds from solar salterns. *Applied and Environmental Microbiology*, **66:** 3052-3057.

Archana, A. and Satyanarayana, T., (1999). Bacterial thermostable cellulase- free xylanases in environment friendly paper pulp bleaching technology. *Advances in Microbial Biotechnology* (eds Tewari, J. P., Lakhanpal, T. N., Singh, J., Gupta, R. and Chamola, B. P.), APH Publishing Corporation, New Delhi, pp. 131–140.

Arguilar, A., Ingemansson, T., Magnien, E. (1998). Extremophile micro organisms as cell factories: support from the European union. *Extremophiles*, **2:** 367-373.

Arthur, J. W., West, C. W., Allen, K. N and Hedke, S. F. (1987). Seasonal toxicity of ammonia to five fish and nine invertebrate species. Bulletin of*Environmental Contamination and Toxicology*, **38:** 324–331.

Asker, D. and Ohta, Y. (1999). Production of canthaxanthin by extremely halophilic bacteria. *Journal of Bioscience and Bioengineering*, **88:** 617-621.

Asker, D. and Ohta, Y. (2002). *Haloferax alexandrinus* sp. nov., an extremely halophilic canthaxanthin-producing archaeon from a solar saltern in Alexandria (Egypt) *International Journal of Systematic and Evolutionary Microbiology*, **52:** 729–738.

Aunstrup, K., Ottrup, H., Andresen, O. and Dambmann, C. (1972). Proceedings of the 4th International Fermentation Symposium. Kyoto, Japan: Society of Fermentation Technology; Proteases from alkalophilic *Bacillus* species; pp. 299–305.

Aygan, A., and Arikan, B., **(2008).** Highly thermostable and alkaline a-amylase from a halotolerant alkaliphilic *Bacillus* sp. AB68. *Brazilian Journal of Microbiology*, **39:** 547-553.

Bakhtiar, S., Estiveira, R. J. and Hatti-Kaul, R. (2005). Substrate specificity ofalkaline protease from alkaliphilic feather-degrading *Nesterenkonia* sp. AL20. *Enzyme and Microbial Technology*, **37:** 534-540.

Baumgarte, S. (2003). Microbial diversity of soda lake habitats. Thesis, Brauchweig, Germany. Technische Universitat Braunschweig.

Baumgarte, S., Mau, M., Bennasar, A., Moore, E. R., Tindall, B. J. and Timmis, K. N. (1999). Archeal diversity in soda lake habitats. (Vortrag) Jahrestagung der VAAM, Gottingen.

Benlloch, S., Acinas, S. G, Martí nez-Murcia, A. J., and Rodrí guez-Valera, F. (**2002**). Description of prokaryotic biodiversity along the salinity gradient of a multipond saltern by direct PCR amplification of 16S rDNA. *Hydrobiologia*, **329:** 19–31.

Benlloch, S., Martí nez-Murcia, A. J., and Rodrí guez-Valera, F. (1995). Sequencing of bacterial and archaeal 16S rRNA genes directly amplified from a hypersaline environment. *Systematic and Applied Microbiology*, **18:** 574–581.

Bock, E., and Koops, H. P. (1992). The genus *Nitrobacter* and related genera. In: Balows A, Trüper H G, Dworkin M, Harder W, Schleifer K-H, editors; Balows A, Trüper H G, Dworkin M, Harder W, Schleifer K-H, editors. The prokaryotes 2nd ed. New York, N.Y: Springer-Verlag. pp. 2302–2309.

Boltyanskaya, V., Antipov, A. N. Kolganova, T. V., Lysenko, A. M. . Kostrikina, N. A. and Zhilina, T. N. (2004). *Halomonas campisalis*, an Obligatorily Alkaliphilic, Nitrous Oxide–Reducing Denitrifierwith a Molybdenum Cofactor–Lacking Nitrate Reductase. *Microbiology*, **73:** *271–278.*

Borsodi, A. K., Micsinai, A., Rusznyak, A., Vladar, P., Kovacs, G., Toth, E. M. and Marialigeti, K. (2005). Diversity of alkaliphilic and alkalitolerant bacteria cultivated from decomposing reed rhizomes in a Hungarian soda lake. *Microbiology and Ecology.* **50:** 9–18.

Bowers, K. J., Mesbah, N. M. and Wiegel, J. (2008). *Natranaerobius 'grantii' and Natranaerobius 'jonesii'*, spp. nov., two anaerobic halophilic alkaliphiles isolated from the Kenyan-Tanzanian Rift [abstract]. *Abstract General American Meeting of the Society for Microbiology Boston, MA* I-007.

Bowers, K. J., Mesbah, N. M. and Wiegel, J. (2009). Biodiversity of poly-extremophilic *Bacteria*: Does combining the extremes of high salt, alkaline pH and elevated temperature approach a physico-chemical boundary for life? *Saline Systems*, **23:** 5-9.

Broderick, N. A., Raffa, K.F., Goodman, R M., Handelsman, J. (2004). Census of the bacterial community of the gypsy moth larval midgut by using culturing and culture-independent methods. *Applied and Environmental Microbiology*, 70 (1)**:** 293-300.

Buchalo, A. S., Nevo, E., Wasser, S. P. and Volz, P. A. (2000). Newly discovered halophilic fungi in the Dead Sea (Israel). *Journey to Diverse Microbial Worlds* (ed. Seckbach, J.), Kluwer, Dordrecht, vol. 2, pp. 241– 252.

Burns, D. G., Camakaris, H. M., Janssen, P. H. and Dyall-Smith, M. (2004). Combined use of cultivation-dependent and cultivation-independent methods indicates that members of most haloarchaeal groups in an Australian crystalliser pond are cultivable. *Applied and Environmental Microbiology* **70:** 5258-5265.

Bursy, J., Kuhlmann A. U., Pittelkow, M., Hartmann, H., Jebbar, M., Pierik, A. J. and Bremer, E. (2008). Synthesis and uptake of the compatible solute ectoine and 5-hydroxyectoine by *Streptomyces coelicolor* A3 (2) in response to salt and heat stresses. *Applied and Environmental Microbiology*, **74:** 7286-7296.

Cánovas, D., Vargas, C. and Laszlo, N.C. (1998). Synthesis of glycine betaine from exogenous choline in the moderately halophilic bacterium *Halomonas elongata*. *Applied and Environmental Microbiology*, **64:** 4095-4097.

Cappuccino, J.G. and Sherman, N. (2002). *Microbiology. A laboratory manual*. 6th edition. Pearson education inc. San Francisco, California. 215-224.

Casamayor, E. O., Massana, R., Benlloch, S., Ovreas, L., Diez, B., Goddard, V. J., Gasol, J. M., Joint, I., Rodriguez-Valera, F. and Pedros-Alio, C. (2002). Changes in archaeal, bacterial and eukaryal assemblages along a salinity gradient by comparison of genetic fingerprinting methods in a multipond solar saltern. *Environmental Microbiology*, **4:** 338-348.

Castro, G. R, Ferrero, M. A., Méndez, B. S. and Sineriz, F. (1993). Screening and selection of bacteria with high amylolytic activity. *Acta Biotechnologica*, **13:** 197–201.

Cavicchioli, R. and Thomas, T. (2003). Extremophiles. *The Desk Encyclopedia of Microbiology* (ed. Schaechter, M.), Elsevier, London pp. 436–453.

Cayol, J. L., Ollivier, B., Patel, B. K. C., Prensier. G., Guezennec, J., Garcia, J.L. (1994). Isolation and characterization of *Halothermothrix orenii* gen. nov., sp. nov., a halophilic, thermophilic, fermentative, strictly anaerobic bacterium. *International Journal of Systematic and Evolutionary Microbiology*, **44:** 534-540.

Chi, E. and Bartlett, D. H. (1993). Use of a reported gene to follow high pressure signal transduction in the deep-sea bacterium *Photobacterium* SS9. *Journal of Bacteriology*, **175:** 7533–7540.

Collins, M. D., Lawson, P. A., Willems, A., Cardoba, J. J., Fernandez-Garayzabal, J., Garcia, P., Cai, J., Hippe, H. and Farrow, J. A. E. (1994). The phylogeny of the genus *Clostridium*: proposal of five new genera and eleven new species combinations. *International Journal of Systematic Bacteriology*, **44:** 812–826.

Cook, G. M., Russell, J. B., Reichert, A. and Wiegel., J. (**1996).** The intracellular pH of *Clostridium paradoxum*, an anaerobic, alkaliphilic, and thermophilic bacterium. *Applied and Environmental Microbiology*, **62:** 4576-4579.

Coronado, M.J., Vargas, C., Hofemeister, J., Ventosa, A. and Nieto, J. J. (2000). Production and biochemical characterization of an a-amylase from the moderate halophile *Halomonas meridiana. FEMS Microbiology Letters*, **183:** 67-71.

DasSarma, S. and DasSarma, P. (2006). *Halophiles*, **Encyclopedia of Life Sciences**, Wiley, London. *Desulfonatronovibrio hydrogenovorans* gen. nov., sp. nov. an alkaliphilic, sulfate reducing bacterium. *International Journal of Systematic Bacteriology*, **47:** 144-149.

David, R. B. and Richard, W. C. (2001). Bergey's Manual of Systematic Bacteriology, vol. 1, second ed., The William's and Wilson Co., Baltimore. Dieffenbach and G. S. Dveksler (ed.), PCR primer: a laboratory manual. Cold Spring.

Doyle, M. P. (2007). Food Microbiology: Fundamentals and Frontiers. ASM Press.

Duckworth, A. W., Grant, W. D., Jones, B. E. and Van Steenburgen, R. (1996). Phylogenetic diversity of soda Lake Alkaliphiles. *FEMS Microbiology Ecology*, **9:** 181–191.

Duckworth, A. W., Grant, W. D., Jones, B. E., Meijer, D., Marques, C. M. and Ventosa, A. (2000). *Halomonas magadii* sp.nov. a New Member of the Genus *Halomonas*, Isolated from a Soda Lake of the East African. *Extremophiles*, **4:** 53-60.

Dussault, H. P. (1955). An improved technique for staining red halophilic bacteria. *Journal of Bacteriology*, **70:** 484–485.

Echigo, A., Hino, M., Fukushima, M., Mizuki, T., Kamekura, M. and Usami, R. (2005). Endospores of halophilic bacteria of the family *Bacillaceae* isolated from non-saline Japanese soil may be transported by Kosa event (Asian dust storm) *Saline Systems* **1:** 8doi:10.1186/1746-1448-1-8.

Ehrich, S., Behrens, B., Lebedeva, A., Ludwig, W. and Bock, E. J. (1995). A new obligately chemolithoautotrophic nitrite-oxidizing bacterium *Nitrospira moscoviensis* sp. nov. and its phylogenetic relationship. *Archives of Microbiology*, **164:** 16–23.

Embley, T. M. (1991). The linear PCR reaction: a simple and robust method for sequencing amplified t-RNA genes. *Letters in Applied Microbial Research*, **13:** I71- 174.

Embley, T. M. and Stackebrandt, E. (1994). The molecular phylogeny and systematics of the actinomycetes. *Annual Review of Microbiology*, **48:** 257-289.

Fatope, M. O., Al-Kindi, M. Z. S. and Abdulnour, O. A. (2000). Research trends: Natural products as pest, microbial disease and tumour control agents. *Science and Technology*, Special Review. Pp 55-71.

Felsenstein J. **(1985).** "Confidence limits on phylogenies: an approach using the bootstrap," *Evolution*, **39**, pp. 783–791.

Gauthier, M. J., Lafay, B., Christen, R., Fernandez, L., Acquaviva, M., Bonin, P. and Betrand, J. C. (1992). *Marinobacter hydrocarbonoclasticus* gen. nov. sp. nov., a new extremely halotolerant, hydrocarbon– degrading marine bacterium. *International Journal of Systematic Bacteriology*, **42:** 568–576.

Geoscience, Australia. **(2002)** http://www.auslig.gov.au/facts/landforms/Larglake.htm Saltlake.

Govender, L., Naidoo, L. and Setati, M. E. (2009). Isolation of hydrolase producing bacteria from Sua pan solar salterns and the production of endo-1, 4-b-xylanase from a newly isolated haloalkaliphilic *Nesterenkonia* sp. *African Journal of Biotechnology*, **8:** 5458-5466.

Grant W.D., (2006). Alkaline environments and biodiversity, in *Extremophilies*, [Eds. Charles Gerday, and Nicolas Glansdorff], in *Encyclopedia of Life Support Systems (EOLSS)*, Developed under the Auspices of the UNESCO, Eolss Publishers, Oxford ,UK.

Grant, S., Grant, W.D., Jones, B. E., Kato, C. and Li, L. (1999). Novel archaeal phylotypes from an East African alkaline saltern. *Extremophiles*, **3:** 139–145.

Grant, W. D. (1992). Alkaline environments in Encyclopedia of microbiology, pp.78-80. Edited by J. Lederberg.Sandiego: Academic press.

Grant. W. D and Tindall, B. J. (1986). The alkaline saline environment. In: Microbes in Extreme Environments (Herbert. R.A. and Codd, G.A. Eds.), Academic Press. London, pp. 25-54.

Grant, W.D and Tindall, **B.J. (1990).** Isolation of alkaliphilic bacteria, in: R.A. Herbert, G.A. Codd (Eds.), Microbial Growth and Survival in Extremes of Environment, Academic Press, London, pp. 27–36.

Grant, W. D., Mwatha, W.E. and Jones, B. E. (1990). Alkaliphiles: ecology. diversity and applications. *FEMS Microbiology Review*, **75:** 255-270.

Gregersen, T. (1978). Rapid method for distinction of Gram–negative from gram–positive bacteria. *European Journal of Applied Microbiology and Biotechnology*, **5**: 123–127.

Gupta, R., Gigras, P., Mohapatran, H., Goswami, K.V. and Chauhan, B. (2003). Microbial a-amylase: a biotechnological perspective. *Process Biochemistry*, **38:** 1599-1616.

Halebian, S., Harris, B., Finegold, S. M. and Rolfe, R. D. (1981). Rapid method that aids in distinguishing Gram-positive from Gram-negative anaerobic bacteria. *Journal of Clinical Microbiology*, **13:** 444–448.

Harold, J. B. (2002). Microbiological applications: *a laboratory manual in general microbiology*. 8th Edition 1- 478.

Hedi, A., Sadfi, N., Fardeau, M. L, Rebib, H., Cayol, J. L., Ollivier, B., and Boudabous, A. (2009). Studies on the Biodiversity of Halophilic Microorganisms Isolated from El-Djerid Salt Lake (Tunisia) under Aerobic Conditions. *International Journal of* Microbiology, Volume (2009) Article ID 731786, 17 pages doi:10.1155/2009/731786.

Hopwood, D. A. (1960). The phase-contrast observations on *Streptomyces coelicolor*. *Journal of General Microbiology*, **22**: 295-302.

Horikoshi, K. (1971). Production of alkaline enzymes by alkalophilic microorganisms. II. Alkaline amylase produced by *Bacillus* No. A-40-2. *Agricultural and Biological Chemistry*, **35:** 1783–1791.

Horikoshi, K. (1996). Alkaliphiles from an industrial point of view. *FEMS Microbiology Reviews*, **18:** 259-270.

Horikoshi, K. (1999). Alkalophiles: Some applications of their products for biotechnology. *Microbiology and Molecular Biology Reviews* **63:** 735-750.

Horikoshi, K. and Akiba, T. (1982). Alkalophilic microorganisms: a new microbial world. Springer, New York.

Horikoshi, K. and Grant, W. D. (eds.) **(1998).** *Extremophiles - Microbial Life in Extreme Environments* (New York: Plenum).

Igarashi, K., Hatada, Y., Hagihara, H., Saeki, K., Takaiwa, M., Uemura, T., Ara, K., Ozaki, K., Kawai, S., Kobayashi, T. and Ito, S. (1998). Enzymatic properties of a novel liquefying α-amylase from an alkaliphilic *Bacillus* isolate and entire nucleotide and amino acid sequences. *Applied and Environmental Microbiology*, **64:** 3282–3289.

Imhoff, J.F and Trüper, H. G. (1977). *Ectothiorhodospira halochloris* sp. nov., a new extremely halophilic phototrophic bacterium containing bacteriochlorophyll b**.** *Archives of Microbiology*, **114:** 114-121.

Imhoff, J. F. and Suling, J. (1990). The phylogenetic relationship among *Ectothiorhodospiracaea*: a reevaluation of their taxonomy on the basis of 16S rDNA analysies**.** *Archives of Microbiology*, **165:** 106-113.

International Journal of Systematic and Evolutionary Microbiology (IJSEM) (2009). **(52):** 1205- 1209.

Jones, B. E, Grant, W. D., Collins, N. C. and Mwatha, W. E. (1994). Alkaliphiles: diversity and identification. In: Priest F.G, Ramos-Cormenzana A, Tindall B.J. (eds) *Bacterial diversity and systematics.* Plenum Press, New York, pp 195–230.

Jones, B. E. (2004). Industrial enzymes: do halophiles and alkaliphiles have a role to play? In: Ventosa A, editor. *Halophilic Microorganisms*. New York, NY, USA: Springer; 2004. pp. 275–284.

Jones, B. E. and Grant, W. D. (1999b). Microbial diversity and ecology of alkaline environments. In: Journey to Diverse Microbial Worlds. Adaptation to Exotic Environments (Seckbach, J., Ed.), pp. 177–190. Kluwer Academic Publishers, Dordrecht.

Jones, B. E. and Grant, W. D. (2000). Microbial diversity and ecology of alkaline environments. In: Journey to Diverse Microbial Worlds. Adaptation to Exotic Environments (Seckbach, J., Ed.), pp. 177–190. Kluwer Academic Publishers, Dordrecht.

Jones, B. E., and Grant, W.D. (1999a). Microbial diversity and ecology of the Soda Lakes of East Africa. Microbial Biosystems: New Frontiers**.***Proceedings of the 8th International Symposium on Microbial Ecology Bell CR, Brylinsky M, Johnson Green P (eds) Atlantic Canada Society for Microbial Ecology, Halifax, Canada.*

Jones, B. E., Grant, W. D., Duckworth, A. W and Owenson, G. G. (1998). Microbial diversity of soda lakes. *Extremophiles,* **2:** 191–200.

Joshi, A. A., Kanekar, P. P., Kelkar, A. S., Sarnaik, S. S., Shouche, Y. and Wani, A. (2007). Moderately halophilic, alkalitolerant Halomonas campisalis MCM B-365 from Lonar Lake, India. *Journal of Basic Microbiology*, **47:** 213-221.

Judicial Commission of the International Committee on Systematic Bacteriology (JCICSB) (1999). Rejection of *Clostridium putrificum* and conservation of *Clostridium botulinum* and *Clostridium sporogenes* Opinion 69. *International Journal of Systematic Bacteriology*, **49:** 339.

Jukes T.H. and Cantor C.R. (**1969**). Evolution of protein molecules. In Munro HN, editor, *Mammalian Protein Metabolism*, pp. 21-132, Academic Press, New York.

Karbalaei-Heidari, H. R., Amoozegar, M. A. and Ziaee, A. A. (2009). Production, optimization and purification of a novel extracellular protease from the moderately halophilic bacterium. *Journal of Industrial Microbiology and Biotechnology*, **36:** 21- 27.

Kastritis, P. L., Papandreou, N. C. and Hamodrakas, S. J. **(2007).** Haloadaptation: insights from comparative modeling studies of halophilic archaeal DHFRs. *International Journal of Biology*, **41:** 447-453.

Keast, D., Rowe, P., Sanfellieu, L., Shhannan, J., Bowra, B., Skates, S., Stapley, E. 0. and Woodruf, H. B. (1984). Use of computers to group actinomycetes for studies on the ecology of soil microorganisms. *Applied and Environmental Microbiology*, **48:** 791-796.

Kelly, C. T., Mctigue, M. A., Doyle, E. M. and Fogarty, W. M. (1995). The raw starch-degrading alkaline amylase of *Bacillus* sp. IMD 370. *Journal of Industrial Microbiology*, **15:** 446–448.

Kevbrin, V.V., Zhilina, T. N., Rainey, F. A. and Zavarzin, G. A. (1998). *Tindallia magadii* gen. nov., sp. nov.: an alkaliphilic anaerobic ammonifier from soda lake deposits. *Current Microbiology*, **37:** 94–100.

Kim, K. K., Jin, L., Yang, H. C. and Lee, S. (2007). *Halomonas gomseomensis* sp. nov., *Halomonas janggokensis* sp. nov., *Halomonas salaria* sp. nov. and *Halomonas denitrificans* sp. nov., moderately halophilic bacteria isolated from saline water. *International Journal of Systematic and Evolutionary Microbiology,* **57:** -681.

Kim, T. U., Jeog, G, B. G., Byun, J. Y. S. M., and Shi, Y. C. (1995). Purification and characterization of a maltotetraose-forming alkaline α-amylase from an alkalophilic *Bacillus* strain, GM8901. *Applied Environmental Microbiology,* **61:** 3105–3112.

Kobayashi, T., Hakamada, Y., Adachi, S., Hitomi, J., Yoshimatsu, T., Koike, K., Kawai, S. and Ito, S., (1995). Purification and properties of an alkaline protease from alkalophilic *Bacillus* sp. KSM-K16. *Applied Microbiology and Biotechnology*, **43:** 473–481.

Koops, H. P. and Möller, U. C. (1992). The lithotrophic ammonia-oxidizing bacteria. In: Balows A, Trüper H G, Dworkin M, Harder W, Schleifer K-H, editors; Balows A, Trüper H G, Dworkin M, Harder W, Schleifer K-H, editors. The prokaryotes 2nd ed. New York, N.Y: Springer Verlag;. pp. 2625–2263.

Korn-Wendisch, F. A. and Kutzner, H. J. (1992). The family Streptomycetaceace, p 921-995 .*In*: A. Balows , H. G. Truper, M. Dworkin ,W. Harder and K. H. Schleifer (ed). The prokaryotes. Springer Verlag, Berlin.

Kovacs, N. (1956). Identification of *Pseudomonas pyocyanea* by the oxidase reaction. *Nature*, **178:** 703–704.

Kumar, S. and Satyanarayana, T. (2004). Purification and kinetics of a raw starch-hydrolyzing, thermostable and neutral glucoamylase of the thermophilic mold *Thermomucor indicae-seudaticae*. *Biotechnology Progress*, **19:** 936–944.

Lacey, M. (2006). An Age-Old Salt Lake May Yield a Washday Miracle. Lake Bogoria Journal, New York Times.

Lane, D. J. (1991). 16S/23S rRNA sequencing In: Nucleic Acid Techniques in Bacterial Systematics, (Stackebrandt, E. and Good fellow, M., Eds.). Pp115-175. John Wiley & Sons Inc., New York.

Lee, J., Jeon, C. O., Lim, J., Lee, S., Lee, J., Song, S., Park, D., Li, W. and Kim, C. (2005). *Halomonas taeanensis* sp. nov., a novel moderately halophilic bacterium isolated from a solar saltern in Korea. *International Journal of Systematic and Evolutionary Microbiology*, **55:** 2027-2032.

Lee, S. and Lee, J. J. (1997). Insoluble dye substrates from screening and assay of Xylan degrading enzymes. *Journal of Microbiological Methods*, **29:** 1-5.

Li, L., Kato, C. and Horikoshi, K. (1998.) Distribution of the pressure regulated operons in deep-sea bacteria. *FEMS Microbiology Letters*, **159:** 159–166.

Liaw, H. J and Mah, R. A. (1992). Isolation and characterization of *Haloanaerobacter chitinovorans* gen. nov., sp. nov., a halophilic, anaerobic, chitinolytic bacterium from a solar saltern. *Appl Env Micro.* **58:**260-266.

Litchfield, C. D. and Gillevet, M. P. (2002). Microbial diversity and complexity in hypersaline environments: A preliminary assessment. *Industrial Microbiology and Biotechnology*, **28:** 48-55.

Louis, P. and Galinski, E. A. (1997). Characterization of genes for the biosynthesis of the compatible solute ectoine from *Marinococcus halophilus* and osmoregulated expression in *Escherichia coli*. Microbiology, **143:** 1141–1149.

Ludwig, W., Strunk, O., Klugbauer, S., Klugbauer, N., Weizenegger, M., Neumaier, J., Bachleitner, M. and Schleifer, K. H. (1998). Bacterial phylogeny based on comparative sequence analysis. *Electrophoresis*, **19:** 554–568.

Madigan, M and Martinko J (editors). (2005). *Brock Biology of Microorganisms* (11th ed.). Prentice Hall. ISBN 0131443291.

Magarvey, N. A., Keller, M. J., Bernan, V., Dworkin, M. and Sherman, D. H. (2004). Isolation and characterization of novel marine- derived Actinomycetes taxa rich in Bioactive metabolites. *Applied and Environmental Microbiology*, **70:** 7520- 7529.

Marcella, C., Maria, S., Mosè, R., and Sabato, D. (2006). Proteins from extremophiles as stable tools for advanced biotechnological applications of high social interest. Institute of Protein Biochemistry, CNR, Italian National Research Council, Via Pietro Castellino, 111, 80131 Naples, Italy

Margesin, R. and Schinner, F. (2001). Potential of halotolerant and halophilic microorganisms for biotechnology. *Extremophiles*, **5:** 73-83.

Marrs, B. W., Brzozowski, A. M., Bjoernvad, M. E., Andersen, K. V. and Schuelein, M. (1999). Phylogenetic diversity of soda lake alkaliphiles. *FEMS Microbiology Ecology*, **18:** 161-171.

Martínez-Cánovas, M. J., Béjar, V., Martínez-Checa, F. and Quesada, E. (2004). *Halomonas anticariensis* sp. nov., from Fuente de Piedra, a saline-wetland wildfowl reserve in Malaga, southern Spain. *International Journal of Systematic and Evolutionary Microbiology*, **54:** 1329–1332.

Martí nez-Murcia, A. J. (1995). Sequencing of bacterial and archaeal 16S rRNA genes directly amplified from a hypersaline environment. *Systematic and Applied Microbiology*, **18:** 574–581.

Mattiasson, B., Virginia, A., Vargas, O., Delgado, D. and Hatti-Kaul, R. **(2004).** Lipase-producing microorganisms from a Kenyan alkaline soda lake. *Biotechnology Letters*, **26:** 81–86.

McGraw-Hill Concise Encyclopedia of Bioscience. (2002). The McGraw-Hill Companies, Inc.

McTigue, M. A., Kelly, C. T., Doyle, E. M. and Fogarty, W. M. (1995). The alkaline amylase of the alkalophilic *Bacillus* sp IMD 370. *Enzyme and Microbial Technology*, **17:** 570–573.

Melack, J. M. (198I). Photosynthetic activity of phytoplankton in tropical African soda lakes. *Hydrobiologia*, **81:** 7 I-86.

Melack, J. M. and Kilham, P. (1974). Photosynthetic rates of phytoplankton in tropical African soda lakes. *Hydrobiologia*, **81:** 71–85.

Mesbah, N. M, Abou-El-Ela S. H., and Wiegel J (2007): Novel and unexpected prokaryotic diversity in water and sediments of the alkaline, hypersaline lakes of the Wadi An Natrun, Egypt. *Microbial Ecology*, **54:** 598-617.

Mesbah, N. M., Cook, G. M. and Wiegel, J. (2009): The halophilic alkalithermophile *Natranaerobius thermophilus* adapts to multiple environmental extremes using a large repertoire of $Na^+(K^+)/H^+$ antiporters. *Molecular Microbiology*, **74:** 270-281.

Mevarech, M., Frolow, F. and Gloss L. M., (2000). "Halophilic enzymes: proteins with a grain of salt," *Biophysical Chemistry*, **86:** (2-3) 155–164.

Michael, T. M. and Barry, L. M. (1997). *Extremophiles.* Scientific American Inc. Woods Hole Oceanographic Institution and University of Regensburg, Germany.

Milford, A. D., Achenback, L. A., Jung, D. O. and Madigan, M. T. (2000). *Rhodobaca bogoriensis* gen. nov. and sp. nov., an alkaliphilic purple nonsulfur bacterium from African Rift Valley soda lakes. *Archives of Microbiology* **174:** 18–27.

Moreno, M. D. L., Garcia, M. T., Ventosa, A. and Mellado, E. (2009). Characterization of *Salicola* sp. IC10, a lipase- and proteaseproducing extreme halophile. *FEMS Microbiology Ecology*, **68:** 59-71.

Mouné, S., Caumette, P., Matheron, R. and Willison J. C. (2003). "Molecular sequence analysis of prokaryotic diversity in the anoxic sediments underlying cyanobacterial mats of

two hypersaline ponds in Mediterranean salterns," *Federation of European Materials Societies Microbiology Ecology*, vol. 44, no. 1, pp. 117–130.

Moun'e, S., Eatock, C., Matheron, R., Willson, J. C., Hirschler, A., Herbert, R. and Caumette, P. (2000). Orenia salinaria sp. nov., a fermentative bacterium isolated from anaerobic bacterium isolated from anaerobic sediments of meditteranean salterns. *International Journal of Sytematical amd Evolutionary Microbiology*, **50:** 721-729.

Mwatha, W. E and Grant, W. D. (1993). Natronobacterium vacuolata sp. nov., a Haloalkaliphilic Archaeon Isolated from Lake Magadi, Kenya. *International Journal of Systematic Microbiology*, **43:** 401-404.

Mwiricha, R., Cousin, S., Mwigai, A., W., Boga, H. I., and Stackebrandt, E. (2010a). Isolation and characterization of bacteria from the haloalkaline Lake Elmenteita, Kenya. *Extremophiles*, **14:** 339-348.

Mwirichia, R., Muigai, A.W., Cousin, S., Boga, H.I. and Stackebrandt, E. (2010b): Bacterial Diversity in the Haloalkaline Lake Elmenteita in Kenya Curr Microbiol. ; **62** (1):209-21.

Narang, S. and Satyanarayana, T. (2001). Thermostable *a*-amylase production by an extreme thermophile *Bacillus thermoleovorans*. *Letters in Applied Microbiology*, **32:** 31–35.

Nazina, T. N., Lebedeva, E. V., Poltaraus, A. B., Tourova, T. P., Grigoryan, A. A. Sokolova, D. S. H., Lysenko A. M. and G. A. Osipov. (2004). *Geobacillus gargensis* sp. nov., a novel thermophile from a hot spring, and the reclassification of *Bacillus vulcani* as

Geobacillus vulcani comb. nov. *International Journal of Systematic and Evolutionary Microbiology*, **54:** 2019-2024.

Nazina, T. N., Taurova, T. P., Poltaraus, A. B., Novikova, E. V., Grigoryan, A. A., Ivanova, A. E., Lysenko, A. M. and Petrunyaka, V. V. (2001). Taxonomic study of aerobic thermophilic bacilli: descriptions of *Geobacillus subterraneus* gen. nov., sp. nov. and *Geobacillus uzenensis* sp. nov. from petroleum reservoirs and transfer of *Bacillus stearothermophilus, Bacillus thermocatenulatus, Bacillus thermoleovorans, Bacilluskaustophilus, Bacillus thermoglucosidasius* and *Bacillus thermodenitrificans* to *Geobacillus* as the new combinations *G. stearothermophilus, G. thermocatenulatus, G. thermoleovorans, G. kaustophilus, G. thermoglucosidasius and G. thermodenitrificans*. *International Journal of Systematic and Evolutionary Microbiology* **51:** 433–446.

NCBI, website, (2011). Bacillus cereus. "Accessed on August 24, 2011"

Ollivier, B., Caumette, P., Garcia, J. L. and Mah, R. A. (1998). Anaerobic bacteria from hypersaline environments. *Microbiology Reviews*, **58:** 27–38.

Oren, A. and Gurevich, P. (1993). Characterization of the dominant halophilic archaea in a bacterial bloom in the Dead Sea. *FEMS Microbiology Ecology*, **12:** 249–256.

Oren, A. (1993). "Ecology of extremely halophilic microorganisms," in *The Biology of Halophilic Bacteria*, R. H. Vreeland and L. I. Hochstein, Eds., pp. 26–53, CRC Press, Boca Raton, Fla, USA.

Oren, A. (2000). "Biological processes in the Dead Sea ace influenced by short-term and long-term salinity changes," *Archives of Hydrobiology Special Issues Advances Limnology*, **55:** 531–542.

Oren, A. (2001). The order Halobacteriales. In: The Prokaryotes. A Handbook on the Biology of Bacteria: Ecophysiology, Isolation, Identification, Applications, 3rd edn. (Dworkin, M., Falkow, Rosenberg S.,, E., Schleifer, K.-H. and Stackebrandt, E., Eds.). Springer-Verlag, New York (electronic publication).

Oren, A. (2002). Diversity of halophilic microorganisms: Environments, phylogeny, physiology and applications. *Journal of Industrial Microbiology and Biotechnology*, **28:** 56-63.

Oren, A. (2006). Life at high salt conditions**.** In *The Prokaryotes. A Handbook on the Biology of Bacteria: Ecophysiology and Biochemistry*. *Volume 2*. Edited by: Dworkin M, Falkow S, Rosenberg E, Schleifer K-H, Stackebrandt E. New York: Springer: 263-282.

Pakdeeto, A., Tanasupawat, S., Thawai, C., Moonmangmee, S., Kudo, T., Itoh, T. (2007): *Salinicoccus siamensis* sp. nov., isolated from fermented shrimp paste in Thailand. *International Journal of Systematic and Evolutionary Microbiology*, **57:** 2004-2008.

Phil, K. R. T. A., Vinitha, D. A. J., Kiran, G. S. and Manjusha, W. A. (2005). Isolation and cultivation of Halophilic Archaea from Solar salterns located in pensular coast of India. *International Journal of Microbiology*, volume 1 number 2.

Pikuta, E. V., Lysenko, A. M. and Zhilina, T. N. (1998). Distribution of *Desulfonatronovibrio hydrogenovorans* in soda lakes of Tuva. *Microbiology*, **66:** 216–221.

Post, F. J. (1977). The microbial ecology of the Great Salt Lake. *Microbial Ecology*, **3:** 143–165.

Prosser, J. I. (1989). Autotrophic nitrification in bacteria. *Advanced Microbiology and Physiology*, **30**: 125–181.

Rees, H. C., Grant, W. D., Jones, B. E. and Heaphy, S. (2003). Diversity of Kenyan soda lake alkaliphiles assessed by molecular methods. *Extremophiles*, **8:** 63-71.

Rheims, H., Schumann, P. Rohde, M. and Stachbrandt, E. (1998). *Verrucosispora gifhornensi* gen. nov., sp.nov. A new member of Actinobacterial family micromonosporaceae. *International Journal for Systematic Bacteriology*, **48:** 119-1127.

Rodriguez-Valera, F. (1992). Biotechnological potential of halobacteria. In: Danson M.J, Hough D.W. and Lunt G.G. (eds) *The Archaebacteria: Biochemistry and Biotechnology*, pp. 135–147. London: Portland Press.

Rohban, R., Amoozegar, M. A. and Ventosa, A. (2009). Screening and isolation of halophilic bacteria producing extracellular hydrolyses from Howz Soltan Lake, Iran. *Journal of Industrial Microbiology and Biotechnology*, **36:** 333-340.

Roux, K. H. (1995). Optimization and trouble shooting in PCR, p. 53-62. In C. W.

Saitou, N. and Nei, M. (1987). The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution*, **4:** 406-425.

Sambrook, K. J., Fritsch, E. F. and Maniatis, T. **(1989).** *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Sánchez-Porro, C., Martin, S., Mellado, E. and Ventosa, A. (2003). Diversity of moderately halophilic bacteria producing extracellular hydrolytic enzymes. *Applied Microbiology*, **94:** 295-300.

Santos, H., and da Costa, M. S. (2002). Compatible solutes of organisms that live in hot saline environments. *Environmental Microbiology*, **4:** 501-509.

Satyanarayana, T., Raghukumar, C., and Shivaji, S. (2005). Extremophilic microbes: Diversity and perspectives: *Current science*, **89:** 78-90.

Setati, M. E. (2009). Diversity and industrial potential of hydrolaseproducing halophilic/halotolerant eubacteria. *African Journal of Biotechnology*, **9:**(11) 1555-1560.

Setyorini, E., Takenaka, S., Murakami, S. and Aoki, K. (2006). Purification and characterization of two novel halotolerant extracellular protease from *Bacillus subtilis* strain FP-133. *Bioscience Biotechnology and Biochemistry*, **70:** 433- 440.

Sharma, A., Archana, A. and Satyanarayana, T. (2000). Reduction in organochlorine pollutants in paper pulp industry using microbial xylanases. *Environmental Protection* (eds Thukral, A. K. and Virk, G. S.), Scientific Publ. (India), Jodhpur, pp. 27–37.

Shivanand, P., Jayaraman G (2009). Production of extracellular protease from halotolerant bacterium, *Bacillus aquimaris* strain VITP4 isolated from Kumta coast. *Process Biochemistry*, **44:** 1088-1094.

Sierra, G. (1957). A simple method for the detection of lipolytic activity of micro-organisms and some observations on the influence of the contact between cells and fatty substrates. *Antonie van Leeuwenhoek*, **23:** 15-22.

Smibert, R. M. and Krieg, N. R. (1981). General; characterization, pp.409-433. In G. B. Philips (ed.), Manual of methods for general bacteriology. *American Society of Microbiology*, Washington, D. C.

Snoke, J. E. and Cornell, N. (1965). Protoplast Lysis and Inhibition of Growth of *Bacillus licheniformis* by Bacitracin. *Journal of Bacteriology.* **89(2):** 415–420.

Sorokin, D. Y., Kuenen, J. G., and Jetten, M. S. M. (2001). Denitrification at Extremely High pH Values by the Alkaliphilic, Obligately Chemolithoautotrophic, Sulfur-Oxidising Bacterium *Thioalkalivibrio denitrificans* Strain ALJD, *Archives of Microbiology*, **175:** 94–101.

Sorokin, D. Y., Tourova, T. P., Lysenko, A. M. and Muyzer, G. (2006): Diversity of culturable halophilic sulfur-oxidizing bacteria in hypersaline habitats**.** *Microbiology UK*, **152:** 3013-3023.

Sorokin, D.Yu. Jones, B.E., and Kuenen, J.G., (2000). An Obligate Methylotrophic, Methane-Oxidizing *Methylomicrobium* Species from a Highly Alkaline Environment. *Extremophiles*, **4:** 145–155.

Stackebrandt,E and Goebel, B.M. (1994). A place for DNA-DNA association and 16S ribosomal RNA sequence analysis in the present species definition in bacteriology. *International Journal of systematioc Bacteriology*, **44:** 846-849.

Svetlitshnyi, V., Rainey. F. and Weigel, J. (1996). *Thermosyntropha lipolytica* gen. nov., sp. nov., a lypolytic, anaerobic, alkalitolerant, thermophilic bacterium utilising short- and

long-chain fatty acids in syntrophic coculture with a methanogenic archaeum. *International Journal of Systematic Bacteriology*, **46:** 1131–1137.

Takami, H., Akiba, T. and Horikoshi, K. (1989). Production of extremely thermostable alkaline protease from *Bacillus* sp. no. AH-101. *Applied Microbiology and Biotechnology*, **30:** 120–124.

Talaro, K. P. and Talaro, A. (2002). Foundations of Microbiology 4Th Edition. McGraw Hill, Newyork, USA. p. 205.

Talling, J. F. and Talling, I. B. (1965). The chemical composition of African lake waters. *International Reviews of Hydrobiology Entire*, **50:** 421-463.

Tamura K., Peterson D., Peterson N., Stecher G., Nei M., and Kumar S. (2011). MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. *Molecular Biology and Evolution* **(In Press)**.

Teske, A., Alm, E., Regan, J. M., Toze, S., Rittmann, B. E. and Stahl, D. A. (1994). Evolutionary relationships among ammonia- and nitrite-oxidizing bacteria. *Journal of Bacteriology*, **176:** 6623–6630.

Tindall, B. J., Mills, A. A., and Grant. W. D. (1980) An alkaliphilic red halophilic bacterium with low magnesium requirement from a Kenyan soda lake. *Journal of General Microbiology*, **116:** 257-260.

Tokunaga H, Arakwa T, Tokunaga M (2008). Engineering of halophilic enzymes: Two acidic amino acid residues at the carboxy-terminalregion confer halophilic characteristics to *Halomonas* and*Pseudomonas* nucleoside diphosphate kinases. *Protein Science,* **17:** 1603-1610.

Utåker, J. B., Bakken, L., Jiang, Q., and Nes, J. (1995). Phylogenetic analysis of seven new isolates of ammonia-oxidizing bacteria based on 16S rRNA gene sequences. *Systematic Applied Microbiology*, **1:** 549–559.

Vargas, C., Argandona, M., Reina-Bueno, M., Rodriguez-Moya, J., Fernandez-Aunion, C. and Joaquin, J. N. (2008). Unravelling the adaptation responses to osmotic and temperature stress in *Chromohalobacter salexigens*, a bacterium with broad salinity tolerance. *Saline Systems,* **4:** 14.

Vargas, V. A., Delgado, O. D., Kaul, R. H. and Mattiasson, B. (2004). Lipase-producing microorganisms from a Kenyan alkaline soda lake. *Biotechnology Letters,* **26:** 81–86.

Veith, B., Herzberg, C., Steckel, S., Feesche, J., Maurer, K. H., Ehrenreich, P., Bäumer, S., Henne, A., Liesegang, H., Merkl, R., Ehrenreich, A. and Gottschalk, G. (2004). The complete genome sequence of *Bacillus licheniformis* DSM13, an organism with great industrial potential. *Journal of Molecular Microbiology and Biotechnology*. **7(4):** 204-211.

Ventosa, **A. (2006).** "Unusual micro-organisms from unusual habitats: hypersaline environments," in *Prokaryotic Diversity: Mecanisms and Significance*, Logan, N. A.

Lappin-Scott, H. M. and. Oyston, P. C. F (Editors), pp. 223–253, Cambridge University Press, Cambridge, UK.

Ventosa, A., Nieto, J. J. and Oren, A. (1998). Biology of moderately halophilic aerobic bacteria. *Microbiology and Molecular Biology Reviews*, **62**: 504–544.

Wagner, M., Erhart, R., Manz, W., Amann, R., Lemmer, H., Wedi, D., and Schleifer, K. H. (1994). Development of an rRNA-targeted oligonucleotide probe specific for the genns *Acinetobacter* and its application for in situ monitoring in acitivated sludge. *Applied and Enviromental Microbiology*, **60:** 792-800.

Wang, Y. X., Srivastava, K. C., Shen, G. J. and Wang, H. Y. (1995). Thermostable alkaline lipase from a newly isolated thermophilic *Bacillus*, strain A30-1 (ATCC 53841). *Journal of Fermentation and Bioengineering*, **79:** 433–438.

Wani, A. A., Surakasi, V. P., Siddharth, J. Raghavan, R. G., Patole, S. M., Ranade, D. and Souche, Y. S. (2006). Molecular analyses of microbial diversity associated with the Lonar soda lake in India: An impact crater in a basalt area.Science direct: *Research in Microbiology*, **157:** 928–937.

Wipat, A. and Colin, R. H. (1999). The Bacillus subtilis genome sequence: the molecular blueprint of a soil bacterium. *FEMS Microbiology Ecology*, **28:** 1-9.

Woese, C. R., Weisburg, W. G., Paster, B. J., Hahn, C. M., Tanner, R. S., Krieg, N. R., Koops, H. P., Harms, H. and Stackebrandt, E. (1984). The phylogeny of purple bacteria, the beta subdivision. *Systematic and Applied Microbiology*, **5:** 327–336.

Xu, X., Yuehong, W. and Zhang, T. (2006). Isolation and phylogenetic Analysis of Halophilic Archaeon AJ6. *Frontiers of Biology in China* **1:** 29-34.

Zavarzin, G. A, Zhilina, T. N. and Kevbrin, V. V. (1999). The alkaliphilic microbial community and its functional diversity. *Microbiology,* **68:** 503–521.

Zhilina, T. N, Garnova, E. S., Tourova, T. P., Kostrikina, N. A., Zavarzin, G. A. (2001). *Halonatronum saccharophilum* **gen. nov., sp. nov.: a new haloalkaliphilic bacterium of the order** *Haloanaerobiales* **from Lake Magadi.** *Microbiology,* **70:** 77-85.

Zhilina, T. N. and Zavarzin, G. A. (1994). Alkaliphilic anaerobic community at Ph-10. *Current trends in Microbiology*, **29:** 109–112.

Zhilina, T. N., Garnova. E. S., Detkora, E. N., Rainey, F. A., Osipov, G. A., Lysenko, A. M., Kostrikina, N. A. and Zavarzin, G.A. **(1998).** ***Natronoincola histidinovorans*gen** nov. Sp. nov., a new halkaliphilic, acetogenic anaerobe. *Current microbiology* **37:** 177-185.

Zhilina, T. N., Zavarzin, G. A., Rainey, F. A., Pikuta, E. N., Osipov, G. A. and Kostrikina. N. A. (1997). *Desulfonatronovibrio hydyogenovorans* gen. nov., sp. nov., an alkaliphilic, sulfate-reducing bacterium. *International Journal of Systematic Bacteriology*, **47:** 144-149.

Zhiyu, L., Yoshiaki, K., Osamu, S., Shuzo, Y., Takashi, D. and Takayuki, E. (2002). *Bacillus okuhidensis* sp. nov., isolated from the Okuhida spa area of Japan**.** *International Journal of Systematic and Evolutionary Microbiology*, 52, 1205–1209.

APPENDICES

APPENDIX A: MEDIA PREPARATION

MODIFIED DUCKWORTH MEDIA (1996)

M1: Glucose 10.0 g, Peptone (Difco) 5.0 g, Yeast extract (Difco) 5.0 g, K_2HPO_4, 1 .O g, $MgSO_4.7H_2O$ 0.2 g, Cycloheximidine 0.01 g, Agar 20.0 g.

M2: Cellulose 10.0 g, Peptone (Difco) 5.0 g, Yeast extract (Difco) 5.0 g, K_2HPO_4 1.0 g, $MgSO_4.7H_2O$ 0.2 g, Cycloheximidine 0.01 g, Agar 20.0 g.

M3: Xylose 10.0 g, Peptone (Difco) 5.0 g, Yeast extract (Difco) 5.0 g, K_2HPO_4 1.O g, $MgSO_4.7H_2O$ 0.2 g, Agar 20.0 g.

M4: Starch 10.0 g, Peptone (Difco) 5.0 g, Yeast extract (Difco) 5.0 g, K_2HPO_4 1.O g, $MgSO_4.7H_2O$ 0.2 g, Agar 20.0 g, Cycloheximidine 0.01 g.

M5:(DSC-97): Contained, in grams per litre of Lake Magadi water: Casamino acids, 7.5 g, Yeast extract, 10.0 g, Trisodium citrate 3.0 g, KCl 2.0 g, $MgSO_4.7H_2O$ 20.0 g, $FeCl_2$ 0.023 g, Agar 15 g, Cycloheximidine 0.01 g (*Phil et al*., 2005).

M6: KCl 1.0 g, $MgSO_4.7H_2O$ 20.0 g, $FeSO4.7H_2O$ 0.02 g, Trisodium citrate 3.0 g, Casamino acids 7.5 g, Yeast extract 0.5 g, Cycloheximidine 0.01 g (Xu et al., 2006).

M8: $MgSO_4.7H_2O$ 9.7 g, Trisodium citrate 3.0 g, KCl 2.0 g, $CaCl_2$ 0.2 g, Bacterial peptone (Oxoid L 37) 10.0 g, Agar 15.0 g, Cycloheximidine 0.01 g.

Preparation : Suspend the components in 700ml of Lake Magadi water and10 g Na_2CO_3 in 300mls of distilled water separately to make 1 litre, mix well to dissolve the medium completely. Sterilize separately by autoclaving at 121°C for 15 minutes. Mix the two sets at 60^0C. Dispense in 3-10mls in sterile tubes under sterile conditions.

Use: For the enrichment of bacteria during culture.

BASAL MEDIA

Formula: 1% KH_2PO_4, 0.01% $MgSO_4.7H_2O$, 0.005% $CaCl.2H_2O$, 4% NaCl, 1% Na_2CO_3, supplemented with 2.5% xylan, 1% cellulose, 1% carbonmethylcelullose, 1% starch, skim milk or 1% olive oil or esterase.

Preparation: Suspend the components in 1 litre of distilled water. Stir well to dissolve the medium completely. Sterilize by autoclaving at 121°C for 15 minutes. Cool to 45-50°C and pour in 15 – 20 ml amounts into Petri dishes.

Use: To test for the release of enzymes that utilizes these substrates respectively.

NUTRIENT BROTH: **Formula:**1.3% nutrient broth, 15% sodium chloride, 1% sodium carbonate.

Preparation: Suspend the components in 1 litre of distilled water. Stir well to dissolve the medium completely. Dispense in 3-10mls in tubes then Sterilize by autoclaving at 121°C for 15 minutes.

Use: General purpose broth media.

NITRATE BROTH

Formula: 0.8% Nutrient, 0.1% KNO_4, 4% NaCl, 1% Na_2CO_3

Preparation: Suspend the components in 1 litre of distilled water. Stir well to dissolve the medium completely. Dispense in 3-10ml in tubes then sterilize by autoclaving at 121°C for 15 minutes.

Use: to test for nitrate reduction to nitrites.

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

Formula: 1.7% MR-VP Media, 4% NaCl, 1% Na_2NO_3

Preparation: Suspend the components in 1 litre of distilled water. Stir well to dissolve the medium completely. Dispense in 3-10ml in tubes then sterilize by autoclaving at 121°C for 15 minutes and measure the pH before inoculation.

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

SULPHUR-INDOLE MORTILITY (SIM) AGAR MEDIA.

Preparation: 3.6% SIM, 4% NaCl, 1% Na_2NO_3.

Use; to test for the production of tryptophanase enzyme and the ability to produce hydrogen sulfide from substrates, such as sulfur containing amino acids and organic sulfur.

APPENDIX B

DNA EXTRACTION REAGENTS

BSS buffer

10mM K_2HPO_4

6.9mM KH_2PO_4

21.5mM KCl

24.5mM NaCl

Adjust PH to 7.4 with KOH, autoclave.

1.0mM DTT (Filter, do not autoclave).

1mg/ml Lysozyme.

CTAB/NaCl solution

10% CTAB in 0.7 M NaCl (Final concentration).

10 % SDS

Lysozyme 20mg/ml

RNase A 20mg/ml

Proteinase K 50mg/ml

Proteinase K: To 1 ml of ddH_2O add 50 mg of Proteinase K (Promega # 52066). This gives a 50mg /ml stock.

SDS 10%: Dissolve 100g of electrophoresis-grade SDS in 800ml ddH_2O. Heat the solution to dissolve. Bring to a final volume of 1000 ml using ddH_2O. Do not autoclave.

Phenol

Chloroform

Absolute ethanol.

5M NaCl

Isopropanol

Isoamyl alcohol

Electrophoresis buffer Working Concentrated stock

TBE buffer 10 ×

Chemical	Volume
Tris	108 g
Boric Acid	55 g
$Na_3EDTA.2H_2O$	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.

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.

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.

TE pH 7.4 or pH 8.0

Chemical	Volume
1 M Tris pH 8.0	2 ml
0.5 M EDTA pH 8.0	400 µl

Bring it to a final volume of 100ml using ddH_2O and Autoclave.

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.