THE DIVERSITY OF EXTREMOPHILIC BACTERIA IN LAKE MAGADI, KENYA

BY

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SIGNED _____

A thesis submitted in partial fulfillment for the award of the degree of Master of Science in Microbiology

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Declaration

I, Bancy N. Muruga, hereby declare that this is my original work, and has not been presented for a degree in any other university.

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Dedication

I dedicate this work to my late mother who passed away in the course of this study and my children who have given me constant encouragement during the study period.

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List of Acronyms/Abbreviations

AE (buffer) ----------Elution buffer
AL (buffer) ----------Lysis buffer
ATL (buffer) ----------Tissue lysis buffer
AW (buffer) ----------Wash buffer
BLAST ------- --------Basic local alignment search tool
CMCases----------Carboxymethylcellulases
DNA----------Deoxyribonucleic acid
DO ----------Dissolved oxygen
dNTP----------Deoxynucleotide triphosphate
EB (buffer) ----------Elution buffer
EC ----------Electrical conductivity
EDTA----------Ethylene diamine tetraacetic acid
FASTA ---------- Pronounced 'fast aye' used for fast nucleotide or protein comparison
G+C----------Guanine + Cytosine
GPS ----------Global Positioning System
NCBI ----------National Centre for Biotechnology Information(USA)
ORP----------Oxidation reduction potential
OTU----------Operational Taxonomic Units
PBI (buffer) ----------Binding buffer
PCR----------Polymerase Chain Reaction
PE (buffer) ----------Protein Extraction & Lysis buffer
PHA----------Polyhydroxyalkanoates
rDNA----------Ribosomal DNA
rRNA----------Ribosomal RNA
RNA----------Ribonucleic acid
Rpm----------Revolution per minute
TDS----------Total dissolved solutes
TAE----------Tris acetate EDTA
TSB ----------Tryptic soy broth
UA ----------Universal Archeal primers

Units

bp----------Basepairs
G/l----------Gram per liter
Kbp----------Kilobasepairs
m----------Meters
Mg/l---------- Milligram per liter
mM----------Millimolar
nm----------Nanometers
PSS----------Practical Salinity Scale

PSU---------- Practical Salinity Units
Ppm---------- Parts per million
S---------- Svedberg units
W/v----------Weight per volume
µl----------Microliters
µm----------Micrometers

Chemical symbols

CO_2----------Carbon (IV) oxide
$CaCO_3$----------Calcium carbonate
Cl^-----------Chloride ions
Ca^{2+}----------Calcium ions
CO_3^{2-}----------Carbonate ions
HCO_3^-----------Hydrogen carbonate ions
KNO_3----------Potassium Nitrate
KCl----------Potassium chloride
K^+----------Potassium ions
KH_2PO_4----------Potassium Hydrogen phosphate
Mg^{2+}----------Magnesium ions
$MgSO_4.7H_2O$---------- Hydrated Magnesium sulphate
Na^+----------Sodium ions
Na_2CO_3----------Sodium Carbonate
$Na_2CO_3.,NaHCO_3.\ 2H_2O$------Sodium sesquicarbonate (Trona)
NaCl----------Sodium chloride
Fe^{2+}----------Iron (II) ions
Fe^{3+}----------Iron (III) ions
$FeS0_4\ .7H_2O$ ----------Hydrated Iron (II) sulphate

Abstract

Lake Magadi, a highly saline lake with a pH range of about 11 to13, harbors extremophilic eubacteria and archaeobacteria that are either halophilic, alkaliphilic, haloalkaliphilic or thermophilic. Water sampling was done in seven hot springs along the shoreline and one sample from along the Western causeway. Physicochemical conditions included a temperature range of 30°C to 47°C , a pH range of 10 to 10.5, hydrogen peroxide concentration of less than 0.5 ppm and dissolved O_2 concentration ranging from 1.1 mg/l to 2.9 mg/l. Morphological characterization indicated presence of gram positive and gram negative bacteria with shapes varying from rods, cocci, spirilla to coccobacilli. Biochemical characteristics of the isolates included catalase production and hydrolysis of casein and starch. The optimal temperature for growth of bacterial isolates ranges from 40°C to 45°C while optimal salt concentration ranges from 6% to 9% w/v. Successful PCR was done on genomic DNA from the bacterial isolates, using 16S primers, and verified by means of agarose gel electrophoresis. On sequencing, four of the PCR products had about 1,500 bp of 16S rRNA. When aligned to existing sequences in the NCBI database, they were found to be closely related to members of the gamma subdivision of the Proteobacteria with over 98% similarity. One of the isolates appeared to have a separate lineage from the ones in the database with only 97% similarity to its closest comparison. Two of the isolates were closely related to but not identical to members of the genus *Halomonas*. Phylogenetic analysis by means of a rooted tree showed that three of the isolates clustered closely with members of gamma Proteobacteria. The multialignments obtained in this study can be used for primer design and the characterized isolates can be used to obtain metabolites for biotechnological applications.

Chapter One

Introduction and Literature review

All cellular organisms are classified into three dormains namely Archaea, Bacteria and Eucarya; Archaea and Bacteria being prokaryotic. The cell strucrure of prokaryotes is simpler and usually small (average diameter, 1μm), and does not possess a distinct nucleus, whereas the eucaryotic cell is larger (5-10μm) and more complex, possessing a distinct nucleus and sub-cellular organelles such as chloroplasts and mitochondria. The archaea are a unique group occurring in extreme environments such as highly saline, highly alkaline, very high temperatures or high pressure. Their cell wall structure, composition of plasma membrane and nucleic acid sequences in small ribosomal subunits are distinctive to this group (Woese *et al*, 1990). Extremophiles which include both bacteria and archaea (and some Eucaryotes) live, grow, and multiply in various extremely harsh environments (reviewed by Rothschild and Mancinelli, 2001).Such environments include the Soda lakes with alkaline saline conditions. Factors leading to formation of alkaline saline lakes are divided into climatic, geological and topographical. The climate controls amount of water entering the system as rainfall or runoff and amount leaving by evaporation. Geological influences determine which ions enter the system such as in the case where a solution of CO_2 forms weak carbonic acid that leaches minerals. Topography allows concentration of salts in a shallow depression forming a closed drainage system where there is little or no outflow except by evaporation (Tindall, 1988).

1.1 Location and general description of Lake Magadi

Lake Magadi is situated in a closed lake basin (Fig 1) in the Southern part of the Gregory Rift, a branch of Africa's Great Rift Valley. The Magadi-Natron basin has been occupied by a larger body of water on several occasions during late Pliocene and Pleistocene times. Both lakes are saline, but Lake Magadi is the most saline of the lakes in the Gregory Rift with salinity of up to 30% w/v (Grant, 1992). The volcanic rocks in the surrounding areas of the lake, generally present high alkalinity and the lake water has pH values of over 11.5 (Duckworth *et al.*, 1996). Ol Doinyo Lengai, which is considered to be the youngest volcano, presents eruption of carbonates consisting of Sodium Carbonate ($Na_2 CO_3$) and Calcium Carbonate ($CaCO_3$). It is famous for being the only volcano known in the world to have erupted carbonatite lava in historical time. The younger the age of the volcanic rock, the higher is its alkalinity (DasSarma, 2006). The lake consists of an almost solid deposit of sodium chloride and sodium carbonate, the latter existing as sodium sesquicarbonate or trona ($Na_2CO_3 NaHCO_3 2H_2O$). It covers an area of 90 km^2, is located about 2° S, 36° 20 E and the elevation above sea level is about 600m. The alkali salt crust on the surface of the lake is often colored red or pink by the salt-loving halophiles that live there. Saltwater can evaporate leaving evaporite deposits consisting of salts such as sodium chloride (halite) and calcium sulfate (gypsum). Within evaporites are fluid inclusions (small trapped pockets of water) that can provide a refuge for microbes for at least six months (DasSarma, 2006). Cyanobacteria trapped within dry evaporite crusts can continue to have low levels of metabolic function such as photosynthesis. These deposits also form fossils of the organisms trapped within. Although highly controversial, it has been claimed that bacteria might survive for millions of years in the fluid inclusions of salt deposits. Intriguingly, such deposits have been found on Mars (DasSarma, 2006).

The climatic zone receives erratic rainfall below 800 mm per year, with substantial annual variation. There is a significant relationship between elevation and rainfall in this area, with every 100m rise in elevation corresponding to an increase of 35 mm in annual rainfall. Most of the rain in this area falls between December and May, followed by a long dry season. Daily temperatures at the lake are frequently above 40°C. These hot, dry conditions expose the lakes to high evaporation rates. Soda brines lack the divalent cations magnesium and calcium because of their low solubility at alkaline pH (DasSarma, 2006). There are no permanent rivers entering the Magadi basin and solutes are supplied mainly by a series of alkaline springs with a temperature as high as 86°C. The springs feed lagoons which support a thriving colony of fish, the *Tilapia grahami* species, which can thrive on pH of 10.5 and temperature of 39°C (Tindall, 1988).

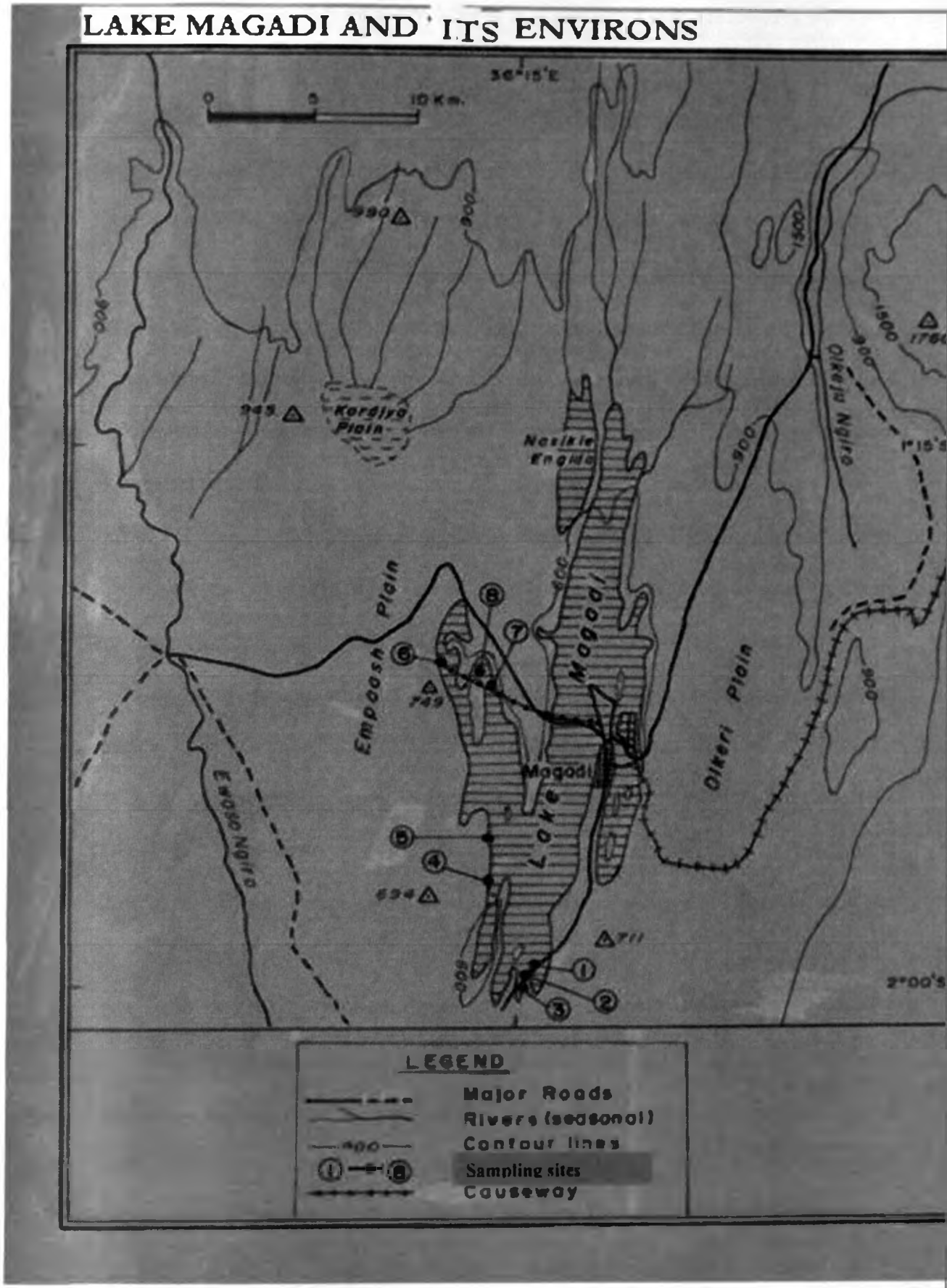


Fig1: Lake Magadi and its environments (sourced from Geographical Dept,UON)

1.2 Extremophiles expected in L. Magadi

Soda lakes harbour considerable taxonomically diverse bacterial populations consisting mainly of aerobic and anaerobic organotrophic prokaryotes (Rees *et al.*, 2003). Lake Magadi harbors a dense and diverse population of aerobic, organotrophic, halophilic, alkaliphilic and alkalitolerant representatives of major bacterial and archeael phyla. The majority of gram negative isolates are the members of the gamma subdivision of Proteobacteria, including many proteolytic organisms related to members of the genus *Halomonas* (Duckworth et al., 2000).

Halophiles

Halophiles are extremophilic microorganisms that thrive well in environments with very high concentrations of salt. Some halophiles are classified in the Archaea dormain, others are Bacteria while others such as the alga *Dunaliella salina* are Eukaryotes. Non-halophiles grow best in media containing less than 1.2%w/v NaCl, slight halophiles (marine bacteria) grow best at 1.2-3.0 %w/v NaCl, moderate halophiles at 3-15%w/v NaCl, extreme halophiles grow in media containing 15-31.2%w/v or saturated NaCl (Dubey and Maheshwari, 2004). Halophilic microorganisms are found in hypersaline lakes such as Magadi in Kenya, the Great Salt Lake in Utah, Owens Lake in California and the Dead Sea. Other habitats for halophilic microorganisms include highly salted foods, saline soils, and underground salt deposits. They have specialized cell walls, and usually incorporate pigmentation in the form of bacteriorhodopsin for photosynthesis, and carotenoids for ultraviolet protection. Waters containing halophiles are often colored red by the dense microbial communities of pigmented halophiles such as *Halobacterium* but sometimes they are colorless. Halophiles are able to live in salty conditions by preventing dehydration of their cytoplasm. They do this by either producing large amounts of an

internal organic solute referred to as a compatible solute or by concentrating a solute from their environment. The archaeon *Halobacterium* concentrates in the cytoplasm large amounts of potassium (K^+, as KCl) from the environment. The dissolved KCl in the cells is present at a concentration equal to or slightly above that of the dissolved NaCl outside, and in this way cells maintain the tendency for water to enter and thereby prevent dehydration. Enzymes that function inside the cells of *Halobacterium* have evolved to require this large dose of K^+ for catalytic activity. Membranes or cell wall-positioned proteins in *Halobacterium* require Na^+ and are typically stable only in the presence of high Na^+ concentration (Horikoshi and Grant, 1991).

Alkaliphiles

Alkaliphilic organisms grow best at pH10-12 while alkali-torelant organisms grow best at pH 7-9 (they cannot grow above pH 9.5) (Dubey and Maheshwari, 2004). Most of the alkaliphilic organisms are anaerobic or facultatively anaerobic. Some alkaliphiles such as *Bacillus alkaliphilus* bear flagella and hence are motile. Extreme alkaliphiles such as *Natronobacterium magadii*, isolated from Lake Magadi, grow optimally at a pH of about 10. Alkaliphiles have to contend with the problems associated with high pH. Above a pH of 8, certain biomolecules, notably RNA, break down and consequently alkaliphiles must maintain their cytoplasm close to neutrality. Proteins found in the cell wall or in the membrane that makes contact with the environment are stable at high pH. Besides keeping their cytoplasm near neutrality, there is the problem of membrane-mediated bioenergetics; protons extruded to the external surface of the membrane enter a sea of hydroxyl ions. A proton motive force is formed by these extreme alkaliphiles that also

drives some of the energy-requiring reactions in the cell, such as motility and transport (Grant and Jones, 2000).

Thermophiles

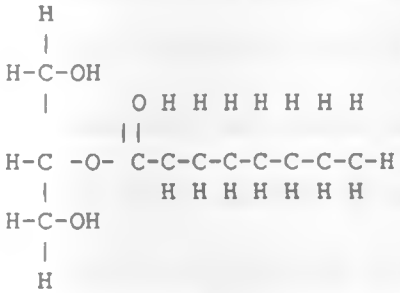
Thermophilic organisms grow at a temperature exceeding 40°C. Moderate thermophiles grow well at 40-60°C while extreme thermohiles grow at 80 -100°C. Hyperthermophiles are Archaea and are unable to grow below 80°C. Most of the hyperthermophiles are anaerobic due to low solubility of oxygen at high temperature. Their membranes are unusually stable at high temperatures. They are found in volcanic areas such as hot springs and solfataric fields. These are high temperature fields located within volcanic zones with a lot of sulfur acidic soil, acidic hot springs and boiling mud. Few of the hyperthermophiles live in shallow submarine hydrothermal systems and abyssal hot vent systems called 'black smokers' having temperature of about 270-380°C. Hydrothermal vents with temperatures of 350°C or greater also show the existence of hyperthermophiles. Some examples of hyperthermophiles are *Thermotoga* which has rod shaped cells surrounded by a characteristic sheath-like structure (the toga) which balloons out at one end, Archaeal coccoid sulphate reducers that are members of the genus *Archeoglobus, Methanopyrus kandleri* which is a rod shaped methanogen (Dubey and Maheshwari , 2004).

1.3 Literature review

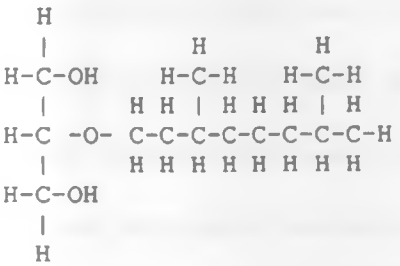
1.3.1 Overview of Archaeobacteria

The Archaeobacteria (Greek *archaios*, ancient, and *bakterion*, a small rod) are quite diverse, both in morphology and physiology. They can stain either gram positive or gram

negative and may be spherical, rod-shaped, spiral, lobed, plate-shaped, and irregularly shaped (pleomorphic). Some are single cells, whereas others form filaments or aggregates. They range in diameter from 0.1 μm to over 15 μm, and some filaments can grow up to 200 μm in length. Multiplication is mainly by binary fission, budding or fragmentation. They can be aerobic, facultatively anaerobic, or strictly anaerobic. Nutritionally, they range from chemolithoautotrophs to organotrophs. Some are mesophiles, others are hyperthermophiles that can grow above 100°C. Archaeobacteria usually prefer extreme aquatic and terrestrial habitats such as anaerobic, hypersaline, and high temperature habitats. A few are symbionts in animal digestive systems. The Archaeobacteria cell wall differs chemically from that of the Eubacteria cell wall by lacking peptidoglycan (Prescott *et al.*, 1996). The membranes of Archaeobacteria have lipid bilayers consisting of branched chain hydrocarbons linked by ether linkages to glycerol (as opposed to ester linkages in Eubacteria). Typical structure of Eubacteria monoglyceride:



Typical structure of Archaeobacteria monoglyceride:



Both Archaeobacteria and Eubacteria have closed circular DNA genomes. However, the average Eubacteria genome may be as much as two or more times larger than the average Archaeobacteria genome. Archaeobacteria, unlike Eubacteria, tend to display few plasmids. Some detailed aspects of the Archaeobacterium molecular genetics resemble those of Eucaryota rather than Eubacteria (the RNA polymerase and to a lesser extent the ribosome). Bergey's Manual (Bergey and Holt, 1994) divides the Archaeobacteria into five major groups: methanogenic archaeobacteria, sulfate reducers, extreme halophiles, cell wall-less archaeobacteria, and extremely thermophilic S-metabolizers (Prescott *et al.*, 1996). Methanogens is the largest group among the Archaeobacteria. They convert various substrates to methane (or methane and CO_2) under strict anaerobic conditions. They contribute significantly to the release of methane, a potent green house gas, into the atmosphere. Sulfate reducers are extreme thermophilic Archaeobacteria which display apparently low diversity (one known genus), found at deep sea hydrothermal vents. The sulfate reducers reduce various sulfur containing compounds but are not capable of reducing molecular sulfur (they cannot use molecular sulfur as a final electron acceptor in anaerobic respiration). Extreme halophiles are aerobic chemoheterotrophs which require complex energy and carbon sources (particularly amino acids). Osmotic requirements exceed 8% w/v NaCl for all extreme halophiles and tolerances range up to 36% w/v. *Halobacterium salinarium* is an extreme halophile capable of a unique form of photosynthesis which does not utilize chlorophyll. There exist cell-wall less Archaeobacteria which live in the high temperature (55°C to 59°C) and acidic conditions. Extreme thermophilic sulfur metabolizers are strictly anaerobic Archaeobacteria that utilize elemental sulfur as a final electron acceptor in anaerobic respiration. Many extreme thermophilic S-metabolizers are also acidophiles. Habitats in which extreme

thermophilic S-metabolizers are found tend to be geothermic or volcanic, where molecular sulfur and high temperatures are found together (Prescott *et al.*, 1996).

1.3.2 Extremophiles in evolution

Prokaryotes have existed for some 3.8 billion years and have had the evolutionary time to show great genetic divergence. However, unlike metazoans, evolutionary change in prokaryotes is not manifest in morphological variation. Bacteria maintained a very small size and changed relatively little (compared with metazoans) in morphology through billions of years of evolutionary history. Molecular sequencing shows that bacteria have indeed evolved but that the product of their evolutionary change is invisible. Thus instead of big changes in size or shape, evolutionary change in the prokaryotes focused on metabolic diversity and the genetic capacities to explore and eventually colonize every conceivable environment on Earth, including extreme environments (Bell and Stillman, 1992). Studies of the genetics and diversity of extremophiles reveal their unique metabolic properties and possibly characteristics of early evolutionary histories of the Earth. Prokaryotes that grow at very high temperatures have cellular components that need to be heat stable. Their evolutionary position is that of the least derived of all known life forms. The knowledge of bacterial diversity has improved, primarily from the introduction of molecular tools for assessing bacterial phylogeny and diversity, and from new advances in isolation and laboratory culture. It is now clear that the bulk of evolutionary diversity on Earth does not reside in plants and animals, but instead in the invisible prokaryotic world. With the advent of comparative ribosomal RNA gene sequencing, microbiologists are now able to experimentally determine the evolutionary relationships of bacteria. One major concept that has emerged from comparative

molecular sequencing of ribosomal RNA gene is that cells evolved along three major lineages, the Bacteria, the Archaea, and the Eukarya (see Fig 2), instead of just two, the prokaryotes and the eukaryotes as earlier believed (Woese *et al.*, 1990).

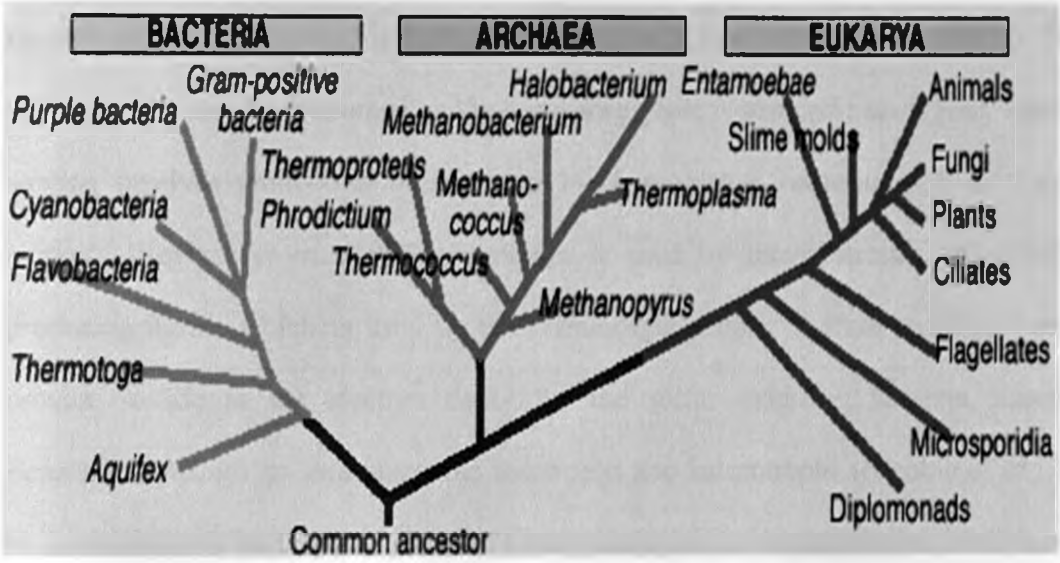


Fig 2: A phylogenetic tree of life based on comparative ribosomal RNA sequences (Woese *et al.*, 1990)

1.3.3 Nutrient cycling in soda lakes

Soda lakes are closed systems that exhibit complete nutrient cycling under anaerobic and aerobic conditions. Photosynthetic *Cyanobacteria* add carbon and nitrogen into the ecosystem through carbon dioxide and nitrogen fixation. Some contribution to primary productivity is also made by anoxygenic phototrophic bacteria of the genus *Ectothiorhodospira* (Jones *et al*, 1998), genus *Halorhodospira* and also *Rhodobaca bogoriensis* (Milford *et al.*, 2000).

Chemoorganotrophs produce hydrolytic enzymes such as proteinases, cellulases, lipases and amylases in order to utilize the products of primary production. They produce sugars and amino acids which form substrates for fermentation by anaerobes such as *Clostridium sp* to produce volatile fatty acids. These fatty acids produced by chemoorganotrophs may be used by acetogenic bacteria such as *Natroniella acetigena* and *Tindallia magadiensis* (Zavarzin *et al.*, 1999). Saccharolytic spirochaetes utilize sugars and some polysaccharides to produce acetate, lactate, ethanol and hydrogen. These products may also be consumed by the homoacetogenic *Natroniella acetogena*. Nitrogen cycling involves production of ammonia by fermentative bacteria such as *Tindalia magadii* (Kevbrin *et al.*, 1998). Ammonia is used by methylotrophs and nitrifiers, producing nitrate which is used by the chemoorganotrophs. Sulfate reducing bacteria provide sulfide as the electron donor for the sulfur oxidizing bacteria, anaerobic *Ectothiorhodospira sp.* and anaerobic autotrophs and heterotrophs (Sorokin *et al.*, 1996 b). Methanogenic bacteria are obligately methylotrophic but use methanol, methyl amine and dimethyl sulfides. Methane is not lost from the system as methane oxidizers such as the *Methylobacter* may be present in soda lakes (Jones *et al.*, 1998)

1.3.4 The biotechnological potential of extremophiles

Extremophilic microorganisms produce extra cellular enzymes with potential applications in biotechnology. The following are some of the applications that the secondary metabolites produced by extremophiles can be put to;

Halophiles

1. Bioplastic or polyhydroxy alkanoates (PHA)

This is a biodegradable heteropolymer that exhibits total resistance to water and is degraded in human tissue; hence it is biocompatible. It has pharmaceutical and clinical importance, including the use in delayed drug release, bone replacement and surgical sutures. PHA is produced by *Haloferax mediterranei*, a halobacterium that possesses high genomic stability which is a prerequisite for industrial purposes (Roderiguez-Valera and Lillio, 1992).

2. Polysaccharides

Microbial exopolysaccharides are used as stabilizers, thickeners, gelling agents and emulsifiers in pharmaceutical industries, paint and oil recovery, paper, textile and food industries. *Haloferax mediterranei* produces a highly sulfated and acidic heteropolysaccharide (up to 3g/l) which contains mannose as a major component. Such a polymer combines excellent rheological properties with a remarkable resistance to extremes of salinity, temperature and pH (Dubey and Maheshwari, 2004).

3. Microbially enhanced oil recovery

The residual oil in natural oil fields can be extracted by injection of pressurized water down in a new well. Bacterial biopolymers are of interest in enhanced oil recovery because of this bio-surface activity and properties of bio-emulsifiers (Bertrand, 1990).

4. Cancer detection

A protein from *Halobacterium halobium* has been used as an antigen to detect antibodies against the human c-Myc oncogene product in the sera of cancer patients suffering from leukaemia. The use of bacterial antigens as probe for some type of cancer seems to be promising (Kushner, 1985).

5. Liposomes

Ether-linked lipid of the *Halobacteria spp* is used in liposome preparation which has great value in the cosmetic industry. Such liposomes would be more resistant to biodegradation, good shelf-life and resistance to other bacteria (Dubey and Maheshwari, 2004).

6. Enzymes

Protease and amylase from *Halobacterium salinarium*, *H. halobium* and lipase from several Halobacteria have been reported. A site-specific endonuclease activity has been reported in *H. halobium* (Kushner, 1985).

7. Bioremediation

Bertrand (1990) observed that the *Halobacterium* strain EH4 isolated from salty water was found to degrade alkanes and other aromatic compounds in the presence of salt.

8. Gas vacuoles or vesicles

Some *Halobacterium spp.* produce intracellular gas filled organelles called vacuoles which provide buoyancy. In future, the genes for such properties could be engineered in other microorganisms to produce gas vacuoles to float in water (Grant *et al.*, 1990).

9. In food

A sauce called 'nam pla' is prepared in Thai from fish fermented in concentrated brine that contains a large population of *Halobacteria.* These are responsible for aroma production because they produce salt-stable extra cellular proteases. Large scale cultivation of the cyanobacterium *Spirulina platensis* in Israel uses brackish water which is unsuitable for agriculture and the *Spirulina* biomass is marketed as a food. *Spirulina* grows optimally in alkaline lakes with a salt concentration ranging from 2 to 7%w/v.

Carotenoids produced by moderate halophiles have major applications in the food industry as food-coloring agents and as additives in health food products. (Dubey and Maheshwari, 2004).

Alkaliphiles

The use of alkaliphilic microorganisms has a long history especially in Japan. From ancient times indigo has been naturally reduced under alkaline conditions in the presence of sodium carbonate. Indigo from indigo leaves is reduced by a particular bacterium that grows under highly alkaline conditions in a traditional process called indigo fermentation. The following are some of the uses that alkaliphiles have been put to;

1. Proteases

Horikoshi (1971 b) reported the production of an extracellular protease by alkaliphilic *Bacillus clausii* 221.Two other *Bacillus strains* AB42 and PB12 producing alkaline proteases were reported by Aunstrup *et al.*, (1972). Proteases are used as detergent additives (detergent enzymes account for about 30% of total worldwide enzyme production) and in the hide-dehairing process. They are a group of enzymes whose catalytic function is to hydrolyze peptide bonds of proteins (also called proteolytic enzymes or proteinases); proteinase K is a common protease used in DNA extraction in molecular biology. Alkaline proteases used in the detergent industry remove stains such as blood, egg, and human sweat. For an enzyme to be used as a detergent additive it must be stable and active in the presence of typical detergent ingredients, such as surfactants (surface active agents), builders, bleaching agents, bleach activators, fillers, fabric softeners, and various other formulations. Most liquid detergents contain builders such as citrate, sodium carbonate or sodium silicate to soften water by sequestration, precipitation or ion exchange. Alkaliphilic bacteria such as *Bacillus licheniformis* are good sources of

alkaline proteases. *Bacillus subtilis* a neutrophilic bacterium produces subtilisin, an alkaline enzyme used in detergent making (Horikoshi, 1999).

2. Starch-degrading enzymes

A haloalkaliphilic archaeon *Natronococcus* sp strain Ah-36 produces an extracellular maltotriose-forming amylase. The gene encoding this enzyme has been cloned and expressed in *Haloferax volcanii*. These starch-degrading enzymes, such as amylase, hydrolyze starch to produce glucose, maltose, and maltotriose (Kobayashi *et al.*, 1992).

3. Pullulanase

Nakamura *et al.* (1975) discovered that alkaliphilic *Bacillus halodurans* produces an extra cellular pullulanase in Horikoshi II medium. This enzyme has a pH optimum at 8.5-9.0 and is stable for 24 hr at pH 6.5-11.0 at 4°C. Pullulanase is a good candidate for a dishwashing detergent additive.

4. Cellulases

Two alkaliphilic *Bacillus* strains no. N-4 and no. 1139, producing extracellular carboxymethylcellulases (CMCases) have been isolated (Horikoshi *et al.*, 1984). Such cellulases are used as laundry detergent additives.

5. Xylanases

Alkaline xylanases are of interest because the enzyme can readily hydrolyze xylan, which is soluble under alkaline conditions. The enzyme is produced by alkaliphilic *Bacillus* sp. no. C-59-2 and *Bacillus halodurans* C125. It has a possible application in biological debleaching processes in pulp-milling factories (Horikoshi and Atsukawa, 1973).

6. Pectinases

Bacillus halodurans C125 also produces pectinases, another group of enzymes that improves the production of paper by making it high-quality, nonwoody, and stronger. They are also used in waste treatment (Horikoshi, 1999).

Thermophiles

1. Amylopullulanases produced by thermophiles are thermo-stable enzymes that have been used to reduce the number of steps needed to transform starch into fructose syrup. Certain bacteria and archaea such as *Thermococcus litoralyse, T. celer* produce amylopullulanase having a temperature optimum of 105°C and pH 6 that is useful for bio-conversion of starch into various useful products (Lin *et al.*, 1994).

2. Taq polymerase is a very important enzyme used in molecular biology for the amplification of DNA in polymerase chain reaction (PCR). This enzyme found in *Thermus acquaticus* is active at 80°C and pH 8 (Chien, 1976).

1.3.5 Previous work on Lake Magadi

Hypersaline soda lakes are populated by alkaliphilic representatives of halophilic archaea, the red-pigmented microbes. A novel haloalkaliphilic archaeon isolated from Lake Magadi has cells containing large gas vacuoles in the stationary phase of growth, and colonies that are bright pink. The G+C content of DNA is 62.7 mol%. The name *Natronobacterium vacuolata* sp. nov. was proposed. The type strain was designated NCIMB 13189. A coccoid, gram positive archeobacterial isolate was isolated from the less saline parts of the lake. This isolate resembled the genus *Natronococcus.* None of the gram positive isolates from the Kenyan soda lakes resembles the only validly named

alkaliphile, *Bacillus alkaliphilus* or *Bacillus alkaliphilus* subsp *halodurans* (Mwatha, 1991).

Kamekura *et al.*, (1997) studied the diversity of alkaliphilic halobacteria on the basis of phylogenetic tree constructions, and sequences of spacer regions between 16S and 23S rRNA genes. They proposed the following changes: *Natronobacterium pharaonis* to be transferred to *Natronomonas gen.* nov. as *Natronomonas pharaonis* gen. comb. nov.; *Natronobacterium vacuolatum* to be transferred to the genus *Halorubrum* as *Halorubrum vacuolatum* gen.comb. nov.; and *Natronobacterium magadii* to be transferred to the genus *Natrialba* as *Natrialba magadii.*

Kevbrin *et al.*, (1997) isolated an alkaliphilic, obligately anaerobic, fermentative, asporogenous bacterium with a gram-positive cell wall structure from soda deposits in Lake Magadi. 16S rRNA gene sequence analysis of this bacterium showed that it belongs phylogenetically to the low G+C gram-positive bacteria. On the basis of its distinct phylogenetic position and unique physiological properties, they proposed a new genus and new species, *Tindallia magadii*, for this strain.

A novel osmolyte, 2-sulfotrehalose, was discovered in several *Natronobacterium* species of haloalkaliphilic archaea. The concentration of this novel disaccharide increased with increasing concentrations of external NaCl, behavior consistent with its identity as an osmolyte. Other common osmolytes (glycine betaine, glutamate, and proline) were not accumulated or used for osmotic balance in place of the sulfotrehalose by these halophilic archaea. (Jones *et al.*, 1998)

Zhilina and Zavarzin (1994) described bacterial communities in alkaline lakes, and in particular the diversity of anaerobic bacteria developing at pH 10. A new obligate

alkaliphilic, *Methylotrophic methanogen* was isolated from Lake Magadi. According to its phenotypic and genotypic properties, the isolate belonged to *Methanosalsus (Methanohalophilus) zhilinaeae.* The strain did not require Cl^- but was obligately dependent on Na^+ and HCO_3^-. It was an obligate alkaliphile and grew within a pH range of 8 to 10.

Zhilina *et al.*, (1996) isolated and characterized *Desulfonatronovibrio hydyogenovorans* gen. nov., sp. nov., an alkaliphilic, sulfate-reducing bacterium, from Lake Magadi. Strain Z-7935(T) is an obligatory sodium-dependent alkaliphile, which grows in sodium carbonate medium and does not grow at pH 7; the maximum pH for growth is more than pH 10, and the optimum pH is 9.5 to 9.7. The optimum NaCl concentration for growth is only 3% (w/v). Several alkaliphilic spirochaetes were also isolated from Lake Magadi. The α-amylase-producing haloalkaliphilic archaeon *Natronococcus sp.* strain Ah-36^T (T = type strain) was isolated from Lake Magadi. Most cells of strain Ah-36^T occurred in irregular clusters, and the colonies were orange-red. The complete nucleotide sequence of the 16S rRNA gene revealed that the closest relative of strain Ah-36^T is *N. occultus* ATCC43101^T (level of similarity, 96.4%), an extremely halophilic archaeon. However, strain Ah-36^T did not exhibit a significant level of DNA homology to *N. occultus* ATCC43101^T, which represents the only previously described species in the genus *Natronococcus.* The name *Natronococcus amylolyticus* was proposed (Haruhiko, 1995). On the physicochemical aspect, Lake Magadi has some seasonal rivers draining into it but no outlet. Apart from the seasonal rivers the lake is supplied with water by the hot springs found along the shoreline. The closed drainage basin is exposed to high rates of evaporation as the temperature during the daytime can go to over 40°C. The molar

concentration of HCO_3^- and CO_3^{2-} ions greatly exceeds that of Ca^{2+} and Mg^{2+} ions. As a result of the high rate of evaporation, saturation of the alkaline earth ions is rapidly achieved and they precipitate out as insoluble carbonates. This leaves Na^+, Cl^-, HCO_3^- and CO_3^{2-} as the major ions in solution leading to hyper saline and alkaline conditions (Grant and Jones, 2000).

The hot springs share a common source of heat within the Earth's crust. The geothermal water can dissolve and carry large amounts of chemicals from the rocks through which it percolates. This creates great disparities in the spring water chemistry. In this way springs have far reaching implications on the hydrochemistry of the lake water (Mwaura, 1999).

1.4 Study Justification

Many extremophiles including those at Lake Magadi are yet to be classified. Conventional classification takes into account various features of morphology, nutrition, pigment, sensitivity to antibiotics, habitat requirements, pathogenicity, symbiosis and immunological characteristics. Molecular approaches, especially DNA base composition, nucleic acid hybridization and sequencing of rRNA gene can also be used. Interest in soda lake microbiology has centered primarily on the isolation and characterization of individual microorganisms with potential industrial applications. Little attention has been paid to the microbial community as a whole. There is a need to understand the biodiversity of the lake for purposes of mining the diverse resources of extremophiles. Industrial microbiologists could use the microorganisms to produce organic chemicals, antibiotics and other pharmaceuticals and supplements. *Dunaliella salina*, a green alga found in soda lakes, is cultivated for beta-carotene a widely used antioxidant and food coloring agent. This is an important industry, and Australia produces 95% of the world's supply of beta-carotene.

Protease enzyme produced by alkaliphiles is used in the detergent industry. Soda lake microorganisms can also be applied in insect and pest control, recovery of metals, and improvement and maintenance of environmental quality (Dubey and Maheshwari, 2004). Extremophiles are useful in construction of phylogenies and discovery of new species. Thus, an understanding of their basic biology may be an opportunity for biologists to look backward in time to a period of early life on Earth. Microbiologists therefore no longer have to propose bacterial phylogenies based on speculation of what type of microbe likely preceded another. The phylogenies themselves are etched in the sequences of molecules and all that has to be done is to read them. Thus, continued and expanded research into the diversity of microbial life in all environments, extreme and otherwise and an understanding of their basic and applied biology is important (Amann *et al.*, 1995). Lake Magadi is a highly productive ecosystem with a natural biodiversity that should be explored, protected and conserved.

1.5 Objectives

Main objective

To describe the diversity of extremophilic bacteria in Lake Magadi and relate it to the prevailing physicochemical conditions.

Specific objectives

1. To determine the physicochemical properties of water from Lake Magadi.
2. To isolate and characterize the microorganisms by microscopy and biochemical tests.
3. To identify and classify some of the microorganisms by nucleic acid analysis.

Chapter Two

Materials and Methods

2.1 Water sampling methods

Samples of water were collected from eight sampling sites which consisted of seven hotsprings on the shoreline of the lake and one sample from along the Western Causeway. Sampling was done in the month of January; 2009. Three samples of water were collected from each site and placed in separate, sterile and labeled bottles. These were carried in a cool box and then kept in the refrigerator at about 4°C prior to analysis. This helps in preservation as it brings down the metabolic rate.

2.2 Determination of physicochemical conditions in the lake

Measurement of physicochemical conditions was done using a water multiparameter Meter (HANNAS HI 769828 model). The instrument measured simultaneously the pH, oxidation-reduction potential (ORP), dissolved oxygen, electrical conductivity, total dissolved solutes (TDS) and salinity. Hydrogen peroxide content was measured using test paper strips (Insta Test Strips model 2984LR), nitrate and nitrite content was measured using test paper strips (Nitrate/Nitrite Test Paper strips model LH-S15). Temperature was taken using a laser beam thermometer and a Global Positioning System (GPS) instrument was used to record the position of the site.

2.3 Bacterial isolation and characterization

The microbial diversity was described in terms of the different microbial isolates that were cultured and identified. Colony characteristics and bacterial morphology and physiology were used in characterisation of the bacterial isolates.

2.3.1 Types of Selective enrichment media used for isolation.

1. Medium for haloalkaliphiles (Designed by Prof Duboise , personal communication)

Reagent	Amount (g/l)
0.5M Na_2CO_3	53.0
0.5M $NaHCO_3$	42.0
TSB	1.5
0.5M NaCl (Sea salt)	29.5
Agar	15.0

2. Haloalkaliphilic medium of Tindall 1980 (Tindall *et al.*, 1984)

Reagent	Amount (g/l)
Yeast extract	10.0
Casamino acids	7.5
$KHPO_4$	1.0
$MgSO_4.7H_2O$	0.2
NaCl	200.0
Na_2CO_3	18.5
Agar	20.0
$FeSO_4.7H_2O$ 50g/l soln	1ml

3. Horikoshi medium (Horikoshi, 1971 b)

Reagent	Amount (g/l)
Glucose	10.0
Peptone	5.0
Yeast extract	5.0
KH_2PO_4	1.0
$MgSO_4.7H_2O$	0.2
NaCl	40.0
Na_2CO_3	18.0
Agar	20.0

For each of the three media, salts were sterilized in an autoclave at 121 °C for 20 min separately then mixed with the rest of the sterilized ingredients at 60 °C just before pouring into the petri dish.

2.3.2 Enrichment and isolation

An enrichment culture technique was employed to build up the population of the microbes in the water samples where broth medium was mixed with each water sample in the ratio of 1: 1 and incubated at 37°C for 12-48 hrs. Enrichment broth cultures were stored at around 4° C for later use. The microbial suspension obtained with the enrichment culture technique was cultured by the pour plate method (Brown, 2005). Exactly 1 ml of the broth culture was spread onto a sterile Petri dish and melted agar media for haloalkaliphiles (after cooling) poured into the culture dish. The plates were incubated at 37°C until well-formed colonies appeared (24-48 hr). This was repeated with broth cultures from all the eight sites using the Horikoshi medium and the Tindall medium as well. The cells from a discrete colony were picked and sub cultured by streaking method in order to obtain pure colonies. The various colonies were distinguished and described by means of traits such as configuration, size, elevation, colour, margin and opacity. The pure colonies were transferred onto agar slants and stored at around 4° C for preservation and later use.

2.3.3 Characterization of isolates

Gram Stain reaction

A thin film of each isolate was smeared and spread over the surface of the slide. The slide was heated gently over a flame in order to fix the material. The entire slide was flooded with crystal violet for about 60 sec then washed for 5 sec with water. The slide was then flooded with the iodine solution for about a minute and rinsed with water for 5 sec. Ethanol was added drop wise until the blue-violet color was no longer eluted from the specimen then rinsed with water for 5 sec. The counterstain, safranin was

added, allowed to stand for about a minute then rinsed with water for 5 sec to remove excess dye (Brown, 2005). Gram positive cells remained blue-violet in appearance. Gram negative bacteria, however, took on the pink color of the counter stain. The slide was allowed to dry before viewing under the microscope.

Motility test

Motility medium, a semisolid medium that barely gels at room temperature (containing 7 g/l of agar) (Brown, 2005), was inoculated with the bacterial isolates. This was done using an inoculating needle, stabbing the media in a straight line and then incubating at 37°C for 12-24 hr. Migration away from the original line of inoculation meant that the test organism was motile (positive test). Lack of migration away from the line of inoculation indicated a lack of motility (negative test result).

Biochemical tests

The physiology of the isolates was determined by means of biochemical tests. Each test was done using the three media in order to select the most suitable one. Once the media was selected, each test was replicated three times for comparison or obtaining an average where necessary.

Salt concentration range for optimal growth

The medium for haloalkaliphiles was made with different NaCl concentrations of 0 % w/v 3 % w/v, 6% w/v, 9% w/v and 15 %w/v and poured onto plates. An inoculation loop was dipped into stock broth cultures of the isolates and streaked onto the plates then incubated for 24 hr at 37°C. Enhanced growth (when compared to growth in 3 % w/v) was recorded as positive and no enhancement as negative (Mwatha, 1991).

Temperature range for optimal growth

Each isolate was cultured in 10 ml of broth medium of haloalkaliphiles at 37°C for 24 hr. Exactly 1 ml of each of the bacterial isolates broth culture was added to exactly 6 ml of freshly prepared nutrient broth and incubated at 30° C for 24 hrs. The procedure was repeated at 35°C, 40°C, 45°C and 50°C. The optical density of each broth culture was measured using a spectrophotometer (Bausch and Lomb Spectronic 20 model) at wavelength of 686 nm (Brown, 2005).

pH range for optimal growth

Using concentrated hydrochloric acid and universal paper strips to estimate pH, 200 ml of haloalkaliphilic broth media was neutralized by titration to adjust the pH from pH 11 to pH 9. Another 200ml had the pH adjusted to pH 7. Exactly 6 ml of each media with different pH values of 11, 9 and 7 was filter sterilized by means of a 0.45 µm pore size filter, poured into sterile tubes and inoculated with each isolate. These were incubated at 37°C for 24 hr and their optical densities measured at wavelength of 686 nm (Brown, 2005).

Utilization of different carbon sources.

The medium used (Mwatha, 1991) had the following composition; Yeast 1.0 g/l; KH_2PO_4 1.0 g/l , $MgSO_4.7H_2O$ 0.2 g/l ; KNO_3 1.0g/l NaCl 40.0g/l; Na_2CO_3 18.0g/l; Agar 2.0g/l . Salts were sterilized separately then mixed with the rest of the sterilized ingredients at 60°C. Each carbohydrate solution was filter sterilized and added to the basal minimum medium to a final concentration of 0.5% w/v just before pouring. The carbohydrates used were glucose, galactose and mannose. The plates were inoculated with each of the isolates and incubated at 37°C for 24hr. Growth was compared to growth

on a plate containing basal minimum medium. Enhanced growth was recorded as positive and no enhancement as negative. A few drops of methyl red were added to check for development of acidity.

Starch hydrolysis

Starch agar was prepared by adding 100g/l starch to the media for haloalkliphiles and poured onto plates. Bacterial isolates were spread onto the plate surface using an inoculating loop and incubated at 37°C for 24 hr. A few drops of Gram's iodine were then added to the plate and the color change observed (Brown, 2005). A reddish color or a clear zone around the bacterial growth meant that starch had been hydrolyzed, a black/blue colour indicated lack of hydrolysis.

Casein hydrolysis

A solution of skim milk powder of 10 % w/v concentration was sterilized by placing in the autoclave for 20 min. After cooling to 55°C (Brown, 2005) it was added to sterile medium for haloalkaliphiles whose salts were sterilized separately then mixed with the rest of the sterilized ingredients at 60°C. The white opaque milk salt agar plates were streaked with the bacterial isolates and incubated at 37°C for 24 hr. Clear zones around the colonies indicated casein hydrolysis.

Indole production

This test was done to determine if bacteria can breakdown the amino acid tryptophan into indole. Tryptic Soy Broth (TSB) media was inoculated using a transfer needle. After incubating the bacteria for at least 48 hrs, Kovac's reagent was added to the media to

detect if indole had been produced by the bacterial isolates. The development of a red/pink layer on top of the media was a positive result (the bacteria could breakdown tryptophan to form indole). Failure to see a red layer was a negative result (indole was not formed from tryptophan). A test on *E. coli* was used as a positive test control (Brown, 2005).

Catalase test

A small amount of each isolate was smeared onto a clean glass slide using an inoculation loop. A drop of 70 % hydrogen peroxide was then added to the smear. Visible bubbles of O_2 bubbling up indicated a positive result meaning that the organism was catalase positive, a lack of bubbles indicated catalase negative (Brown, 2005).

2.4 Nucleic acid analysis (16S rRNA gene sequencing)

Due to the highly conserved nature of the translational material of cells, the 16S rDNA that encodes for the small ribosomal subunits (the 16S rRNA), provide a nucleotide sequence that is highly useful as a molecular basis for phylogeny. The degree of similarity between the DNA sequences encoding the 16S rRNA of two organisms was used as a measure of the relatedness between the two organisms (Dubey and Maheshwari, 2004).

2.5.1 DNA Isolation

Total DNA of the microbial isolates was isolated using a DNEasy Blood and Tissue kit, from Qiagen Inc,(sourced from USA courtesy Prof Duboise) according to the manufacturer's instructions (http//www.qiagen.com), Bacterial cells were harvested in the early log phase of growth in liquid broth (about 24 hr after innoculation), placed in a

micro centrifuge tube and centrifuged for 10 min at 7500 rpm and the supernatant discarded. The pellet obtained was resuspended in 180µl Buffer ATL (Tissue lysis buffer). Exactly 20µl proteinase K was added and mixed thoroughly by vortexing and incubated at 56°C until the cells were completely lysed. RNase A was added to degrade RNA and the mixture was incubated for 2 min at room temperature. Vortexing was done for 5 seconds and 200µl Buffer AL added to sample and vortexed. About 200µl of ethanol (96-100%) was added and mixed by vortexing. The mixture was pipetted into the DNeasy mini spin column which was placed in a 2ml collection tube. Centrifugation was done at 8000 rpm for one minute. The flow through was discarded. The DNeasy mini spin column was placed in a new collection tube and 500µl AW 1 buffer (Wash buffer) added centrifuging for one minute at 8000 rpm. This step was repeated with 500µl buffer AW 2 at 14000 rpm for 3 min to dry the DNeasy membrane by removing residual ethanol. Flow through and collection tube were discarded. The mini column was placed in a clean, labeled micro centrifuge tube and 200µl of buffer AE (Elution buffer) added onto the membrane. It was incubated at room temperature for one minute and centrifuged for one minute at 8000 rpm to elute the DNA. The genomic DNA obtained was stored at -25°C.

2.5.2 Polymerase Chain Reaction (PCR)

Base sequences of the primers used for amplification were:

16S F 5'- AGA GTT TGA TCH TGG CTY AG - 3'

16S R 5'- ACG GNT ACC TTG TTA CGA CTT – 3'

UA 751 F 5' – CCG ACG GTG AGR GRY CAA – 3'

UA 1406 R 5' – ACG GGC GGT GWG TRC AA – 3'

The reaction mix contained the following components; 38.25 µl PCR water; 4µl DNA template diluted 1: 10 in PCR water; 5µl thermopol – 10 x buffers; 1.25µl dNTP mix; 0.5µl each of forward and reverse Universal Archaea or 16S primer diluted 1: 10 with PCR water; 0.5µl Taq polymerase.

The total volume of reaction mix in the PCR tubes was 50µl. A second set-up was done with higher concentration of magnesium ions. The PCR tubes were placed in a programmed thermocycler. The program consisted of the following steps; 95°C for 3 min, to thoroughly denature the DNA at the beginning of the reaction, 95°C for 30 sec to denature the DNA, 55°C for 30 sec to anneal the primers, 72°C for 1 min for extension of the PCR product, 30 cycles were performed with a final extension of 3 min at 72°C. The reaction was held at 4 °C and the product removed and stored under -25°C

PCR Clean up

To purify the PCR products a QIAquick Purification KIT from Qiagen Inc was used (http//wwwl. qiagen.com), Exactly 5 volumes of buffer PBI was added to 1 volume of PCR product and mixed well. The quick spin column was placed in a 2ml collection tube. The PCR product was pipetted into the column and centrifuged for 60 sec at 13000rpm. The flow through was discarded and column returned onto the same collection tube. About 0.75ml of buffer PE (Protein Extraction & Lysis buffer) was added and centrifuged for 60 sec at 13000rpm. The flow through was discarded and the mini column put in the same collecting tube and centrifuged for an additional 60 sec. This removes residual ethanol from buffer PE. The column was placed in a clean 1.5 ml micro centrifuge tube and about 50µl of buffer EB (Elution buffer) was added to the centre of the quick column membrane. It was centrifuged for 1 minute after standing in room temperature for 1 minute and about 50µl of eluate was collected.

2.5.3. Agarose gel electrophoresis

To make 1 Litre TAE , 242 g Tris –base, 57.1 ml glacial acetic acid, 18.6 g EDTA was mixed with 900 ml distilled water .

To make 1% agarose gel, 0.5 g agarose was dissolved in 50ml TAE. About 0.014% ethidium bromide was added. The agarose solution was heated in a microwave oven until fully dissolved. On cooling, it was poured into an electrophoresis cassette fitted with sample well forming combs. The cassette was placed on the tank and covered with TAE. Exactly 6µl of each PCR product was placed on a parafilm on the bench and about 1.5µl of loading dye (bromothynol blue) added to it and mixed well. Using a micropipette, each PCR product was loaded into a well in the agarose gel. A DNA molecular marker of 0.12-23.1 Kbp was loaded in the outermost well. The electrophoresis was left to run for about 20 min at 5V/cm keeping track of the tracking dye front. The gel was observed in a transilluminator and band images taken using a digital camera and the Doc-It LS Image Analysis Software.

2.5.4 Sequencing of the PCR Products

PCR product obtained was sequenced using the same primers used in the polymerase chain reaction. This was done in the laboratories of University of Maine, courtesy of Prof. S.M. Duboise. The raw sequence data obtained was edited using the BioEdit program before analysis with the FASTA database query program available on the World Wide Web (http://www.ncbi.nlm.nih.gov/). The sequences were compared to sequences available in the NCBI GenBank databases using the Basic Local Alignment Tool (BLAST). The rDNA sequences of the isolates together with sequences of their close relatives were aligned with each other using the multialin program (http; // www-archbac.u-psud. fr/genomics/multialin.html). The differences in the nucleotides were

converted into distance matrices using the Jukes and Cantor (1969) neighbor joining method. Construction of a phylogenetic tree was done using Kimura two-parameter calculation model (Van de Peer and De Wachter, 1994).

Chapter Three

Results

3.1 Physicochemical conditions in the sampled sites

Table 1 shows the physicochemical conditions of water from the eight sampling sites. Hydrogen peroxide content was measured using test paper strips (Insta Test Strips model 2984LR), nitrate and nitrite content were measured using test paper strips (Nitrate/Nitrite Test Paper strips model LH-S15). Temperature was taken using a laser beam thermometer and a Global Positioning System (GPS) instrument was used to record the position of the site. The rest of the parameters were measured using a water multiparameter Meter (HANNAS HI 769828 model). The water had alkaline saline conditions with a pH of 10-11 and salinity of up to 25.6 in site 1.The temperature of the water ranged from 30°C in site 6 to as high as 44°C in site 4. Nitrate, nitrite and dissolved oxygen content were highest in site 2 where presence of animal dung indicated animal interference. Total dissolved solutes were high in all the sites and were a reflection of the relatively high electrical conductivity. The oxidation - reduction potential values were negative showing a tendency for reduction in all the sites.

Table 1: Physicochemical conditions in the sampled sites 1-4

SITE	1	2	3	4	5	6	7	8
G.P.S	1°59.30S 36°15.877E	1°59.320S 36°15.89E	01°59.54S 36°15.89E	01°57.24S 36°13.88E	1°55.880S 36°13.880E	1°51.026S 36°13.030E	1°51.392S 36°14.200E	1°51.800S 36°14.552E
Elevation	606 m	606 m	603 m	625 m	601	601	605	605
Temp(°C)	37	34	42	47	44	30	43	34
pH	11	10	10.3	10.5	10.2	10.5	10	10
H_2O_2 (ppm)	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
NO_3 (ppm)	0	1	0	0	0 ppm	0	0	0
NO_2 (ppm)	0	0.2	0	0	0 ppm	0	0	0
ORP (mV)	-47.7	-80.9	-29.1	-43.7	-40.8	-40.5	-41.2	-43.6
DO%	30.5	51.1	22.7	25.7	24.5	24.2	25.5	24.6
DO (mg/l)	1.9	2.9	1.1	1.6	1.6	1.3	1.6	1.7
EC (mS/cm)	37.7	20.3	39.8	14.1	33.0	34.2	36.3	36.9
TDS (mg/l)	20.4	10.0	21.5	9.6	16.5	18.2	18.5	18.6
Salinity	25.6	11.7	24.2	7.9	20.5	23.5	18.2	20.3

Key:

Elevation----------in meters above sea level

G.P.S----------Geographic Positioning System

H_2O_2 (ppm) ------- Hydrogen peroxide in parts per million

NO_3 (ppm)----------Nitrate content in parts per million

NO_2 (ppm) ----------Nitrite content in parts per million

ORP (mV) ----------Oxidation-Reduction Potential in millivolts

DO%----------Dissolved Oxygen in percentage

DO (mg/l) ----------Dissolved oxygen in milligrams per liter

EC (mS/cm) -------Electrical conductivity in millisiemens per centimeter

TDS (mg/l) ----------Total dissolved solutes in milligrams per liter

The study site: Sampling site 1

The photograph, Plate 1a, shows the environs of Lake Magadi which have semiarid conditions with temperatures of about 40°C. These high temperatures cause a lot of evaporation leading to drying up of the lagoons created by the hotsprings. The lagoon seen in this photograph had very little water.

Plate 1a: The semi arid conditions seen in the background of Site 1

The study site: Sampling site 2

The photograph, Plate 1b, shows the observable green coloration in which is due to algal growth. This site had animal dung which may have provided nutrients for the algal growth.

Plate1b: Green coloration of lake water due to algal bloom in site 2

Study site: Non sampling site

The photograph, Plate1d, shows the red /pink coloration of the lake water an indication of presence of halophilic archaea which have the pigment bacteriorhodopsin used in photosynthesis hence primary productivity.

Plate 1c: Red /pink coloration of lake water due to presence of halophilic Archaea

3.2 Characterization of bacterial isolates

3.2.1 Colony characteristics of bacteria isolated from Lake Magadi

The isolates were cultured in the media for haloalkaliphiles at 37°C for 24 hr and colonies were distinguished by characteristics such as configuration and elevation where some colonies were hilly as a mound, raised above the surface of the media or flat on the surface. A total of 26 pure isolates were obtained and named as M1-1 where 'M' stands for Magadi, '1' for site 1 and '1' for colony number 1 or MC-5 where 'M' stands for Magadi, 'C' for Causeway and '5' for isolate number 5. The characteristics were summarized in Table 2 below.

Table 2: Colony characteristics

COLONY CODE	CONFIG URATION	MARGIN	ELEVAT ION	COLOR	OPACITY	SIZE OF COLONY
M1-1	round	smooth	raised	cream	opaque	Large
M1-2	round	smooth	flat	white	translucent	Small
M1-3,M2-1, M4-3,M5-2	round	smooth	hilly	white	opaque	Small
M2-2,MC-3	round	wavy	hilly	white	opaque	Small
M2-3	round	smooth	raised	white	opaque	Small
M3-1	round	wavy	flat	white	translucent	Large
M3-3,M4-1	round	wavy	raised	white	opaque	Large
M4-2	round	wavy	flat	cream	translucent	Large
M4-4,M5-1	round	smooth	flat	white	translucent	Large
M5-3	round	smooth	hilly	clear	translucent	Small
M6-2	round	smooth	hilly	brown	opaque	Small
M6-3	rhizoid	smooth	hilly	brown	opaque	Small
M6-4,MC-1	round	smooth	flat	brown	opaque	Small
M6-5	rhizoid	smooth	raised	brown	opaque	Small
M7-1	round	smooth	flat	white	opaque	Large
M7-3	round	smooth	flat	cream	opaque	Small
MC-2	round	smooth	raised	white	opaque	Large
MC-4	round	wavy	raised	cream	opaque	Large
MC-5	rhizoid	wavy	raised	cream	opaque	Large

Large colony >2mm diameter, Small colony ≤ 2mm diameter

3.2.2 Morphological characteristics of bacterial isolates from Lake Magadi

Out of twenty six isolates, two were spirillum shaped, two were coccobacilli, five were coccus and the rest were rods. The rods were of relatively small, medium or large size. Gram stained isolates were observed under high power (X 1000) magnification.

Only three of the isolates were Gram positive while the rest twenty three were Gram negative. Three of the isolates were observed to be motile. The characteristics were summarized in Table 3 below.

Table 3: Morphology of isolates

Colony code	Cell shape	Cell Arrangement	Relative size	Gramstain Reaction	Motility
M1-1,M4-2	Rod	Single	Small	-	-
M1-2,M2-1,M4-4	Rod	Single	Small	-	-
M1-3,M5-2	Spirillum	Single	Small	+	-
M2-2,M4-1	Rod	Single	Medium	-	-
M2-3,M5-3	Rod	Single	Small	-	+
M3-1,M3-3, M6-2,MC-2	Coccus	Single	Small	-	-
M4-3,M6-4,MC-1	Rod	Single	Large	-	-
M5-1	Coccobacillius	Paired	Small	-	-
M6-3	Rod	Single	Large	-	+
M6-5	Rod	Single	Large	+	-
M7-1	Rod	Single	Medium	-	+
M7-3	Coccobacillus	Single	Small	-	-
MC-3	Coccus	Single	Small	-	-
MC-4	Rod	Single	Large	-	-
MC-5	Rod	Paired	Large	-	-

Gram stain reaction;
(+) refers to Gram positive bacteria: (-) refers to Gram negative bacteria
Motility;
(+) refers to motile bacteria: (-) refers to non-motile bacteria

Gram stains at x1000 magnification

Photographs of pure bacterial isolates cultured in the media for haloalkaliphiles, Gram stained and observed under high power (x1000 magnification).

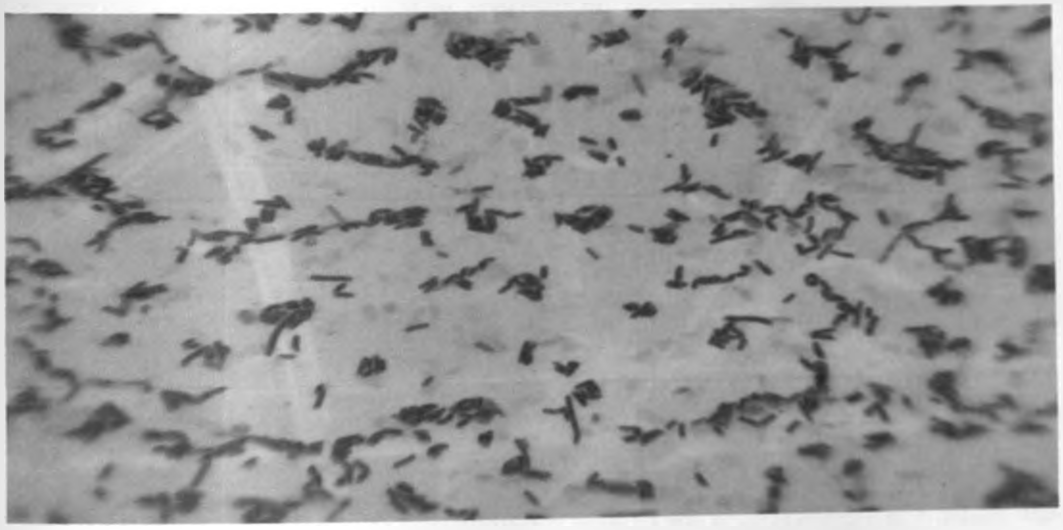


Plate2a: Large rods

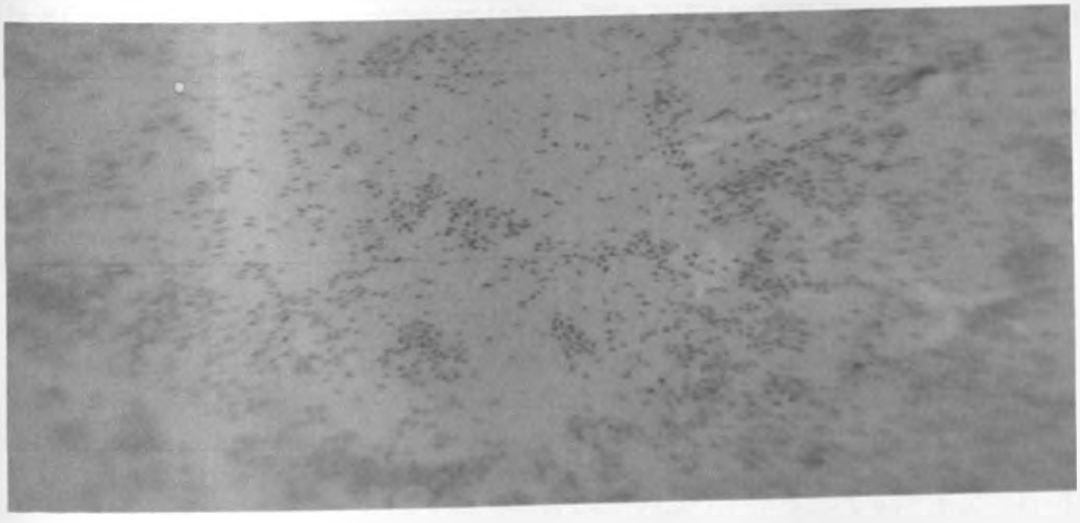


Plate2b: Coccus

3.2.3 Salt Concentration range for growth of bacterial isolates from Lake Magadi

Growth was compared to growth on a plate containing media with 3 % w/v NaCl concentration

Enhanced growth in 0%-9% w/v NaCl indicated halotolerance; enhanced growth in 3% w/v NaCl indicated slightly halophilic; enhanced growth in 6-9% w/v NaCl indicated moderately halophilic; enhanced growth in 15% w/v NaCl would have indicated extremely halophilic bacteria.

Table 4: Optimal Salt concentration ranges for growth of isolates

Isolate	0% w/v	6% w/v	9% w/v	15% w/v	Description
M1-1,M2-2,M4-4,M6-5,M7-3	+	+	+	-	Halotolerant
M1-2,M2-1,M2-3,M3-1,M4-2	-	-	-	-	Slight halophile
M1-3,M3-3,M4-1,M5-3,M6-2,MC-1,MC-5	-	+	+	-	Moderate halophile
M4-3,M5-1,M5-2,M6-3,MC-2,MC-4	-	+	-	-	Moderate halophile
M6-4,M7-1,MC-3	+	+	-	-	Halotolerant

Key

(+) refers to enhanced growth; (-) refers to lack of enhancement of growth

M1-1 to MC-5 refers to bacterial isolates

3.2.4 Effect of incubation temperature on growth of the bacteria isolated from Lake Magadi

Isolates were cultured in 10 ml broth media for halloalkaliphiles and incubated at different temperatures of 30°C, 35°C, 40°C, 45°C and 50°C for 24 hrs. Exactly 1ml of stock broth culture was used as inoculum. Optical densities were measured using a spectrophotometer at a wavelength of 686 nm (Brown 2005). The data obtained (see Appendix I) was used to draw graphs of optical density (OD) against temperature (°C).

Graphs of Optical density against Temperature °C

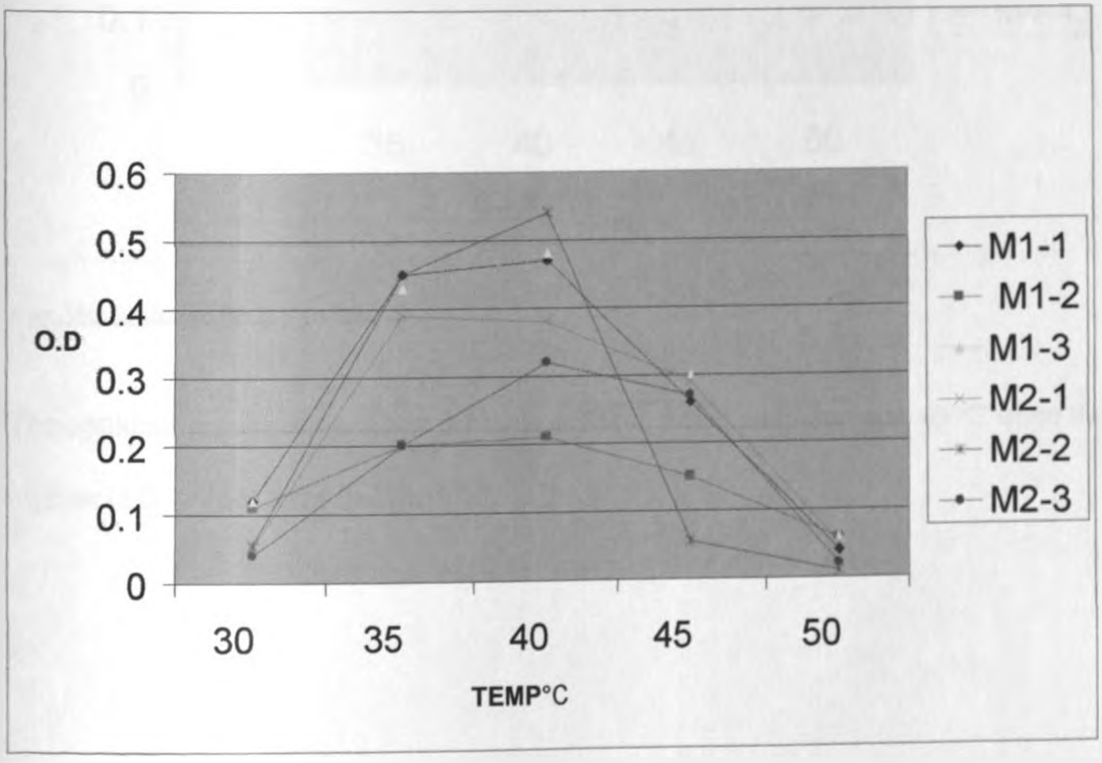


Fig. 3a: Isolates M1-1 to M2-3

For most of the isolates the optimum growth temperature was 40 °C when the highest O.D. was recorded. The optimum growth temperature for isolate M2-1 was 35 °C.

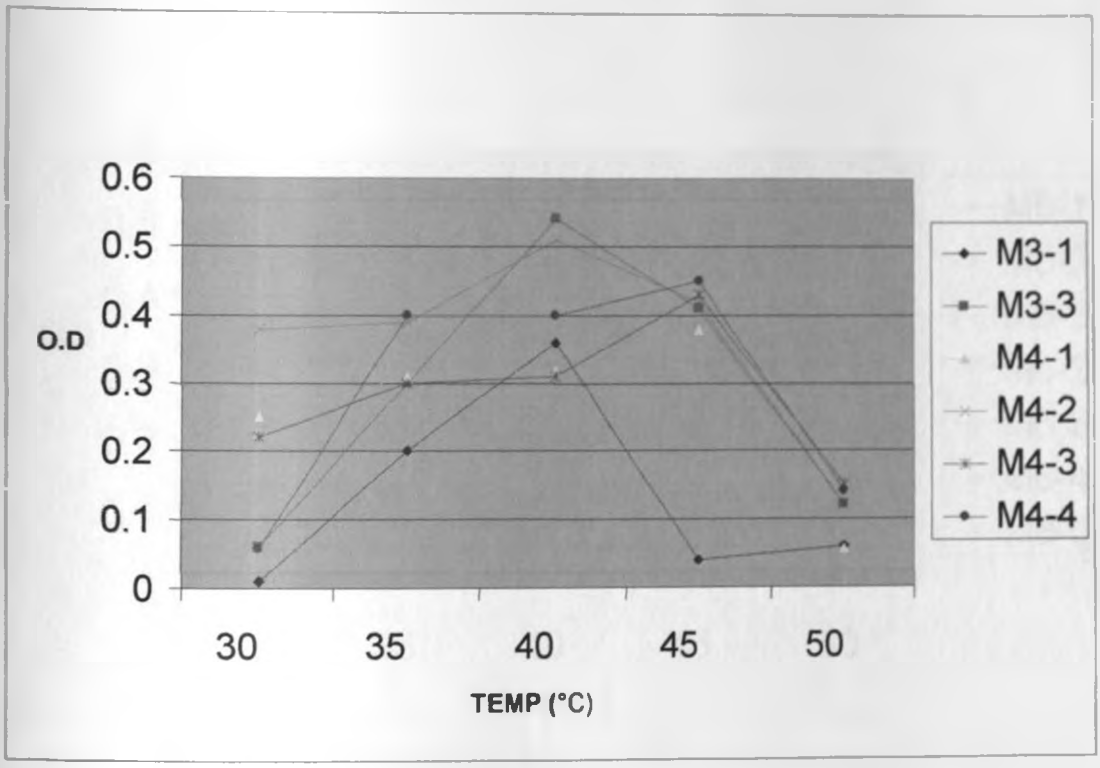


Fig.3b: Isolates M3-1 to M4-4

The optimum growth temperature for isolates M4-1; M4-3 and M4-4 was 45 °C when the highest O.D. values were recorded.

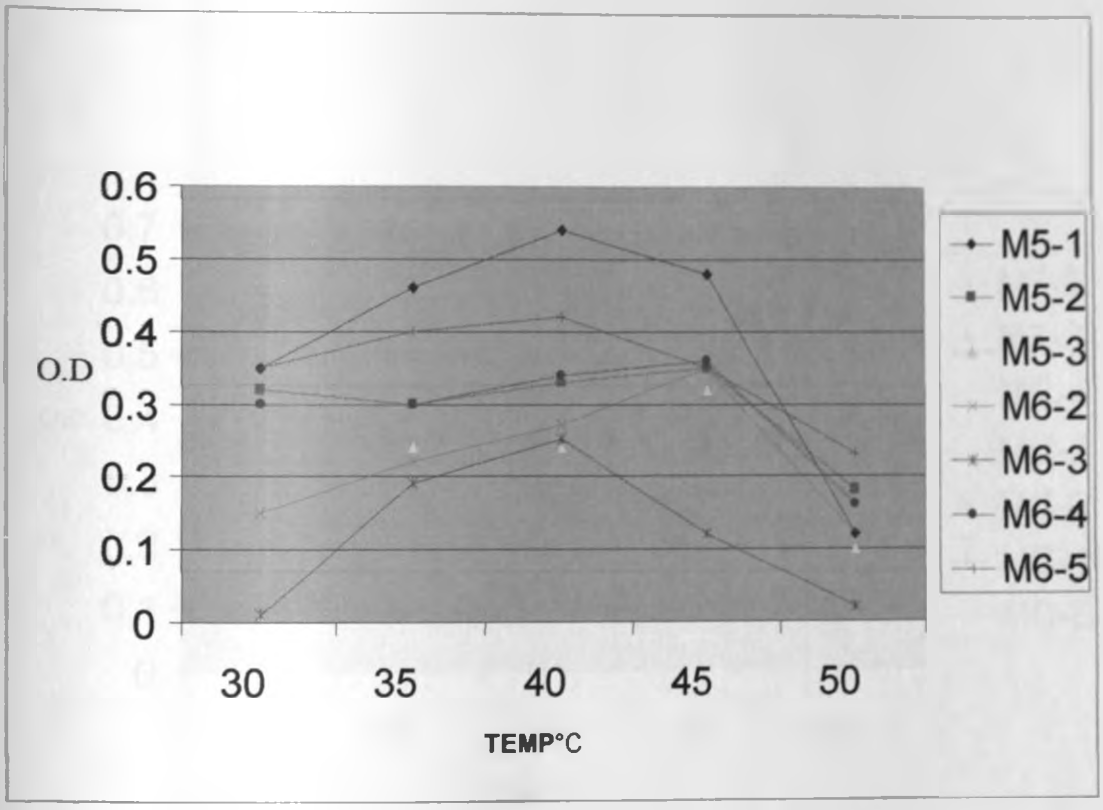


Fig.3c: Isolates M5-1 to M6-5

Optimum growth temperature for most isolates was 40 °C. Isolates M6-4, M5-2, M6-2 and M5-3 had an optimum growth temperature of 45°C.

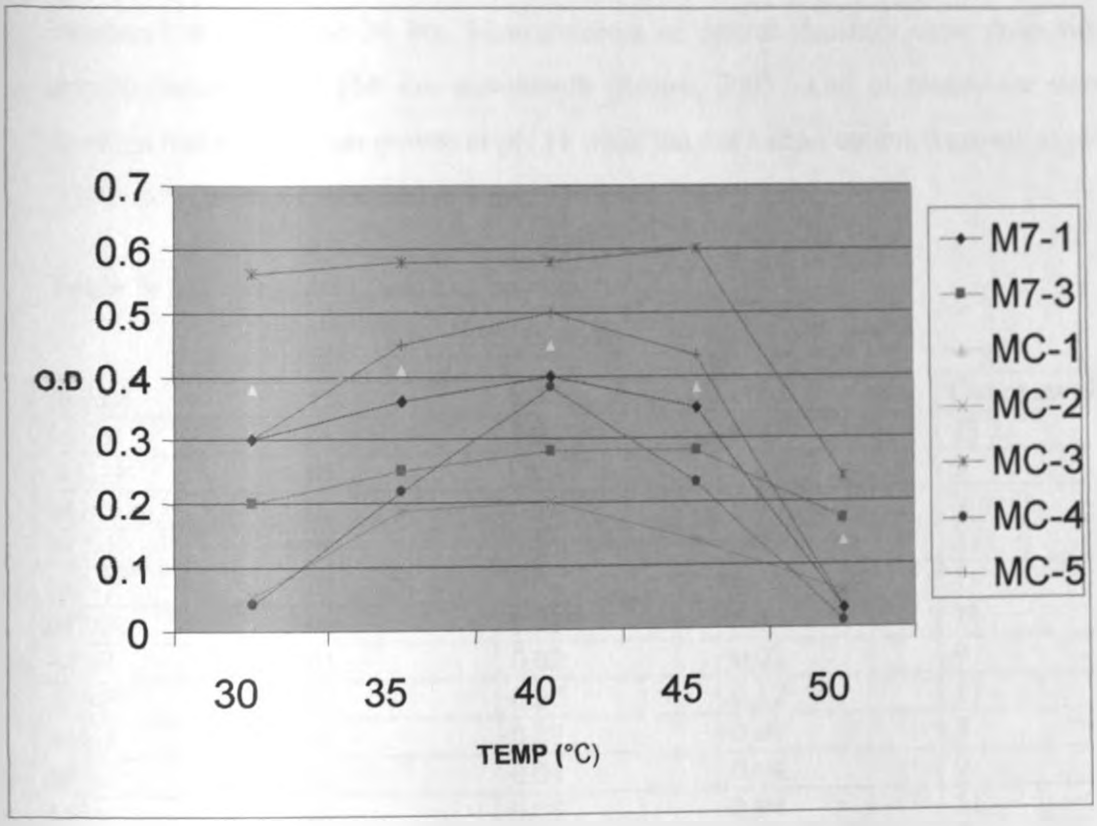


Fig.3d: Isolates M7-1 to MC-5

Optimum growth temperature for most isolates was 40 °C. Isolate MC-3 and M7-3 had optimum growth temperature of 45 °C.

3.2.5 Optimal pH ranges for growth of bacteria isolated from Lake Magadi

Optimum pH for growth of isolates was determined by measurement of optical densities of broth cultures (in the medium for haloalkaliphiles) with pH values of 7, 9 and 11 incubated at 37°C for 24 hrs. Measurements of optical densities were done with a spectrophotometer at 686 nm wavelength (Brown, 2005). Out of twenty six isolates fourteen had an optimum growth at pH 11 while the rest had an optimum growth at pH 9. The results were summarized in Table 5 below.

Table 5: pH range for growth of isolates

Isolate	pH 7	pH 9	pH 11	Optimum pH
M1-1	0.01	0.01	0.03	11
M1-2	0.01	0.04	0.04	9
M1-3	0.02	0.14	0.09	9
M2-1	0.05	0.01	0.02	11
M2-2	0.03	0.04	0.06	11
M2-3	0.01	0.01	0.03	11
M3-1	0.01	0.02	0.02	9
M3-3	0.03	0.07	0.12	11
M4-1	0.02	0.07	0.07	9
M4-2	0.01	0.04	0.04	9
M4-3	0.03	0.05	0.04	11
M4-4	0 01	0.09	0.05	9
M5-1	0 05	0.09	0.15	11
M5-2	0.01	0.15	0.13	9
M5-3	0.02	0.06	0.05	9
M6-2	0.04	0.14	0.15	11
M6-3	0.02	0.03	0.06	11
M6-4	0.05	0.07	0.13	11
M6-5	0.02	0.05	0.12	11
M7-1	0.03	0.07	0.11	11
M7-3	0.01	0.06	0.06	9
MC-1	0.02	0.08	0.07	9
MC-2	0.02	0.09	0.11	11
MC-3	0.01	0.05	0.07	11
MC-4	0.03	0.12	0.10	9
MC-5	0.03	0.09	0.08	9

Key:

M1-1 to MC-5 refers to the bacterial isolates

3.2.6. Utilisation of carbohydrates by bacterial isolates from Lake Magadi

Bacterial isolates were cultured on media containing glucose, galactose or mannose as the only carbon source and incubated at 37°C for 24 hrs. Growth was compared to growth on a plate containing medium for haloalkaliphiles with tryptic soy broth as the carbon source. Most of the isolates had enhanced growth in glucose but few had enhancement in galactose and mannose. Isolates M3-1 and M3-3 had no growth in galactose and mannose while M5-2 had no growth in mannose. The results were summarized in Table 6 below.

Table 6: Carbohydrate utilization by bacterial isolates

ISOLATE	GLUCOSE	GALACTOSE	MANNOSE
M1-1,M1-2,M1-3,M2-1,M2-2,M5-1	-	-	-
M3-1,M3-3	-	o	o
M4-1,M4-2,M6-2,M6-3,M6-4,M6-5,MC-3,MC-4, M2-3,M4-3	+	-	-
M4-4,M7-1,M7-3	+	+	+
M5-2	+	-	o
M5-3,MC-5	+	+	-
MC-1,MC-2	+	-	+

(o) no growth, (-) no enhancement of growth, (+) Enhanced growth

3.2.7 Metabolic characteristics of bacteria isolated from Lake Magadi

To determine hydrolysis of casein, isolates were cultured in milk salt agar and incubated at 37°C for 24hrs. Clear zones in the milk agar showed casein hydrolysis. To check for starch hydrolysis isolates were cultured on starch agar plates and incubated at 37°C for 24 hrs. Iodine was then added onto the plates and clear zones that did not turn blue black colour indicated starch hydrolysis. Catalase production was detected by adding drops of hydrogen peroxide onto isolates cultured in the media for haloalkaliphiles. Formation of gas bubbles indicated catalase production. To determine indole production isolates were grown in media for haloalkaliphiles culture broth and incubated at 37°C for 24hr. Kovac's reagent was then added and development of a red/pink layer on top of the media indicated indole production. Out of twenty six isolates, nine were able to hydrolyze casein, twelve hydrolyzed starch, seventeen were catalase positive and all of them were indole negative. The results were summarized in Table 7 below.

Table 7: Casein and Starch hydrolysis, Catalase and Indole production by bacterial isolates

ISOLATE	RESULT
M1-1, M1-3, M2-1, M2-2, M3-3, M5-2, M7-3, MC-2, MC-3.	Casein hydrolyzing isolates
M3-1, M3-3, M4-1, M4-2, M4-3, M4-4, M5-1, M6-4, M6-5, M7-3, MC-3, MC-5	Starch hydrolyzing isolates
M1-3,M3-3,M4-4,M6-3,MC-2	Catalase negative isolates
All isolates	Indole negative isolates

Key:

M1-1 to MC-5 refers to bacterial isolates

Casein hydrolysis

Bacterial isolates were cultured on milk salt agar plates and incubated at 37°C for 24 hrs. The clear zones (see X) correspond to areas of casein hydrolysis by the isolates.

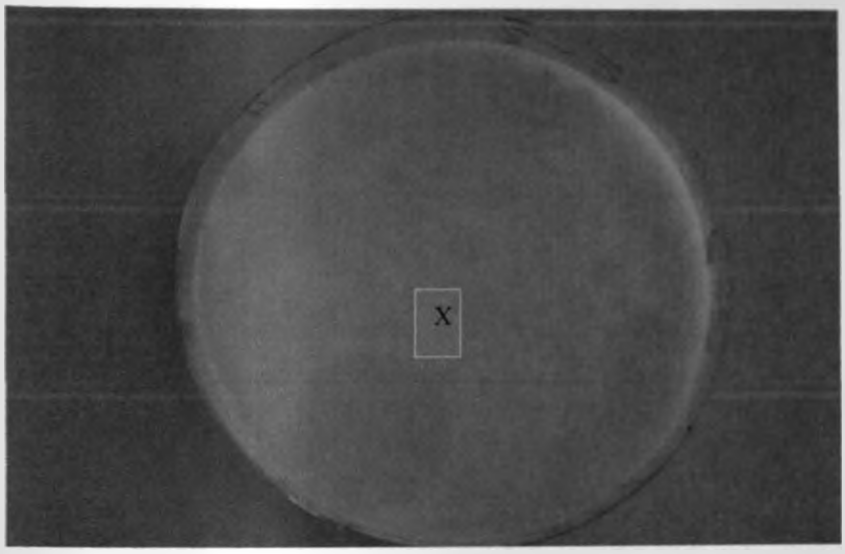


Plate 3: Photograph of Casein hydrolysis by bacterial isolates.

Starch hydrolysis

Bacterial isolates were cultured on starch agar plates and incubated at 37°C for 24 hrs. Iodine was then added onto the plates to test for presence of starch. The clear zones (see X) are areas of starch hydrolysis by isolates.

Plate4: Photograph of Starch hydrolysis by bacterial isolates.

Table 8: Summary of morphological and biochemical characteristics of isolates from Lake Magadi cultured in media for haloalkaliphiles and incubated at 37°C for 24 hrs.

Isolate	shape	G.S.	M	S.O.	T.O	P.O	S.H.	C.H	I.P.	C.P
M1-1	rod	-	-	H	ST	11	-	+	-	+
M1-2	Rod	-	-	SH	ST	9	-	-	-	+
M1-3	spirilla	+	-	MH	ST	9	-	+	-	-
M2-1	Rod	-	-	SH	ST	11	-	+	-	+
M2-2	Rod	-	-	H	ST	11	-	+	-	+
M2-3	rod	-	+	SH	MT	11	-	-	-	+
M3-1	Coccus	-	-	SH	ST	9	+	-	-	+
M3-3	Coccus	-	-	MH	ST	11	+	+	-	-
M4-1	Rod	-	-	MH	MT	9	+	-	-	+
M4-2	Rod	+	-	SH	ST	9	+	-	-	+
M4-3	Rod	-	-	MH	MT	11	+	-	-	+
M4-4	Rod	-	-	H	MT	9	+	-	-	-
M5-1	Coccobacilli	-	-	MH	ST	11	+	-	-	+
M5-2	Spirillum	+	-	MH	MT	9	-	+	-	+
M5-3	Rod	-	+	MH	ST	9	-	-	-	+
M6-2	Coccus	-	-	MH	ST	11	-	-	-	+
M6-3	Rod	-	+	MH	ST	11	-	-	-	-
M6-4	Rod	-	-	H	MT	11	+	-	-	+
M6-5	Rod	+	-	H	MT	11	+	-	-	+
M7-1	Rod	-	+	H	ST	11	-	-	-	+
M7-3	Coccobacilli	-	-	H	MT	9	+	+	-	+
MC-1	Rod	-	-	MH	ST	9	-	-	-	+
MC-2	Coccus	-	-	MH	ST	11	-	+	-	-
MC-3	Coccus	-	+	MH	MT	11	+	-	-	+
MC-4	Rod	-	-	MH	ST	9	-	+	-	+
MC-5	Rod	-	-	MH	ST	9	+	-	-	+

KEY

G.S.- Gram stain; M-Motility; S.O - Salt Concentration optima; T.O-Temperature optima; P.O- pH optima S.H-Starch hydrolysis; C.H-Casein hydrolysis; I.P-Indole production; C.P-Catalase production

H - Halotolerant, SH - Slightly halophilic, MH - Moderately halophilic, ST - Slightly thermophilic, MT - Moderately thermophilic

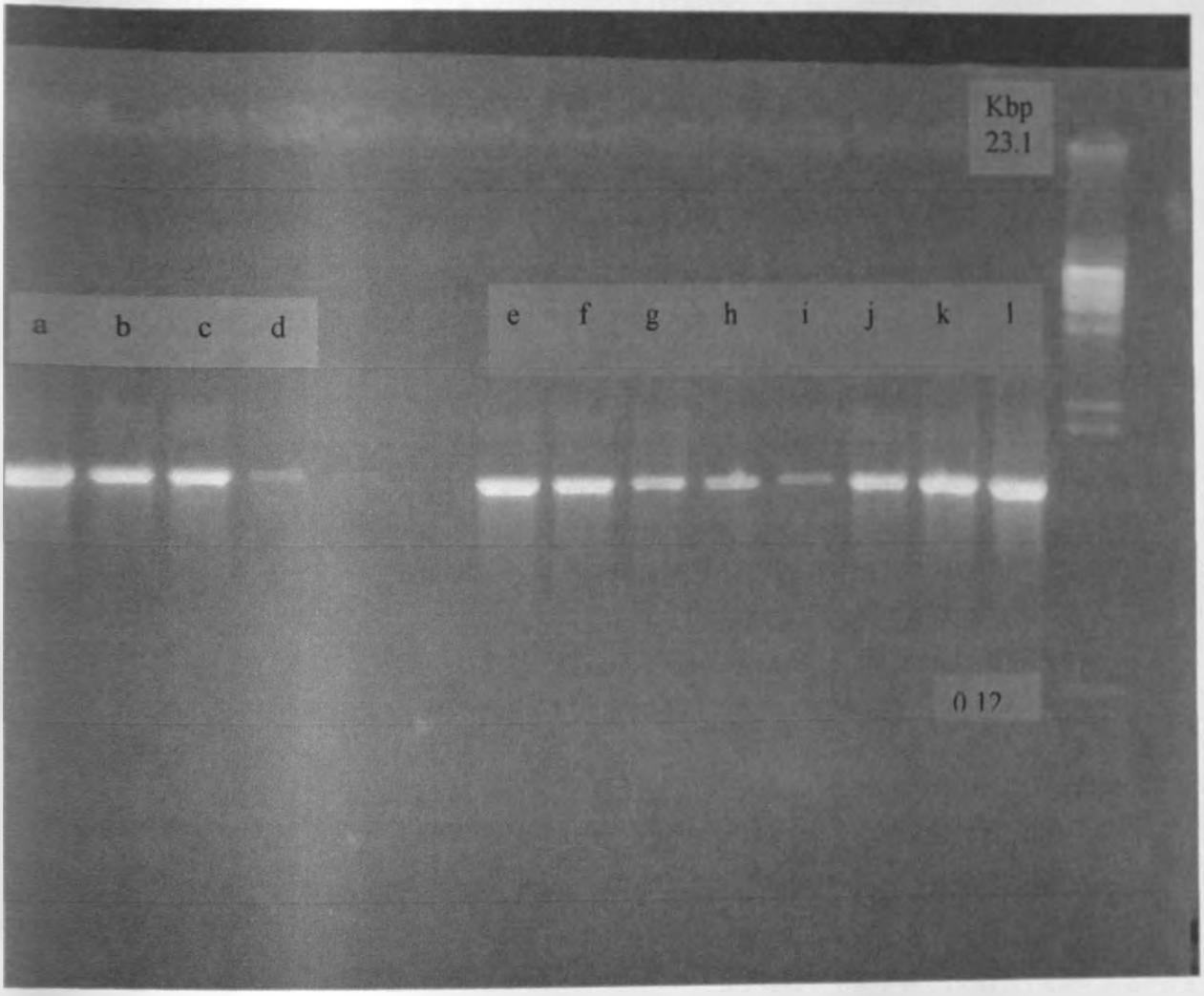


Plate 8: Image of Ethidium Bromide Stained Gel Electrophoresis of PCR product on 1% agarose. Lanes labeled a-l correspond to PCR product generated from genomic DNA of the following bacterial isolates. Lane a, M7-1; Lane b, M7-3; Lane c, M6-2; Lane d, M1-3; Lane e, M2-3; Lane f, M3-3; Lane g, M4-3; Lane h, M6-1; lane I, MC-3; Lane j, M5-1; Lane k, M4-1 Lane l, MC-1; Last Lane had DNA molecular marker of 0.12-23.1 kbp

3.3 Nucleic acid analysis of bacteria isolated from Lake Magadi

The G+C mol% of the rRNA gene was used as an estimation of the G+C mol% of the genomic DNA. The maximum identity shows the magnitude of similarity of the isolate to a species which had been characterized previously and enlisted in the database with the given accession number. The comparisons were summarized in Table 9 below.

Table 9: A comparison of 16S rRNA gene sequences of the isolates with sequences of close relatives from NCBI GenBank

Isolates	G+C Mol%	Accession no	Names of comparative isolates	Maximum identity %
M4-1	55.50	EU870505.1	*Halomonas sp. Sua-BAC009*	99
		EF527873.1	*Halomonas salifodinae strain BC7*	98
M7-1	53.29	AY730234	Gamma Proteobacterium M6-24A	99
		EU723884.1	Gamma Proteobacterium CF12-14	98
		FJ170028.1	*Idiomarina sp. CF12-14*	98
M5-1	55.98	DQ077911.1	*Halomonas campisalis strain LL6*	97
		DQ077910.1	*Halomonas campisalis strain LL5*	96
		DQ077909.1	*Halomonas campisalis strain LL4*	96
		DQ077908.1	*Halomonas campisalis strain LL3*	96
MC-1	53.35	FJ170017.1	*Idiomarina sp. CF11-10*	99
		EU723884.1	G. Proteobacterium CF12-14	98
		EF554894.1	*Idiomarina sp. JK16*	98
		AY914068.1	Gamma Proteobacterium A-7B	98

Isolate M4-1 is 98% similar to *Halomonas salifodinae*, M7-1 and MC-1 are 98% similar to *Idiomarina sp* and M5-1 is 97% similar to *Halomonas campisalis*

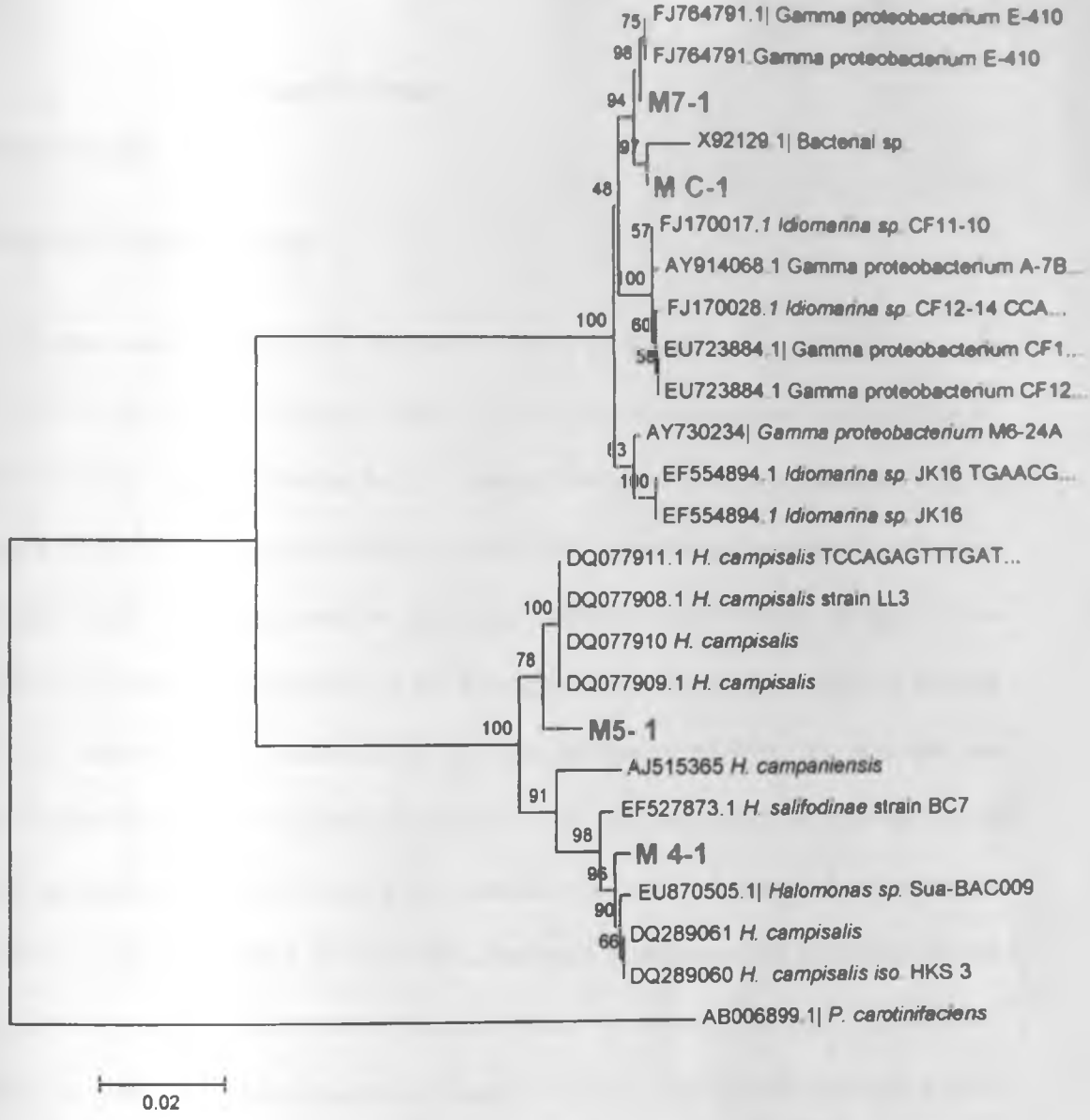


Fig. 4 Rooted phylogenetic tree to show relationship between 16S rRNA sequences of isolates and their comparisons from NCBI GenBank

The tree was generated by the neighbor joining method. Distance matrices were calculated by Jukes and Cantor (1969). Bootstrap values at the nodes of the branches are based on 1,000 replications. Accession numbers of the comparable isolates are provided in the text. The scale bar represents 2 nucleotide substitutions per 100 nucleotides.

Chapter Four

Discussion

Physicochemical conditions

The temperature of the water in the sampled sites varied from as low as 30°C to as high as 47°C in one of the hot springs (Table 1). The pH lies within a narrow range of 10 to 11 while salinity, which is reflected by the electrical conductivity and total dissolved solutes, ranges from 7.9 to 25.6 practical salinity units (PSU is used to indicate that the Practical Salinity Scale (PSS) was used to determine conductivity salinity. Oceanographers in UNESCO (1985), defined salinity in the Practical Salinity Scale as the conductivity ratio of a sea water sample to a standard KCl solution. Salinity therefore being a ratio does not have physical units. The bacterial population in the Kenyan soda lakes is limited by the total phosphate ions, total nitrogen and conductivity levels. A study of organotrophic bacteria in the saline lakes showed that eubacterial haloalkalophiles dominated at low conductivity and archeobacteria haloalkalophiles dominated at high conductivity (Mwatha, 1991). Despite the apparently harsh conditions, soda lakes are home to a large population of prokaryotes, a few types of which may dominate as permanent or seasonal blooms. The organisms range from alkaliphilic cyanobacteria to haloalkaliphilic archaeobacteria. It is not unusual to find common types of alkaliphilic organisms inhabiting soda lakes in various widely dispersed locations throughout the world such as in the East African Rift Valley, in the western U.S., Tibet, China and Hungary. An example is *Natronobacteria* which has been isolated and identified in soda lakes located in China (Wang and Tang, 1989), Western U.S. (Morth and Tindall, 1985) and in India (Upasani and Desai, 1990).

Phenotypic characteristics

Three culture media were used for culturing isolates in order to carry out the biochemical tests. The medium for haloalkaliphiles contained TSB as a carbon source, sodium carbonate and sodium hydrogen carbonate to create alkaline conditions and sodium chloride (sea salt) as a source of sodium ions and other ions required as trace elements for growth of microbes. The haloalkaliphilic medium of Tindall has all the trace elements and casamino acids for carbon source. The haloalkaliphilic medium of Tindall had a brown tinge presumably from the presence of green Fe^{2+} ions which turn into brown Fe^{3+} ions after oxidation. Both the Horikoshi and the Tindall media had a proliferation of bacterial growth within the first 12 hours. The medium for haloalkaliphiles on the other hand had a slower rate of growth and appeared to be a more favorable medium for slow growers. Observation of colony characters helped to redeem twenty six bacterial isolates. However some colonies may have appeared different yet could belong to the same species and vice versa. The isolates had growth pH optima ranging from 9 to11 (Table 5) and an optimum NaCl concentration ranging from 3 % to 9 % w/v (Table 4). Most of them were slight or moderate thermophiles with growth temperature optima ranging from 40° C to 45° C (Fig 3a – d). The isolates were able to utilize glucose, galactose or mannose as carbon sources with no production of acid from the substrate. They are chemoorganotrophs growing on complex substrates such as yeast extract and peptone. Out of 26 isolates 17 were catalase positive (Table 7), an indicator of aerobic respiration. When aerobic bacteria grow by respiration, they produce hydrogen peroxide as a by product of reducing oxygen to water. They therefore produce catalase which converts the toxic hydrogen peroxide to harmless oxygen and water. The presence of catalase is a way of differentiating strict anaerobes and aerotolerant from aerobic bacteria. About 50% of

the isolates hydrolyzed starch (Table 7), a large molecule consisting of amylose, a straight chain polymer of 200 to 300 glucose units and amylopectin, a larger branched polymer with phosphate groups (Brown, 2005). The isolates that hydrolyse starch produce amylases that yield molecules of glucose, maltose and dextrins. About 30% of the isolates hydrolyze casein and about 10 % hydrolyze both starch and casein. Casein is the predominant protein in milk and these isolates that hydrolyze casein produce proteases that degrade proteins such as casein into peptides and individual amino acids. A few of the Gram negative rods were motile. All isolates were indole negative indicating that they do not produce tryptophanase, the enzyme that hydrolyses the amino acid tryptophan into indole and pyruvic acid.

Genotypic and phylogenetic characteristics

Taxonomists today are using more direct means of classifying organisms by analyzing the genetic relatedness between organisms as opposed to numerical taxonomy which compares a large number of phenotypic characteristics. The more characteristics they have in common the more closely related the organisms are. Numerical taxonomy defines taxospecies as a group of strains with high mutual phenetic similarity. Such groups of strains for which no term (species or genus name) is available are called operational taxonomic units (OTUs) (Dubey and Maheshwari, 2004). Ribosomal RNA (rRNA) which represents about 80% of total RNA of the cell has proved to be a useful tool in phylogenetic and interrelationship analysis among organisms. It is ancient, universally distributed and is the most conserved region in the genome of the microorganisms. In Prokaryotes, rRNA is encoded by genes located in the chromosomal DNA referred to as

ribosomal DNA (rDNA). The 70 S ribosome in prokaryotes is made up of two subunits, 50 S and 30 S. The 50 S subunit contains 23 S rRNA which has about 2,900 nucleotides, which are too many for convenient sequencing and 5 S rRNA with only about 120 nucleotides; whereas the 30 S contains 16 S rRNA which has about 1,500 nucleotides which are convenient for sequencing. This classification of the rRNA into 23S, 16S and 5S is done in reference to their sedimentation rates during ultracenrifugation (Dubey and Maheshwari, 2004).

Genomic DNA was isolated for all the twenty six isolates and twelve PCR products were successfully obtained. This may have been due to non-specific primers for some of the isolates. Sequencing was successfully done for six of the PCR products. The PCR products obtained with Universal Archaea primers had sequences of less than 400 bp and were too small to be used for comparison as they scored maximum identity that was too low for sensible alignment to be done. The longest sequences were obtained with 16S primers and had about 1400 bp. These were sequences of isolates M4-1, M5-1, M7-1, and MC-1 which were later compared to sequences of closely related organisms in the NCBI GenBank. Clones with sequence maximum identity of over 98% to known organisms may represent the same species. Sequences that share an identity between 88% and 98% are usually considered to be part of the same genus. Where the identity is less than 88% the rDNA could represent new genera (Stackerbrandt and Gobel, 1994). The phylogenetic tree obtained (Fig. 3) had bootstrap values in several places indicating high confidence in the tree structure.

If the G+C content of the 16S rRNA gene is used as an estimation of the G+C content of the genomic DNA then isolate M4-1 had an estimated G+C content of 55.55 mol% (Table 9).

Comparisons of 16S rRNA gene sequences with sequences in the NCBI GenBank database showed that the isolate was 99% similar to *Halomonas sp.* Sua-BAC009 of accession no EU870505.1 and 98% similar to *Halomonas salifodinae* strain BC7 accession no EF527873.1. The phylogenetic comparisons indicated that the isolate clustered closely with *Halomonas campisalis iso. HKS 3.* Isolate M4-1 was sampled from site 4 whose water had a temperature of 47°C, pH of 10.5 and salinity of 7.89.The isolate was a Gram negative, aerobic, rod growing optimally at a temperature of 45°C, 6-9 % w/v NaCl and a pH range of 9-11. These characteristics match those of *Halomonas* species belonging to the *Halomonadaceae* family that are heterotrophic, gram negative, rod shaped, slight or moderate halophiles and aerobic (Duckworth *et al.* 2000).

Isolate M5-1 had a DNA G+C content of 55.98 mol%. It showed highest similarity of 97 % with *Halomonas campisalis* strain LL6 of accession no DQ077911.1; 96% with *Halomonas campisalis* strain LL5 of accession no DQ077910.1 ; 96% with *Halomonas campisalis* strain LL4 of accession no DQ077909.1; and 96% with *Halomonas campisalis* strain LL3 of accession no DQ077908.1.The isolate formed a distinct out group in the phylogenetic tree without any close affinity to known taxa, but showed some clustering with *Halomonas campisalis.* Isolate M5-1 which was isolated from site 5 with a temperature of 44°C, salinity of 20.4 and a pH of 10.2, was a gram negative aerobic coccobacillus, growing best at a temperature optimum of 40°C, NaCl concentration of 3-6%w/v and a pH of 11. This is likely to be a novel species and further analysis needs to be done to verify this.

The estimated G+C content for isolate M7-1 was 53.29 mol%. It showed a 99 % similarity to AY730234.1 *Gamma Proteobacterium* M6-24A; 98 % similarity to EU723884.1 *Gamma Proteobacterium* CF12-14; 98% similarity to FJ170028.1

Idiomarina sp. CF12-14; the isolate clustered closely with a *Gamma Proteobacterium* E-410 strain which has not been characterized. Isolate M7-1 was isolated from site 7 where the water temperature was 43°C, pH of 10.0 and salinity of 18.19. It was a motile Gram negative aerobic rod, that grew optimally at a temperature of 40°C, 0%-6%w/v NaCl and a pH of 11.

Isolate MC-1 had a G+C content of 53.35 mol%. Close similarity of sequence was 99 % with FJ170017.1 *Idiomarina sp.* CF11-10; 98% with EU723884.1 *Gamma proteobacterium* CF12-14; 98% with EF554894.1 *Idiomarina sp.* JK16; 98% with AY914068.1 *Gamma proteobacterium* A-7B. Highest clustering was with isolate M7-1 which clustered with *Gamma proteobacteria.* Isolate MC-1, sampled from the Western causeway, was a gram negative aerobic large rod, growing best at a temperature range of 35°C-40°C, 6-9%w/v NaCl and at a pH of 9.

Most of the isolates are obligately alkaliphilic or alkalitolerant. The majority of Gram negative isolates were members of the gamma subdivision of the Proteobacteria. Organisms that are related to but not identical to members of the *Halomonas* group were well represented. Other gram negative isolates were closely related to *Idiomarina spp.* Species similar to the ones identified in this study have been found elsewhere in the world. The haloalkaliphile *Halomonas campisalis*, isolated near Soap Lake, Washington, grows under both aerobic and denitrifying conditions from 0 to 26% w/v NaCl, with optimal growth occurring at 2 to 3% w/v NaCl, respectively. *Halomonas campisalis* produces high concentrations of compatible solutes, most notably ectoine (up to 500 mM within the cytoplasm), but also hydroxyectoine and glycine betaine. The types and amounts of compatible solutes produced depend on salinity and specific growth rate, as well as on the terminal electron acceptor available (O_2 or NO_3^-) (Aston and Peyton,

2007). Strains of *Halomonas campisalis* have also been isolated from the alkaline Lonar Lake, India, identified and confirmed by 16S rRNA sequencing. They were capable of using a variety of electron donors and were found to grow in the presence of sodium chloride up to 24% w/v, at pH from 7 to 11, 9 being the optimum. The isolates could grow over a wide range of temperatures (from 4 to 45 °C). One of the isolates, ARI 351, was able to produce lipase at pH-9.0, while two isolates, ARI 351 and ARI 360, could accumulate polyhydroxyalkanoic acid (PHA) granules when grown in a medium containing maltose. Thus the *H. campisalis* isolated from Lonar Lake was different from the previously reported ones, with respect to its biotechnological potential for production of Lipase and PHA (Joshi *et al.*, 2007). *Halomonas salifodinae*, a Gram-negative, aerobic, motile, halophilic bacterium, designated strain BC7^T was isolated from a salt mine in China with optimum growth at 3 % (w/v) NaCl, pH 7.0 and 30 °C. The c ells were long rods, 0.8–1.2 μm wide and 4.0–6.0 μm long. The DNA G+C content was 65.5 mol%. Phylogenetic analysis based on 16S rRNA gene sequences showed that strain BC7^T belonged to the genus *Halomonas* and showed highest sequence similarity to the type strain of *Halomonas pacifica* (99.2 %) (Wang et al., 2008). Two bacterial strains, F23^T and R22^T, isolated from hypersaline habitats in Málaga and Murcia (Spain) are novel strains, similar to previously described *Idiomarina* species. They are slightly curved rods, Gram-negative, chemo-organotrophic, strictly aerobic and motile by a single polar flagellum. Both strains produce catalase and oxidase and hydrolyze gelatin, casein but not starch or tyrosine. The strains differ from the other described *Idiomarina* species in their capacity to produce extracellular polysaccharides and their different patterns of carbon sources and antimicrobial susceptibility. They are moderate halophiles capable of growing in NaCl concentrations of 0·5 to 25 % w/v, the optimum being 3–5 % w/v.

The DNA G+C composition is 46·0 mol% in strain FP23^T and 48·7 mol% in strain R22^T. Phylogenetic analyses indicate conclusively that the two strains belong to the genus *Idiomarina*. It was proposed that they be classified as novel species of the genus *Idiomarina*, with the names *Idiomarina fontislapidosi* sp. nov. and *Idiomarina ramblicola* sp. nov. (Martínez-Cánovas *et al.*, 2004).

Red anoxigenic phototrophic halophilic *Ectothiorhodospira*, a genus of bacteria in the family Chromatiaceae, have been isolated at Lake Magadi and other soda lakes. They are the major primary producers in the lake (Grant and Tindall, 1986). Normally the trona crusts are colored red by the haloalkaliphilic Archaea which are classified in two genera *Natronococcus* and *Natronobacterium* (Kamekura *et al.*, 1997). Gram positive isolates from the hypersaline environment could be either of the high G+C content or of the low G+C divisions. High G+C isolates redeemed from the Rift Valley soda lakes are of the genus *Dietzia* and are loosely associated with *Arthrobacter sp* and *Terrabacter spp.* The low G+C isolates are associated with the *Bacillus* spectrum, especially *Bacillus alcalophilus* (Duckworth *et al.*, 1996). Cyanobacteria of the genus *Cyanospira* have been isolated from Lake Magadi. However, cyanobacterial blooms occur only occasionally after extensive rainfall causing dilution of the brine (Florenzano *et al.*, 1998).

Conclusions and Recommendations

The work presented here has produced some insight into the diversity of microbes in Lake Magadi but much more needs to be brought out. More sites could be sampled to include a larger diversity of species. Culturing the microorganisms is a major hurdle to the complete understanding of the microbial diversity. There is evidence that if experiments are conducted *in situ* then a different spectrum of organisms can be isolated (Mwatha, 1997). Such studies on the springs of Lake Magadi have hinted at uncultured thermoalkaliphiles. One cloned gene showed only 7 % similarity to all known Archeal sequences thus representing a new distinct Archeal line (Grant *et al.*, 1998). There remains therefore the challenge to obtain more of the microbes in culture in order to study their physiology and to classify them to the species level. Such classification would require analyses of the polar lipids, fatty acid profiles and isoprenoid quinones, which are characteristic components of the plasma membrane, as well as the G+ C content of the DNA. Phylogenetic analyses based on 16S rRNA gene sequence comparisons are of utmost importance as they have helped to reorganize species into defferent genera. A good example is the family Halomonadaceae within the Gamma Proteobacteria whose species were previously assigned to other genera such as *Deleya*, now extinct, *Alcaligenes, Pseudomonas, Halovibrio and Volcaniella* (David and Ventosa, 1998).

The East African soda lakes, among the most productive aquatic environments in the world, has water with shades of red and green reflecting on the primary productivity. This is due to the unlimited supply of carbon dioxide, high ambient temperature and high daily light intensities (Melack and Kilham, 1974). Their productivity, as well as accessibility demands that they be regularly sampled for studies of microbial ecology. The soda lakes are not entirely closed ecosystems. This is due to disturbance by wildlife including

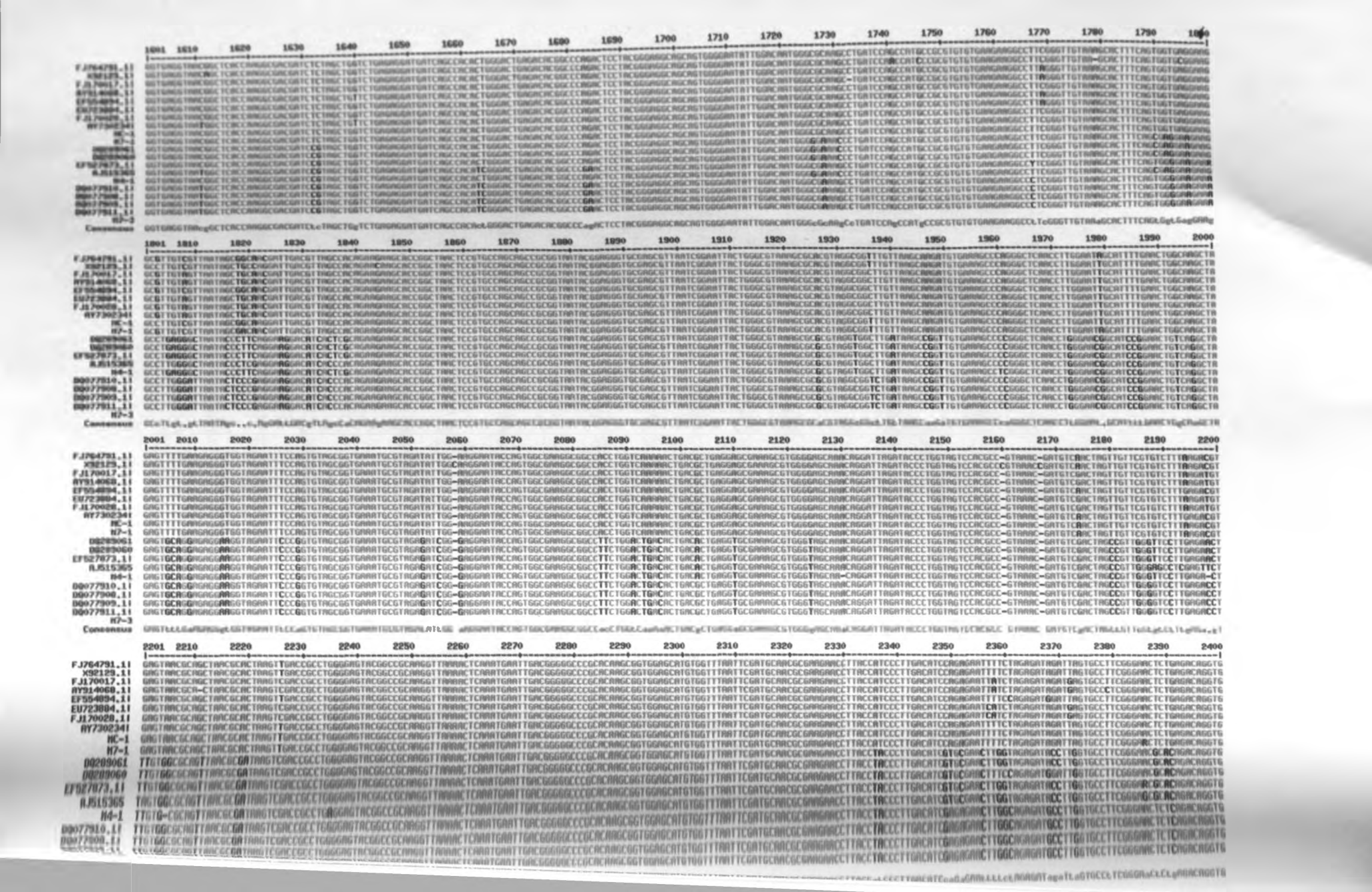
migratory birds such as flamingoes that use the Rift Valley as a migratory route and contribute to the spread of haloalkaliphiles. Constant sampling raises the chances of redeeming novel species which may be of commercial importance. Alkaliphilic bacterial strains have the potential to produce different kinds of enzymes which are expressed under specific cultural conditions. A good example is the facultatively alkaliphilic *Bacillus halodurans C-125* which produces at least five kinds of enzymes of industrial interest (Takami and Horikoshi, 1999). The amount of each enzyme produced varies depending on the culture conditions particularly with different combinations of nitrogen and carbon sources. It is also important to determine all the information on the genome of the bacterial strains in order to control the extracellular enzyme production (Takami and Horikoshi, 2000). The genes encoding these alkali-tolerant enzymes may be isolated, cloned and brought to expression in compatible expression hosts. This would provide a source of larger volumes of enzyme products especially if the wild-type strain has failed to produce sufficient amounts of the desired enzyme. Halophiles, having the largest plasmid so far known among all the known bacteria, (Dubey and Maheshwari, 2004) could be used for genetic manipulation.

The isolates characterized in this study could be put to commercial use by optimizing production of metabolites for biotechnological applications and the multialignments (see Appendix II) obtained could be used for primer design.

Appendix I

Optimum growth temperature of isolates (Optical Densities of broth cultures incubated for 24 hr at varying temperatures of 30°C,35°C,40°C,45°C,50°C)

Isolate	30°C	35°C	40°C	45°C	50°C	Optima°C	Description
M1-1	0.12	0.45	0.47	0.26	0.04	35-40	Slightly thermophilic
M1-2	0.11	0.20	0.21	0.15	0.058	35-40	Slightly thermophilic
M1-3	0.12	0.43	0.48	0.30	0.06	40	Slightly thermophilic
M2-1	0.052	0.39	0.38	0.29	0.050	35-40	Slightly thermophilic
M2-2	0.054	0.45	0.54	0.056	0.01	40	Slightly thermophilic
M2-3	0.04	0.24	0.32	0.27	0.02	40-45	Moderately thermophilic
M3-1	0.01	0.20	0.36	0.04	0.06	40	Slightly thermophilic
M3-3	0.06	0.30	0.54	0.41	0.12	40	Slightly thermophilic
M4-1	0.25	0.31	0.32	0.38	0.058	45	Moderately thermophilic
M4-2	0.38	0.39	0.51	0.42	0.12	40	Slightly thermophilic
M4-3	0.22	0.30	0.31	0.43	0.15	45	Moderately thermophilic
M4-4	0.058	0.40	0.40	0.45	0.14	45	Moderately thermophilic
M5-1	0.35	0.46	0.54	0.48	0.12	40	Slightly thermophilic
M5-2	0.32	0.30	0.33	0.35	0.18	30-45	Moderately thermophilic
M5-3	0.01	0.24	0.24	0.32	0.10	45	Slightly thermophilic
M6-2	0.15	0.22	0.27	0.35	0.12	45	Slightly thermophilic
M6-3	0.01	0.19	0.25	0.12	0.02	40	Slightly thermophilic
M6-4	0.30	0.30	0.34	0.36	0.16	30-45	Moderately thermophilic
M6-5	0.35	0.40	0.42	0.35	0.23	30-50	Moderately thermophilic
M7-1	0.30	0.36	0.40	0.35	0.03	40	Slightly thermophilic
M7-3	0.20	0.25	0.28	0.28	0.17	40-50	Moderately thermophilic
MC-1	0.38	0.41	0.45	0.38	0.14	40	Slightly thermophilic
MC-2	0.054	0.19	0.20	0.14	0.055	35-40	Slightly thermophilic
MC-3	0.56	0.58	0.58	0.60	0.24	45	Moderately thermophilic
MC-4	0.04	0.22	0.38	0.23	0.01	40	Slightly thermophilic
MC-5	0.30	0.45	0.50	0.43	0.03	40	Slightly thermophilic



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