Genetic Diversity of Actinobacteria Isolated from Lake Magadi

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DECLARATION

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

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DEDICATION

I dedicate this thesis to my family for their moral support and words of encouragement.

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ABBREVIATIONS AND ACRONYMS

ATCC	American Type Culture Collection
bp	Base Pairs
BLAST	Basic Local Alignment Search Tool
dNTP	2'-deoxynucleoside-5' triphosphate
EDTA	Ethylene-Diamine-tetra-Acetic Acid
G+C	Guanine-Cytosine content
ISP	International *Streptomyces* project
LB	Luria Bertani
MEGA	Molecular Evolutionary Genetic Analysis
NCBI	National Centre for Biotechnology Information
nm	Nanometers
OD	Optical Density
PCR	Polymerase Chain Reaction
rDNA	Ribosomal Dioxyribonucleic Acid
rRNA	Ribosomal Ribonuclaic Acid
Tris	Tris-(hydroxymethyl)- aminomethane

ABSTRACT

Actinobacteria are morphologically and phylogenetically diverse Gram positive bacteria generally aerobic and have high genomic guanine-plus-cytosine (G+C) contents (>55 mol %). Many studies on alkaliphilic microbial communities have been done on Kenyan soda lakes focusing on general microbial diversity. However, actinobacterial communities have not been documented on Lake Magadi, a hyper saline lake with up to 34% salinity levels. Four different sampling points were selected randomly within the lake to cover a broad range of sample diversity. Sediments and water samples were collected from each sampling point. Serially diluted samples were plated on alkaline growth media containing complex substrates such as malt and chitin. The media was prepared using filtered lake water. DNA was extracted from the isolates and the 16S rRNA genes amplified using primers described to be domain specific for actinobacteria. The PCR based method was used to screen actinobacteria from other isolates. Eleven isolates produced amplifications and were presumed to be actinobacteria and thus characterized further. The amplified products were used for sequence analysis. Five actinobacteria isolates were obtained belonging to the families *Norcadiaceae, Microbactericeae, Streptomycetaceae* and *Dietziaceae*. BLAST analysis showed that isolates MS-2, MS-3, MS-5, MM-10 and MS-11 had between 97% to 99% similarity to their close relatives. The isolates were affiliated with the genera *Dietzia, Microbacterium, Rhodococcus, Streptomycetes*

and *Nocardia* respectively. Screening of the isolates for type-1 polyketide synthases showed isolates MS-1, MS-2, MS-5, MS-9, MM-10 and MS-11 to contain these biosynthetic systems involved in synthesis of biologically active compounds. All the domains obtained had below 80% identity to GenBank sequences. This may imply chemical and functional novelty. The isolates were characterized both physiologically and biochemically and were tested for production of extra-cellular enzymes. The physiological results from the study showed that growth was possible up to a pH of 11. Isolates MS-2, MS-5, MSL-7, MM-9, MM-10, and MS-11 were alkaliphiles and isolates MS-1, MS-3, MS-4 and MM-8 were alkalitolerants. Optimum growth temperature was at 30°C and the highest at 40°C. All isolates were able to grow in media with Sodium chloride. Eight isolates grew optimally at 5% w/v salt and the rest in media without salt. The isolates were able to utilize various carbon sources and produced hydrolytic enzymes such as lipase, amylase, xylanase, proteinase and cellulase. They showed antagonistic activity against both Gram negative and Gram positive bacteria. These findings show that isolates from Lake Magadi are metabolically active and adapted to life in the alkaline environment.

CHAPTER ONE

1.0 INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Lake Magadi is a saline alkaline soda lake with a pH of 9-12 and salt concentration of up to 34% w/v. Increase in evaporative concentration leads to almost equal carbonate and chloride ions compared to more dilute soda lakes where carbonates dominate (Grant, 2006). Halobacteria are the most halophilic organisms known and form the dominant microbial population when hypersaline waters approach saturation, frequently imparting a red colouration to the brines because of C_{50} carotenoids. Functionally they have a specific trophic position and flourish on the organic matter concentration arising from evaporation of brine and the death of its microbial population (Zavarzin *et al*., 1999). An understanding of distribution of actinobacteria which have always been associated with terrestrial habitats is important in deciphering their ecological role in the haloalkaline environment.

Previous studies focused attention to investigation of the general microbial communities in soda lakes including Lake Magadi using both culture dependent methods (Duckworth *et al*., 2000; Sorokin *et al*., 2004; Sorokin and Kuenen, 2005) and molecular techniques (Hiorns *et al*., 1997; Methe *et al*., 1998; Rappe *et al*., 1999; Crump *et al*., 1999; Glochner *et al*., 2000; Donachie *et al*., 2002; Zwart *et al*., 2002; Humayoun *et al*., 2003; Rees *et al*., 2004; Scholten *et al*., 2005; Foti *et al*., 2007). Few reviews summarize the findings, showing that soda lakes contain representatives of the major trophic and phylogenetic groups of microorganisms, and that they can be considered as autonomous

systems, in which cycling of nutrients is almost complete (Zavarzin *et al*., 1999; Jones *et al*., 1998).

Studies have shown that actinobacteria are widespread in nature and are able to occupy a wide range of habitats including extreme environments such as hot springs and deep sea sediments (Allgaier and Grossart, 2006; Holmfeldt *et al*., 2009). They have attracted attention because of their biotechnologically important secondary metabolites including antibiotics and enzymes and their role in decomposition of hazardous chemical compounds (Sahin and Ugur, 2002). Isolation programs have now focused on aquatic environments where several strains have been cultured and lineages of 16S rRNA sequences of uncultivated strains have been obtained from various aquatic environments (Crump *et al*., 1999; Humayoun *et al*., 2003). Most of these findings were documented from marine and fresh water environments. Data on the ecology of actinobacteria endemic to soda lakes are scanty.

The phylum actinobacteria is made up of Gram-positive micro-organisms with a high mole% G+C composition (> 55% G+C), generally aerobic bacteria noted for a filamentous and branching growth pattern that results, in most forms, in an extensive colony, or mycelium (Lauro and Jensen, 2008). The mycelium in some species may break apart to form rod- or coccoid-shaped forms. The mycelia may be of two types, one prostrate, forming a vegetative growth, sometimes referred to as substrate mycelium and the other an erect or aerial mycelium. The spore-bearing hyphae of the aerial mycelium usually have a somewhat greater diameter than the hyphae of the substrate mycelium.

Actinobacteria typically produce two types of spores; true conidia and arthrospores. Growth of a new colony from a single spore (developed on sporophore), a sporangium, a fragment of hypha or a small part of an old colony, account for their ubiquity (Maldonado *et al*., 2005).

Among gram-positive bacteria, the actinobacteria exhibit the greatest morphological differentiation, from coccoid, for example *Micrococcus* or rod-coccoid such as *Arthrobacter*, fragmenting hyphal forms mostly *Nocardia* to those with permanent and highly differentiated branched mycelium which include *Streptomyces* (Ward and Bora, 2006). It is nearly impossible to summarize all the variations of colonial characters as far as known for the different species. Colonies may be raised or flat, sometimes covered with a leathery layer. Their consistency varies from very soft and pasty to extremely hard. They have a color range, which includes white, yellow, orange, rose, red, purple, blue, green, brown, and black. Surfaces may be smooth, rigid wrinkled, granular or squamous. The colonies may be completely compact or may demonstrate different zones of growth, in concentric rings with a radial orientation and frequently a combination of the two. Colony size depends on the species, age and growth conditions, varying from a fraction of a millimeter up to the diameter of centimeters (Maldonado *et al*., 2005). In general actinobacteria will grow on ordinary laboratory media, but their growth is usually slower than that of ordinary bacteria. A division cycle in actinobacteria may take 2-3 hours as compared with 20 minutes of *Escherichia coli*. Some may take even longer. Other actinobacteria such as *Mycobacterium leprae*, have never been grown on laboratory

media. Others such as plant endophytes *Frankia* grow only with the greatest difficulty (Schaal *et al*., 2006).

There is no single selective procedure for isolation of actinobacteria. Good success is obtained using a variety of strategies. The strategies are designed to select against fast growing bacteria and fungi. Incorporation of antifungal antibiotics such as nystatin eliminates fungi. Samples serially diluted and plated into growth media, usually containing complex substrates such as casein, starch, chitin, malt. These selective media promote the growth of actinobacteria and suppress the growth of accompanying organisms. The selective qualities of these synthetic media can be altered by changing various parameters like pH, salinity, temperature and period of incubation (Schaal *et al*., 2006).

They are also physiologically very diverse as evidenced by their production of numerous extracellular enzymes and by the thousands of metabolic products including antibiotics they synthesize and excrete (Berdy, 2005).

1.2 Literature review

1.2.1 Life in Alkaline Environment

There are environments which are thought to be extreme habitats for microorganisms to exist. In these habitats, environmental conditions such as pH, temperature and salinity concentrations are extremely high or low. Extreme environments are populated by groups of organisms that are specifically adapted to these particular conditions and these types of

extreme micro-organisms are usually referred to as alkaliphiles, halophiles, thermophiles and acidophiles, reflecting the particular type of extreme environment which they inhabit (Ciaramella *et al*., 2003; Oarga, 2009). The condition of high pH which occurs in nature in soda lakes such as Lake Magadi is referred to as alkaline environment (Valverde *et al*., 2012).

There are two kinds of naturally occurring stable alkaline environments in the world. First, high Ca^{2+} environments (ground waters bearing high Calcium carbonate) and second, low Ca^{2+} environments which include soda lakes and deserts dominated by sodium carbonate (Grant, 2003). Soda lakes and soda deserts represent the most stable, naturally occurring alkaline environments found worldwide. These environments are characterised by high concentrations of sodium carbonate (usually as $Na_2CO_3.10H_2O$ or $Na_2CO_3.NaHCO_3.2H_2O$).The distinguishing feature of soda lakes is depleted Mg^{2+} and the presence of carbonate provides buffering capacity to the lake waters (Duckworth *et al*., 2000).

The formation of alkalinity in the soda lake environment requires a combination of geographical, topographical and climatic conditions: firstly, the presence of geological conditions which favour the formation of alkaline drainage waters; secondly, suitable topography which restricts surface outflow from the drainage basin; and thirdly, climatic conditions conducive to evaporative concentration. Such conditions are found in arid and semi-arid zones of tropical or subtropical areas. A vital condition necessary for the formation of a soda lake is that significant amounts of Ca^{2+} and Mg^{2+} must be absent so

that ground waters containing HCO_3 are produced where the molar concentrations of -HCO_3/-CO_3 greatly exceed those of Ca^{2+} and Mg^{2+}. Through evaporative concentration, such waters rapidly achieve saturation with respect to alkaline earth cations which precipitate as insoluble carbonates, leaving Na^+, Cl^- and HCO_3^-/CO_3^{2-} as the major ions in solution (Fujiwara, 2002; Grant 2006; Stal, 2007). Alkalinity evolves concomitant with the precipitation of other ions, especially Na^+ and Cl^-, leading to the development of alkaline and saline conditions. The relative salinity of any lake is dependent on the local geologic and climatic conditions, resulting in saline, alkaline lakes (Bowers *et al*., 2009). In lakes of lower salinity, the concentration of CO_3^{2-} usually exceeds that of Cl^-, but in brines of higher salinity like in the case of Lake Magadi Cl^- exceeds CO_3^{2-} concentrations. Lakes Magadi and Wadi An Natrun and the Dead Sea are examples of athalassohaline lakes worldwide (Mesbah *et al*., 2007).

Micro-organisms adapted to alkaline environments can be classified into two main categories; alkaliphiles (also called alkalophiles) and alkalitolerants. The term alkaliphiles is generally restricted to those micro-organisms that actually require alkaline media for growth. The optimum growth rate of these micro-organisms is observed in at least two pH units above neutrality. Organisms capable of growing at pH values more than 9 or 10, but with optimum growth rates at around neutrality or less, are referred to as alkalitolerant (Le Romancer *et al*., 2006; Magan, 2007).

Most of our concepts about microbial life in both naturally and artificially generated alkaline environments are based on studies in which the pH of the environment and of the growth media used in laboratory studies is constant. However, in naturally occurring

environments, the pH values are often far from constant and are subject to short-term changes. Microbial communities thriving in them must adapt to the changing conditions. One of the major barriers to the study of biodiversity is our inability to culture more than a relative small number of species from any one environment. Studies have so far concentrated on the cultivable population of most environments not only artificially but also in naturally occurring environments. Clearly, sampling methods and culture conditions have a major effect on the range of microorganism types encountered in the laboratory.

1.2.2 Soda Lakes Ecology

Soda lakes are a specific type of salt lake with high to extremely high alkalinity and a moderate to extremely high salinity. They are spread all over the world, but located, as most inland salt lakes, in arid and semi-arid areas where the evaporative climate favors accumulation of salts in local depressions. The major ions in soda lakes brines are sodium (Na^{+}), carbonate (CO_3^{-2})/ bicarbonate (HCO_3^{-1}), chloride (Cl^{-}) and sulfate (SO_4^{-2}), whereas calcium (Ca^{+2}) is virtually absent and magnesium (Mg^{+2}) only present at very low concentrations. In contrast to other alkaline environments, such as low-salt alkaline springs, soda lakes maintain a stable alkaline pH due to the high buffering capacity of the soluble carbonates. These double extreme conditions (high pH and high salinity) make soda lakes a unique ecosystem (Foti *et al*., 2008).

One of the unique features of many soda lakes is the appearance of the water body. Depending on the water chemistry of the individual lakes, they are likely to be green,

pink, red or orange, due to massive permanent or seasonal blooms of microorganisms. This is reflected in the extremely high primary productivity associated with some of these lakes. Despite the extreme nature of such environments, they are characterized by exceptionally rich productivity rates presumably because of the relatively high ambient temperatures, high light intensities, availability of phosphate, and unlimited access to carbon dioxide in these carbonate-rich waters (Mwirichia *et al*., 2010). They are also regarded as naturally eutrophic reservoirs and, like all eutrophic bodies of water, they feature considerable microbial diversity (Zavarzin *et al*., 1999).

Prokaryotic photo-synthetic primary production is probably the driving force behind all nutrients recycling in soda lakes. Culture dependent studies have revealed a remarkable diversity of prokaryotes with alkaliphiles represented in most of the major taxonomic groups. Major trophic groups responsible for the recycling of carbon, sulfur and nitrogen in the lakes have been documented. Many of the microorganisms so far characterized from soda lakes have relatives in salt lakes except that they are all alkaliphilic or at least highly alkali-tolerant (Grant, 2006).

In the moderately saline lakes cyanobacteria are responsible for primary production which supports organotrophic bacteria. The essential role of primary producers is the fixation of nitrogen and production of oxygen. Species of cyanobacteria that have been recorded include *Spirulina* as the main primary producer and *Cyanospira*, *Chroococcus*, *Synechococcus*, as the minor producers (Zavarzin *et al*., 1999). In hypersaline lakes like Magadi it is rather uncertain what organisms are responsible for primary production,

especially in the trona beds that are usually dominated by organotrophic archaea. Cyanobacterial blooms do occur occasionally in the lagoon waters, but only after an unusually wet rainy season has caused substantial dilution of the brine (Foti *et al*., 2008).

The aerobic environment contains prokaryotic groups of considerable phylogenetic diversity, including haloalkaliphilic archaea (Grant *et al*., 1999). Chemoorganotrophic populations are biochemically very active, hydrolyzing many different polymers and producing sugars and amino acids. These may be used as substrates for the fermentation of simple compounds by anaerobic fermentors. Fatty acids produced by anaerobes may be consumed by other groups such as the acetogenic bacteria. Organic material degraded by anaerobic digestion produces substrates for methanogens. The methane produced is oxidized by methane-oxidizers (Sorokin *et al*., 2000).

The sulfur cycle in these lakes utilizes sulfur and sulfate presumably generated by *Ectothiorhodospira* and *Halorhodospiria* species (the link between the carbon and sulfur cycles), and also aerobic sulfur-oxidizers. Sulfur-oxidizing bacteria belong to two groups, those similar to the non-lithotrophic *Halomonas deleya* and those assigned to the novel genus *Thioalcalovibrio* (Sorokin *et al.,* 2001). Sulfate-reducing bacteria then complete the cycle. The nitrogen cycle in these lakes involves the production of ammonia by fermentative anaerobes. Ammonia is utilized by methanotrophs and nitrifiers, producing nitrate. Nitrate, in turn, is utilized by the chemoorganotrophs, creating a link between the nitrogen and carbon cycles (Sorokin *et al*., 2007).

1.2.3 Microbial Diversity of Soda Lakes

Despite the extreme nature of soda lakes environment previous studies on Kenyan soda lakes have shown a rich diversity of microbial communities. However salinity is an important defining factor in the alkaline lakes. There is a distinct difference in microbial community composition between the hypersaline, alkaline lakes such as parts of Lake Magadi with salinity approaching saturation, compared with the more dilute lakes like Nakuru with 5% salt concentration (Zavarzin *et al*., 1999).

Gram positive aerobic isolates of both high G+C and low G+C divisions have been found on the Kenyan soda lakes, with the low G+C isolates forming the largest population. The low G+C were associated mainly with members of the diverse *Bacilli* taxon, especially rRNA group 6 and rRNA group 7 (Duckworth *et al*., 2000). Within the high G+C division of the actinobacteria two alkaliphilic organotrophic isolates from a moderately saline and alkaline East African soda lake (Lake Oloiden) were affiliated with the members of the genus *Dietzia* (Duckworth *et al*., 1998). Other Gram positive high G+C isolates were loosely associated with known species of the genera *Athrobacter* and *Terrabacter* (Duckworth *et al*., 1996).The least halotolerant strains belonging to group XI *Clostridium* spectrum and gamma division proteobacteria were isolated from Lake Bogoria. In the less saline Lake Elementaita archaea belonging to the phylum Euryarchaeota have been isolated and uncultured members of *Microbacteriacea* in the Actinobacteria (Mwirichia *et al*., 2010).

Despite these differences in microbial diversity photosynthetic primary production by cyanobacteria appears to play an important role in the soda lake environment, and presumably supports the rest of the microbial community (Jones *et al*., 1994). In the hypersaline lakes of Magadi-Natron basin it is unclear what organisms are responsible for primary production (Mesbah *et al*., 2007). Different microbial populations have been reported from the hypersaline Lake Magadi, where haloalkalophilic archaea were reported to be dominant. The only other organisms that have been cultivated from this hypersaline environment are haloalkaliphilic *Bacillus* species (Grant, 2006).

It seems probable that some organisms are unique to soda lakes, especially haloalkaliphiles found in the hypersaline lakes such as Lake Magadi. However the diversity of these haloalkaliphiles has not yet been adequately explored and most attention has been paid to few groups of microorganisms.

1.2.4 Polyketide Synthase-I genes (PKS-I)

Type I polyketide synthases are a large highly modular proteins. Polyketide synthase genes for a certain polyketide are usually organized in one operon in bacteria (Ayuso and Genilloud, 2004). Structurally, PKS-I are multifunctional polypeptides encoded by a variable number of modules with multiple enzymatic activities. Each PKS-I module encodes at least three domains corresponding to a ketosynthase, acyltransferase, and acyl carrier protein involved in the selection and condensation of the correct extender unit. They can also include additional enoylreductase, dehydratase, and ketoreductase activities involved in the reduction of the b-keto group formed in the condensation. All

these domains are involved in a programmed synthesis of the new polyketide chain. The substrate specificity of each elongating carbon unit is determined by the AT domain where two main groups of acyl transferases, malonyl-CoA transferases and methyl-malonyl-CoA transferases, can be clearly distinguished at sequence level (Zhao *et al*., 2008).

Type I polyketide synthases (PKS-I) are biosynthetic systems involved in the synthesis of a large number of important biologically active compounds produced by microorganisms, among others by actinobacteria. These structurally diverse metabolites include antibiotics, carotenoids and siderophores.

Actinobacteria produce over two-thirds of clinically useful antibiotics including antibacterial, antifungal and antiparasitic drugs of natural origin. They also produce a wide range of other bioactive compounds such as immunosuppressants (Berdy, 2005). *Streptomyces* is the largest antibiotic producing genus. Among the important antifungals are nystatin from *Streptomyces noursei*, natamycin from *S. natalensis* and amphotericin B from *S. nodosus*. Antibacterials include streptomycin from *S. griseus* and neomycin from *S. fradiae*. *Streptomyces avermitilis* is responsible for the production of one of the most widely employed drugs against nematode and arthropod infestations, ivermectin. Less commonly, S*treptomyces* produce compounds used in non-pathogenic medical treatments. Migrastatin from *S. platensis* and bleomycin from *S. verticillus* are antineoplastic (anticancer) drugs (Miao, 2005). The natural herbicide bialaphos is produced by *S. hygroscopicus* and *S. viridochromeogenes* (Woodyer, 2006).

Carotenoids are important source of vitamin A. They also have been reported to have a considerable anti-carcinogenic effect (Lee and Schmidt-Dannert, 2002). A lycopene – type of carotenoid has been documented from an alkaliphilic actinobacteria *Microbacterium arborescens* isolated from sediments (Godinho and Bhosle, 2008).

Siderophores are low molecular weight compounds that have an affinity for Fe (III) and facilitate in solubilizing iron before transportation into the cell. They have been suggested to have potential applicability in therapeutics, agriculture and environment (Chincholkar *et al*., 2004). Biofilms composed of bacterial aggregates, can cause problems for human health and industry. These biofilms display resistance to conventional control methods such as antibiotics. Hence siderophores are postulated to have the potential to inhibit biofilm formation by making iron unavailable to the biofilm constituents (Budzkiewicz, 2001; Martinez, 2002).

The majority of bioactive metabolites have been isolated from *Streptomyces* species. Nevertheless, microorganisms belonging to the families *Micromonosporaceae, Pseudonocardiaceae, Streptosporangiaceae* and *Thermomonosporaceae* also produce valuable compounds, including erythromycin, gentamicin, rifamycin, teicoplanin and vancomycin (Lazzarini *et al*., 2000). It is not currently known whether the higher number of streptomycete metabolites reflects an increased capacity to produce secondary metabolites, a larger strain diversity, or if it simply depends on a higher number of strains screened for bioactive metabolites. This latter possibility may reflect their ease of isolation in comparison to many other actinobacteria genera. Searching for new microbial

metabolites requires strategies directed at decreasing the probability of identifying known compounds. One approach is to use cultured isolates from extreme habitats such as soda lakes and hot springs. Since the microbial community composition is likely to contain unusual phylogenetically divergent microorganisms with unique adaptations to their habitats, which might be correlated in some cases with synthesis of unusual natural products (Pantham-Aree *et al*., 2006). And besides, the microorganisms living in extreme environments are still largely unknown.

1.2.5 Application of Actinobacteria in Enzymes Production

Many enzymes of microorganisms, including actinobacteria, have found widespread application in industry such as food and detergent industries (Sarethy *et al*., 2011). Important enzymes that have found industrial application include catalases, xylanases, proteases, cellulases and lipases among others.

Proteases constitute a very important group among industrial enzymes. Their global sales amount to the order of 60% of the total enzyme market, of which alkaline proteases from *Streptomyces clavuligenus* constitute 25% (Thumar and Singh, 2009). The enzyme is resistant to detergent components making it suitable for industrial use in commercial detergent formulations. It is also being used in contact lens solutions, cheese production, processing of meat products, and for the recovery of silver from photographic films (Yang, 2010). The usage of these enzymes has also contributed to making production processes more environmentally sustainable by lowering the generation of toxic waste.

For instance in contrast to conventional silver recovery techniques such as burning of films which produces toxic fumes, utilization of the enzymatic process is eco-friendly.

Cellulose, an important component of plant cell walls is made of D-glucose units that are joined by *b*-1,4-glucosidic bonds (Jagta and Rao, 2005). While available as components of agricultural and municipal waste, cellulose is not effectively utilized due to the considerable expenses associated with the conversion processes (Guo *et al*., 2008). Among the microorganisms being utilized for production of cellulases is *Streptomyces gulbargensis* (Dustager *et al*., 2009). Cellulases from this microorganism are also being used in fabrics in a technique known as bio-polishing. Cellulase eliminates the rough cellulose lumps formed on cloth, thereby providing an even finish to the fabric as well as bright colours. A similar finishing effect is produced when the enzyme is included in laundry detergent as an additive (Serethy *et al*., 2011).

Catalase is an anti-oxidant biocatalyst facilitating conversion of hydrogen peroxide to oxygen and water (Brioukhanov *et al*., 2006). Catalases from *Rhodococcus* species have found use in biotechnological applications in food, medical and textile industries (Phucharoen *et al*., 2005). In the food processing industry during microbial fermentation, hydrogen peroxide accumulates as a toxic by-product. To counter this catalase is added to foods stored in closed containers (Sivasankar, 2005). In textile, catalase is added during bleaching of fabrics before dyeing (Amorim *et al*., 2002; Wang *et al*., 2009).

Xylanases are hydrolases that catalyses breakdown xylan found in plants. Xylanases from microorganisms such *Nocardiopsis albus* have found applications in diverse biotechnological platforms (Niwani and Kapadnis, 2003). These applications include pre-bleaching of pulp, conversion of lignocellulosic biomass to serve as source of biofuel, improvement of cereal food products and animal feed-stocks, and degumming of plant fibers (Sanghi *et al.,* 2010).

Commercial lipases are obtained from microorganisms as they are more stable compared to plant or animal lipases and they can be obtained cheaply. Lipases from microbial origin have been obtained from *Micromonospora carbonacea* and *Streptomyces* species (Panchagnula and Terli, 2011). They constitute the most important group of biocatalysts having tremendous biotechnological potential because, firstly, they can be produced in large quantities from microbial sources, secondly, they display exquisite chemo selectivity, regioselectivity and stereo selectivity and thirdly, the crystal structures of many lipases have been solved, facilitating considerably the design of rational engineering strategies. These properties make lipases the most widely used group of biocatalysts in organic chemistry. Important applications of lipases in biotechnology include their supplementation to detergents, in the manufacture of food ingredients, pitch control in the pulp and paper industry, and biocatalysis of stereo selective transformations (Jaeger, 2002).

Bioprospection for novel isolates from unexplored habitats in conjunction with high-throughput screening technologies for specific products having suitability for broad

technological platforms can provide environmentally friendly and cost-effective solutions.

1.3 Statement of the problem

The distribution of aquatic actinobacteria is largely unexplored and the presence of indigenous soda lakes actinobacteria has not been documented. This is partly caused by the lack of effort spent in exploring soda lakes actinobacteria whereas terrestrial actinobacteria have been, until recently, a successful source of novel bioactive metabolites. Furthermore, skepticism regarding the existence of indigenous populations of soda lakes actinobacteria arises from the fact that the terrestrial bacteria produce resistant spores that are known to be transported from land into the lakes where they can remain available but dormant for many years. Thus, it has been frequently assumed that actinobacteria isolated from aquatic samples are merely of terrestrial origin.

Lake Magadi contains almost equal concentration of chloride and carbonate ions in solution due to high evaporation unlike the more dilute soda lakes where carbonate anions dominate. As a consequence different populations of prokaryotes may be found in the concentrated brines. Previous studies on Lake Magadi have shown haloalkaliphilic archaea to be the dominant microbial population. The only other organisms that have been cultivated from this hypersaline environment are haloalkaliphilic *Bacillus* species. No phylogenetic study has been documented on actinobacteria. Indeed, the haloalkaline lake environment is a virtually untapped source of novel actinobacteria diversity and, therefore, of new metabolites.

1.4 Justification

Microbial communities in the soda lakes have attracted attention as a possible source of novel enzymes and metabolites for use in biotechnology. Enzymes from alkaliphiles have long term stability and have been utilized in manufacture of detergent products. Use of microbial based enzymes is environmentally friendly as there is reduced effluent during the industrial processes. Due to the unusual properties of these enzymes they are expected to fill the gap between biological and chemical processes.

Actinobacteria are the most economically and biotechnologically valuable prokaryotes. They are responsible for the production of half of the discovered bioactive secondary metabolites. Few members of the actinobacteria have been studied with reference to alkaline aquatic habitat. Soda lakes samples as a source of bioactive actinobacteria are therefore less exploited. As soda lakes environmental conditions are extremely different from fresh water and terrestrial ones, it is expected that soda lake actinobacteria have different characteristics from those of the less hostile environments, and, therefore, might produce different types of bioactive compounds. The living conditions to which soda lakes actinobacteria have to adapt during evolution include extremely high pH and salinity and also temperatures in hot springs. It is likely that this is reflected in the genetic and metabolic diversity of soda lakes actinobacteria, which remains largely unknown. They should therefore be isolated, characterized and identified for a broader understanding of their diversity and decipher their ecological role and biotechnological

bioprospecting. In the present study, the haloalkaline Lake Magadi was sampled to determine actinobacteria diversity.

1.5 Hypothesis

Lake Magadi harbours diverse actinobacteria isolates which are metabolically active and adapted to life in an alkaline environment.

1.6 Objectives

1.6.1 General Objective

To determine the genetic diversity of actinobacteria isolated from Lake Magadi and bioprospect for production of bioactive compounds.

1.6.2 Specific objectives

1. To isolate pure cultures from Lake Magadi samples.
2. To characterize the isolates using molecular, physiological and biochemical techniques.
3. To screen the isolates for production of extracellular enzymes and their antimicrobial activity.
4. To determine the diversity of polyketide synthase genes among the isolates.

CHAPTER TWO

2.0 MATERIALS AND METHODS

2.1 Sampling site

Lake Magadi (1°52′S 36°16′E; 1.867°S 36.267°E) is the southernmost lake in Kenya and is located in the Rift Valley (Figure 1, top right). The lake is 2000 feet below sea level forming one of the lowest points in the valley. The lake acts as a sink for seasonal streams. Lake Magadi is a highly alkaline aquatic environment with a pH of 8-12 where evaporative concentration results in carbonate as a major dissolved anion. As evaporative concentration continues, chloride ions also dominate in solution. Salinity ranges from 5%w/v salt concentration to saturation in the concentrated salt lagoons (Gierlowski, *et al*., 2004). Lake Magadi is famous for its cherts from sodium silicate precursors. Mineral composition consists mostly of trona (sodium sesquicarbonate) mixed with halite and either kogarkoite or villaumite. The large presence of trona causes the lake water to form a sodium carbonate brine. The high basicity of sodium carbonate makes the lake alkaline (Behr, 2002).

2.2 Description of Sampling Points

Samples were collected in four different locations selected to cover a broad range of sample diversity (Figure 1). The first station (S2° 00.060mins; E36° 13.925mins±3) was a shallow area of the Lake with hot springs. Microbial mats were sampled near the hot springs. Mud was collected from where the Lake had receded. The second station (S01° 57.879mins; E36° 16.594mins±3) was an area where the lake had dried out. Mud and dry soil samples were collected. The third station (S01° 43.802mins; E36° 16.843±3) was

near the river banks inhabited by flamingoes. Sediments were sampled. Dry soil was also collected on the dry areas of the river bank. The fourth station (S01° 53.931mins; E36° 17.614mins±3) was at the salt lagoons where salt pans are concentrated. Salt liquor and crystals were sampled. Three samples were collected at every sampling point and put in 250 ml sterile plastic containers and immediately placed in a cool box with dry ice. Water pH readings were measured in situ using pH meter. Salinities were measured using a hand held salinity refractometer with automatic temperature monitor. The measurements were done in triplicates and average recorded. Working samples were kept at 4°C and long term storage done in a deep-freezer.

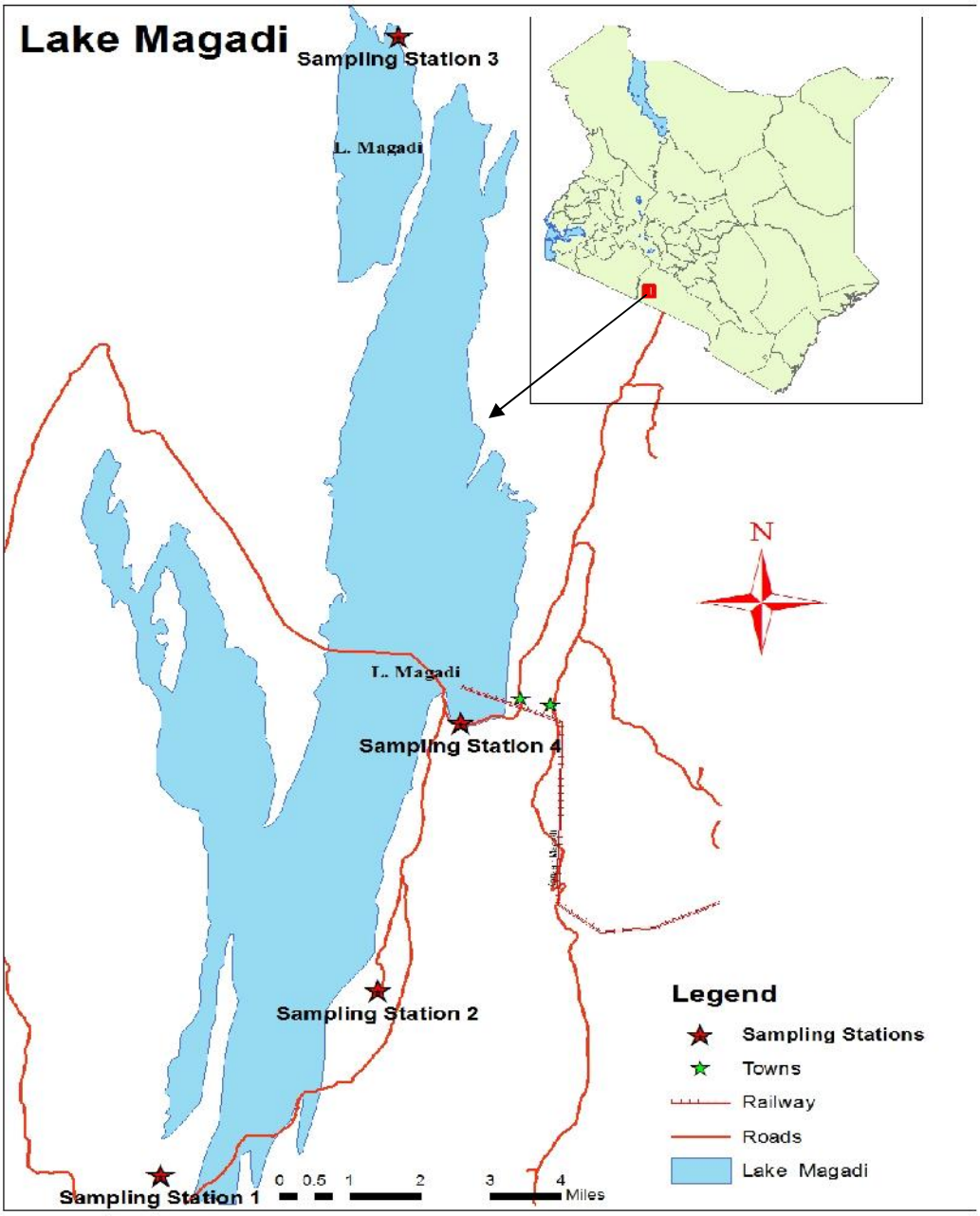

Figure 1: Map of Lake Magadi showing sampling sites: Surface area- 100 km². Top right is map of Kenya showing the location of Lake Magadi.

2.3 Media and Cultivation of Cultures

Isolation of cultures was performed by the serial dilution plate technique using International *Streptomyces* Project (ISP) medium 5 (Isenberg and Garcia, 2007), ISP medium 2 (Murray *et al*., 2007) and chitin agar (Martiny *et al*., 2006). All plates were incubated aerobically at 28ºC for 2-7 days depending on the rate of growth (Panchagnula and Terli, 2011).

2.4 Molecular Characterization of Isolates

2.4.1 DNA Isolation

DNA extraction protocol used was a modification of the method described by Sambrook and Russel (2001). Isolates were sub-cultured in tryptic soy broth medium and 500 µl of the sample pipetted into sterile 1.5 ml Eppendorf tubes then centrifuged at 13 000 rpm for five minutes and the supernatant discarded. The pellet was suspended in 250 µl TE buffer (ph 8.0) to wash away the media, and centrifuged at 13, 000 rpm for five minutes. The supernatant was discarded and the pellet re-suspended in solution A (50 mM Tris pH 8.5, 50 mM EDTA pH 8.0, 25% sucrose solution). 5 µl of lysozyme (20mg/ml) was added and mixed gently, then incubated for one hour at 37ºC. Solution B (10 mM Tris pH 8.5, 5 mM EDTA pH 8.0 and 1% SDS) was added mixed gently and incubated at 65ºC for one hour. The crude sample was then extracted two times with phenol: chloroform (1:1 v/v) centrifuging each time at 13, 000 rpm for fifteen minutes. The clear top layer was pipetted into fresh Eppendorf tubes and extracted two times with chloroform: isoamyl (24:1 v/v) centrifuging each time at 13, 000 rpm for fifteen minutes. The supernatant was then pipetted into fresh tubes and 0.1 volume of 5M sodium chloride and an equal

volume of isopropanol added and stored at -80ºC overnight. After de-freezing the crude sample, it was centrifuged at 13 000 rpm for fifteen minutes. The supernatant was discarded and the pellet washed twice with 70% ethanol for five minutes and centrifuged at 13 000 rpm for 10 minutes. The supernatant was discarded using pipette to get rid of all ethanol. The pellet was air dried for twenty minutes at room temperature and eluted in 50µl pre-warmed TE buffer. The presence of DNA was verified in 0.8 % agarose gel. The concentration of DNA was estimated by spectrophotometry by measurement of the A_{260}/A_{280} ratio. Samples with less than a ratio of 1.8 were re-purified by 70% ethanol precipitation and re-suspending in TE buffer.

2.4.2 Amplification of 16S rRNA Genes

PCR based method was used to screen for actinobacteria using group specific primers described by Monciardini *et al*. (2002). The actinobacterial-specific primers F-Act 243 (5′-GGATGAGCCCGCGGCCTA-3′)/ R-Act A3 (5′-CCAGCCCCACCTTCGAC-3′) and M2F (5′-SAGAAGAAGCGCCGGCC-3′)/ A3R (5′-CCAGCCCCACCTTCGAC-3′) were used to screen all isolates. The second pair of primers was used because 243F/A3R primers may not amplify all actinobacteria groups.

The reactions were performed in a final volume of 50 µl containing; 3 µl of DNA, 5 µl of 10x Genescript *Taq* Buffer, 5 µl of 2.5 mM dNTPs, 0.4 µl of 20 mg/ml BSA, 0.4 µl of 5U Genescript Taq polymerase and 2.5 µl of 20 pmole each primer. Amplification was carried out according to the following profile; 35 cycles of 94 °C for 1 min, 60 °C for 1 min for primers 243F/A3R and 58°C for primers M2F/A3R, 72 °C for 1 min 45 sec. and 72 °C for 10 min. Amplification products were separated on a 2 % agarose gel in 1× TBE

buffer and visualized under ultraviolet by staining with ethidium bromide. The samples were run alongside a molecular mass marker to show the sizes of the fragments.

Negative control used the same master mix but without DNA template. Only isolates that produced amplification were presumed as actinobacteria and were characterized further. Following confirmation of PCR products, successful reactions were cleaned using the QUAquick® PCR purification Kit from Qiagen as was specified by the protocol sent with the kit.

2.4.3 Sequencing and Phylogenetic Analysis

Partial sequences of 16S rDNA were generated at the sequencing facility at ILRI, (BecA-Hub Services, SegoliP) using the primer M13R and M13F. The sequence data were BLAST analysed against the GenBank 16S rRNA gene sequence database in the National Centre for Biotechnology Information (NCBI) website (htt://www.ncbi.nih.gov). The sequences were then aligned using the CLUSTAL W program against the nearest neighbours (Higgins, 1988). Phylogenetic analyses were conducted in MEGA software version 4 (Tamura, *et al*., 2007). The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei 1987). The bootstrap consensus tree inferred from 1000 replicates (Felsenstein, 1985) was taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap tests (1000 replicates) were indicated next to the branches. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood

(Robertson *et al*., 2009) method and in the units of the number of base substitutions per site. The rate variation among sites was modeled with a gamma distribution. The differences in the composition bias among sequences were considered in evolutionary comparisons. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). Phylogenetic analysis was done based on 16S rRNA gene sequences affiliated with actinobacteria.

2.5 Diversity of Polyketide Synthase Genes Type-1(PKS-I)

2.5.1 Amplification of PKS-1 Genes

DNA preparations were used as template DNA for Taq polymerase. Degenerate primers K1F (5′-TSAAGTCSAACATCGGBCA-3′), and M6R (5′-CGCAGGTTSCSGTACCAGTA-3′) were used for amplification (Ayuso-Sacido and Genilloud, 2004). The K1F and M6R primers anneal to the ketosynthase and acyl transferase (methyl-malonyl-CoA for type-I PKS) modules respectively. The size range of the amplified fragments depends on the variable inter-domain region extension.

Reactions were performed in a final volume of 50 µl containing 3 µl of isolates DNA, 5 µl of 10x Genescript *Taq* Buffer, 5 µl of 2.5 mM dNTPs, 0.4 µl of 20 µg/ml BSA, 0.4µl of 5U Genescript Taq polymerase , 2.5 µl of 20 pmole each primer. Amplifications were then performed according to the following profile: 5 min at 95°C and 35 cycles of 1min at 95°C, 1 min at 55°C, 2 min at 72°C and a final extension of 5 min at 72°C. Amplification products were analyzed by electrophoresis in 2% (w/v) agarose gels stained with ethidium bromide. Following confirmation of PCR products, successful

reactions were cleaned using the QUAquick® PCR purification Kit from Qiagen as was specified by the protocol sent with the kit.

2.5.2 Sequence Analysis of Polyketide Synthase Genes

Full sequences of the PCR products were generated using the universal primers M13F and M13R. The sequences were edited and translated with the program ChromasPro© version 1.6 (Technelysium pty ltd). The translated FASTA sequences were compared with protein database in the NCBI GenBank using blastP algorithm. The BLAST results were then tabulated.

2.6 Physiological and Biochemical Characterization

The Gram's stain reaction was carried out following the protocol described by Cappuccino and Sherman (2002). Biochemical characterization was carried to determine enzyme activity and carbohydrate utilization. Physiological tests were carried out to determine optimum temperature, pH and salt concentration.

2.6.1 Growth of Isolates at different pH

Hydrogen potential is one of the important physical factors that influence the growth and survival of microorganisms. The pH of the extracellular environment greatly affects cell's enzymatic activity. This physiological parameter was tested in universal tubes with LB broth media with the pH levels adjusted to 3, 5, 7, 9 and 11, in triplicates. Tubes without inoculates were used as negative control. Incubation was done in a shaking incubator at 100 rpm for three days at 28°C. Turbidity was measured at optical density of 600

nanometers to determine the optimum pH for growth (Sorokin and Kuenen, 2005). The average of the readings was calculated and results tabulated as mean ± standard deviation.

2.6.2 Determination of optimum Growth Temperature

Temperature influences the rate of chemical reactions through its action on cellular enzymes. The isolates were inoculated in universal tubes with LB broth media in triplicates and incubated at 20°C, 30°C, 40°C, 50°C, and 60°C. Incubation was done in a shaking incubator at 100 rpm for three days. Tubes without inoculums were used as negative control. Turbidity was measured at optical density of 600 nanometers (Nazina *et al*., 2001). The average of the readings was calculated and results tabulated as mean ± standard deviation.

2.6.3 Determination of Sodium Chloride Requirement

Salt requirement by isolates was determined by using various concentration of sodium chloride in media. LB broth media in universal tubes were supplemented with 0, 5, 10, 15, 20 and 30 %w/v sodium chloride. The inoculation was done in triplicates. Tubes without inoculums were used as negative control. After three days of incubation at 28°C in shaking incubator at 100 rpm the highest concentration of salt that allows growth was recorded. Measure of growth was taken at OD_{600} (Sorokin and Kuenen, 2005). The average of the readings was calculated and results tabulated as mean ± standard deviation.

2.6.4 Heat Resistance

Tolerance to heat was done following the methods of Schaal *et al*. (2006). The isolates in LB broth were pippeted into sterile 2 ml eppendorf tubes and placed in a heating block with temperature adjusted to 100°C. They were then heated for ten, twenty and thirty minutes. After cooling they were streaked on malt-yeast extract agar and incubated at 28°C for 2-3 days. Presence and absence of growth was recorded. Those which were able to grow were heat resistant.

2.6.5 Lysozyme Resistance

Lysozyme resistance was performed following the method of Cappuccino and Sherman (2002). Gram positive cells are in principle sensitive to lysozyme. Because of additional layers like teiconic acids or modifications of the peptidoglycan molecule some Gram-positive bacteria got resistant against lysozyme. The lysozyme stock solution consisted of 100 mg lysozyme in 100ml 0.1N HCL. The isolates were inoculated in glycerol broth containing 10% lysozyme stock solution. The control did not contain lysozyme. The isolates which were able to grow in lysozyme broth were considered lysozyme resistant.

2.6.6 Utilization of Carbohydrates

The utilization of carbon sources plays an important role in species differentiation of bacteria and likewise also for actinobacteria .The ability of strains to use carbohydrate compounds was tested in ISP medium 9 (Schaal *et al*., 2006). The following carbon sources were used: Arabinose, Sucrose, Xylose, Inositol, Mannitol, and Fructose. 100ml of the carbohydrate solutions were sterilized by filtration and added to the basal medium after autoclaving and cooling to 60°C. The tubes with glucose served as positive control

and the tubes with pure basal medium and water as negative control. The valuation was carried out after the following scheme: Growth not better than the negative control as (-) Growth like the positive control (+) and growth better than negative control but not as good as positive (±).

2.6.7 Nitrate reduction

Tryptic soy broth containing 1% potassium nitrate was supplemented with 4% NaCl and 1% Na_2CO_3 according to the protocol of Harold (2002). After incubation at 28°C for 48hrs 0.2ml of acetic acid sulfanilic acid and 0.2ml of dimethyl-alpha-naphthylamine reagent were added. Those that produced a cherry red coloration indicated positive results while those that did not were negative.

2.6.8 Urea Hydrolysis

Urea hydrolysis was performed following the method of Cappuccino and Sherman (2002) to test presence of urease. Urease is a hydrolytic enzyme that attacks the nitrogen and carbon bond in amide compounds such as urea to form alkaline end product ammonia. Inoculation was done in urea broth containing the pH indicator phenol red. Basal medium consisted of KH_2PO_4 9.1 g/l, Na_2HPO_4 9.5 g/l, Yeast extract 0.1 g/l Phenol red 0.01 g/l and urea 2% w/v. pH was adjusted to 6.8. Presence of the end product ammonia creates an alkaline environment that causes phenol to turn to a deep pink. This is a positive reaction for the presence of urease. Failure of this colour to develop indicated absence of urease.

2.6.9 Catalase Test

Catalase enzyme degrades hydrogen peroxide to produce oxygen and water. Catalase production was determined by adding hydrogen peroxide to incubated trypticase soy agar slant cultures (Cappuccino and Sherman, 2002). Presence of catalase was deduced by production of bubbles indicating free oxygen gas.

2.6.10 Gelatine Hydrolysis

Gelatine is a protein produced by hydrolysis of collagen. Below temperatures of 25°C, gelatin maintains its gel properties and exists as a solid. At a higher temperature gelatin is a liquid. Liquefaction is also accomplished by microorganisms capable of producing gelatinase. Once this degradation occurs, even low temperatures of 4°C will not restore the gel characteristics. The medium consisted of glucose and peptone supplemented with 12% gelatin. This high concentration results in a stiff medium and also serves as the substrate for the activity gelatinase. Following inoculation and incubation for forty eight hours, the cultures were placed in a refrigerator at 4°C for 30 min. Cultures that remained liquefied produced gelatinase and demonstrated rapid gelatin hydrolysis (Harold, 2002).

2.7 Test for Production of Extra-Cellular Enzymes

Isolates were tested for production of exo-enzymes such as lipase, xylanase, cellulase, amylase and protease. Basal media supplemented with starch, xylan, cellulose, skimmed milk, and olive oil was used. Incubation was done at 28°C for three days.

2.7.1 Test for Amylase.

Starch hydrolysis was done using the method of Castro (1993). Starch is a high molecular weight polymer and is hydrolyzed by the enzyme amylase. Starch agar was used to determine the hydrolytic activities of the exo-enzyme. The detection of the hydrolytic activity following growth period was made by performing the starch test to determine the presence or absence of starch in the medium. Starch in the presence of iodine imparts a blue-black colour to the medium, indicating the absence of the enzyme. When starch has been hydrolyzed a clear zone of hydrolysis surrounds the colonies. This is a positive result.

2.7.2 Test for Protease

Casein is a large protein found in milk. This test was conducted on casein agar which is a complex media containing skimmed milk and agar (10g skim milk powder/90ml distilled water, 3g agar/97ml distilled water). Following inoculation and incubation, hydrolysis was demonstrated by clear zones around and/or below the colonies (Lee *et al*., 2005).

2.7.3 Test for Lipase

Olive oil (source of natural triglyceride, triolein) was used as substrate. Lipase positive bacteria break down triglyceride into fatty acid and glycerol. 1% of the substrate was added to the basal media which consisted of 1% potassium di-hydrogen phosphate, 0.01% magnesium sulfate, 0.005% calcium chloride, 4% sodium chloride, and 1% sodium carbonate. After incubating for three days observations were made. The media was observed for zones of precipitation of calcium crystals around each isolate. Positive

isolates for lipase production were indicated by the precipitation of calcium crystals around the colonies (Lee and Lee, 1997).

2.7.4 Test for Xylanase

Xylanase is the name given to a class of enzymes which degrade the linear polysaccharide beta-1,4-xylan into xylose, thus breaking down hemicellulose, one of the major components of plant cell walls. Xylan was used as substrate. 1% of the substrate was added to the basal media which consisted of 1% potassium di-hydrogen phosphate, 0.01% magnesium sulphate, 0.005% calcium chloride, 4% sodium chloride, and 1% sodium carbonate. Observation was made after an incubation period of three days. Xylan degradation was demonstrated by clear halos around the colonies after flooding the culture plates with 1% Congo red dye then rinsing with distilled water (Lee and Lee, 1997).

2.7.5 Test for Cellulase

The major component in the rigid cell walls in plants is cellulose. Cellulose is a linear polysaccharide polymer with many glucose monosaccharide units. Cellulase refers to a suite of enzymes produced chiefly by microorganisms that catalyze cellulolysis, that is the hydrolysis of cellulose. Cellulase production was determined using basal media containing 1% potassium di-hydrogen phosphate, 0.01% magnesium sulphate, 0.005% calcium chloride, 4% sodium chloride, and 1% sodium carbonate. 1% cellulose was used as substrate. Observation was made after an incubation period of three days. Cellulose degradation was demonstrated by clear halos around the colonies after flooding the

culture plates with 1% Congo red dye then rinsing with distilled water (Lee and Lee, 1997).

2.8 Antimicrobial Activity

A cell based technique was employed to screen the isolates for antimicrobial activity against both Gram-positive and Gram-negative microorganisms. The test organisms were *Streptococcus pyogenes* ATCC 12385, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 10273, *Staphylococcus aureus* NCTC 25923, and *Pseudomonas aeruginosa* ATCC 27853. The isolates were cultured in production media broth which consisted of glucose 0.5g/l, glycerol 2.5ml/l, oatmeal 5g/l, soybean meal 5g/l, yeast extract 0.5g/l, casaminoacids 2g/l, and calcium carbonate 2g/l. They were incubated in shaking incubator (100 rpm) for seven days. The pH was kept at neutral (pH 7). The broth cultures were then centrifuged to spin down cells and supernatant membrane filtered. The filtrate was then assayed for antimicrobial activity using sterile paper discs. The test organisms which were standardized at optical density 0.1 were plated on Muller Hinton agar and the paper discs containing 1µl of the filtrate placed after air drying for 30 minutes in a safety cabinet. The preparations were then incubated at 37°C for 48 hours. The diameters of the zones of complete inhibition were measured, including the diameter of the discs. Zones were measured to the nearest millimeter using a ruler on the back of the inverted petri-plates (Fatope *et al.*, 2000).

CHAPTER THREE

3.0 RESULTS

3.1 Physical Parameters at the Sampling Stations

Sampling was done at four different points. Measurements of the physical parameters of the sampling stations were done in triplicates and average calculated. Sampling sites parameters are as shown in table 1. Temperature ranged from a low 37°C to the high of 85°C in the hot springs. Salt lagoon (trona) had the highest salt concentration. pH measurements showed Lake Magadi to be an alkaline environment, with the pH readings ranging from 8.6 to 10.3.

Table 1: Physical parameters of sampling sites in Lake Magadi

Sampling station	Temperature (°C)	Sodium chloride concentration (ppt)	pH
1	45.0	152	9.44
2	37.6	50	8.65
3	85.6	159	9.43
4	47.5	305	10.34

3.2 Isolation of Cultures

Morphological characteristics of the isolates showed them to be diverse in form and pigmentation (Plate 1).

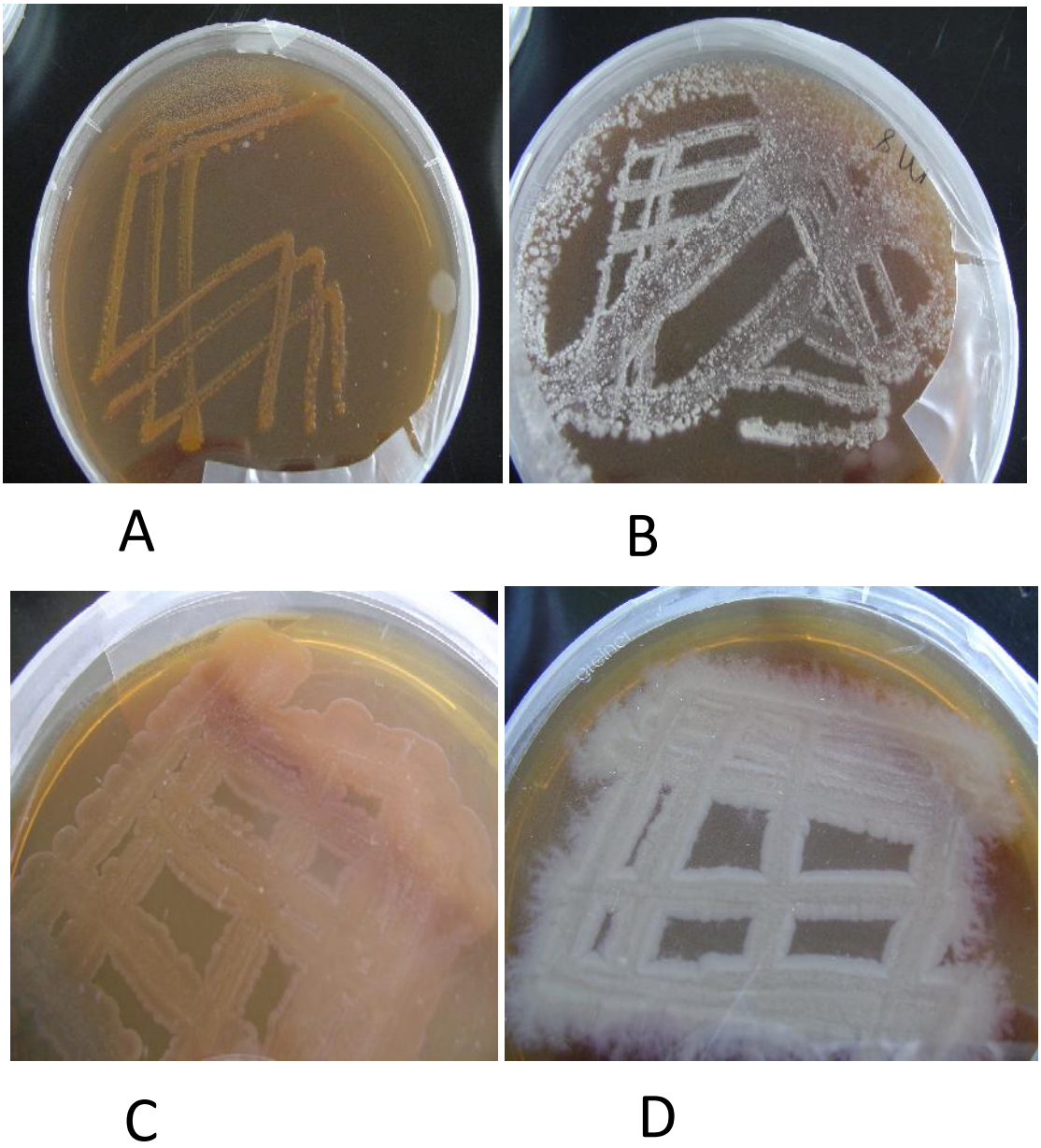

Plate 1: Isolates form Lake Magadi cultured on malt-yeast extract agar. Picture A is isolate MS-3, B is isolate MS-2, C isolate MS-11, and D isolate MSL-7.

The isolates were coded according to the sample type they were isolated from. Isolates cultured from sediment samples were coded S, those from microbial mats were coded M,

and SL for salt liquor. All isolates have code M at the beginning to describe the source of the samples-Magadi (Table 2).

Table 2: Morphological and Cell Form Characterization of Isolates from Lake Magadi

Isolate ID	Colour	Form	Margin	Elevation	Cell form	Gram reaction
MS-1	Cream	Irregular	Filamentous	Raised	Rods	Positive
MS-2	White	Circular	Entire	Flat	Rods	Positive
MS-3	Orange	Circular	Serrated	Raised	Rods	Positive
MS-4	White	Irregular	Serrated	Flat	Rods	Positive
MS-5	White	Circular	Serrated	Raised	Rods	Positive
MS-6	Grey	Irregular	Serrated	Flat	Rods	Positive
MSL-7	Grey	Irregular	Filamentous	Flat	Rods	Positive
MM-8	Pink	Circular	Serrated	Raised	Cocci	Positive
MM-9	Grey	Circular	Entire	Flat	Rods	Positive
MM-10	White	Irregular	Filamentous	Flat	Rods	Positive
MS-11	Orange	Lichenoid	Undulate	Flat	Rods	positive

3.3 Molecular Characterization of Isolates

Group specific primers for actinobacteria were used for amplification. Primer 243F/A3R yielded four amplifications from isolates MS-2, MS-3, MS-5, and MS- 11. The rest of the isolates amplified with the primer pair M2F/A3R. A molecular fragment of about 1200bp and 1500bp respectively was obtained (Plate 2).

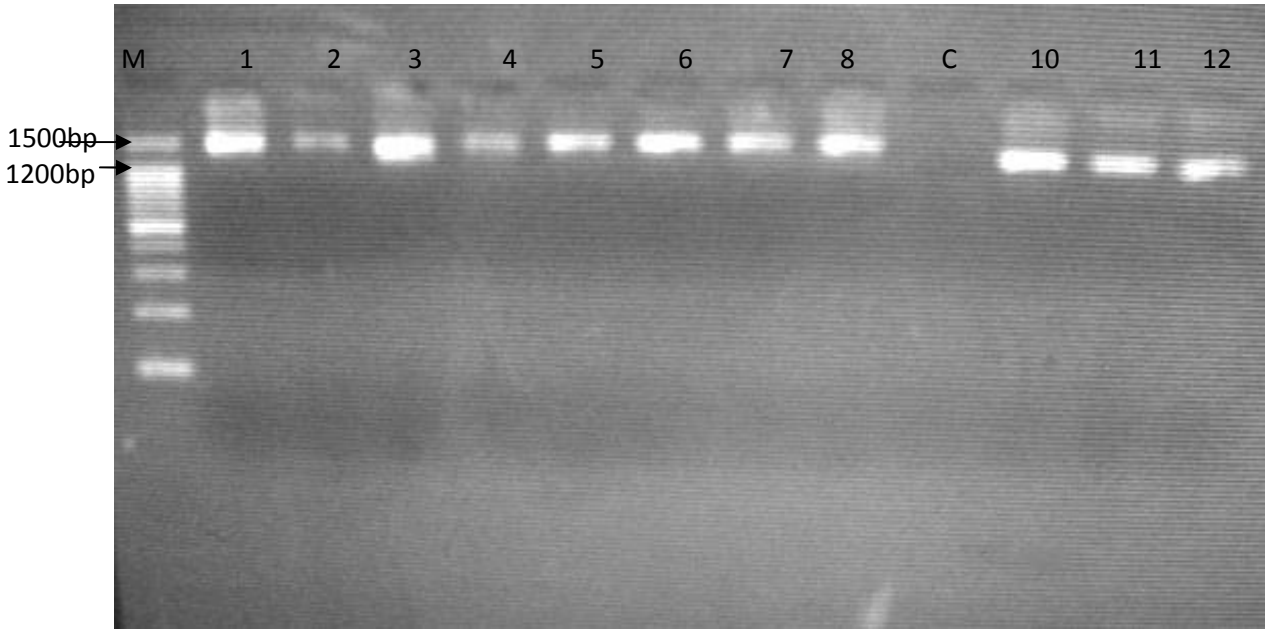

Plate 2: Amplification of 16S rDNA from isolates from Lake Magadi. Lane 1 contain PCR product from DNA of isolate MS-1, 2: isolate MS-4, 3: isolate MS-6, 4: isolate MSL-7, 5: isolate MM-8, 6: isolate MM-9, 7: isolate MM-10, 8: isolate MS-11, C is negative control, 10: isolate MS-2, 11: isolate MS-3 and 12: isolate MS-5. M; Molecular ladder**.**

3.4 Phylogenetic Analysis of 16S rRNA Gene Sequences

The BLAST results showed that about 45% of the isolates belonged to the phylum actinobateria, with 97-98% similarity to their close relatives (Table 3). The high G+C isolates were affiliated to the genera *Dietzia, Rhodococcus, streptomyces, Norcadia,* and

Microbacterium. This was supported by high bootstrap values in the phylogenetic analysis (Figures 2-5). Isolate MS-11 had a similarity of 97% and could represent a novel species. About 40% of the isolates were affiliated with the family *Bacillaceae* with 98-99% similarity. They include *Bacillus clausii, B. halodurans,* and *B. pumilus.* Isolate MS-6 had a 100% similarity to *Clostridium spp.*

Table 3: BLAST Results of Eleven Isolates from Lake Magadi and their Close Relatives

Isolate ID	Nearest neighbor of partial 16s rDNA sequence	GenBank accession number	% Similarity
MS-1	*Bacillus clausii*	FN397454	99
MS-2	*Dietzia maris*	X79290	98
MS-3	*Microbacterium lacus*	AB286030	98
MS-4	*Bacillus halodurans*	HM484173	99
MS-5	*Rhodococcus erythropolis*	JF327477	98
MS-6	*Clostridium venatiosis*	EU089966	100
MSL-7	*Bacillus pumilus*	JQ319539	99
MM-8	*Stenotrophomonas spp*	JF711012	99
MM-9	*Bacillus amylolyticus*	EU308313	99
MM-10	*Streptomyces albixiallis*	AY999901	98
MS-11	*Norcadia cerradoensis*	AF060790	97

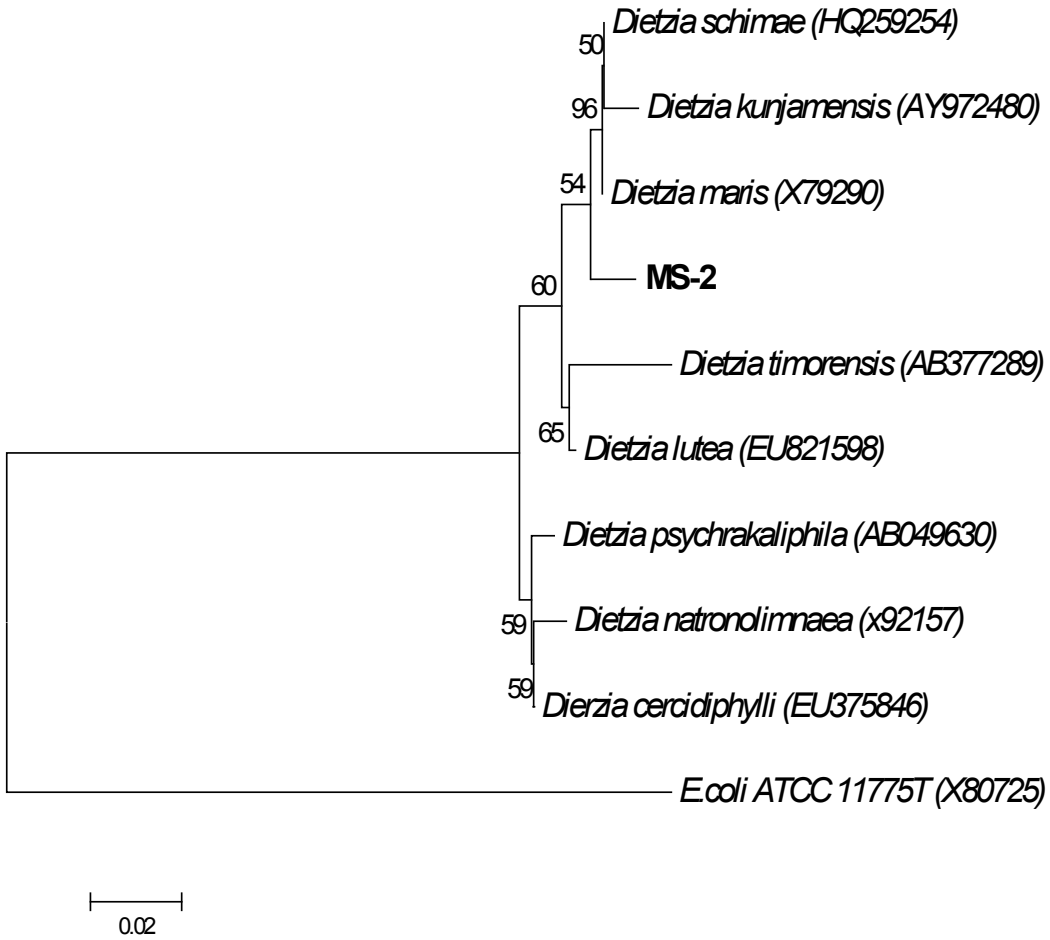

Figure 2: Neighbor joining tree derived from 16S rRNA gene sequences from *Dietziaceae*. Numbers at branch nodes refer to bootstrap values. Only values above 40% significance are indicated. The scale bar represents the expected number of changes per homologous nucleotide position. The tree was rooted using *E. coli* 16S rDNA as outgroup.

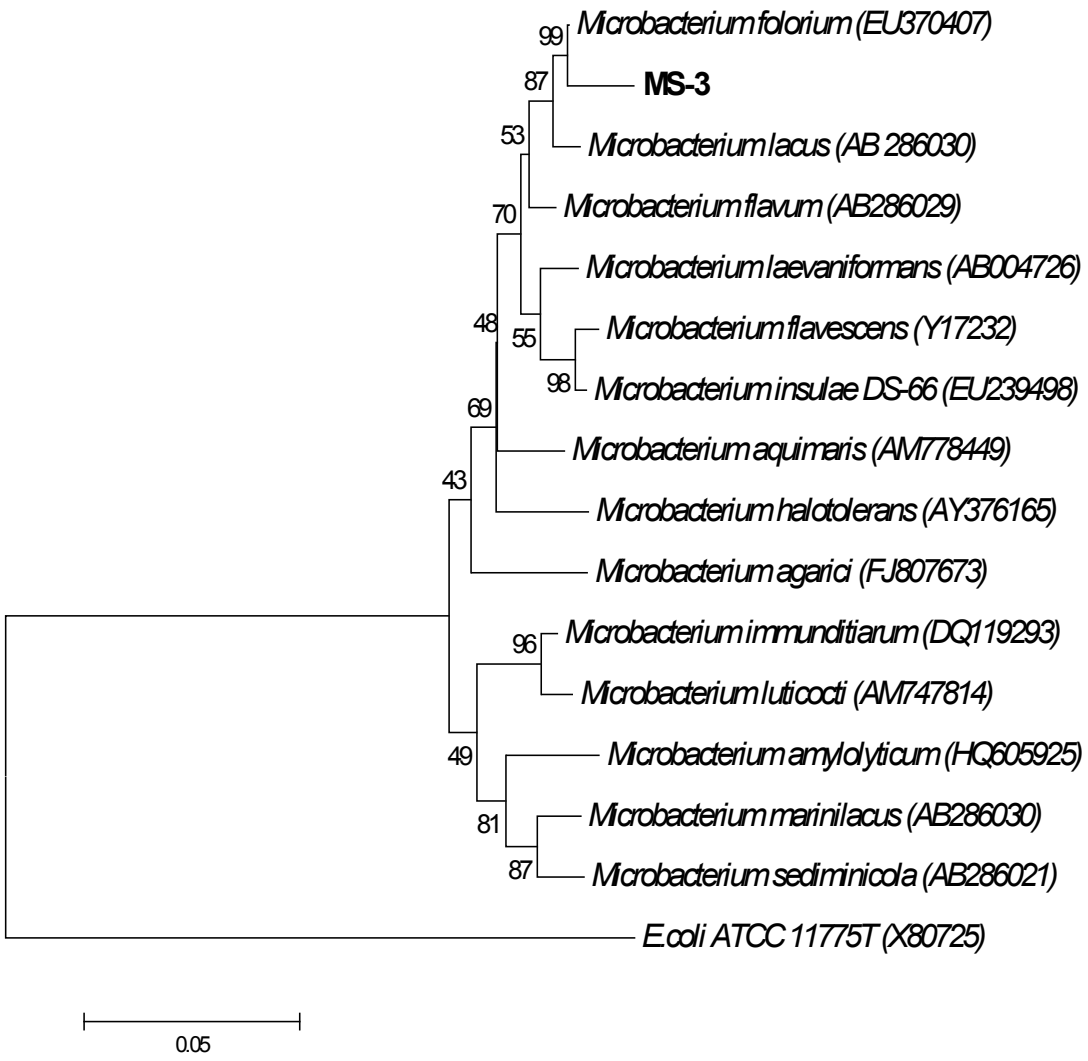

Figure 3: Neighbor joining tree derived from 16S rRNA gene sequences from *Microbacteriaceae.* Numbers at branch nodes refer to bootstrap values. Only values above 40% significance are indicated. The scale bar represents the expected number of changes per homologous nucleotide position. The tree was rooted using *E. coli* 16S rDNA as outgroup.

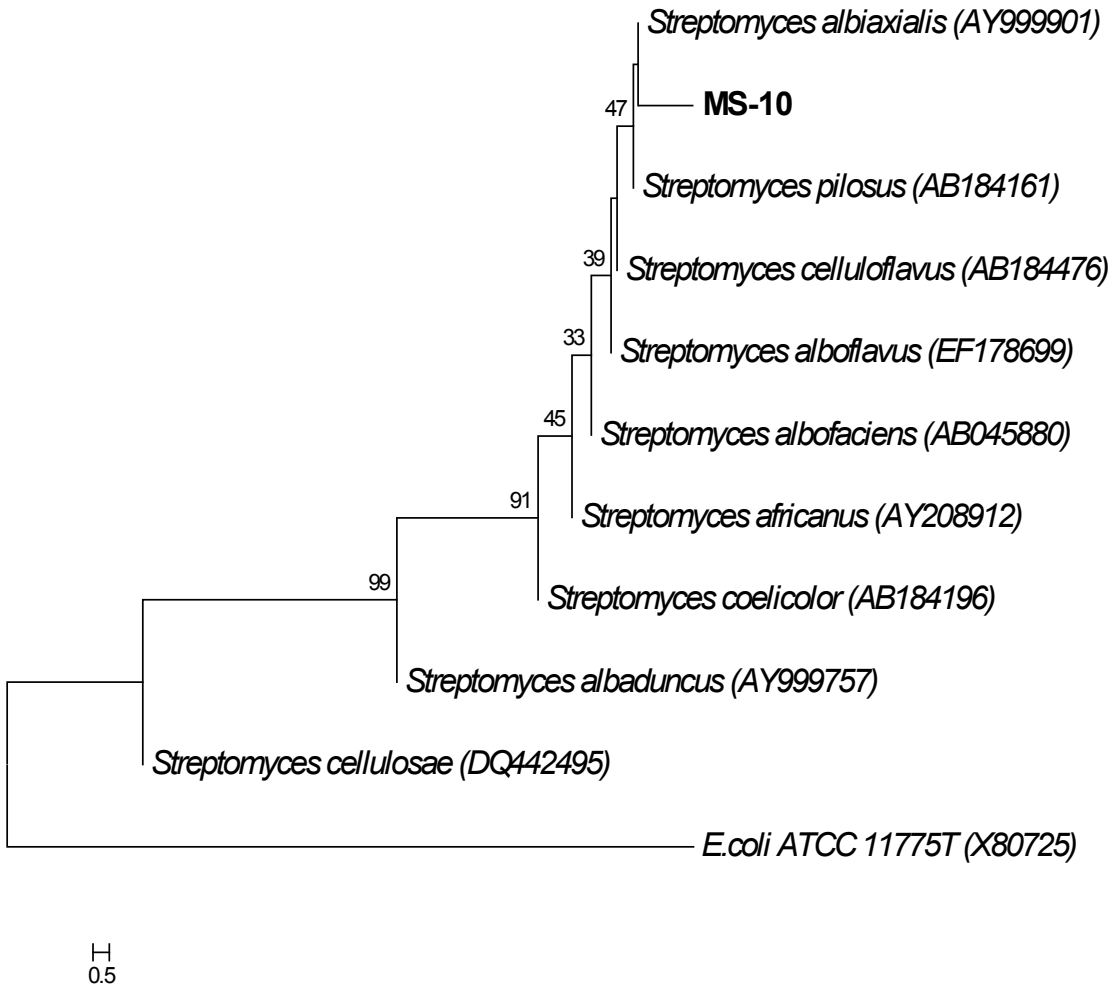

Figure 4: Neighbor joining tree derived from 16S rRNA gene sequences from *Streptomycetacea* rooted using *E. coli* 16S rDNA as outgroup. Numbers at branch nodes refer to bootstrap values. Only values above 30% significance are indicated. The scale bar represents the expected number of changes per homologous nucleotide position.

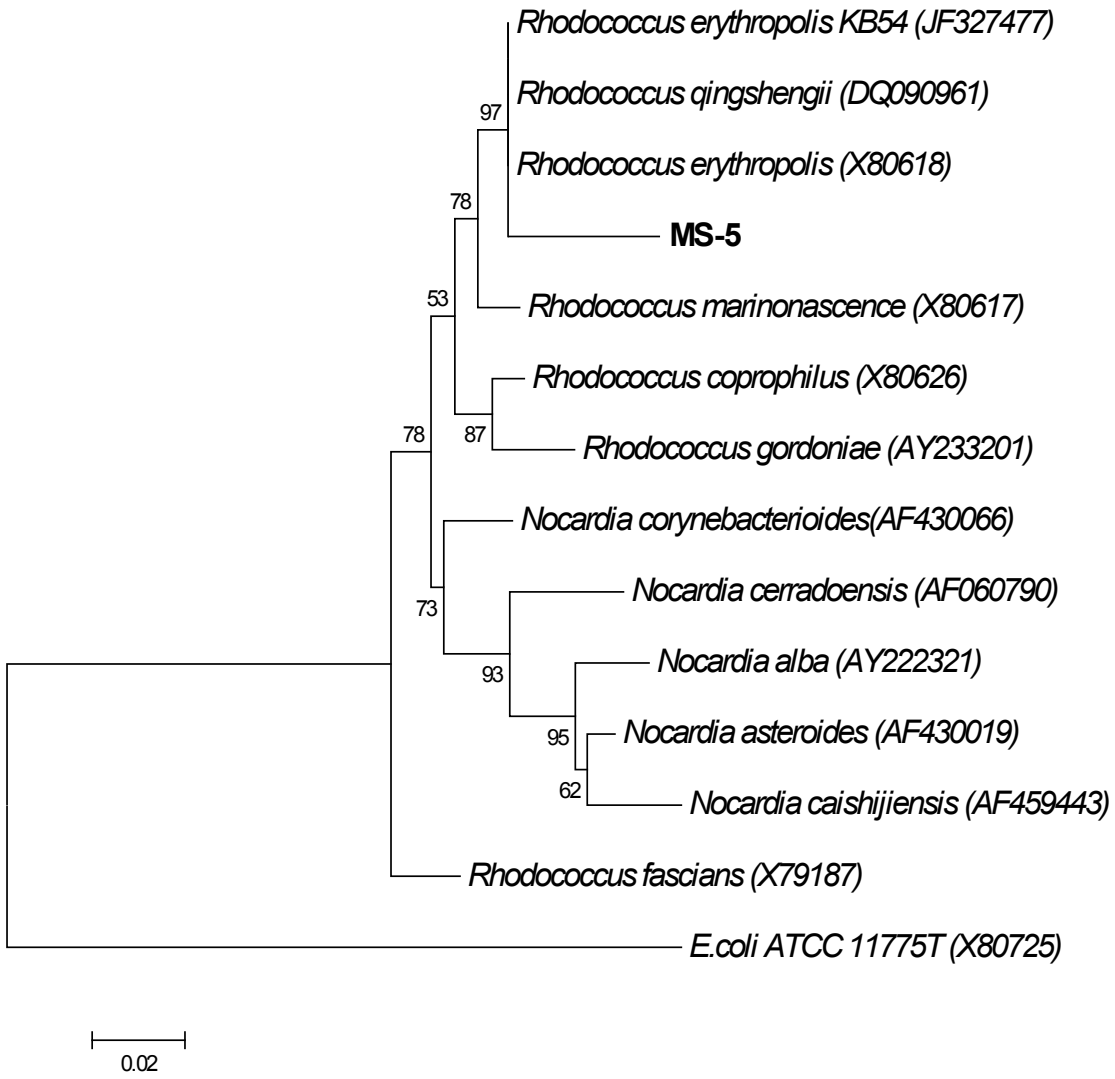

Figure 5: Neighbor joining tree derived from 16S rRNA gene sequences from *Nocardiaceae* rooted using *E. coli* 16S rDNA as outgroup. Numbers at branch nodes refer to bootstrap values. Only values above 40% significance are indicated. The scale bar represents the expected number of changes per homologous nucleotide position.

3.5 Amplification of Polyketide Synthase Genes

Six isolates gave an amplification product with the K1F/M6R specific primers for PKS-1 ketosynthase and methyl-malonyl-CoA transferase domains (Plate 3). An expected fragment of about 600 bp was obtained.

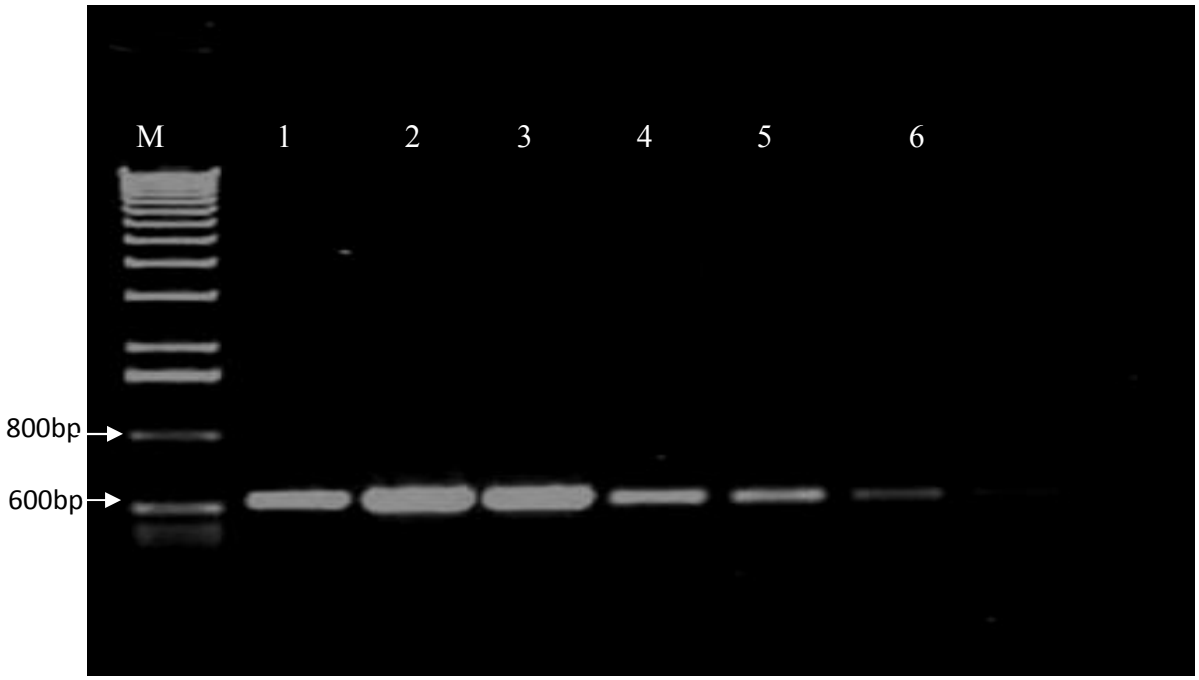

Plate 3: Amplification of polyketide synthase genes from isolates from Lake Magadi. Lanes 1-6 contain PCR products from DNA of isolates MS-1, MS-2, MS-5, MM-9, MM-10 and MS-11 respectively. M- Molecular marker.

3.6 Polyketide Synthase Genes Sequence Analysis

The protein algorithm BLAST results showed sequence homology to peptide and transferase families of enzymes (Table 4). All the obtained fragments had below 80% similarity to referenced sequences from the GenBank database at AA level.

Table 4: BLAST Results of Polyketide Synthase Gene Sequences using Protein Algorithm

Isolate ID	Protein database homology	% similarity
MS-1	Glycosyl transferase	56
MS-2	Adamalysin (Peptidase M10A)	48
MS-5	Matrixin (M12B)	48
MM-9	Prolyl oligopeptidase	58
MM-10	Antibiotic biosynthesis monooxygenase	58
MS-11	Dihydropteroate synthase	52

3.7 Physiological Characterization

3.7.1 Growth of Isolates at Different pH Levels

Growth of the isolates was possible up to pH 11 (Table 5). Isolates MS-2, MS-5, MSL-7 and MM-9 had their optimum growth at pH 9. Isolates MS-1 MS-3, MS-4 and MM-8 grew optimally at pH 7. Isolates MM-10 and MS-11 had optimum growth at pH 11.

Table 5: Turbidity at OD_{600} of Isolates from Lake Magadi Grown at Different pH Levels

Isolate ID	PH 3	PH 5	PH 7	PH 9	PH 11
MS-1	0.066±0.005	1.253±0.08	1.780±0.004	1.303±0.004	1.266±0.004
MS-2	0.213±0.003	1.357±0.008	1.392±0.001	1.399±0.001	1.047±0.002
MS-3	0.108±0.002	0.735±0.005	0.774±0.004	0.159±0.001	0.118±0.004
MS-4	0.087±0.003	1.251±0.009	1.355±0.002	1.294±0.003	1.213±0.002
MS-5	0.142±0.003	0.927±0.003	0.963±0.04	1.421±0.003	0.974±0.002
MS-6	0.147±0.005	1.026±0.007	0.889±0.001	0.841±0.003	0.108±0.003
MSL-7	0.058±0.003	0.400±0.002	0.489±0.005	1.434±0.004	1.403±0.002
MM-8	0.080±0.002	0.642±0.003	0.866±0.002	0.822±0.003	0.344±0.003
MM-9	0.164±0.003	0.334±0.001	0.804±0.002	1.447±0.003	1.025±0.002
MM-10	0.068±0.003	0.231±0.007	0.232±0.001	0.590±0	1.080±0.002
MS-11	0.122±0.004	0.705±0.005	0.706±0.005	0.945±0.003	1.113±0.003

M ± SD

3.7.2 Determination of Sodium Chloride Requirement

Growth was observed between 0 -20% sodium chloride concentrations for most isolates (Table 6). Isolates MS-4, MM-10 and MS-11 had their optimum growth in media without sodium chloride, with the highest tolerable concentration at 5%. Isolate MS-6 could grow up to concentration of 10% with its optimum growth at 0%. Isolates MS-1, MS-2, MS-3, MSL-7, MM-8, and MM-9 had their optimum growth at 5% sodium chloride concentration.

Table 6: Turbidity at OD_{600} of Isolates from Lake Magadi Grown at Different Sodium Chloride Concentrations

Sample ID	0%	5%	10%	20%	30%
MS-1	0.636±0.002	0.731±0.002	0.687±0.002	0±0	0±0
MS-2	0.759±0.006	0.910±0.002	0.302±0.003	0±0	0±0
MS-3	0.717±0.004	0.742±0.003	0.272±0.001	0±0	0±0
MS-4	0.874±0.004	0.791±0.003	0±0	0±0	0±0
MS-5	0.647±0.002	0.813±0.002	0.655±0.002	0±0	0±0
MS-6	1.111±0.003	1.104±0.001	0.586±0.003	0±0	0±0
MSL-7	0.594±0.003	1.275±0.002	0.162±0.004	0.018±0.005	0±0
MM-8	0.594±0.003	0.726±0.005	0.118±0.002	0.022±0.002	0±0
MM-9	0.891±0.002	1.000±0.06	0.056±0.004	0.028±0.004	0±0
MM-10	0.827±0.005	0.729±0.003	0±0	0±0	0±0
MS-11	0.822±0.004	0.710±0.003	0.6±0.003	0.081±0.001	0.069±0.002

M ± SD

3.7.3 Determination of Optimum Growth Temperature

All the isolates had a temperature preference of between 20°C and 40°C (Table 7). Isolates MS-2, MS-3, MS-4, MS-5 had their optimum growth at 30°C. The rest of the isolates had optimum growth at 40°C.

Table 7: Turbidity at OD_{600} of Isolates from Lake Magadi Incubated at Different Temperatures

Sample ID	20°C	30°C	40°C	50°C	60°C
MS-1	0.548±0.004	0.640±0.002	0.841±0.001	0.063±0.003	0.001±0.001
MS-2	0.504±0.002	0.570±0.002	0.406±0.003	0.081±0.001	0.002±0
MS-3	0.361±0.003	0.839±0.002	0.503±0.003	0.044±0.002	0.002±0.002
MS-4	0.761±0.004	1.144±0.002	0.897±0.002	0.207±0.003	0.017±0.002
MS-5	0.342±0.003	1.185±0.004	0.519±0.003	0.044±0.003	0.006±0
MS-6	0.510±0.003	0.702±0.004	1.399±0.002	0.041±0.004	0.006±0.003
MSL-7	0.936±0.003	0.944±0.002	1.192±0.002	0.141±0.002	0.106±0.003
MM-8	0.441±0.002	0.521±0.003	0.642±0.003	0.064±0.003	0.058±0.002
MM-9	0.601±0.002	0.654±0.002	0.859±0.002	0.094±0.003	0.073±0.003
MM-10	0.363±0.002	0.461±0.004	0.708±0.003	0.044±0.001	0.041±0.003
MS-11	0.261±0.002	0.550±0.004	0.707±0.002	0.133±0.003	0.131±0.002

M ± SD

3.8 Biochemical Tests

Carbohydrate utilization test showed that all isolates were able to utilize fructose. Isolates MS-1, MS-2, MS-4, and MSL-7 were able to utilize all the carbohydrate substrates tested. Isolates MS-5 and MS-9 were able to utilize arabinose, xylose, and fructose, and minimal growth on mannitol. Apart from fructose isolate MS-3 was able to utilize sucrose and arabinose. Isolate MS-11 was able to utilize all the carbohydrates tested except innositol, with minimal growth observed with xylose and mannitol. Isolates MM-8 and MM-10 tested positive for arabinose, xylose, fructose, and sucrose utilization. Isolate MS-6 was able to utilize arabinose, xylose and fructose (Table 8).

All the isolates except MM-8 were catalase positive. Eight of the eleven isolates were able to reduce nitrate to nitrite. None of the isolates was able to hydrolyze urea, this shows they were all urease negative. Isolates MS-2, MS-4, MM-8 and MM-9 could hydrolyze tyrosine and therefore was tyrosinase positive. Isolate MM-8 was the only isolate that tested positive for gelatinase. All the isolates except isolates MS-2 and MS-4 were able to grow in presence of 100 mg lysozyme (Table 8).

Heat is lethal to microorganisms, but each species has its own particular heat tolerance. When treated in dry heat at 100°C for thirty minutes then streaked on agar plates, isolates MS-1, MS-3, MS-4, MS-6, and MM-9 were able to grow. Isolates MS-2 and MS-5 were able to grow when heated for twenty minutes. Isolates MS-11 could grow only when heated for ten minutes. Isolates MSL-7, MS-8, and MM-10 were killed by the heat treatment and did not grow when streaked on agar plates (Table 8).

Table 8: Biochemical Tests of Isolates from Lake Magadi

Isolate ID	MS-1	MS-2	MS-3	MS-4	MS-5	MS-6	MSL-7	MM-8	MM-9	MM-10	MS-11
Nitrate reduction	+	+	+	+	+	-	+	-	+	+	-
Catalase production	+	+	+	+	+	+	+	-	+	+	+
Urease production	-	-	-	-	-	-	-	-	-	-	-
Gelatinase production	-	-	-	-	-	-	-	+	-	-	-
Lysozyme resistance	+	-	+	-	+	+	+	+	+	+	+
Tyrosinase production	-	+	-	+	-	-	-	+	+	-	-
Carbohydrates utilization;											
Arabinose	+	+	+	+	+	+	+	+	+	±	+
Xylose	+	+	-	+	+	±	+	+	±	±	±
Inositol	+	±	-	+	-	-	+	-	-	-	-
Mannitol	+	+	-	+	±	-	+	-	±	-	±
Fructose	+	+	+	+	+	+	+	+	+	+	+
Sucrose	+	+	+	+	-		+	+	-	+	+
Heat resistance (100°C)											
10mins	+	+	+	+	+	+	-	-	+	-	+
20mins	+	+	+	+	+	+	-	-	+	-	-
30mins	+	-	+	+	-	+	-	-	+	-	-

Hydrolysis tests; + ≡ hydrolysis, - ≡ no hydrolysis

Utilization test; + ≡ growth, - ≡ no growth, ± ≡ minimal growth

3.9 Bio-polymers Hydrolysis

Four isolates, MS-1, MS-2, MS-6, and MM-9 tested positive for amylase, lipase, cellulase, xylanase, and protease enzymes (Table 9). Isolate MS-11 did not hydrolyze any of the biopolymers. Isolate MS-3 could only hydrolyze skim milk and tested negative for the other substrates. Isolate MM-10 hydrolysed only starch. Isolates MS-4, MS-5 and MS-8 hydrolyzed starch and skim milk. Isolate MSL-7 was able to hydrolyze starch, olive oil, cellulose, and skim milk, but not xylan. Plates 4 and 5 show some of the isolate that tested positive for production of extracellular enzymes.

Table 9: Production of Extra-Cellular Enzymes by Isolates from Lake Magadi

Sample ID	Amylase	Lipase	Cellulase	Xylanase	Protease
MS-1	+	+	+	+	+
MS-2	+	+	+	+	+
MS-3	-	-	-	-	+
MS-4	+	-	-	-	+
MS-5	+	-	-	-	+
MS-6	+	+	+	+	+
MSL-7	+	+	+	-	+
MM-8	+	-	-	-	+
MM-9	+	+	+	+	+
MM-10	+	-	-	-	-
MS-11	-	-	-	-	-

+ ≡ hydrolysis; - ≡ no hydrolysis

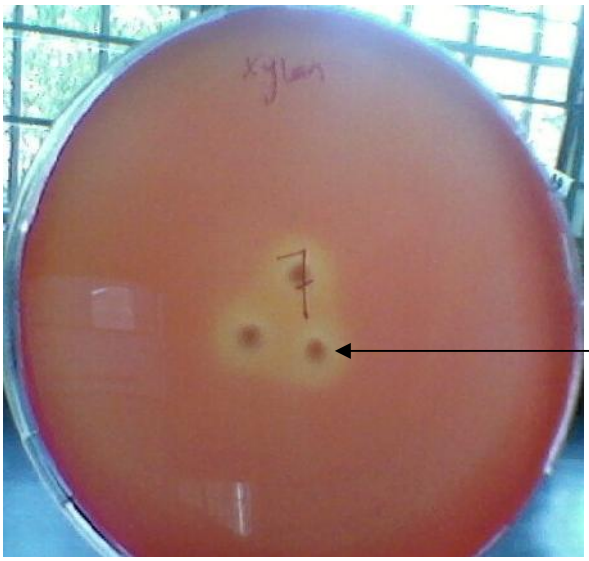

Plate 4: Xylan hydrolysis by isolate MS-2

Plate 5: Skim milk hydrolysis by isolate MS-5

3.10 Antimicrobial Activity

Both Gram positive and Gram negative bacteria were used to test antagonistic activity (Table 10). All isolates had varying degree of growth inhibition against different organisms (Plates 6 and 7). *Staphylococcus aureus* was the least susceptible being inhibited by only three isolates. Isolate MM-9 showed growth inhibition against all the bacterial test organisms showing a greater inhibition zone against *Pseudomonas aeruginosa.* Isolate MS-11 had the highest activity against *Streptococcus pyogenes*. It was able to inhibit all the bacterial test organisms except *P. aeruginosa*. All isolates except MM-8 showed antagonistic activity against *Escherichia coli*. *Streptococcus pyogenes* and *E. coli* were the most susceptible being inhibited by all except two isolates.

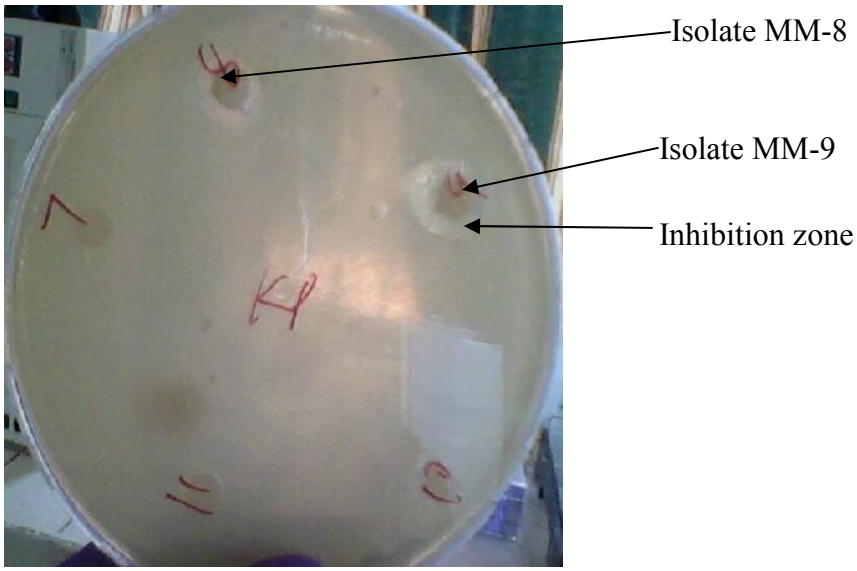

Plate 6: Inhibition zones by isolates on *K.pneumoniae*

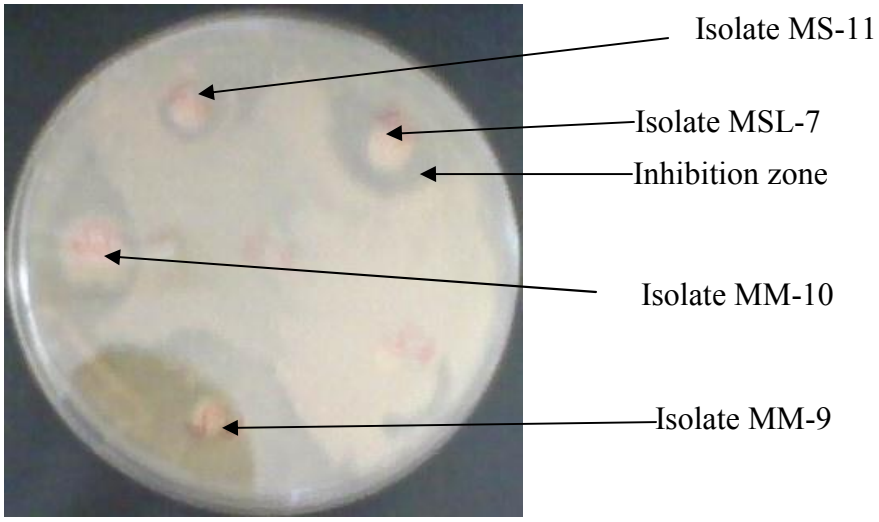

Plate 7: Inhibition zones by isolates on *E. coli*

Table 10: Antagonistic Activity of Isolates from Lake Magadi (cm)

Isolate ID	*Streptococcus pyogenes*	*Escherichia coli*	*Klebsiela pneumoniae*	*Staphylococcus aureus*	*Pseudomonas aeruginosa*
MS-1	1.0±0.07	0.7±0	1.3±0.14	0±0	0±0
MS-2	1.4±0.07	1.1±0.14	0±0	0±0	0±0
MS-3	0±0	1.3±0	0.9±0	0±0	0±0
MS-4	1.85±0.07	1.55±0.07	0±0	0±0	0.8±0.07
MS-5	0±0	0.9±0	0±0	0±0	1.55±0.14
MS-6	1.95±0.07	1.0±0.07	0±0	0±0	1.45±0.07
MSL-7	1.3±0	1.4±0.14	0±0	1.2±0.07	1.3±0.14
MM-8	1.5±0.14	0±0	0.8±0.07	0±0	1.7±0
MM-9	0.9±0	1.45±0.14	0.9±0	1.1±0	1.6±0
MM-10	1.0±0.07	1.35±0.14	0±0	0±0	1.1±0.07
MS-11	2.3±0.14	1.1±0.14	0±0	1.3±0	0±0

CHAPTER FOUR

4.0 DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

4.1 Discussion

Eleven isolates produced amplification with primers known to be specific for actinobacteria. Phylogenetic analysis of 16S rRNA gene sequences showed five isolates to be in the phylum actinobacteria and the rest of the isolates were affiliated to *Bacillus*. This shows that group specific primers may amplify non-actinobacteria targets. In agreement with these findings, Song *et al*. (2009) also reported some degree of non-specificity of the primers. Isolates MS-2, MS-3, MS-5, MM-10 and MS-11 were affiliated to *Dietzia*, *Microbacterium, Rhodococcus, Streptomyces* and *Nocardia* genera respectively. Previous studies have documented actinobacteria isolates from other Kenyan soda lakes. Members of the genera *Arthrobacter* and *Terrabacter* (Duckworth *et al*., 1996) have been described from Lake Oloiden. Others include *Bogoriella caseilytica* (Groth *et al*., 1997) *Dietzia natronolimnaea* (Duckworth *et al*., 1998) and *Cellulomonas bogoriensis* (Jones *et al*., 2005) from Lake Bogoria.

Isolate MS-2 had a 98% similarity to *Dietzia maris* in the family *Dietziaceae,* phylum actinobacteria. *Dietzia* is aerobic, mycolic acid-containing actinobacteria that lack aerial mycelium (Rainey, 1995). The genera *Dietzia* have been found in multiple habitats both terrestrial and aquatic. The isolate under study was able to degrade xylan, starch, cellulose, olive oil, and skim milk. It grew optimally at 5% NaCl concentration, temperature of 30°C and a pH of 9. It was able to produce tyrosinase and good growth was attained with arabinose, xylose, mannitol, fructose and sucrose. It clustered together with *Dietzia maris* (X79290) and *Dietzia schimae* (HQ259254) both from alkaline soil.

Physiological and biochemical tests results showed the isolate to be more similar those documented for *D. natronolimnea* (X92157) (Duckworth *et al*., 1998). The isolate and the reference strain *D. natronolimnea* were both cultivated from soda lake samples.

Isolate MS-3 had 98% similarity to *Microbacterium lacus* (AB286030) in the family *Microbactericeae,* phylum actinobacteria. *Microbacteria* are non-spore forming Gram positive rods found in a wide range of environments (Kageyama *et al*., 2007). Growth was possible up to 10% salt concentration with an optimum growth at 5%. The isolate grew optimally at 30°C and was alkalitolerant. Phylogenetic analysis grouped it together with *M. folorium, M. lacus* and *M. flavum* cultured from marine samples (Kageyama *et al*., 2007). The reference strains documented lower values for optimal physiological requirements than what was observed for isolate MS-3. This could be associated with the original source of the isolates. Marine environments are more acidic (Lam, 2006), whereas soda lakes are alkaline due to buffering action of carbonate ions.

Isolate MS-5 had a 98% similarity to *Rhodococcus erythropolis* (JF327477). *Rhodococcus* is a genus of aerobic, nonsporulating, nonmotile Gram positive bacteria closely related to *Mycobacteria* and *Corynebacteria* (Van der Geize and Dijkhuizen, 2004). While a few species are pathogenic, most are benign and have been found to thrive in a broad range of environments, including soil, water, and eukaryptic cells. Fully sequenced in October 2006, *Rhodococcus* genome is known to be 9.7 mega-base pairs long and 67% G/C content (McLeod *et al*., 2006). An important use of *Rhodococcus* species is in bioremediation. This is borne out of their ability to metabolize harmful environmental pollutants, such as toluene, naphthalene and herbicides. They are

able to transform these compounds into their less toxic intermediaries (Manzoo, 2011). The reference strain *R. erythropolis* cultured from alkaline desert soil (Yoon *et al*., 1997) documented similar optimum physiological requirements as those which were observed for isolate MS-5. The isolate in this study grew optimally at 30°C and 5% salt requirement. Growth was possible up to pH 11 with an optimum at pH 9. This implies that environmental conditions may influence phenotypic characteristic of microorganisms.

Isolate MM-10 had 98% similarity to *Streptomyces albixiallis* (AY999901). The isolate grew optimally in alkaline media of pH 9. Growth was possible up to 60°C with an optimum growth at 40°C and in media without salt. Members of this genus are spore-forming and can therefore survive in extreme environments like polluted soils. They are characterized by the tough, leathery frequently pigmented colonies and their filamentous growth (Zhuh *et al*., 2011). They are capable of using complex organic materials and are involved in the breakdown of the products in the soil. This degrading ability makes these bacteria pivotal in production of fertile soil (Farris *et al*., 2011). The genus *Streptomyces* is known for production of a vast array of secondary metabolites, including antibiotics (Sahin and Ugur, 2003). Most isolates that have been utilized for production of secondary metabolites have been screened from soil, and thus *Streptomyces* have always been associated with soil.

Isolate MS-11 had 97% similarity to *Nocardia* and could represent a new species. They are Gram positive rods which form orange colonies. Most species are found as saprophytes in water, soil and decaying vegetation while others are responsible for

nocardiosis which occur as an opportunistic infection (Kiska *et al*., 2002). The isolate in was able to grow in a wide range of pH but optimally at pH 11 and media without salt. However growth was possible up to 30% salt concentration. This could be an adaptation to salt fluctuation within the lake where dilution occurs when it rains. The isolate was able to grow when incubated in temperatures up to 60°C with optimum growth at 40°C. *Nocardia* species are known to grow in a wide range of temperature and can easily out-compete other microbial communities in decaying organic matter when temperature increases (Conville *et al*., 2000).

The sequence results of the poliketide synthases included peptidases and transferases families of proteins. The polyketide synthases among the isolates revealed specificity with no overlap among any of the enzymes produced. This indicates that a suite of secondary metabolites is dependent on the type of strain producing it. This observation conforms to a model of selection-driven pathway fixation, thus implying that secondary metabolites have important functions in the survival of the producing strain. Six of the eleven isolates were positive for polyketide synthase genes. Lack of amplification may be an indication of the absence of the PKS-1 system or occurrence of methyl-malonyl-CoA transferase domain with less conserved sequences and therefore lower homology with the primers.

The sequence homology of PKS-1 gene from the isolate MS-2 belonged to adamalysin family. This is a family of peptide proteins (Wolfsberg *et al*., 1995). Isolate MM-10 was positive for antibiotic biosynthesis monooxygenase. This domain found in monooxygenases is involved in the biosynthesis of several antibiotics (Sciara *et al*., 2003). The other gene sequence homology was M12B which belong to the matrixin

group of proteins. Sequences having this domain are extracellular metalloproteases which degrade components of the extracellular matrix (Edwards *et al*., 2008). Transferases are used in biomodification of antibiotics (Sciara *et al*., 2003). The obtained fragments exhibited low similarity to sequences in the GenBank closest neighbours. None of the sequences showed higher than 80% identity to GenBank sequences. This may imply their chemical and functional novelty. It may also indicate that the isolates with these domains have not yet been analyzed for their PKS genes pathways (Zhao, 2008).

The pH range of growth was found to be between 5 and 11. Six of the isolates grew optimally at pH above 8 and were therefore alkaliphiles. Five isolates were alkalitolerant and grew optimally between pH 7 and 8. This could be attributed to their adaptation to alkaline environment in the lake where the pH was found to be between 8 and 10. The isolates are therefore good prospects for production of alkaline stable enzymes which are important in industrial bioprocesses such as detergent manufacture. Isolates that have been cultivated from Kenya soda lakes were found to grow optimally at pH 9 ((Jones *et al*., 2005; Groth *et al*., 1997). Isolate MS-6 grew optimally at pH 5 far below that of the source of origin. It might have existed as a spore in the environment and resumed vegetative growth after being introduced to favourable conditions.

Seven isolates grew optimally at 40°C and four at 30°C. Temperatures from the sampling sites ranged from 37°C to a high of 85°C in the hot springs. In such thermobiotic alkaline environment suitable conditions may exist in micro-niches for short periods of time and require microorganisms to have a fast growth response (Oarga, 2009). It has also been demonstrated that in response to high temperatures microorganisms accumulate

compatible solutes such as mannosylglycerase which is highly efficient in the protection of enzymes against thermo inactivation. These molecules could play a key role in thermo-protection of cell components *in vivo* (Rossi *et al*., 2002).

The isolates were able to grow at sodium chloride concentration of up to 20%, with optimum growth at 5% for five isolates and in media without salt for four isolates. This wide range of salt tolerance could be an adaptation to salt fluctuation within the lake when there is dilution due to rain and high concentration as a result of increased rates of evaporation. Moderate halophiles are solute-tolerant and are able to grow in a wide range of solute concentration. This is in contrast to extreme halophiles which lyse the moment the salt concentration in their environment drops below 10 to 15% (Kamekura, 1998).

Phenotypic characteristics in terms of utilization of a range of carbon sources varied across the isolates. Carbohydrate utilization systems are of particular importance to provide carbon and energy to bacteria. The ability to use different carbon sources is important in actinobacteria identification (Schaal *et al*., 2006). Non-fermentation method was used in this study, whereby bacteria were able to utilize carbohydrates without an increase in hydrogen-ion concentration; this means no change in pH. Glucose is the preferred carbon source for actinobacteria and was used as a positive control. Abundant growth was attained with arabinose, xylose and fructose. Other studies on carbohydrate utilization by actinobacteria have documented the same observations (Panchagnula and Terli, 2011). However minimal growth was attained with inositol and mannitol. The differences in utilization may be attributed to the different biochemical pathways used for metabolism due to differing structures of the carbohydrates. Disaccharides such as

sucrose and fructose are abundant in the environment, and most microorganisms posses catalytic enzymes that enable them to metabolize the carbohydrate.

Isolates MS-2, MS-4, MM-8 and MM-9 exhibited tyrosinase reaction. Tyrosinase is responsible in melanin biosynthesis. Nine isolates were lysozyme resistant. Gram positive bacteria are in principle lysozyme sensitive but resistance may be achieved by additional layers like teiconic acids or modifications of the peptidoglycan molecule (Schaal *et al*., 2006). Melanin production and resistance to lysozyme are important defense mechanisms and survival of the microorganisms. Eight isolates were able to reduce nitrate to nitrite. Nitrate reduction plays an important role in nitrogen cycle within the lake ecosystem. Chemoorganotrophs utilize nitrate creating a link between nitrogen and carbon cycles (Duckworth *et al*., 2000).

Isolates in the study were able to produce hydrolytic enzymes that were able to degrade biopolymers. Isolates MS-1, MS-2, MS-6, MSL-7 and MM-9 were able to hydrolyze all the substrates tested and were therefore positive for amylase, lipase, cellulase, xylanase and protease. The degradability is important in nature since the complex biopolymers are hydrolyzed to simpler forms like sugars and amino acids which can be utilized by other microorganisms, and therefore playing an important role in nutrient cycling. This attribute can be harnessed to solve environmental issues such as bioremediation and in biotechnology for industrial bio-processes (Serethy *et al*., 2011).

Antagonistic activity results showed that the isolates were active against both Gram negative and Gram positive bacteria. The widest activity and largest inhibition zone was shown by isolate MS-11. It was able to inhibit *Streptococcus pyogenes, Escherichia coli,*

Klebsiela pneumoniae and *Staphylococcus aureus* with the largest inhibition zone of 2.3 cm against *S. pyogenes*. The antimicrobial activity of the isolates is probably due to an antibacterial complex active against prokaryotic organisms. Antibiotics derived from actinobacteria which are in use are active against both Gram positive and Gram negative bacteria (Nediakova and Naidenova, 2005).

As might be expected considering the nutrient rich habitat that they inhabit, the soda lake isolates are biochemically active. Besides hydrolyzing proteins and polymeric carbohydrates, they utilize a very wide range of sugars, organic and amino acids. The hypothesis is therefore true that isolates from Lake Magadi are metabolically active and adapted to life in an alkaline environment.

4.2 Conclusions

Lake Magadi harbours diverse actinobacteria isolates. Isolates MS-2, MS-3, MS-5, MM-10 and MS-11 were affiliated with *Dietzia, Microbacteria, Rhodococcus, Streptomyces* and *Nocardia* genera respectively.

FASTA sequences analysis of 16S rRNA genes showed high similarity values of 97% to 99% to established genera. This was supported by their relationships with their GenBank relatives in the inferred phylogenetic trees. Six isolates were alkaliphiles and five were alkalitolerants. This shows that isolates from Lake Magadi can be utilized for production of alkaline stable enzymes, which are of industrial importance for example as detergent additives. All the eleven isolates were able to grow in a wide range of temperature up to

60°C with optimum growth at 40°C and 30 °C. This ability to withstand a wide temperature range is important in biodegradation. Eight of the eleven isolates grew optimally at a salt concentration of 5% w/v which is high for most bacteria. This makes them easy to culture from other microorganisms.

Biochemical tests showed that the isolates could utilize various sources of carbon and nitrate reduction. This shows they play an important ecological role in nutrient cycles in soda lake environment. Reduction of nitrate to nitrite is important in nitrogen cycle. All isolates were able to hydrolyze biopolymers by producing enzymes such as xylanase, amylase, protease, cellulase and lipase. These enzymes have various uses in biotechnology and as biocatalysts in industrial processes. Hydrolysis of complex substrates in the environment into simpler components makes them available to other microorganisms. All isolates showed antimicrobial activity against both Gram positive and Gram negative bacteria. They can be used as sources of antibiotics which affect both Gram positive and Gram negative pathogenic bacteria.

Six isolates were positive for type-1 polyketide synthase genes responsible for synthesis of secondary metabolites. All the obtained domains were below 80% similarity to GenBank sequences. This may imply their chemical and functional novelty.

4.3 Recommendations

New techniques should be developed to capture new taxa which may not be cultured using the conventional media.

Further research on the alkaliphiles should be done for biotechnological bioprospecting.

More studies should be done to investigate different parameters of enzyme production in order to establish optimum conditions for their production.

More research should be done to establish chemical structures and functional properties of the novel polyketide synthase genes.

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APPENDICES

APPENDIX A: ISOLATION MEDIA

A-1: International *Streptomyces* Project Medium 2 (ISP medium 2) (per liter)

Yeast extract 1.0 g

Malt extract 1.0 g

Glucose 1.0 g

Peptone 1.0g

Glycerol 1ml

Agar 20.0 g

Adjust ph as desired.

Dispensed into containers and sterilized by autoclaving at 121°C for 15 minutes.

A-2: Starch Casein Agar

Approximate Formula* Per Liter

Soluble Starch..... 0.5 g

Dipotassium hydrogen Phosphate..... 0.5g

Sodium proprionate…..... 0.5 g

Casein……..... 0.3 g

Asparagine……..... 0.1 g

Potassium nitrate…..... 0.1g

Ferrous Sulfate..... 1.0 mg

Vitamin B solution…… 1ml

Agar..... …20.0 g

A-3: Chitin Agar

Chitin 1.0 g

Dipotassium hydrogen phosphate 0.5 g

Ferrous sulphate 0.01 g

Vitamin B solution 1ml

Agar 15.0 g

Adjust ph as desired

100X vitamins

Thiamine hydrochloride 0.5 g

Riboflavin 0.5 g

Niacin 0.5 g

Pyridoxin 0.5 g

Inositol 0.5 g

Calcium pantothenate 0.5 g

p-Aminobenzoic acid 0.5 g

Biotin 0.25 g

Suspended the vitamins in 10 ml of distilled water and sterilized by 0.22 µm filtration and kept at 4°C.

APPENDIX B: MEDIA FOR PHYSIOLOGICAL TESTS: LB BROTH (/Litre)

10 g Sodium Chloride

10 g Tryptone

5 g Yeast extract

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

APPENDIX C: BASAL MEDIA FOR EXTRACELLULAR ENZYMES PRODUCTION TESTS

1 % KH_2PO_4

0.01 % $MgSO_4.7H_2O$

0.005 % $CaCl_2.2H_2O$

4 % NaCl

1 % Na_2CO_3

Supplemented with 1% % xylan, 1 % cellulose, 1 % starch, 1 % Skim milk 1 % olive oil.

APPENDIX D: BIOCHEMICAL TEST MEDIA

D-1: International *Streptomyces* Project Medium 9 (ISP Medium 9) (per liter)

$K_2HPO_4 \cdot 3H_2O$ 5.65 g

$(NH_4)_2SO_4$ 2.64 g

KH_2PO_4 2.38 g

$MgSO_4 \cdot 7H_2O$ 1.0 g

Carbohydrate solution 100.0 ml

Pridham and Gottlieb trace salts 1.0 ml

pH 6.8-7.0

Carbohydrate solution:

Composition per 100 ml:

carbohydrate 10.0 g

Preparation of carbohydrate solution:

Add carbohydrate to distilled water and bring volume to 100.0 ml. Use glucose, arabinose, sucrose, xylose, inositol, mannitol, fructose, rhamnose, raffinose, or cellulose. Mix thoroughly. Filter sterile.

Pridham and Gottlieb trace salts:

Composition per 100 ml:

$MnCl_2 \cdot 7H_2O$ 0.79 g

$CuSO_4 \cdot 5H_2O$ 0.64 g

$ZnSO_4 \cdot 7H_2O$ 0.15 g

$FeSO_4 \cdot 7H_2O$ 0.11 g

Preparation of medium:

Add components, except carbohydrate solution, to distilled water and bring volume to 900 ml. Mix thoroughly. Gently heat and bring to boiling with frequent agitation. Autoclave at 121°C for 15 minutes. Cool to 45°-50°C. Aseptically add sterile carbohydrate solution. Mix thoroughly. Aseptically distribute into sterile tubes or flasks.

D-2: Urea degradation

Basal medium

KH_2PO_4 9.1 g

Na_2HPO_4 9.5 g

Yeast extract 0.1 g

Phenol red 0.01 g

Distilled water 1000 ml

pH 6.8

Autoclave at 121°C for 15 minutes.

Urea solution in distilled water was filter sterile using cellulose acetate membrane filters (pore size 0.45 m) then added to the basal media to give a final concentration of 2.0% (w/v). Three ml amounts were aseptically dispensed into sterile tubes.

D-2: Tyrosine Agar

Formulation:

Basal medium, sterile melted 100 ml

Tyrosine 0.5 g

Distilled water 10 ml

Preparation:

(1) Prepare a sterile tyrosine solution by adding the tyrosine to the distilled water and filter sterilizing through a 0.22 u filter.

(2) Allow the sterile melted basal medium to cool almost to solidification

(3)Add 10 ml of the sterile tyrosine solution to the flask or bottle of basal medium. Mix.

(4) Aseptically dispense aliquots into sterile Petri plates

Basal Medium Tyrosine and Agar

Formulation:

Beef extract 3.0 g

Peptone 5.0 g

Agar 20.0 g

Distilled water 1000.0 ml

Preparation:

(1) Add beef extract, peptone, agar and water together in a flask.

(2) Heat the solution until the ingredients are dissolved.

(3) Dispense 100 ml aliquots into 250 ml flasks or bottles.

(4) Sterilize by autoclaving for 10 minutes at 121 C

(5) Hold the medium at 50 °C in a water bath until ready to add tyrosine solution.

(6) The basal medium can also be stored at 4°C and later re-melted for use

D-3: Lysozyme Broth

Formulation:

(1) Basal glycerol

(2) broth Beef extract 3 g

(3) Peptone 5 g

(4) Glycerol 70 ml

(5) Distilled water 1000 ml

Lysozyme solution

Lysozyme 100 mg

0.01 N HCl 100 ml

Preparation:

(1) Prepare the basal glycerol broth by adding the ingredients together and mixing.

(2) Dispense 500 ml of the broth of 5 ml aliquots into screw cap tubes.

(3) Sterilize the tubes and flask of remaining broth by authoclaving for 15 minutes at 121 C

(4) Prepare the lysozyme solution by adding the lysozyme and 0.01 N HCl together.

(5) Sterilize by filtration through 0.22 µ filter.

(6) Prepare the lysozyme broth by adding 5 ml of the sterile lysozyme solution to 95 ml of the sterile basal glycerol broth. Mix well.

(7) Aseptically dispense 5 ml aliquots into sterile screw cap tubes.

APPENDIX E: DNA EXTRACTION REAGENTS

- Solution 1
  - 50 mM Tris pH 8.5
  - 50 mM EDTA pH 8.0
  - 25 % Sucrose solution

- Solution 2
  - 10 mM Tris pH 8.5
  - 5 mM EDTA pH 8.0
  - 1 % SDS
- Lysozyme 20 mg/ml

- RNase A 20 mg/ml
- Proteinase K 20 mg/ml
- Phenol/chloroform/isoamyl (24:24:1)
- Isopropanol
- 3 M NaCl
- 70% ethanol

APPENDIX F: BUFFERS

F-1: TE/100ml

1ml of 1M tris-HCl

2ml of 0.5 EDTA

F-2: TAE (X50)

Tris 242g

Glacial acetic 57.1ml

0.5M EDTA

F-3: TBE (X10)

Tris 108g

Boric acid 55g

Na_2EDTA 9.3g

Ethidium Bromide l0 ×

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