

**Isolation and Characterization of Fungi from Lake Magadi of the
Kenyan Rift Valley**

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**A thesis submitted in partial fulfilment for the degree of Master of
Science in Biotechnology in the Jomo Kenyatta University of
Agriculture and Technology**

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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 work to my family. My dear parents; Mrs. Wilfrida Tero and the late Ernest Tero Lihanda, beloved husband, Fred Manoa Salano; my daughter Debbie and sons; Beckenbeaur and Gullit Nelson Salano. My sisters; Jane, Adelaide, Audrey, Mercy, Nifrey and brothers, Fredrick, Henry and Felix. Without your encouragement and support this journey would have been long and tough. You put your heart in all that I did, supported, facilitated, encouraged and prayed for me.

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LIST OF ABBREVIATIONS AND ACRONYMS

Bp	Base pairs
ddH₂O	Double distilled water
DNA	Deoxyribonucleic Acid
dNTP's	Deoxynucleotide Triphosphates
EDTA	Ethylene diamine tetra-acetic acid
EtBr	Ethidium Bromide
JKUAT	Jomo Kenyatta University of Agriculture and Technology
MEA	Malt Extract Agar
PCR	Polymerase Chain Reaction
PDA	Potato dextrose Agar
rDNA	ribosomal Deoxyribonucleic Acid
rRNA	ribosomal ribonucleic Acid
SDA	Sabourand dextrose Agar
SDS	Sodium dodecyl Sulphate
TBE	Tris Boric acid Ethylene diamine tetra-acetic acid
TE	Tris Ethylene diamine tetra-acetic acid

ABSTRACT

Lake Magadi is the southernmost lake in the Kenya Rift Valley, lying in a catchment of faulted volcanic rocks, north east of Lake Eyasi. Lake Magadi is a saline, alkaline lake, approximately 100 square kilometers in size that lies in a graben. Soda lakes harbor diverse groups of microorganisms that have developed mechanisms to thrive at different temperature ranges according to their optimal growth requirements. The objectives of this study were to isolate, characterize and identify fungi from Lake Magadi, a soda Lake of the Kenyan Rift Valley and then screen the isolates for the production of useful metabolites. Samples from the lake were isolated on malt extract agar, potato dextrose agar and Sabourand dextrose agar media at pH 10, 30°C. Thirty isolates were isolated, characterized using cultural, biochemical and molecular approaches, and screened for production of extracellular enzymes as well as potential for production of bioactive metabolites. The fungi grew at pH ranging from 5 – 10, temperature range of 25 – 35 °C and sodium chloride range of 5- 30 %.

All the thirty isolates produced different extracellular enzymes such as amylases, lipases, proteases and esterases. Antimicrobial assays done to determine the isolates range of *in vitro* activity against test organisms; *Staphylococcus aureus* (NCTC 10788), *Escherichia coli* (NCTC 10418), *Pseudomonas aeruginosa* (ATCC 27853), *Bacillus subtilis* (ATCC 55732), and fungi; *Candida albicans* (ATCC 90028) exhibited a range of inhibitory effects. Isolate LM13 produced coloured pigments into the media.

Analysis of partial sequences using Blast showed that about 60% of the isolates were affiliated to microorganisms belonging to the genus *Penicillium* and *Aspergillus* .7% and 10% belonged to the genus *Polyzellus* and *Fusarium* respectively while 7% affiliated to the genus *Neurospora* and 16% clustered closely with uncultured fungus. The DNA sequences of LM3 showed identity of 95 % similarity with the previously known sequences in the GenBank database. These could represent a novel species of organisms within the lake's ecosystem. Isolates LM12 and LM17 showed DNA sequence identity of 89 % and 82% respectively and could represent novel genera of organisms.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background information

Researchers all over the world are spending much labour and time searching for microorganisms with new abilities. Consideration of microorganisms which grow well in alkaline media, alkaliphilic microorganisms, has initiated a new aspect of microbiology. The microorganisms are widely distributed on the earth and they were found to produce new products (Horikoshi, 1991). Extreme alkaline environments include naturally occurring soda lakes, deserts, soils and artificial industrial-derived waters. Lake Magadi in Kenya and the Wadi Natrun in Egypt, are probably the most stable highly alkaline environments on Earth, with a consistent pH of 10.5 to 12.0 depending on the site (Mesbah *et al.*, 2007). In these habitats, environmental conditions such as pH, temperature and salinity concentrations are extremely high or low (Horikoshi, 1991). The soda lake environments are characterized by high concentrations of sodium carbonate (Na_2CO_3) (usually as $\text{Na}_2\text{CO}_3 \cdot 10\text{H}_2\text{O}$ or $\text{Na}_2\text{CO}_3 \cdot \text{NaHCO}_3 \cdot 2\text{H}_2\text{O}$). The carbonate provides buffering capacity to the lake waters (Grant *et al.*, 1990). It has been proposed that 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 (Behr and Röhricht, 2000).

Soda lakes are also very productive presumably as a consequence of relatively high surface temperatures (30-45 °C), high light intensities and unlimited reserves of HCO₃ for photosynthesis. This high productivity (Melack, 1983), is the driving force behind all biological processes occurring in soda lakes (Jones *et al.*, 1998). Major contributors to primary production are the autophototrophic cyanobacteria, mostly represented by the planktonic *Spirulina spp.* *Cyanospira*, *Choococcus* and *Synechococcus* (Grant *et al.*, 1990; Mwatha and Grant, 1993; Grant, 2006) may also be present, but their importance is probably minor in comparison with *Spirulina spp.* (Jones *et al.*, 1998). Cyanobacteria are not only responsible for primary production, but also for the fixation of Nitrogen and the production of O₂. However, it is still uncertain which organisms in soda lakes are responsible for Nitrogen fixation, since *Spirulina spp.* is not capable of doing that. A molecular study made on Lake Magadi (Baumgarte, 2003) showed the domination of cyanobacteria among the planktonic prokaryotic community, mostly represented by members of the order *Chroococcales*. This order belongs to a new group based on physiological characteristics (Garcia-Pichel *et al.*, 1998), the *Halotheca* cluster, which can stand extreme high salinity conditions and probably is the most important primary producer in hypersaline lakes. These lakes support large standing crops of a diverse range of micro-organisms that undoubtedly proliferate *in situ* (Grant, 1992; 1993).

Although soda lakes have a worldwide distribution, they are mainly confined to (sub) tropical latitudes in continental interiors or rain-shadow zones (Jones and Grant, 1999). Owing to their hostile nature they are often remote from the main centers of

human activity and perhaps for this reason they have been little studied. The best studied regions are the lakes and solonchaks of the Central Asian (Siberia) steppes and the Rift Valley of Eastern Africa (Baumgarte 2003; Grant *et al.*, 2004). The lakes of East African valleys have revealed a typical and predominance of dense blooms of Cyanobacteria in less saline alkaline Lakes. The predominant filamentous species are *Spirulina platensis*, *Spirulina maxima* and *Cyanospira (Anabaenopsis)* (Melack and Kilham, 1974; Tindall *et al.*, 1984; Florenzano *et al.*, 1985) and unicellular species *Chorococcus spp.*, *Synechococcus sp.* or *Synechocystis* have also been found, and in some cases they may be the dominant primary producers (Grant *et al.*, 1990; Mwatha and Grant, 1993; Grant, 2006).

Microbes have been found thriving in many other alkaline environments as well, including the slag dumps of the Lake Calumet region in southeast Chicago, Octopus Spring in Yellowstone National Park, and the East African Rift Desert. One of the most striking features of many alkaline, saline lakes is the coloration of the waters (Grant *et al.*, 1990). Depending on a variety of conditions related to water chemistry, dense populations of micro-organisms may colour the lakes green, orange, purple or claret. In many cases it has been possible to show that this overt indication of microbiological activity is due to blooms of specific algae, cyanobacteria, eubacteria or archaeobacteria (Tindall, 1998). Many organisms isolated from alkaline and highly saline environments such as soda lakes also require high salinity, which is achieved by adding NaCl to the isolation medium (Vargas *et al.*, 2005). 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 (Jones *et al.*, 1994). There is a distinct difference in microbial community composition between the hypersaline, alkaline lakes such as parts of Lake Magadi with salinity approaching saturation or higher, compared with the more dilute lakes like Nakuru (Zavarzin 1999, Grant *et al.*, 2004).

Hypersaline environments are found in a wide variety of aquatic and terrestrial ecosystems. They are inhabited by halotolerant microorganisms but also halophilic microorganisms ranging from moderate halophiles with higher growth rates in media containing between 0.5 M and 2.5 M NaCl to extreme halophiles with higher growth rates in media containing over 2.5 M NaCl (Ventosa *et al.*, 1998). Moderate and extreme halophiles have been isolated not only from hypersaline ecosystems (salt lakes, marine salterns and saline soils) but also from alkaline ecosystems (alkaline lakes). The most widely studied ecosystems are the Great Salt Lake (Utah, USA), the Dead Sea (Israel), the alkaline brines of Wadi Natrun (Egypt) and Lake Magadi (Kenya) (Oren *et al.*, 1977; 1993; 2002). It is noteworthy that low taxonomic biodiversity is observed in all these saline environments (Kamekura, 1998), most probably due to the highly salt concentrations measured in these environments.

Alkaliphiles isolated in soda lakes have been analyzed and used for their various alkali-tolerant enzymes in many industrial processes (Jones, 1998). Because these enzymes have the ability to function at high levels of pH, they are particularly useful in processes that require these extreme conditions. These alkaliphiles are thought to

have significant economic potential because their specialized enzymes are already “used in detergent compositions and in leather tanning, and are foreseen to find applications in the food, waste treatment and textile industries; additionally, (they) are potentially useful for biotransformations, especially in the synthesis of pure enantiomers” (Jones, 1998). Specific examples of such enzymes are proteases (used as detergent additives), starch-degrading enzymes, cellulases (laundry detergent additive), and pectinases (used to improve production of paper as well as waste treatment) (Bordenstein and Sarah, 2008). Another important application is the industrial production of cyclodextrin by alkaline cyclomaltoamylase. This enzyme has reduced the production cost and paved the way for cyclodextrin use in large quantities in foodstuffs, chemicals, and pharmaceuticals (Menuel *et al.*, 2006). This project intended to isolate, characterize and identify fungi from Lake Magadi, Kenya and targeted screening the isolates for the production of valuable enzymes and secondary metabolites such as antibiotics with antagonistic effect against other organisms.

1.2 Statement of the problem

One potentially optimistic approach to slowing the loss of biological resources is to explore and characterize these resources in order to sustainably use them. Producing immediate revenue and recognizing their value as long term biological resources might protect them from destruction (Reid *et al.*, 1993). The loss of biological resources might be slowed if the economic value of those resources were realized through an increased knowledge about its immediate usefulness to humanity.

It was generally assumed that microbial life in concentrated sea water was composed only of algae, protozoa, archaea and bacteria and not fungi (Brock, 1979; Ventosa and Nieto, 1995). However fungi have subsequently been described in moderately saline environments, such as salt marshes (Newell, 1996), saline soil (Guiraud *et al.*, 1995) and sea water (Kohlmeyer, 1991), but were considered to be unable to grow in highly saline waters.

The highest diversity of fungi currently known is found in tropical regions, mainly in tropical forests (Hawksworth, 1991). However, many fungi can adapt to extreme environmental conditions of water activity, temperature, pH and salinity (Griffith, 1994). Most of the fungi that can be found in extreme environments belong to the imperfect stage of the Ascomycota, which have been reported in mangroves, saline soils, marine sediments, seawater, salt marshes, and sand dunes (Moubasher *et al.*, 1990; Kohlmeyer and Volkmann-Kohlmeyer, 1991; Domsch, *et al.*, 1993; Guiraud *et al.*, 1995; Newell 1996; Hyde *et al.*, 2000). It was only recently that fungi were isolated from hypersaline environments; their function under these extreme conditions is still unclear (Gunde-Cimerman *et al.*, 2004). Most of the studies of fungi in hypersaline environments have been performed in northern latitudes, in the Mediterranean area and the Dead Sea (Buchalo *et al.*, 1998; Kis-Papo *et al.*, 2003; Gunde-Cimerman *et al.*, 2004). Butinar *et al.*, 2005) described fungi isolated from several natural and man-made hypersaline environments including those found in France, Namibia, Portugal, Slovenia, Spain and Dominican Republic.

Gunde-Cimerman *et al.*, (2004) reported that fungi belonging to the *Dermaticeae* can populate salterns with extreme NaCl concentrations (up to 320 psu). In addition, meristemic black yeasts and *Cladosporium sp.* were identified among the most common species identified along with various species from the genera *Aspergillus* and *Penicillium* in this environment. It was thought that the occurrence of fungi in hypersaline environments was due to random events caused by airborne spores; therefore, the fungi were believed to have no specific ecological function.

The fungi from the extreme environment have a great potential to produce natural antimicrobials and enzymes. The importance of the microorganisms in enzyme production is due to high production capacity, low cost and susceptibility to genetic manipulation. Actually, the enzymes of microbial origin have high biotechnological interest such as in the processing of foods, manufacturing of detergents, textiles, pharmaceutical products, medical therapy and in molecular biology (Pilnik and Rombouts, 1985; Falch, 1991; Rao *et al.*, 1998).

1.3 Justification

The earth's biological diversity is disappearing at an ever increasing rate (Wilson, 1988). The documentation of this loss has been based primarily on large organisms, such as mammals and vascular plants; however, evidence exists of an increasing decline in the diversity of the less conspicuous organisms such as the fungi including lichenized fungi, and bryophytes (Arnolds, 1988 and 1989; Richardson, 1990; Pittam, 1991; Lizon, 1993). These poorly known but speciose groups of organisms may

actually be more vital to long term ecosystem survival than the well-known macrofauna and flora.

Fungi have a worldwide distribution, and grow in a wide range of habitats, including extreme environments such as deserts or areas with high salt concentrations (Vaupotic *et al.*, 2008) or ionizing radiation (Dadachova *et al.*, 2007), as well as in deep sea sediments. Fungi have been isolated from saline lakes in the world such as the Dead Sea (Buchalo *et al.*, 2000).

Historical interest in soda lake microbiology has focused primarily on the isolation and characterization of individual microorganisms with potential industrial applications (Horikoshi, 1999), although anaerobic strains with hypothesized ecological roles have also been described (Zhilina *et al.*, 1996; Zhilina *et al.*, 1997; Moser *et al.*, 2001). Recent surveys of soda environments indicate elevated microbial phylogenetic diversity, including: the Wadi Natrun lake system in Egypt (Mesbah *et al.*, 2007), soda lakes in the Kenyan-Tanzanian Rift Valley (Rees *et al.*, 2004), soda lakes in Inner Mongolia in China (Ma *et al.*, 2004), saline, meromictic Lake Kaiike in Japan (Koizumi *et al.*, 2004), saline Qinghai Lake, China (Dong *et al.*, 2006), and athalassohaline Lake Chaka, China (Jiang *et al.*, 2006).

Extensive studies on bacterial isolation, characterization and identification have been carried out in the Kenyan soda lakes. Kenyan soda lakes have revealed a typical and predominance of dense blooms of *Cyanobacteria* in less saline alkaline lakes (Mwirichia *et al.*, 2010). Studies on the low saline lakes of the Kenyan Rift Valley

such as Bogoria, Crater Lake Sonachi, Elementeita and Nakuru revealed the presence of diverse populations of aerobic sulfur oxidizing bacteria of genera *Thioalkalimicrobium* and *Thioalkolivibrio* (Sorokin *et al.*, 2001). Anaerobic alkalithermophiles from Lake Bogoria include *Thermosyntropha lipolytica* (Svetlitsnyi *et al.*, 1996), *Anaerobranca gottschalkii* (Prowe and Antranikian, 2001) and other strains not yet described. Several *Bacillus* strains such as M8 C22, M8-C11 (FJ 764771), M14-C16 (FJ 764778), M4-C7 (FJ 764769), M10-C8 (FJ 764774), M14-C6 (FJ 764777), M1-C6 (FJ 764768), M8-C14 (FJ 764772), M9-C3 (FJ 764773) and M10-C17 (FJ 764775) were isolated from Lake Elmenteita (Mwirichia, 2009; Mwirichia *et al.*, 2009). A Eukaryotic micro-alga was isolated from Lake Magadi water samples; that was identified, by morphology, as *Chlorella minutissima* (Gerasimenko *et al.*, 1999). Information on isolation and characterization of fungi from the Kenyan soda lakes is very scanty. It is for this reasons that the goal of this study was to isolate, characterize and identify fungi from Lake Magadi, identify the enzymes produced by the fungi and the screen for antimicrobial metabolites that could be of commercial importance hence improving industrial and pharmaceutical applications as well as other sectors where they may be of use. This study will contribute to the taxonomy and biodiversity of fungi and moreover support fungal biodiversity research. Data generated from this research will also provide information for researchers to understand the Lake Magadi's microbial ecology and biotechnological benefits.

1.4 Hypothesis

Lake Magadi, a soda Lake in the Kenyan Rift valley harbors novel and previously uncultured micro-organisms with biotechnological potential.

1.5 Objectives

1.5.1 General objective

1. To isolate and characterize fungi from Lake Magadi, a soda lake of the Kenyan Rift Valley.

1.5.2 Specific objectives

1. To isolate fungi from Lake Magadi
2. To characterize and identify the fungal isolates
3. To screen the isolates for useful secondary metabolites

CHAPTER TWO

2.0. LITERATURE REVIEW

2.1 Microbial diversity and ecology of the Soda Lakes in Kenya

Kenya's Rift Valley Soda Lakes are Bogoria, Nakuru, Elmenteita and Magadi. They are all saline and alkaline and similar in scale. Lake Bogoria National Reserve covers an area of 107 km² while Lake Nakuru and Lake Elmenteita have surface areas 40km² and 20km² respectively.

Wildlife and microbial diversity are analogous amongst all these lakes (Gierlowski-Kordesch *et al.*, 2004). Williams, (2002) described these lakes as very important natural assets with considerable aesthetic, cultural, economic, recreational, scientific, conservation and ecological values for mankind. Lake Magadi is the most studied of the alkaline East African soda lakes. The alkaline lake niche limits diversity in microbial life due to high pH and high salinity (Joan and Nielson, 1999). Microbes living in this niche must have modified proteomes in order to survive in such an environment. The specificity for microbial life in this niche has attracted researchers to study how microbial life exists in this environment.

The Kenya soda lakes remain as fragile ecosystem due to low water level through evaporation, pollution through human activities in the catchment's areas, domestic effluent and industrial waste. This could mean the microbial diversity in the lakes is

changing due to human activities, which may be resulting to loss of certain species (Kimunya, 2007; UNESCO, 2007).

2.2 Microbial diversity and ecology of the Soda Lakes in the world

The most widely studied ecosystems are the Great Salt Lake (Utah, USA), the Dead Sea (Israel), the alkaline brines of Wadi Natrun (Egypt), and Lake Magadi (Kenya) (Oren, 2002). It is noteworthy that low taxonomic biodiversity is observed in all these saline environments (Kamekura, 1998) most probably due to the highly salt concentrations measured in these environments.

Fungi are ubiquitous in most ecosystems where they usually colonize a diverse range of substrates. The highest diversity of fungi is found in tropical regions, mainly in tropical forests (Hawksworth, 1991). In Puerto Rico, fungal studies have concentrated on forest and coastal ecosystems (Acevedo, 1987 and 2001; Lodge *et al.*, 2002; Cantrell *et al.*, 2004; Nieves-Rivera, 2005). However, many fungi can adapt to extreme environmental conditions of water, temperature, pH and salinity (Griffith, 1994). Most of the fungi that can be found in extreme environments belong to the imperfect stage of the Ascomycota, which have been reported in mangroves, saline soils, marine sediments, sea water, salt marshes, and sand dunes (Moubasher *et al.*, 1990; Kohlmeyer and Volkmann-Kohlmeyer, 1991; Domsch *et al.*, 1993; Guiraud *et al.*, 1995; Newell 1996; Hyde and Pointing 2000). Because fungi were only recently isolated from hypersaline environments, their function in these extreme conditions is

still unclear (Gunde-Cimerman *et al.*, 2004). Most of the studies of fungi in extreme salt conditions have been performed in the northern latitudes, in the region of Russia and the Dead Sea (Buchalo *et al.*, 1998; Kis-Papo *et al.*, 2001 and 2003; Gunde-Cimerman *et al.*, 2004). Butinar *et al.*, (2005a and b) described fungi isolated from several natural and man-made hypersaline environments including those found in France, Namibia, Portugal, Slovenia, Spain and Dominican Republic.

Reports on presence of filamentous fungi in the hypersaline waters of the Dead Sea (340 g L⁻¹ total dissolved salts), survival of their spores and mycelia in this hostile environment have invoked great interest (Buchalo *et al.*, 1998; Kis-Papo *et al.*, 2003). Further, a gene responsible for High Osmolarity Glycerol (HOG) response pathway from one such Dead Sea-fungus *Eurotium herbariorum* has been identified for stress tolerance to freezing and thawing (Jin *et al.*, 2005). This has further led to production of a recombinant yeast *Saccharomyces cerevisiae* containing the gene HOG (Norbeck *et al.*, 1996). The genetically transformed yeast proved to withstand high salinity and also extreme heat and cold. However, high salt tolerance is governed by several genes and this might be the first step towards genetic engineering of salt-tolerant agriculture crops (Turk and Plemenitas, 2002).

A diverse fungal fauna was recently discovered in environments with salinities ranging between 15–32 %, where it was so far assumed that bacteria only were able to grow. These fungi were first isolated in hypersaline waters of Secovlje salterns in Slovenia (Gunde-Cimerman *et al.*, 2000), and subsequently in the salterns of La

Trinitat (Ebro Delta, Catalonia, Spain) and Bonmatí (Santa Pola, Valencian Community, Spain), with the occurrence of the same dominant species. The majority of species isolated belonged to melanized meristematic and yeast-like fungi, and a few different genera of filamentous fungi were also identified (Méjanelle *et al.*, 2001).

Among the isolated halotolerant and halophilic mycobiota, dematiaceous Ascomycetales of the following genera were found: *Hortaea*, *Phaeotheca*, *Trimmatostroma*, *Aureobasidium*, *Alternaria*, and *Cladosporium* (Zalar *et al.*, 1999). Five melanized species were isolated from La Trinitat salterns (Spain) and further enriched in cultures, as well as *S. cerevisiae* (Jean-Luc Legras, 2007) a pertinent reference for *Ascomycetes*.

A study of coastal mycology in Puerto Rico was performed by Nieves-Rivera, (2005) including many aspects of fungi in marine environments. Also, a study of arenicolous filamentous fungi in the Mayaguez Bay shoreline was performed by Ruiz-Suarez, (2004) who reported the presence of *Aspergillus*, *Cladosporium*, *Dreschlera*, *Fusarium*, *Geotricum*, *Penicillium*, *Trichoderma*, *Mucor* and *Rhizopus*. The genus *Aspergillus* represented 80 % of total fungal abundance. Her data suggested that salinity concentration may regulate the abundance of fungi in the shoreline.

Salt marshes, a moderate salt environment, are another habitat for halotolerant fungi (Abdel-Hafez *et al.*, 1978). Fungi have an important role in the decomposition of salt marsh grasses. Halophytes from salt marshes in Europe were examined for the presence of mycorrhizal fungi (Hildebrandt *et al.*, 2001). Large quantities of spores

were found in saline soil; 80 % of them were identified as *Glomus geosporum*. Also, they have found that several plants from the salt marsh were mycorrhizal. Another study suggests that these fungi confer salt tolerance to plants based on the occurrence of *G. geosporum* and *G. caledonium* spores in salt marshes (Landwehr *et al.*, 2002). This suggestion was supported later by Carvalho *et al.* (2004). They indicated that fungi in salt marsh plants may have the potential to confer salt tolerance to them and may influence the distribution of this vegetation. This study was made in a salt marsh from Portugal and showed that fungal adaptation is one of the possible reasons for the existence of mycorrhizal fungi in salt marshes (Carvalho *et al.*, 2004).

The first record for filamentous fungi in Dead Sea by Buchalo *et al.*, (1998) reported a novel species, *Gymnascella marismortui* (Ascomycota). This fungus was shown to be an obligate halophile that grows optimally in a range of 10-30 % of Dead Sea water. Moreover, they isolated *Ulocladium chlamydosporum* and *Penicillium westlingii* with salt tolerance in the range of 3 to 15 % NaCl at 26 °C. In another study, *Aspergillus versicolor*, *Chaetomium globosum*, *Eurotium herbariorum*, *E. amstelodami* and *E. rubrum* were isolated from Dead Sea waters and together with *Gymnascella marismortui* were tested for survival of spores and mycelia in Dead Sea water for prolonged time (Kis-Papo *et al.*, 2001 and 2003a). After the first record of fungi, Kis-Papo *et al.* (2003b) performed a study of genomic diversity of *A. versicolor* and their results suggested that genomic diversity was positively correlated with stress. The species *Trichosporon mucooides*, *Rhodotorula larynges*, a *Candida glabrata*-like strain and a *Candida atmosphaerica*-like strain were also isolated from these waters.

Candida glabrata was not known for their halotolerance (Butinar *et al.*, 2005b). *Candida atmosphaerica* was found associated with another extreme environment, the deep-sea hydrothermal systems of the Mid-Atlantic Rift (Gadanhó and Sampaio, 2005).

The Mono Lake in California, an alkaline, hypersaline and closed basin, was the site of a study performed by Steiman *et al.*, (2003). This lake is compared with the Dead Sea due to its high salinity. Those conditions permit the mineral formation named tufa. Steiman *et al.*, (2003) isolated many species of the genera *Aspergillus*, *Achaetomium*, *Acremonium*, *Alternaria*, *Chaetomium*, *Cunninghamella*, *Ulocladium*, *Embellisia*, *Fusarium*, *Phoma*, *Sporothrix*, *Penicillium*, *Papulaspora*, *Geotricum*, *Curvularia*, *Mucor*, *Phialophora*, *Phaeoramularia*, *Sporormiella* and *Thelebolus*. Interestingly, in this study they were not able to isolate fungi from water using culture methods. They suggested that the salinity, and most important, the pH (very alkaline), are not favorable for fungal growth (Steiman *et al.*, 2003).

2.4 Fungi in Biotechnology

Fungal biotechnology or ‘mycotechnology’ has advanced considerably in the last five decades. Terrestrial fungi are used in the production of various extracellular enzymes, organic acids, antibiotics and anti-cholesterolemic statins (Pointing and Hyde, 2001). They have been used as expression hosts as well as a source of new genes. With modern molecular genetic tools, fungi have been used as “cell factories” for

heterologous protein production (Punt *et al.*, 2002) and human proteins (Bretthauer, 2003).

2.4.1 Application in Enzymology

Enzymes from extremophiles, extremoenzymes, have a great economical potential in agricultural, chemical and pharmaceutical processes. They are used in biological processes by increasing specificity and catalytic activity, and are stable at extreme incubation conditions (Chadha and Patel, 2008). The main industrial application of alkaliphilic enzymes is in the detergent industry, and detergent enzymes account for approximately 30 % of total worldwide enzyme production. Alkaline enzymes have been used in the hide-dehairing process, where dehairing is carried out at pH values between 8 and 10.

An interesting application of alkaline protease was developed by Fujiwara and coworkers (Fukumori *et al.*, 1985). They reported the use of an alkaline protease to decompose the gelatinous coating of X-ray films, from which silver was recovered. Protease B189 had a higher optimum pH and temperature, around 13.0 and 85 °C. The enzyme was most active toward gelatin on film at pH 10. Ligninolytic enzymes from members of Basidiomycetes have a potential in several industrial and biotechnological processes within a wide variety of organic and inorganic substrate specificities (Couto and Herrera, 2006). Such applications include the detoxification of industrial effluent (Nilsson *et al.*, 2006; Zhao, 2007; Hardin and Hardin, 2007), mostly from, textile and petrochemical industries, bleaching and delignification processes in the paper and pulp

industries (Archibald and Bourbonnais, 1997), removing the phenolic compounds from the beer and wine in the food industry (Minussi *et al.*, 2002). In addition, their capacity to remove xenobiotic substances and produce polymeric products makes them a useful tool for bioremediation purposes (Pointing, 2001).

Recent studies have suggested that lignin-degrading or white-rot fungi (note: decay caused by these species gives wood a bleached appearance) such as *Phanerochaete chrysosporium* and *Trametes versicolor* could replace some of the chemical steps used in paper making (Kis-Papo *et al.*, 2003b). An industrial bio-pulping/ bio-bleaching process would eliminate the pollution problems associated with the use of chemicals. Lignin-degrading fungi or their enzymes also have the ability to degrade highly toxic organic compounds such as dioxins and PCB's (polychlorinated biphenyls), and could have an important role to play in the remediation of contaminated soils and the disposal of chemical wastes. A recent report also indicates that lignin-degrading fungi can even degrade synthetic textile polymers such as nylon previously thought to be non-biodegradable (Deguchi *et al.*, 1997).

2.4.2 Food industry

The growth of the mould *Penicillium roqueforti* in the body of blue-veined cheeses and the surface growth of the moulds *Penicillium candidum*, *Penicillium caseicolum*, or *Penicillium camemberti* on Camembert, Brie and related types of cheeses play an important role in the development of the characteristic flavours of these cheeses

(Wolf, 1997). The principal fungus involved, *Aspergillus oryzae*, is now also used to produce a range of commercially important enzymes.

A recent innovation in food technology has been the development of Quorn mycoprotein (Choi *et al.*, 2004) from a filamentous fungus by Rank Hovis, McDougall PLC and ICI (now Zeneca). The culture employed, a strain of *Fusarium graminearum*, was isolated from a field in Marlow, Buckinghamshire. Probably the most thoroughly tested food ever to appear on supermarket shelves, annual sales of Quorn are now in excess of £15 million in the UK.

2.4.3 Biological control agents

Recent concerns over the effect of pesticides on the environment have intensified efforts to use biological control agents rather than toxic chemicals for the control of pests, Butt *et al.*, (2001). Considerable research has been devoted to the possibility of using fungi to control fungal pathogens (mycofungicides), insects (mycoinsecticides) and weeds (mycoherbicides), a trend which will be enhanced by recent developments in the production of genetically engineered strains of fungi (Jackson *et al.*, 2000). Several species of fungi have now been commercially formulated as mycoinsecticides.

Fungal biocontrol agents are particularly effective when used in greenhouses where the target pathogen is confined and where environmental parameters are relatively constant (Shah and Pell, 2003).

2.4.4 Production of metabolites

The main industrial use of fungi has been associated with the production of fermentation products, including antibiotics, enzymes and a range of biochemicals. Ethanol, citric acid, gluconic acid, itaconic acid, amino acids, vitamins, nucleotides and polysaccharides provide examples of primary metabolites produced by fungi, while antibiotics such as penicillin, the cephalosporins, fusidic acid and griseofulvin are important secondary metabolites (Namikoshi *et al.*, 2002).

Fungi are well known as a source of antibiotics but new therapeutic compounds with novel pharmacological activities have also been developed in recent years (Jensen and Fenical, 2000). One such example is the cyclosporins first isolated from *Tolypocladium inflatum* in 1976 as antifungal compounds and later shown to possess immunosuppressive activity. Cyclosporin A is currently the most widely used drug for preventing rejection of human organ transplants (Borel, 2002).

2.4.5 Soil bioremediation

The use of fungi in bioremediation of soils receiving hazardous or otherwise problematic waste is based largely on the versatile enzymic abilities of wood-rotting fungi, most notably *Phanerochaete chrysosporium* (Pointing, 2001). These fungi produce ligninases which degrade complex aromatic polymers like those present in wood. Fortunately ligninases are not specific to lignin, but will degrade a wide variety of toxicants including chlorinated biphenyls, aromatic hydrocarbons and chlorinated dibenzodioxins.

Microorganisms including bacteria, algae, fungi and yeast are found to be capable of efficiently accumulating heavy-metal ions (Mullen *et al.*, 1989; Gadd, 2010). Fungi in particular have demonstrated unique metal adsorption characteristics and are easy to cultivate (Gadd, 1987). A large number of studies for the removal of metal ions have been reported using strains of *Penicillium*, *Rhizopus* and *Aspergillus* (Kapoor *et al.*, 1999; Say *et al.*, 2004; Ahmad *et al.*, 2005). White-rot fungi like *Pleurotus* species have been known for their ability to degrade lignin, a non-repeating structural polymer found in woody plant and this ability enables them to degrade xenobiotic pollutants (Bumpus and Aust, 1987). Adenipekun and Fasidi, (2005) reported the ability of *Lentinus subnudus* to mineralize soil contaminated with various concentrations of crude oil.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study site

Lake Magadi is a saline-alkaline lake in the southern part of Kenya, and is located in the Rift Valley. The lake is located 2000 feet below sea level forming one of the lowest points in the valley (Gierlowski-Kordesch *et al.*, 2004). Lake Magadi is. It is approximately 2 ° S and 36 ° E of the Equator at an elevation of about 600 m above sea level, and lies in the lowest part of the trough in a naturally formed closed lake basin. The lake covers an area of 90 km² and a depth that ranges from 1-5 m. It is one of the smaller Rift Valley lakes. Evaporation is intense during the dry season (3500 mm per annum), the range of temperature being between 22 °C and 34 °C. The Loita Hills and the Mau Escarpment to the west shield the valley floor from rainfall resulting in an annual total of approximately 500 mm of rainfall in the two rainy seasons (Behr and Röhricht, 2000).

The lake acts as a sink for seasonal streams and is famous for its cherts from sodium silicate precursors. It has a pH of 10 and alkalinity of 380 mmol L⁻¹. Mineral composition consists mostly of trona mixed with halite and either kogarkoite or villaumite, resulting in fluoride concentrations up to 8.7 mg F. The high fluoride concentration in Lake Magadi is strongly related to the weathering of volcanic rocks enriched in fluoride and alkalis which are found in the same area as Lake Magadi.

This has affected the local communities who have customarily used the chert from Lake Magadi as a meat tenderizer and has inadvertently ingested high levels of fluorine (Nielson, 1999). The large presence of trona (sodium sesquicarbonate) causes the lake water to form sodium carbonate brine. The high basicity of sodium carbonate makes the lake alkaline (Nielson, 1999).

A gel of Sodium-aluminium silicate as thick as 5 centimeters is found in Lake Magadi. These gels are formed through the interaction of hot alkaline spring waters (67 °C – 82 °C with a pH of 9) and alkali trachyte flows. Algal mats from the lake protect the gels from erosion. It is believed that this gel was a precursor for the cherts of rocks in Lake Magadi. It is also believed that hydrous sodium silicates and their chemical precipitates may form bedded chert deposits in the lake as well. Cycling alkaline levels are represented in alternating bands of silica rich and iron rich segments (Eugster, 1968).

Lake Magadi is a popular destination for many animals due to the fact that it is situated between Maasai Mara and Amboseli National Parks, but very few animals actually have any contact with, or live in, the lake itself. However, the lake is a popular destination for wading birds during the dry season including flamingos, heron, pelicans, and spoonbills. The birds congregate in streams of fresh water that run into the lake because this water brings in large amounts of diverse food. There is only one species of fish that can actually be found in the lake itself. *Tilapia grahami*, a type of small tilapia, has adapted to live in the harsh conditions of the lake, and is normally

found in the lagoons on the lake's periphery. An abundance of research has been performed on these fish, indicating that the fish have adapted to live in temperatures up to and possibly above 44 °C, a pH varying between 5-11 (though the lake pH naturally ranges from pH 9-11), low oxygen levels in the water (as low as 1.1 mg O₂/L of water), and a salinity concentration up to 4 % (Reite *et al.*, 1974). These tilapia have many adaptations to survive in this lake, and due to the lakes extreme conditions, little other non-microbial life exists.

Lake Magadi

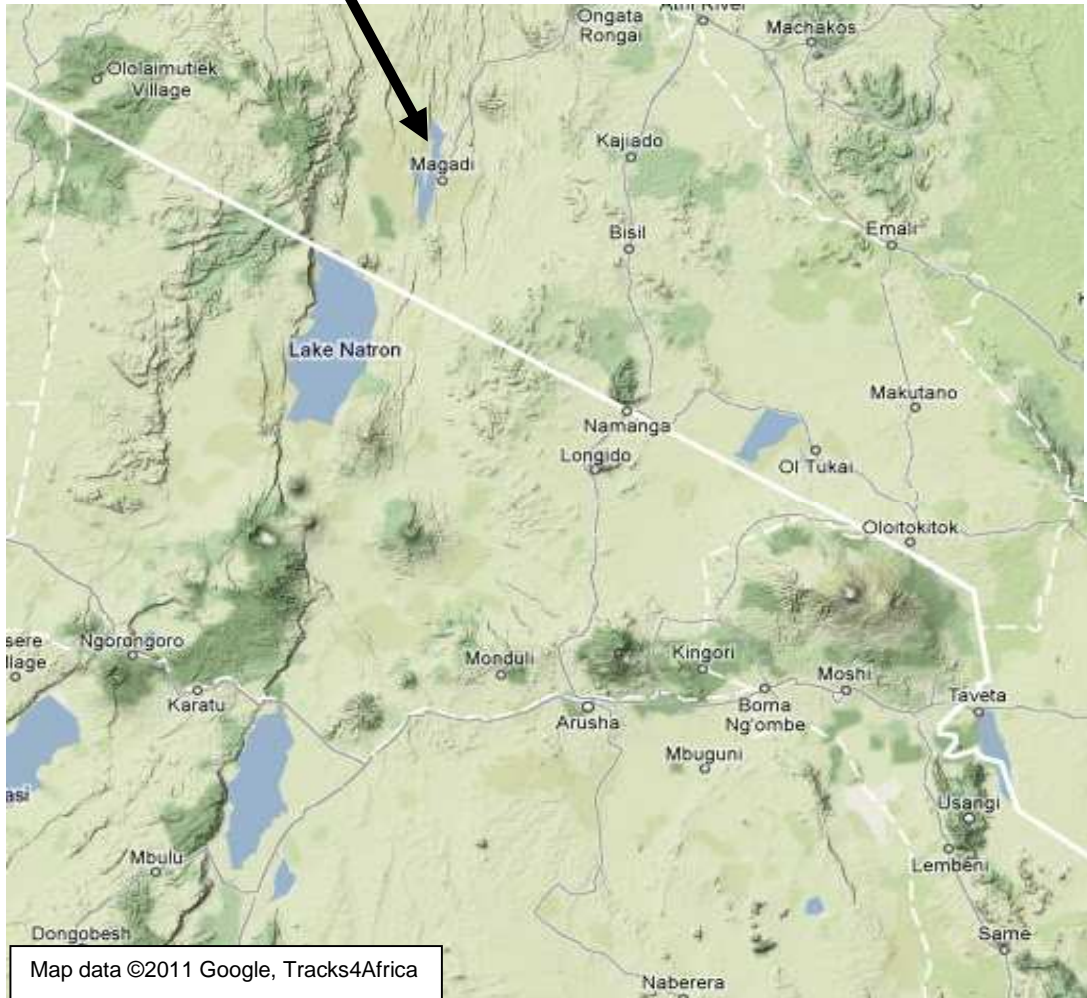


Figure 3.1a: A Google map showing the location of Lake Magadi



Figure 3.1b: Sampling point station 1 (hot springs, (48 °C))



Figure 3.1c: Sampling point station 2 (39.4 °C)

3.2 Sample collection

Sampling was done on 10th and 11th March 2010 at different sites at Lake Magadi. The pH, dissolved solids, temperature, and dissolved oxygen of each sampled site were noted. Microbial mat, mud sediment, water, salt, and foam were collected from the lake at three points that differed in alkalinity levels.

Sediment, water samples, foam and microbial mats were collected from Lake Magadi (2 °00'S and 36 °13'E) in sterile bottles, preserved in dry ice and transported to the Institute for Biotechnology Research Laboratory at Jomo Kenyatta University of Agriculture and Technology for analysis.

Once in the laboratory, water samples were filtered through 0.45µm and 0.22µm membrane filters (Whatmann) and preserved at -70 °C. The collected mud, sediment, foam and microbial mats were all preserved at -80 °C.

3.3 Isolation of fungi

Fungi were isolated from the different samples obtained at different sampling sites of Lake Magadi. The lake water was used to prepare culture medium. Isolation of fungi from sediments and microbial mats was performed using the serial dilution technique in combination with three selective media prepared in the laboratory: Malt extract agar (MEA), Sabourand dextrose agar (SDA) and Potato dextrose agar (PDA) separately prepared with lake water. Rose Bengal was added as bacteriostatic agent (Smith and Dowson, 1944). Antibiotics such as penicillin (100 mg/litre) and streptomycin (100 mg/litre) were used to inhibit bacterial growth and were added to the media after autoclaving. The plates were inoculated with a mass of fungal cells scooped using a cork borer of a diameter of 0.65cm and were then incubated at 30°C for one to two weeks during which the growing fungal colony were examined and identified. Individual colonies which grew on the plates were re-inoculated on MEA, PDA, and SDA media several times until pure colonies were obtained. The pure colonies were selected based on morphological characteristics.

3.4 Morphological characteristics of the isolates

3.4.1 Colonial morphology

Colony colour, shape, size, elevation and form of the pure cultures were observed and noted.

3.4.2 Cellular morphology

Preliminary characterization by simple staining (using lactophenol cotton blue dye) of each of the isolates were done and observed under a light microscope at $\times 100$ (Keast *et al.*, 1984). The staining technique was used to divide isolates on morphological basis (Cappuccino and Sherman, 2002). The most commonly used prior art method and apparatus comprised of a round petri-dish which was filled with water to a depth partially submerged by spaced pair of toothpicks. A standard microscope slide was placed atop the toothpicks and a block of suitable culture nutrient medium (MEA) was applied to the upper surface of the slide. The culture medium was then inoculated with the fungi and a standard cover slip placed atop the inoculated medium. The petri dish was then covered with a standard petri-dish lid, and after 4-7 days incubation period, the petri-dish lid was removed and the cover slip, cultured fungi, agar, and microscope slide lifted as a unit from the petri-dish and placed upon the stage of a microscope for examination purposes. Cell characteristics of the cultured fungi were observed after staining with lactophenol cotton blue dye.

3.5 Physiological characterization

3.5.1 Growth at different temperature

To determine the ability of the isolates to grow at varied range of temperature, plates with malt extract agar media were inoculated with the isolates and incubated at the six different temperature levels (25 °C, 30 °C, 35 °C, 40 °C, 45 °C and 50 °C) separately for 7 days (Nazina *et al.*, 2001). Two uninoculated plates for each temperature were

used as controls. Experiments were done in duplicates. Positive isolates had growth detected by measuring the diameter of the colony in millimeters and recorded.

3.5.2 Growth at different pH

The aim of this experiment was to check for the ability of the isolated fungi to grow in acidic, neutral and alkaline pH. Malt extract agar media was prepared separately according to manufacturer's instructions (Difco Laboratories) in conical flasks and the pH of each set of experiments was adjusted to 5.0, 7.0, 8.5 and 10.0 with a pH meter respectively. The media was then autoclaved and dispensed in petri dishes. The plates were then inoculated with the isolates and incubated at 30 °C for a period of 7 days and the diameter of the growth measured in millimeters and recorded. Two uninoculated plates were used as controls.

3.5.3 Growth on different media

The growth requirements for fungi may vary from strain to strain, although cultures of the same species and genera tend to grow best on similar media. An optimal nutrient medium should provide not simply adequate growth but the best possible growth in order to allow the fungi to grow without restriction and express all phenotypes. This experiment was to determine the effect of media on the cultivation of the fungal isolates. Three types of media were used namely Sabourand dextrose agar (SDA), malt extract agar (MEA) and potato dextrose agar (PDA). The isolates were incubated at 30 °C for a period of 7 days and the diameter of the growth measured in millimeters.

3.5.4 Growth at different sodium chloride concentration

Malt extract agar was prepared in 1 litre of distilled water and supplemented with 5 %, 10 %, 20 % and 30 % sodium chloride concentration separately. This was to determine the ability of the isolates to grow at different sodium chloride concentration. Three sets of experiments for each salt concentration, with 30 plates containing the media were inoculated with each of the obtained 30 isolates respectively for replication. This was incubated at 30 °C then checked for growth after 7 days by measuring the diameter of the colony in millimeters.

3.6 Biochemical characterization of the isolates

3.6.1 Screening for production of enzymes

The fungal isolates were screened for their ability to produce enzymes i.e. amylases, proteases, xylanases, lipases, esterases and cellulases.

3.6.2 Determination of amylolytic activity

The methodology used was one described by Hankin and Anagnostakis, (1975). The isolates were inoculated in nutrient agar (NA) with 0.2 % of soluble starch (g l^{-1}), pH 8.0. After incubation, the cultures were treated under iodine vapours, which allowed the visualization of clear halos around the colonies while negative isolates indicated a blue black colour all over the plate (Castro *et al.*, 1993).

3.6.3. Determination of the esterase activity

The media used is described by Sierra, (1957), containing (g l⁻¹): peptone 10.0, NaCl 5.0, CaCl₂ 2H₂O 0.1, agar 18.0, pH 8.0. To the sterilized culture media, previously sterilized Tween 80 was added in a final concentration of 1 % (v/v). This medium was inoculated with the isolates in duplicates and the presence of a precipitation of calcium crystals around the colonies showed positive results.

3.6.4 Determination of the lipolytic activity

A previously described methodology to determine the esterase activity (Sierra, 1957) was used. In this determination, Tween 80 was substituted by Tween 20. This medium was inoculated with the isolates in duplicates for replication. Positive isolates for lipase production were indicated by a precipitation of calcium crystals around the colonies while the negative isolates did not have the crystals around the colonies.

3.6.5. Determination of the proteolytic activity

For the determination of casein hydrolysis, the media used was according to the method of (Vieira, 1999). The isolates were cultured on a media containing g/l Nutrient Broth 8.0, glucose 1.0, agar 18.0, and the pH was adjusted to 8.0. After autoclaving, 15.0 ml of skimmed milk, separately autoclaved was added. The microorganisms were inoculated in duplicates and incubated at 30 °C and after the growth period of 7 days, 2.0 ml of HCl 0.1 mol l⁻¹ was added to the plates. Positive isolates for protease production exhibited a zone of proteolysis as demonstrated by

clearing zones while the negative isolates did not have the clearing zones (Cappuccino and Sherman, 2002).

3.6.6 Determination of the cellulolytic activity

The media used contained 7.0g KH_2PO_4 , 2.0g K_2HPO_4 , 0.1g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.0g $(\text{NH}_4)_2\text{SO}_4$, 0.6g yeast extract, 10g microcrystalline cellulose and 15g agar per liter (Stamford *et al.*, 1998). The plates were inoculated in duplicates for replication and incubated at 30 °C for 7 days. For best viewing area for clarification the plates were stored at 50 °C for one night after the incubation period. The presence of a clear halo around the fungal growth indicated positive results.

3.6.7 Determination of the xylanolytic activity

The fungal isolates were screened for their abilities to produce extracellular xylanases during their growth on Czapek's agar medium containing xylan as the sole carbon source. The composition of the medium was g/l Birch wood xylan 5.0g, Peptone 5.0g, Yeast extract 5.0g, K_2HPO_4 1.0g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.2g and agar 20.0g (Nakamura *et al.*, 1993). The inoculated plates were incubated for 7 days at 30 °C. The clearing zones formed around the fungal growth were more visible when the plates were flooded with 0.1 % (w/v) Congo red dye. After 30 minutes of incubation, the plates were washed with 1M NaCl. The colour around positive isolates for xylanase production changed from red to orange while the colour around the negative isolates remained red.

3.6.8. Screening the isolates for production of antibiotics.

Fungal isolates were grown on malt extract broth at 30 °C in an incubator shaker at 100rpm for 7 days and the crude extracts used to screen the isolates for antibiotic activity against the test organisms; *Pseudomonas aeruginosa* (ATCC 27853), *Bacillus subtilis* (ATCC 55732), *Escherichia coli* (NCTC 10418), *Candida albicans* (ATCC 90028) and *Staphylococcus aureus* (NCTC 10788). The test organisms were spread on malt extract agar plates. Three sterile cellulose discs of a diameter of 0.5cm were then dipped in each isolates crude product and placed at three points on the plates. The plates were then incubated at 30 °C for a period of 96 hrs. Isolates that tested positive for antibiotic production were indicated by clearing zones around them while the negative isolates were indicated by lack of the clearing zones around them (Cappuccino and Sherman, 2002).

3.7 Molecular characterization of the isolates

3.7.1 DNA extraction

This was done to identify the fungal isolates. Each of the 30 isolates was grown on malt extract agar for 7 days. Total genomic DNA of the isolates was extracted from these cells in duplicate using two lysis buffers as solution A (50mM Tris pH 8.5, 50mM EDTA pH 8.0 and 25 % sucrose solution) and solution B (10mM Tris pH 8.5, 5mM EDTA pH 8.0 and 1 % SDS). The cells were scrapped aseptically using a sterile surgical blade taking care not to pick the media. These were crushed separately

in 200µl solution A using sterile mortar and pestle, and resuspended in 100µl of solution A. This was followed by addition of 30µl of 20mg/l Lysozyme and 15µl of RNase, gently mixed and incubated at 37 °C for two hours to lyse the cell wall. 600µl of Solution B was then added and gently mixed by inverting the tubes severally, followed by the addition of 10µl of Proteinase K (20mg/l) and the mixture incubated at 60 °C for 1 hour. Extraction followed the phenol/chloroform method (Sambrook *et al.*, 1989). The presence of DNA was checked on 1 % agarose and visualized under ultraviolet by staining with ethidium bromide. The remaining volume was stored at -20 °C. The genomic DNA was used as templates for subsequent PCR amplification.

3.7.2 PCR amplification of 18S rDNA.

Total DNA from each isolate was used as a template for amplification of the 18S rRNA genes. Nearly full-length 18S rDNA gene sequences were PCR-amplified using fungal primer pair Fung5f forward 5'-GTAAAAGTCCTGGTTCCCC-3' and FF390r reverse, 5'-CGATAACGA ACGAGA CCT-3'(Vainio and Hantula, 2000) and Lueders et al. (2004). Amplification was performed using Peqlab primus 96 PCR machine. Amplification was carried out in a 40µl mixture containing 5µl of PCR buffer (×10), 3µl dNTP's (2.5mM), 1µl (5 pmol) of Fung5f forward primer, 1µl (5pmol) of FF390r reverse primer, 0.3µl taq polymerase, 1.5µl of template DNA and 28.2µl of water. The control contained all the above except the DNA template. Reaction mixtures were subjected to the following temperature cycling profiles repeated for 36 cycles: Initial activation of the enzyme at 96 °C for five minutes,

denaturation at 95 °C for 45 seconds, primer annealing at 48 °C for 45 seconds, chain extension at 72 °C for 1.30 minutes and a final extension at 72 °C for 5 minutes. Amplification products (5µl) were separated on a 1 % agarose gel in 1× TBE buffer and visualized under ultraviolet by staining with ethidium bromide (Sambrook *et al.*, 1989).

3.7.3 Purification of PCR products

The PCR products were purified using the QIAquick PCR purification Kit protocol (Qiagen, Germany) according to manufacturer's instructions. Five volumes of buffer PB (Qiagen, Germany) was added to 1 volume of the PCR sample and thoroughly mixed. The QIAquick spin column was placed in a 2ml collection tube; the sample was applied to the QIAquick column to bind the DNA, and then centrifuged for 60 seconds at 13000 rpm. The flow-through was discarded, and the QIAquick column placed back into the same tubes. To wash the DNA, 740 µl buffer PE was added to the QIAquick column and centrifuged for one minute. The flow-through was discarded and the column centrifuged again for an additional one minute at 13000rpm to remove residual ethanol from buffer PE. The Qiaquick column was placed in a 1.5ml micro centrifuge tube and 30µl of buffer EB (10mM Tris-Cl, pH 8.5) added to elute DNA. The tubes were then centrifuged for one minute, the spin column removed and DNA stored at -20 °C for application (Sambrook *et al.*, 1989).

3.7.4 DNA Sequencing

Partial sequences were generated at the sequencing facility at ILRI, (BecA-ILRI Hub Services, SegoliP).

3.7.5 Phylogenetic data analysis

Partial sequencing of purified PCR products was done at ILRI, (BecA-ILRI Hub Services, SegoliP using the reverse primer FF390r. The CHROMAS-LITE program (<http://www.technelysium.com.au/chromas>) was used to check for the presence of possible chimeric artifacts. Alignments were checked and corrected manually where necessary, based on conserved regions. The 18S rDNA gene sequences were compared to sequences in the public database using Basic Local Alignment Search Tool (BLAST) in the National Center for biotechnology Information (NCBI) website (<http://www.ncbi.nih.gov>) in order to determine similarity to sequences in the Gene bank database (Altschul *et al.*, 1990; Shayne *et al.*, 2003). The 18S rDNA gene sequences with high similarities to those determined in the study were retrieved and added to the alignment based on BLAST results. Sequencing alignment was done using Mega 4 (Tamura *et al.*, 2007).

The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). 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 method (Tamura *et al.*, 2004). Phylogenetic analyses were conducted in MEGA4 (Tamura *et al.*, 2007).

CHAPTER FOUR

4.0. RESULTS

4.1 Physical characteristics at sampling site

Sampling was done on 10-11th of February 2010 at Lake Magadi. Three study sites were considered in the sampling process. These values (**Table 4.1**) are the raw figures of the conditions at the three sampling sites of Lake Magadi during the sampling times (seasons).

Table 4.1: Summary of physical parameters at Lake Magadi during sampling

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

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

Table 4.2: Summary of the three sampling stations, the sample type collected and their codes

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

4.2 Isolation of fungi

A total of thirty (30) fungal isolates were isolated from the three sampling stations at Lake Magadi. Isolates LM1, LM4, LM12, LM23, LM24, LM26, LM29 were isolated from water, LM3, LM21, LM28, from microbial mat near the hot springs, LM5, LM9, LM17, were collected from mud. LM13 was isolated from the salt; LM7, LM16, LM18 and LM19 were from biofilms, and lastly LM8, LM14, and LM20 from microbial mat in the hot springs.

Six (6) out of the thirty (30) isolates from the saline Lake Magadi were isolated from the second sampling station. LM10, LM11, LM22, LM30, LM25 were from the foam on the water surface and LM15 was collected from mud below the water columns. Three (3) isolates LM2, LM6, LM27 were sampled from the salt at sampling station three (3). The fungal isolates were of different colours, margin, elevation and form.

4.3 Morphological characterization of isolates

4.3.1 Colony Morphology

Morphological characterization was based on classical macroscopic techniques of color, form, shape, margin and elevation of the pure colonies. Most colonies were able to grow within 4-7 days of incubation at 30 °C. The colony characteristics recorded for the various isolates are shown on (**Table 4.3**) whereas figure 4.1 to 4.3 shows the pigmentation of isolates LM14, LM16 and LM9 respectively on malt extract agar media and potato dextrose agar media.

Table 4.3: Morphological characteristics of the 30 isolates from Lake Magadi

Isolate	Colour		Margin	Elevation	Sample
	Top	Bottom			
LM1	White	yellow	Entire	Flat	S17
LM2	Grey	cream	Entire	Flat	S31
LM3	Cream	Brown	Undulate	Flat	S14
LM4	Purple, green, cream	Purple and cream	Undulate	Umbonate	S17
LM5	Brown	Blackish Brown	Entire	Raised	S16
LM6	Cream	Cream	Entire	Flat	S31
LM7	Orange, cream	Orange	Entire	Raised	S13
LM8	Pinkish cream	Brown	Entire	Flat	S12
LM9	Orange	Cream	filamentous	Flat	S16
LM10	Cream and green	Orange	Entire	Raised	S22
LM11	Greenish cream	Brown	Undulate	Crateriform	S22
LM12	Green	brown	Entire	Raised	S17
LM13	Grey	Black	Undulate	Convex	S11
LM14	Green and white	Yellow	Entire	Raised	S12
LM15	Green with cream ends	cream	Entire	Flat	S25
LM16	Green	Purple	Entire	Convex	S13
LM17	Green(slimy)	Black	Undulate	Convex	S16
LM18	Greenish brown	Black	Entire	Flat	S13
LM19	Greenish brown	Black	Entire	Raised	S13
LM20	Green and cream	brown	Entire	Flat	S12
LM21	Green and white	Brown	Entire	Umbonate	S14
LM22	Brown and cream	Brown	Curled	Umbonate	S22
LM23	Cream	brown	Undulate	Flat	S17
LM24	Green and cream	Green and cream	Curled	Umbonate	S17
LM25	White and cream	White	Entire	Umbonate	S22
LM26	White	cream	Entire	Umbonate	S17
LM27	White and green	white	curled	Raised	S31
LM28	White and black	Black	Entire	flat	S14
LM29	Cream coiled	cream	curled	Raised	S17
LM 30	Cream	Brown	Entire	Raised	S32

The following diagrams are representatives of the 30 isolates observable characteristics (**Plate 4.1a-t (i)**) and as viewed under a compound microscope magnification x100 (**Plate 4.1a-t (ii)**).



Plate 4.1a (i): Isolate LM 1-white powdery flat colony

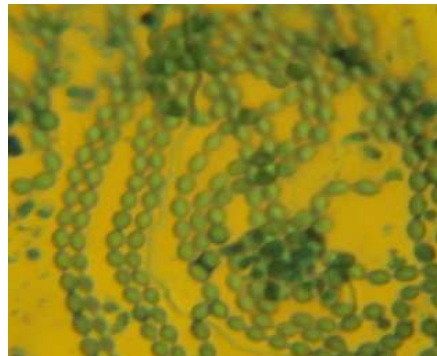


Plate 4.1a (ii): Isolate LM1-spores arranged in a chain

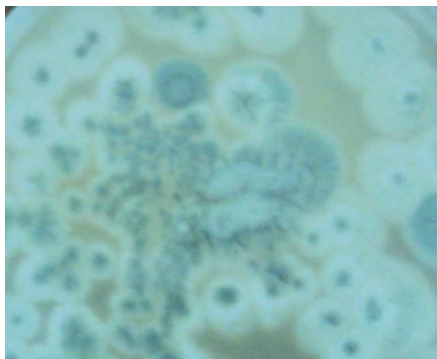


Plate 4.1b (i): LM 2 showing round colonies with raised margin



Plate 4.1b (ii): LM2 chain of spores at tip of mycelia

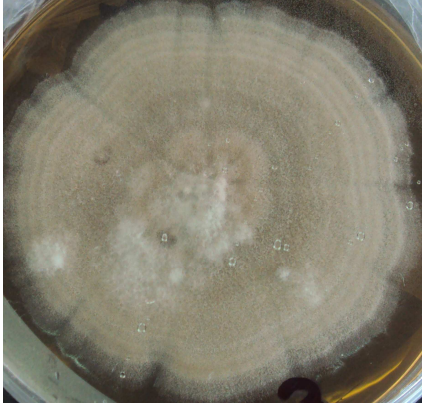


Plate 4.1c (i): LM 3 showing a powdery flat colony with circular folds

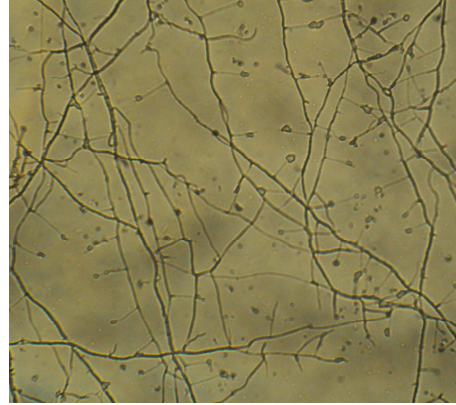


Plate 4.1c (ii): LM33 spores on long branching mycelia.



Plate 4.1d (i): A white lateral colony of Isolate LM 4

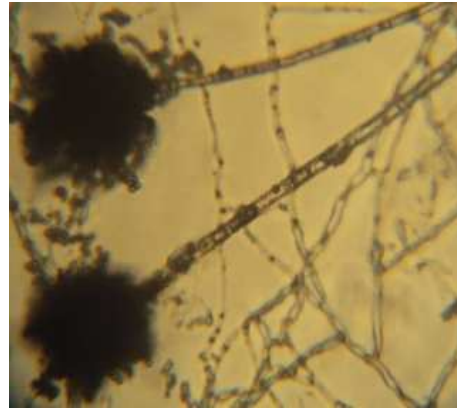


Plate 4.1d (ii): LM4 long branching mycelia

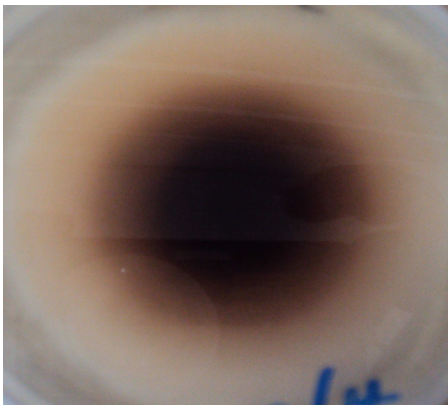


Plate 4.1e (i): Isolate LM 5 with radial folds, circular form and an entire margin

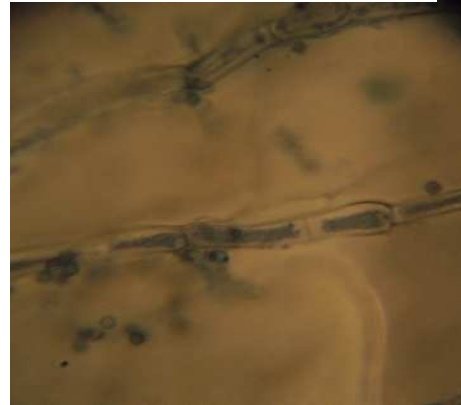


Plate 4.1e (ii): LM5 showing distorted macroconidia

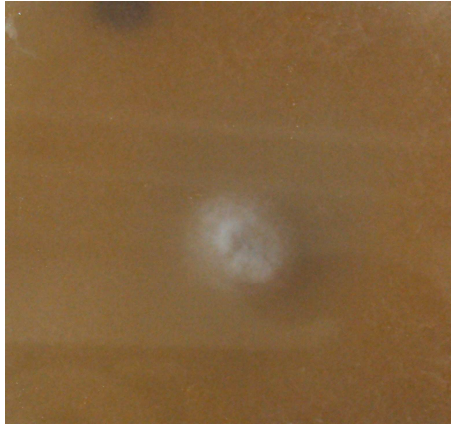


Plate 4.1f (i): LM 6 showing a cotton white heaped colony

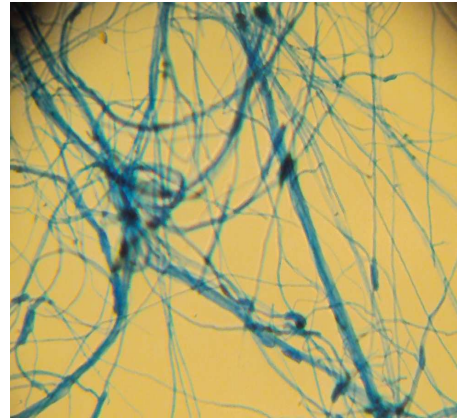


Plate 4.1f (ii): Isolate LM6 showing long branching mycelia with spiral tips.



Plate 4.1g (i): orange, round margin colony of isolate LM9

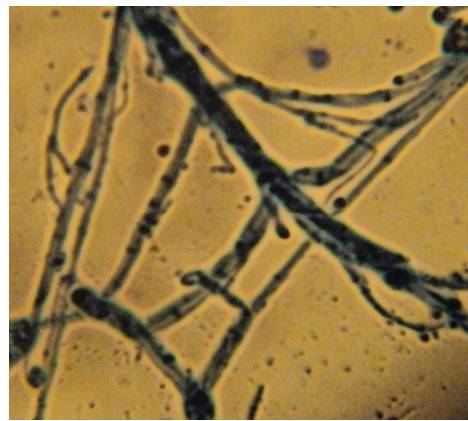


Plate 4.1g (ii): LM9-Filamentous intertwined mycelia with oval spores

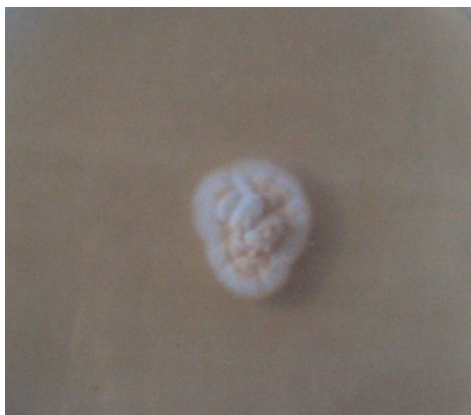


Plate 4.1h (i): LM 10 tough, raised leathery white brown mycelia with wrinkled margins

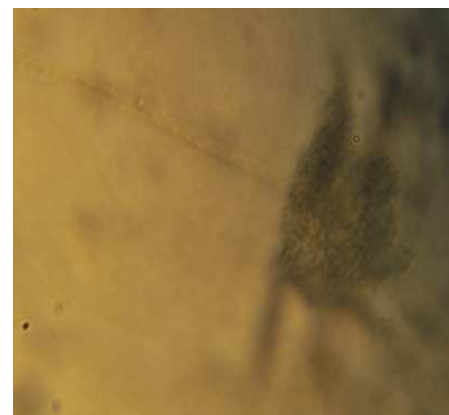


Plate 4.1h (ii): LM 10 showing a septate hyphae with bushy spores



Plate 4.1i (i): LM 11 showing a Crateriform elevation

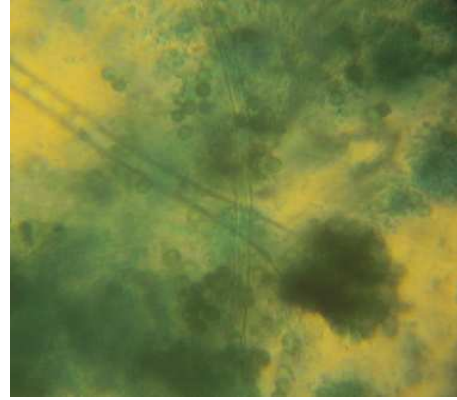


Plate 4.1i (ii): Isolate LM11 with spores scattered in the media

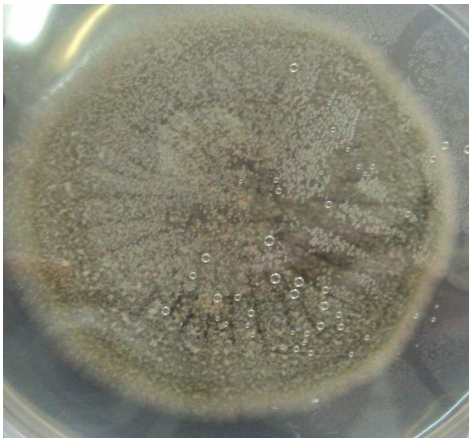


Plate 4.1j (i): LM 12 a granular flat colony with an entire margin

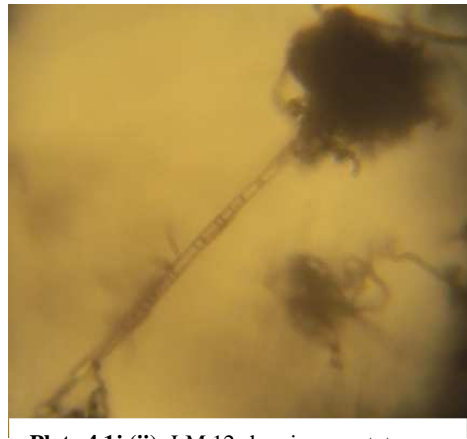


Plate 4.1j (ii): LM 12 showing aseptate hyphae with a brush like terminal



Plate 4.1k (i): LM13. The colony forms a complex shape and secretes brown pigment into the media



Plate 4.1k (ii):LM13 irregular shaped spores scattered in the media

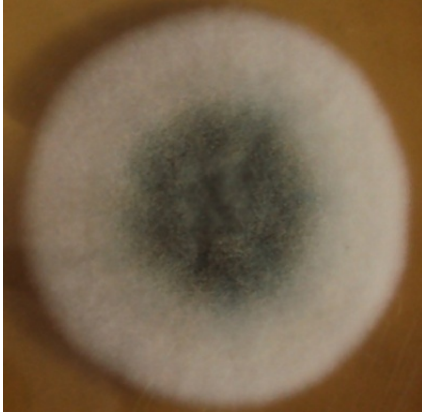


Plate 4.11 (i): LM14 showing a green and white raised colony with an entire white margin



Plate 4.11 (ii): LM 14 showing a fruiting body at the terminal end of a filamentous mycelia



Plate 4.1m (i): wrinkled, heaped colonies of isolate LM16 on potato dextrose agar media

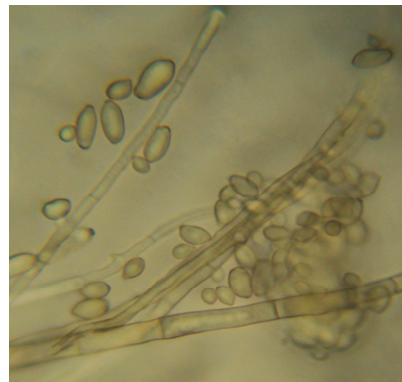


Plate 4.1m (ii): LM16 Spores are oval and disseminate from mycelia at the terminal.

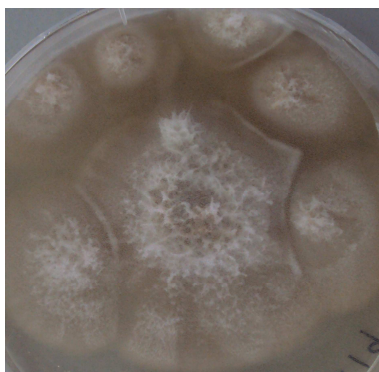


Plate 4.1n (i): Isolate LM 19 cottony white colonies

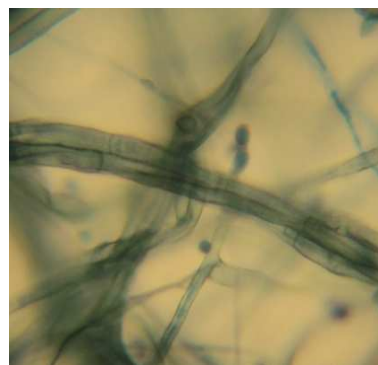


Plate 4.1n (ii): LM19 oval spores at the terminal of aerial mycelia

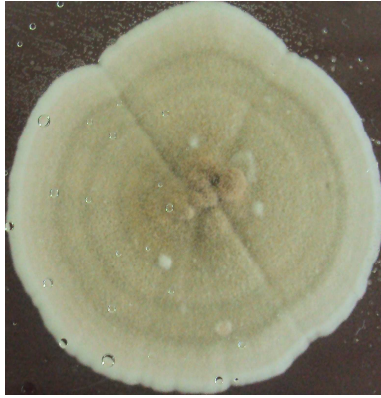


Plate 4.1o (i): LM 20 curled colony with a flat elevation

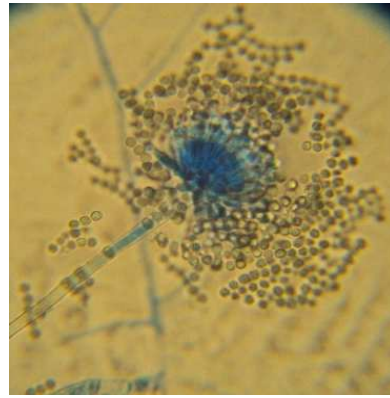


Plate 4.1o (ii): LM20 conidia released from conidiophores on aseptate hyphae

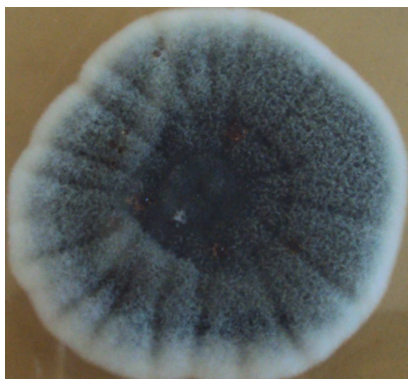


Plate 4.1p (i): Isolate LM21 showing a flat, granular Texture



Plate 4.1p (ii): LM 21 long mycelia with round spores

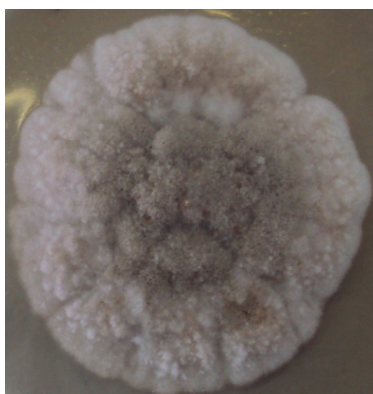


Plate 4.1q (i): LM 24 tough, leathery white heaped colony

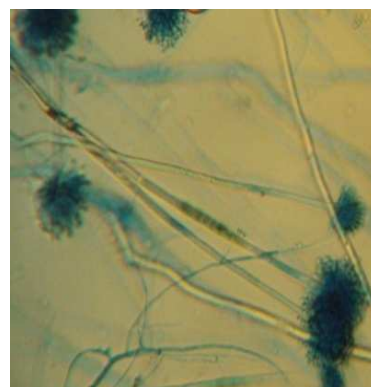


Plate 4.1q (ii): LM24 showing long branching spiral mycelia with fruiting body at the terminal.



Plate 4.1r (i): Isolate LM 27 showing flat entire margin



Plate 4.1r (ii): LM 27 spores scattered in the media



Plate 4.1s (i): LM26 waxy or glabrous, flat colony



Plate 4.1s (ii): LM 26 Conidia are produced in a chain at the end of aerial hyphae called conidiophores.

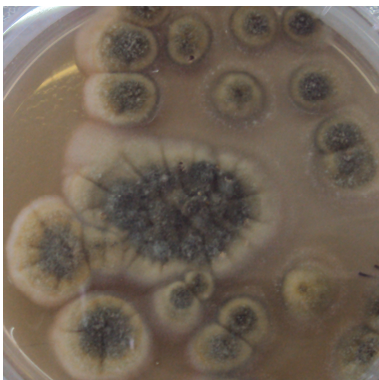


Plate 4.1t (i): LM 29-Circular heaped granular colonies with wrinkled surfaces



Plate 4.1t (ii): LM 29 Long septate mycelia

4.3.2 Cell morphology

Microscopic characterization was done by simple staining (using lacto phenol cotton blue dye) and observed under a light microscope at $\times 100$ (Keast *et al.*, 1984). The isolates showed reproductive spores, mycelia and hyphae. The hyphae were either septate or aseptate. In some the spores or conidia were in a chain at the end of aerial hyphae or in a sac like structure and in some the spores were formed externally on a base or just scattered in the media.

4.4 Physiological characterization

4.4.1: Growth at different temperature

All the isolates grew at temperatures 25 °C, 30 °C, and 35 °C. Growth at 40 °C was observed only for the isolates LM2, LM7, LM16 and LM17. Generally the isolates did not show any growth at temperatures 45 °C and 50 °C. The optimum growth of the isolates was observed between 30 °C – 35 °C (**Table 4.4**).

Table 4.4: Effect of temperature on the growth of the isolates

Isolates	25 °C	30 °C	35 °C	40 °C
LM1	++	+++	++	-
LM2	+++	+++	+++	+
LM3	+	+	+	-
LM4	+	+	+	-
LM5	++	+++	++	-
LM6	++	++	++	-
LM7	+++	+++	+++	+
LM8	+	+	+++	-
LM9	+	++	+	-
LM10	+	+	+	-
LM11	+	+	+	-
LM12	+	+	+	-
LM13	++	+	+	-
LM14	+	+	+	-
LM15	+	+++	+++	-
LM16	+	+	+++	+
LM17	++	+++	+++	+
LM18	+	+	+	-
LM19	+	+	+	-
LM20	+++	+	+	-
LM21	+	++	++	-
LM22	+	+	+++	-
LM23	+++	+	+	-
LM24	+	+	+	-
LM25	+	+	+	-
LM26	+	++	++	-
LM27	+	+	+	-
LM28	++	++	+	-
LM29	+	+	+	-
LM30	+	+	+	-

- = no growth

+ = slight growth (0cm-0.3cm)

++ = moderate growth (0.31cm-0.6cm)

+++ = abundant growth (0.61cm-above)

4.4.2: Growth at different pH

This was to check for the ability of the fungal isolates to grow in acidic, neutral and alkaline pH. Although the isolates were from an alkaline environment, all showed growth at all the tested pH values including acidic pH 5 and neutral pH (Table 4.5).

Table 4.5: Effect of pH on the growth of the isolates from Lake Magadi

Isolates	pH 5.0	pH 7.0	pH 8.5	pH 10.0
LM1	+++	+++	+++	+++
LM2	++	++	+++	+++
LM3	+	+	++	++
LM4	+	+	+	+
LM5	+	++	+++	+++
LM6	++	+	++	++
LM7	+++	+++	+++	+++
LM8	++	++	+	+
LM9	+	+	+	+
LM10	+	+	++	++
LM11	+	+	+	+
LM12	+	+	+	+++
LM13	+	+	+++	+
LM14	+	+	++	+++
LM15	+	+	++	+
LM16	+	+	+	+
LM17	+	++	+++	+++
LM18	+++	+	++	++
LM19	+	+	+	+++
LM20	+	+	+	+
LM21	+	+	+	+
LM22	+	++	+++	+++
LM23	+	+	+	++
LM24	+	+	+	+++
LM25	+	+	+++	+
LM26	+	+	+	+
LM27	++	+	++	++
LM28	++	++	++	+
LM29	+	+	+	++
LM30	+	+	++	++

- = no growth
+ = slight growth (0cm-0.3cm)
++ = moderate growth (0.31cm-0.6cm)
+++ = abundant growth (0.61cm-above)

4.4.3: Growth on different media

Different isolates showed different growth in the three types of media used. Isolates LM1, LM2, LM5, LM7 and LM17 recorded abundant growth in malt extract agar,

Sabourand dextrose agar and potato dextrose agar. Generally Malt extract agar recorded the highest results in millimeters among the three types of media used.

Table 4.6: Effect of media on the growth of the isolates

Isolates	MEA	PDA	SDA
LM1	+++	+++	+++
LM2	+++	+++	+++
LM3	++	++	+
LM4	+	+	+
LM5	+++	+++	+++
LM6	++	++	+
LM7	+++	+++	+++
LM8	++	+	+++
LM9	++	+	+
LM10	+	+	+
LM11	++	+	+
LM12	++	+	++
LM13	++	+	++
LM14	+++	+	+
LM15	+	+	+
LM16	++	+	+
LM17	+++	+++	+++
LM18	++	++	+
LM19	+++	+	++
LM20	++	+	+
LM21	+	+	++
LM22	+	+	++
LM23	++	+	+
LM24	++	+	++
LM25	+	+	+
LM26	+	+	++
LM27	+++	++	+
LM28	++	+	++
LM29	++	+	+
LM30	++	+	+

- = no growth
 + = slight growth (0cm-0.3cm)
 ++ = moderate growth (0.31cm-0.6cm)
 +++ = abundant growth (0.61cm-above)

4.4.4: Growth at different sodium chloride concentration

All the 30 isolates were able to grow in varying concentrations of sodium chloride. Growth improved with decrease in salt concentration from 30 % up to 5 % sodium chloride. The highest growth was recorded at 5 % sodium chloride followed by 10 % then 20 % and gradually decreased towards 30 %.(Table 4.7)

Table 4.7: Effect of sodium chloride concentration on the growth of the isolates

Isolates	5 %	10 %	20 %	30 %
LM1	+	+	+	+
LM2	+++	+++	+	+
LM3	++	++	+	+
LM4	+	+	+	+
LM5	+	+	+	+
LM6	+	+	+	+
LM7	+++	++	+	+
LM8	++	++	+	+
LM9	++	++	+	+
LM10	+	++	+	+
LM11	+	++	+	+
LM12	+++	++	+	+
LM13	+	+	+	+
LM14	++	++	+	+
LM15	++	++	+	+
LM16	+	+	+	+
LM17	++	+	+	+
LM18	+	+	+	+
LM19	+	+	+	+
LM20	+++	++	+	+
LM21	++	++	+	+
LM22	++	++	+	+
LM23	++	++	+	+
LM24	++	++	+	+
LM25	+	++	+	+
LM26	+++	++	+	+
LM27	++	++	+	+
LM28	++	+	+	+
LM29	+	+	+	+
LM30	+	+	+	+

- = no growth

+ = slight growth (0cm-0.3cm)

++ = moderate growth (0.31cm-0.6cm)

+++ = abundant growth (0.61cm-above)

4.5: Biochemical characterization of the isolates

4.5.1 Screening for production of enzymes

All the isolates apart from isolates LM2, LM5, LM6, LM7 and LM9, were positive for amylase test. Only isolates LM2 and LM7 gave negative results for esterase test. Ten (10) isolates out of the 30 isolates tested negative for lipolytic activity while 6 isolates gave negative results for protease test. Isolates LM1, LM4, LM7, LM9 and LM30 were negative for xylanases while the rest of the isolates were positive. All the 30 isolates tested negative for the production of cellulases enzyme (**Table 4.8**).



Figure 4.5a: Isolate LM27 showing positive results for starch utilization.

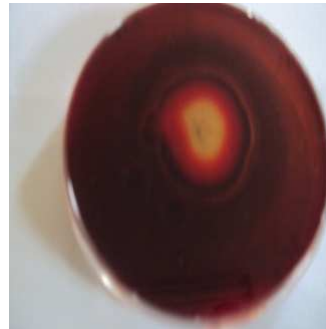


Figure 4.5b: Isolate LM2 showing positive results for xylan utilization



Figure 4.5c: Isolate LM9 showing production of protease enzyme.



Figure 4.5d: Isolate LM6 showing precipitates of calcium salts around the colony

Table 4.8: Biochemical characterization of the 30 isolates from Lake Magadi

Isolates	Amylases	Esterases	Lipases	proteases	Xylanases	Cellulases
LM1	+	+	+	+	-	-
LM2	-	-	-	-	+	-
LM3	+	+	+	+	+	-
LM4	+	+	+	+	-	-
LM5	-	+	+	-	+	-
LM6	-	+	-	+	+	-
LM7	-	-	-	-	-	-
LM8	+	+	-	+	+	-
LM9	-	+	+	-	-	-
LM10	+	+	+	+	+	-
LM11	+	+	-	+	+	-
LM12	+	+	+	+	+	-
LM13	+	+	+	+	+	-
LM14	+	+	+	+	+	-
LM15	+	+	+	+	+	-
LM16	+	+	+	+	+	-
LM17	+	+	-	-	+	-
LM18	+	+	-	+	+	-
LM19	+	+	+	+	+	-
LM20	+	+	-	+	+	-
LM21	+	+	-	+	+	-
LM22	+	+	+	-	+	-
LM23	+	+	+	+	+	-
LM24	+	+	+	+	+	-
LM25	+	+	+	+	+	-
LM26	+	+	-	+	+	-
LM27	+	+	+	+	+	-
LM28	+	+	-	+	-	-
LM29	+	+	+	+	+	-
LM30	+	+	+	+	-	-

4.5.2 Screening the isolates for production of antibiotics

Isolates LM1, LM13, LM19, LM25 and LM30 showed inhibitory activity against the two test organisms; (*Pseudomonas aeruginosa* and *Staphylococcus aureus*). Isolates LM13, LM26, LM28, LM30, showed inhibitory activity against the test organisms *Escherichia coli* and *Bacillus subtilis*. All the isolates tested negative for activity against *Candida albicans* (**Table 4.9**). This shows that some of the isolated fungi had the ability to produce useful antimicrobial metabolites that could be used against bacterial microorganisms.



Plate 4.5e: Antagonistic activity of LM 25 on *Pseudomonas aeruginosa*. The zone of inhibition around the paper disc shows antagonistic effect



Plate 4.5f: Antagonistic activity of LM 28 on *Escherichia coli*. The zone of inhibition around the paper disc shows antagonistic effect

Table 4.9: Selected isolates that showed inhibition zones around them with antibiotic activity against test organisms

Isolate/Test organism	<i>E. coli</i>	<i>P. aeruginosa</i>	<i>S. aureus</i>	<i>C. albicans</i>	<i>B. subtilis</i>
LM1	-	+	+	-	+
LM3	-	+	+	-	-
LM4	-	-	+	-	+
LM13	+	+	+	-	+
LM17	+	-	+	-	+
LM 25	-	+	+	-	-
LM28	+	-	-	-	+
LM30	+	+	+	-	+

4.6 Molecular characterization of the isolates

4.6.1 DNA extraction and PCR amplification of 18s rRNA gene from isolates

Genomic DNA was extracted from all the 30 isolates. From the PCR amplification a product of approximately 1800 base pairs was gotten as shown on **Figure 4.6a** below.

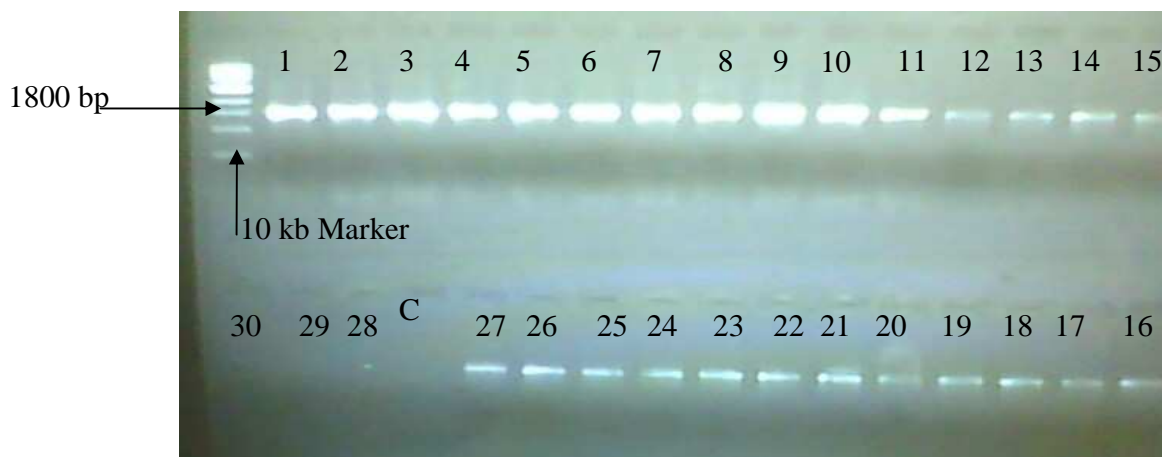


Figure 4.6a: A 1% agarose gel showing PCR amplification of 18S rDNA of the isolates LM1-LM 27 visualized after ethidium bromide staining.

C = Control

4.6.2 Phylogenetic analysis of sequences

Sequenced PCR amplicons of the thirty (30) isolates of Lake Magadi were used in phylogenetic analysis. The BLAST search results showed that all the isolates belong to the fungal domain and were clustered within two phyla; Basidiomycota and Ascomycota (**Table 4.10**). Basidiomycota together with the Ascomycota, comprise the subkingdom Dikarya (often referred to as the "higher fungi") within the Kingdom Fungi. Isolates LM2, LM13, LM15, LM16, LM18, LM25, LM26, and LM29 clustered closely with members of the genus *Penicillium/ Eupenicillium* with a score of between 98 % and 100 % similarity (**Table 4.10**). Among these were *Penicillium janthinellum*, *Eupenicillium sp.*, *Penicillium decumbens*, *Eupenicillium limosum*, *Penicillium charlesii*, *Penicillium sacculum*, *Penicillium glabrum* and *Penicillium sp.* (**Figure 4.6d**).

Seven of the isolates from Lake Magadi namely LM4, LM8, LM10, LM20, LM21, LM22 and LM27 were closely clustered with members of the genus *Aspergillus* (**Table 4.10**) with a score of between 99 % and 100 %. This included *Aspergillus versicolor*, *Aspergillus oryzae*, *Aspergillus parasiticus*, *Aspergillus fumigatus*, *Aspergillus nomius*, *Aspergillus ochraceus*, *Aspergillus silvaticus* and *Aspergillus sparsus* (**Figure 4.6d**).

The phylogenetic tree constructed showed the phylogenetic position of each isolate (**Figure 4.6b**). However, isolate LM3 had sequence similarity of 95 % and these could represent novel species, while isolates LM12 and LM17 had sequence similarities of

89 % and 82 % respectively, could represent novel genera and are highlighted in red (Table 4.10).

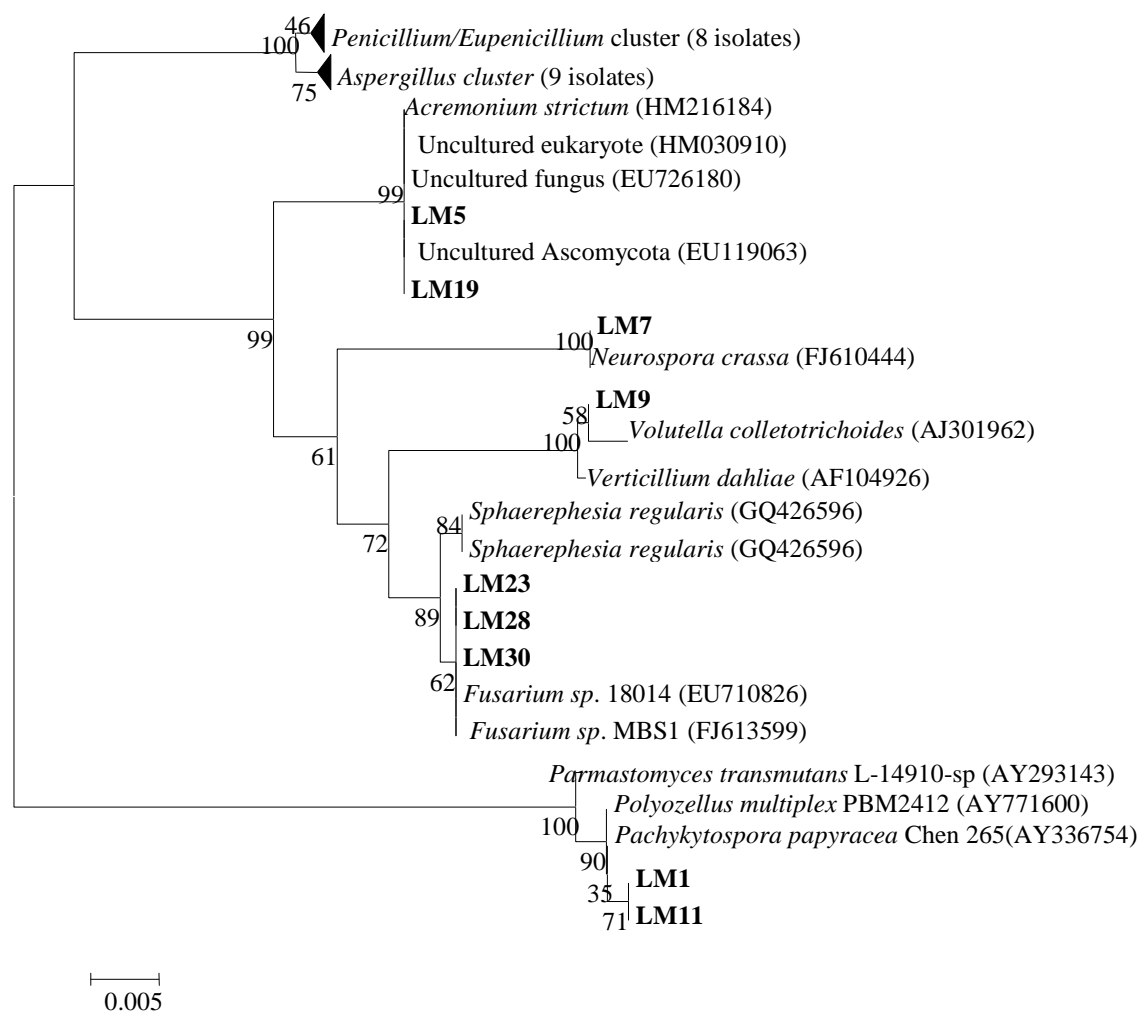


Figure 4.6b: Phylogenetic relationships of 26 isolates from this study and the closest relatives from BLAST analysis.

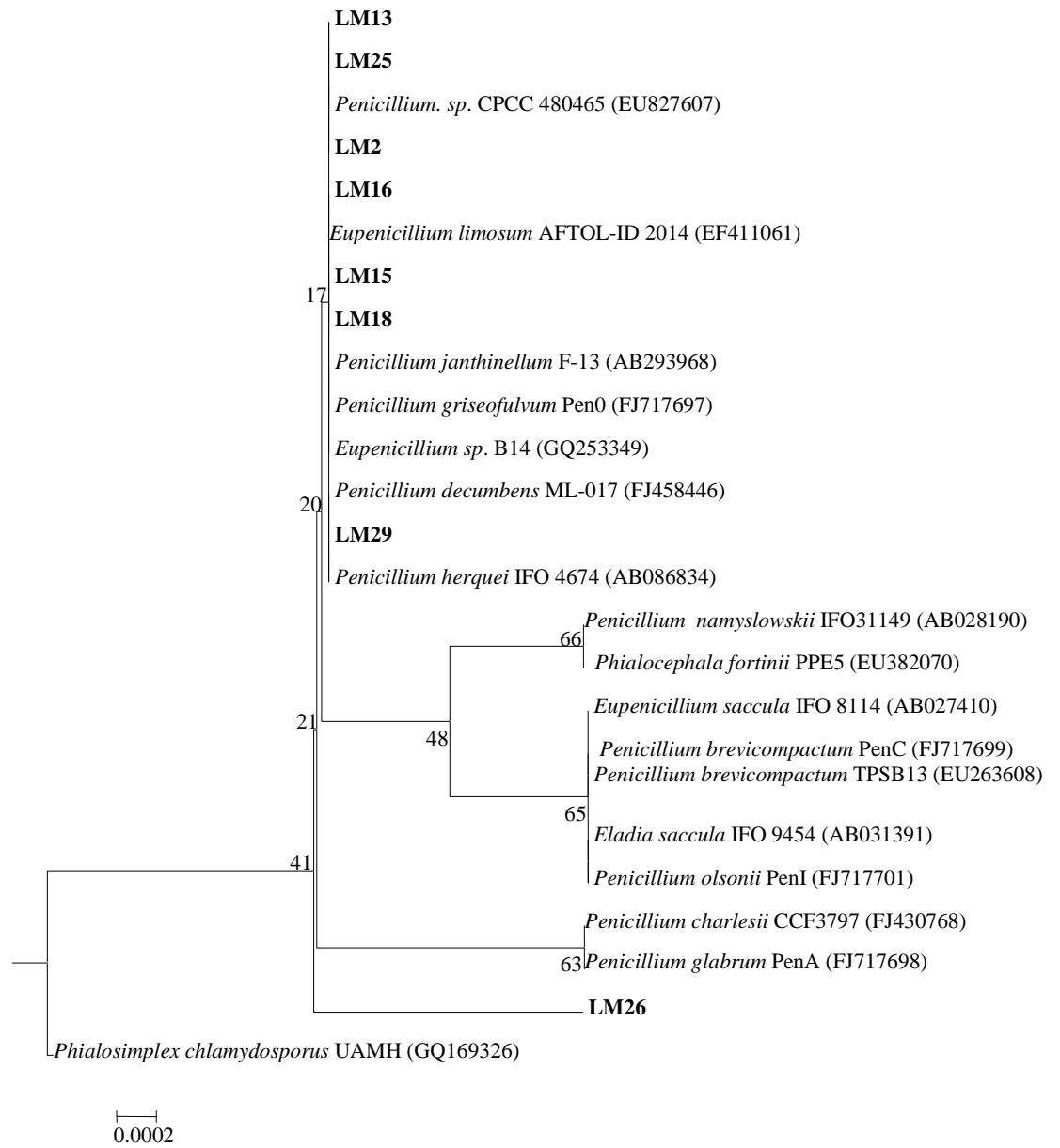


Figure 4.6c: *Penicillium/Eupenicillium* sub tree

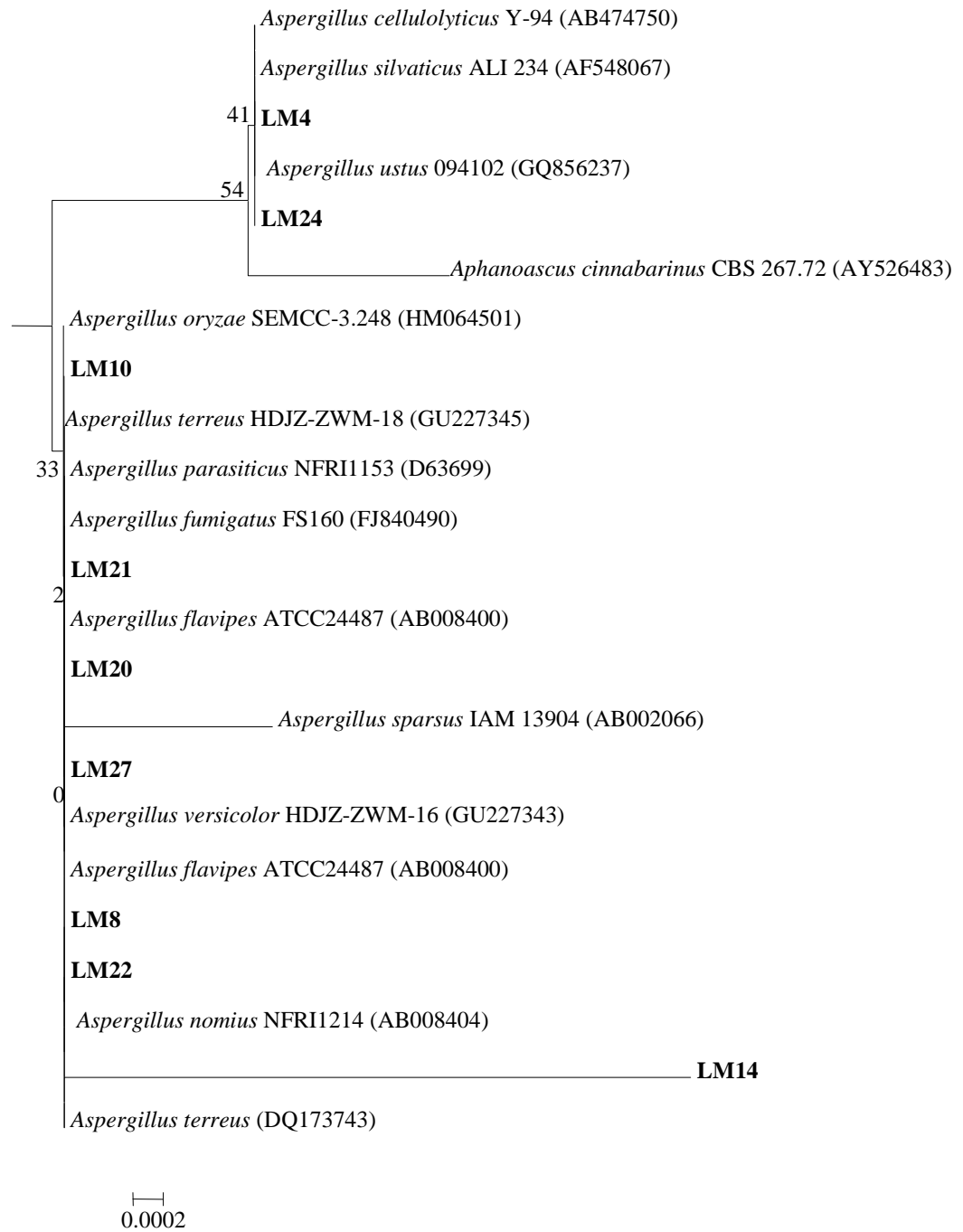


Figure 4.6d: *Aspergillus* sub tree

Table 4.10: Blast results of isolates from Lake Magadi and their close relatives.

Sample ID	Station	BP	Acc No.	Next neighbour in BLAST	% Identity
LM1	S17	545	EU888830	<i>Pycnoporus sp.</i> SYBC-L1	99%
LM2	S13	542	AB293968	<i>Penicillium janthinellum</i> strain: F-13	99%
LM3	S14	538	AY973269	<i>Acremonium cellulolyticus</i> Y-94	95%
LM4	S17	552	AF548069	<i>Aspergillus versicolor</i> strain UPSC 1532	99%
LM5	S16	513	HM216184	<i>Acremonium strictum</i> strain DS1bioAY4a	100%
LM6	S13	722	AY856952	<i>Helicoon richonis</i> strain CBS 282.54	98%
LM7	S13	599	FJ610444	<i>Neurospora crassa</i>	99%
LM8	S12	542	HM064501	<i>Aspergillus oryzae</i> strain SEMCC-3.248	100%
LM9	S16	539	AJ301962	<i>Volutella colletotrichoides</i>	99%
LM10	S22	543	D63699	<i>Aspergillus parasiticus</i>	99%
LM11	S22	549	AY771600	<i>Polyozellus multiplex</i> isolate AFTOL-ID 677	98%
LM12	S17	553	EU888830	<i>Pycnoporus sp.</i> SYBC-L1	89%
LM13	S11	549	GQ253349	<i>Eupenicillium sp.</i> B14	99%
LM14	S12	552	AB002071	<i>Petromyces alliaceus</i>	98%
LM15	S25	542	FJ458446	<i>Penicillium decumbens</i> strain ML-017	100%
LM16	S13	553	EF411061	<i>Eupenicillium limosum</i> isolate AFTOL-ID 2014	100%
LM17	S16	748	GU214604	<i>Teratosphaeria mexicana</i> strain CBS 110502	82%
LM18	S13	542	FJ430768	<i>Penicillium charlesii</i> strain CCF3797	98%
LM19	S13	538	HM216184	<i>Acremonium strictum</i> strain DS1bioAY4a	99%
LM20	S12	533	FJ840490	<i>Aspergillus fumigatus</i> strain FS160	99%
LM21	S14	539	AB008404	<i>Aspergillus nomius</i>	99%
LM22	S22	544	AF548065	<i>Aspergillus ochraceus</i> strain UPSC 1983	99%
LM23	S17	518	EU710826	<i>Fusarium sp.</i> 18014	99%
LM24	S17	529	AF548067	<i>Aspergillus silvaticus</i> strain ALI 234	99%
LM25	S22	525	AB027410	<i>Penicillium sacculum</i>	99%
LM26	S17	533	FJ717698	<i>Penicillium glabrum</i>	99%
LM27	S31	525	AB002066	<i>Aspergillus sparsus</i>	99%
LM28	S14	528	AF141950	<i>Fusarium merismoides</i>	98%
LM29	S17	539	EU827607	<i>Penicillium sp.</i> CPC 480465	100%
LM30	S32	526	FJ613599	<i>Fusarium sp.</i> MBS1	100%

CHAPTER FIVE

5.0. DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 DISCUSSION

The aim of this study was to isolate, characterize and identify fungi from Lake Magadi, identify the enzymes produced by the fungi and then screen for antimicrobial metabolites. The study will contribute to the understanding of fungal diversity and their possible role in Soda Lakes. There is also a high probability of discovering novel enzymes and antimicrobial agents.

Characterization of the isolates was based on morphological features, physiological and biochemical properties and molecular analysis, using 18S rDNA gene (Cappa and Cocconcelli, 2001; Henry *et al.*, 2000). The molecular analysis is an important tool to fungal taxonomy (Turenne, 1999; Henry *et al.*, 2000; Pařericová *et al.*, 2001; Zhao *et al.*, 2001; Cappa and Cocconcelli, 2001), because it is based in the study of important genes which are conserved during evolution. Morphological characteristics indicated that the isolates belonged to the domain fungi. The isolates exhibited different colours like green with a white ring, orange and white. Cellular morphology showed long, branching filamentous structures of the fungus called hyphae (Madigan *et al.*, 2005). In most fungi, hyphae are the main mode of vegetative growth. A hypha consists of one or more cells surrounded by a tubular cell wall. Some isolates had their hyphae partitioned by septa while others did not.

Microorganisms are now known to thrive over a broad range of physical extremes in temperature. The isolates grew within temperature range of 25°C – 40°C. The optimum growth was recorded between 30 – 35 °C Isolates LM2, LM6, LM7, LM16 and LM17 showed slight growth at 40 °C. However, no growth was recorded at temperatures above 40 °C. In natural ecological systems, temperature is one of the most important factors that determine the occurrence of fungi, and influences its growth and metabolism (Theodorou, 1971). Usually, fungi cannot grow well at some extreme temperatures (Bi *et al.*, 1989). In some regions, the fruiting bodies occur, when the ground temperature ranges in 25–30 °C. Based on the laboratory experiment result, the optimum temperature for the growth of isolates studied was 25–40 °C. This indicated that the highest richness of fungi species may occur when the temperature consisted with the optimum temperature. On the contrary, if the temperature is too high or too low, the formation of fruiting bodies will be influenced.

Life also occupies an equally broad range of salinity. The isolates grew well in the presence of sodium chloride (lake condition) and were able to tolerate concentrations up to 20 %. The optimum growth was recorded when sodium chloride concentration was between 5 % - 10 %. However, the isolates showed poor growth in media with high salt concentration. Growth reduced with increasing salt concentration (**Table 4.7**). Growth of the isolates in culture medium at different sodium chloride levels (varying between 0 % and 30 %) indicated tolerance to salinity and an adaptability of these isolates to adverse growth conditions. Salt-loving halophiles live in salt plains, salt marshes (moderate salt environments), evaporation ponds at salt works, and

natural salt lakes (e.g. the Dead Sea, Israel and the Great Salt Lake Utah). Recently it has been shown that fungi are not only able to sustain, but also to propagate at different environmental extremes such as hypersaline waters (Gunde-Cimerman *et al.*, 2000), dry rock surfaces (Stefflinger, 1998) and ocean depths (Lopez-Garcia *et al.*, 2001). Fungi can tolerate higher salinities than the one of sea water. Pitt and Hocking, (1977), examined the growth of xerophilic fungi (*Aspergillus ochraceous*, *Aspergillus flavus*, *Chrysosporium fastidium*, *Eurotium chevalieri*, *Xeromyces bisporus* and *Wallemia sebi*) in the presence of glycerol, sodium chloride and glucose/fructose mixture. Salt marshes, a moderate salt environment, are another habitat for halotolerant fungi (Abdel-Hafez *et al.*, 1978). Fungi have an important role in the decomposition of salt marsh grasses.

In nature, microorganisms have been shown to occupy nearly the entire range of pH. Physiochemical characterization of the isolates showed that the highest growth was observed at pH 10. However, the isolates were able to grow at pH 5 which suggests that they are tolerant to acidic conditions (Moreira and Siqueira, 2002) (**Table 4.5**). This growth at pH range of 5 to 10 is consistent with an earlier study by Horikoshi, (1998) which showed that a low to high pH range of 5.7 to 9.0, favours growth of alkaliphiles and that a pH range 9.0 to 10 may serve as their selective optimum pH.

Enzymes from extremophiles, extremoenzymes, have a great economical potential in agricultural, chemical and pharmaceutical processes. They are used in biological processes by increasing specificity and catalytic activity, and are stable at extreme incubation conditions (Chadha and Patel, 2008). Halophilic enzymes, while

performing identical enzymatic functions as their non-halophilic counterparts, have been shown to exhibit different properties such as a requirement for high salt concentrations, increased activity and variable stability (Mevarech *et al.*, 2000). Usually, halophilic enzymes are not only able to deal with high ionic strength in their environment but also able to maintain their function and structure (Dym *et al.*, 1995).

Screening the isolates for useful secondary metabolites showed that they have the ability to produce enzymes and antibiotics. The thirty (30) isolates from Lake Magadi produced various extracellular enzymes such as amylases, lipases, proteases, esterases and xylanases. This is a characteristic that confirm their role in the decomposition of organic matter in the habitats (Crawford, 1988; Kieser *et al.*, 2000). Studies have shown that during vegetative growth and subsequent sporulation, a variety of proteases are produced (Priest, 1977), indicating their role in decomposition of organic matter in nature. Of the 30 isolates screened in this study, 25 isolates hydrolyzed starch and skim milk. Twenty eight (28) and nineteen (19) out of the thirty (30) isolates were able to utilize Tween 80 and Tween 20 respectively. Besides starch and skim milk hydrolysis, twenty four (24) of the studied isolates hydrolyzed xylan. All the 30 Isolates were non – cellulolytic (**Table 4.8**).

The hydrolysis of the various substrates was an indication of the ability in the various isolates to produce amylases, lipases, proteases, esterases and xylanases that are of industrial importance. The enzymes of microbial origin have high biotechnological interest such as in the processing of foods, manufacturing of detergents, textiles, pharmaceutical products, medical therapy and in molecular biology (Pilnik and

Rombouts, 1985; Falch, 1991; Rao *et al.*, 1998). An interesting application of alkaline protease was developed by Fujiwara and coworkers (Fukumori *et al.*, 1985). They reported the use of an alkaline protease to decompose the gelatinous coating of X-ray films, from which silver was recovered. Protease B189 had a higher optimum pH and temperature, around 13.0 and 85 °C. The enzyme was most active toward gelatin on film at pH 10.

Fungi are well known as a source of antibiotics but recently, new therapeutic compounds with novel pharmacological activities have been developed. One such example is the cyclosporin first isolated from *Tolypocladium inflatum* in 1976 as antifungal compounds and later shown to possess immunosuppressive activity (Leung *et al.*, 2006). Cyclosporin A is currently the most widely used drug for preventing rejection of human organ transplants (note: 20 years later the sexual stage of *T. inflatum* was found to be *Cordyceps subsessilis* (Hodge *et al.*, 1996).

In a research study carried out in Turkey, microorganisms isolated from the alkaline saline Lake Acigol were screened for their activity against other micro-organisms. The preliminary results indicated that alkaline-saline lake isolates exhibited antimicrobial activity against *Bacillus subtilis*, *Staphylococcus aureus*, *Micrococcus luteus*, *Mycobacterium smegmatis*, and *Candida albicans* (Eltem and Ucar, 1998).

In this study, isolates were studied for the production of antimicrobial compounds. The ability of individual isolates to inhibit the growth of Gram-positive and Gram-negative test organisms was tested. The isolates showed antagonistic activity that differed from one isolate to another. This could be due to the expected different modes

of action and activity of the individual biochemical constituents of the respective isolates (Mao *et al.*, 2006).

The selected isolates showed antagonistic activity against test organisms and were found to have antibiotic activity against *Escherichia coli*, *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Twelve (12) of the studied isolates had inhibitory effects on *Staphylococcus aureus* (**Table 4.9**). Isolate LM3, a probable novel species that clustered with *Acremonium cellulolyticus* Y-94 at 95 % similarity inhibited growth of *Staphylococcus aureus* and *Pseudomonas aeruginosa* but had no inhibition against *E. coli* and *Bacillus subtilis*. This was consistent with earlier studies that have shown that most of the antibiotics are active against Gram-positive micro organisms, although there are exceptions (Eltem and Ucar, 1998). Ten (10) of the studied isolates showed antagonistic effect against the Gram negative *Escherichia coli*, while nine (9) of the studied isolates had antagonistic effect against *Pseudomonas aeruginosa*. Eight (8) of the studied isolates had antagonistic effect against *Bacillus subtilis*. Isolate LM17, a probable novel genus that clustered with *Teratosphaeria mexicana* strain CBS 110502 at 82 % similarity inhibited growth of both gram positive *Staphylococcus aureus* and Gram negative *E.coli*. None of the isolates had antagonistic activity against test fungi, *Candida albicans* (**Table 4.9**).

Isolate LM13 that clustered closely with *Eupenicillium sp.* B14 at 99 % similarity produced dark brown pigments into the media. This was an indication that the isolate could have an ability to produce diverse secondary metabolites. Species of the fungus, *Eupenicillium spp.* are capable of producing many natural products which have

potential bioactivity. The endophytic species of *Eupenicillium*, isolated from the forest tree *Glochidion ferdinandi*, was found to produce four polyketides: phomoxin, phomoxin B, phomoxin C and eupenoxide (Davis *et al.*, 2005). Previously, phenopyrrozin had been isolated from *Penicillium* sp. FO-2047 (Shiomi *et al.*, 1995). In 2006, p-hydroxyphenopyrrozin and phenopyrrozin had been elucidated from the marine fungus *Chromocleista* sp. by Park *et al.* (2006).

Filamentous fungi are attractive organisms for production of useful protein and biological active secondary metabolites. These fungi produce high levels of polysaccharide-degrading enzymes and are frequently used for the production of industrial enzymes. Fungi have high secretion capacity and are effective hosts for the production of foreign proteins (Tsukagoshi *et al.*, 2001). Amylases have varied industrial applications and are classified as α -1-4-glucanase and α -1-6-glucanase according to specific glucosidic bond cleavage. In brewing industry, the products of starch obtained after enzymatic hydrolysis are used as nutrients in microbial fermentation (Stewart and Russel, 1978) for ethanol production (Matsumoto *et al.*, 1982). The major markets for amylases are food industries for the preparation of sweeteners and syrups (Nigam and Singh, 1995). During solid-state fermentation *Penicillium restrictum* produced amylase, lipase and protease on basal medium of industrial waste of babassu oil (Palma *et al.*, 2006). A high maltose producing amylase was secreted from *Penicillium expansum* (Doyle *et al.*, 1989) and increased secretion of xylanase and pectinase was observed when grown on xylan and pectin as respective carbon sources (Kimura *et al.*, 2002). The pigments produced by *Penicillium* (PP-V and PP-R) and *Monascus* (monascorubrine and monascorubramine) were

structurally similar (Ogihara and Oishi, 2002). Lovastatins or monacolins produced by *Penicillium*, *Monascus*, *Aspergillus* and *Rhizopus* (Endo *et al.*, 1979) inhibits cholesterol biosynthesis by binding to catalytic site of HMG-CoA reductase a key enzyme in cholesterol biosynthesis (Albert, 1990) and scavenged DPPH radicals (Aniya *et al.*, 1999; Dhale *et al.*, 2007a and b). Penicilllenols secreted by *Penicillium sp* showed biological activity against HL-60 cell lines (Lin *et al.*, 2008). The production of pigment and amylase by *Penicillium sp* NIOM-02 indicated its importance in food, pharmaceutical and nutraceutical industries (Parekh *et al.*, 2000).

Isolate LM7 clustered closely with *Neurospora crassa* (FJ610444) and scored 99 % similarity. *Neurospora crassa* is Ascomycetes, the red bread mold. Like all fungi, it reproduces by spores. It is particularly well suited for genetic studies over the past 60 years because it can be grown quickly on simple culture medium. It spends most of its life cycle in the haploid condition so any recessive mutations will show up in its phenotype (Galagan, J.E, 2003). It is important in the elucidation of molecular events involved in circadian rhythms, epigenetics and gene silencing, cell polarity, cell fusion, development, as well as many aspects of cell biology and biochemistry. Because its characteristics make it ideal for scientific research, *Neurospora* is considered a model organism (Davis 2000; Davis and Perkins 2002).

Isolate LM5 and LM19 clustered closely with *Acremonium strictum* strain DS1bioAY4a (HM216184) with a similarity of 99 %. *A. strictum* is a filamentous, cosmopolitan fungus commonly isolated from plant debris, soil and water (Domsch *et al.*, 1980). This is in line with the study as isolate LM5 was isolated from mud and

isolate LM19 from microbial mat. The *A. strictum* taxon is genetically diverse (Novicki *et al.*, 2003). It is one of the keratinophilic fungi and a dermatophyte (Ali-Shtayeh *et al.*, 2002), a pathogen causing opportunistic infections in immunocompromised patients (Schell and Perfect, 1996; Warris *et al.*, 2000), and is a pathogen of maize (Tagne *et al.*, 2002) and button mushroom (Kang *et al.*, 2002). Meanwhile, it is also important in industry. The biotransformation of hydrocortisone (Faramarzi *et al.*, 2002), the production of a novel glucooligosaccharide oxidase (Lin *et al.*, 1991) and Xenovulene A, a novel GABA-benzodiazepine receptor-binding compound (Ainsworth *et al.*, 1995), degradation of thiocyanate (Hyouk *et al.*, 2002) and hydroxylation of progesterone (Faramarzi *et al.*, 2003), by *A. strictum* have been reported. Also Cephalosporin C (CPC) is a major precursor of semisynthetic cephalosporin antibiotics, which is naturally produced in a secondary metabolite form in *Acremonium sp* (Velasco *et al.*, 2001).

Isolates LM2, LM13, LM15, LM16, LM18, LM25, LM26, and LM29 clustered closely with members of the genus *Penicillium/Eupenicillium* with a score of between 98 % and 100 % similarity (**Table 4.10**). The species include *Penicillium janthinellum* strain: F-13, *Eupenicillium sp.* B14, *Penicillium decumbens* strain ML-017, *Eupenicillium limosum* isolate AFTOL-ID 2014, *Penicillium charlesii* strain CCF3797, *Penicillium sacculum*, *Penicillium glabrum* and *Penicillium sp.* CPCC 480465. Gunde-Cimerman *et al.* (2004 and 2005b), carried out several studies on the isolation and characterization of fungi isolated from salterns in Slovenia. They observed a surprisingly rich diversity of fungi. The fungi most frequently observed were species of the genera *Aspergillus*, *Penicillium* with teleomorphic stages and *Wallemia*,

Scupolariopsis and *Alternaria*. An exhaustive study carried out by Buchalo et al. (2000) permitted the taxonomic characterization of filamentous fungi isolated from the Dead Sea. They included 26 species representing 13 different genera of Zygomycotina (*Absidia glauca*), Ascomycotina (most representative were species of *Aspergillus*, *Chaetomium*, *Cladosporium*, *Penicillium* and *Eurotium*, as well as a new species of the genus *Gymnascella*, designated *Gymnascella marismortui*) and mitosporic fungi (four species belonging to the genera *Acremonium*, *Stachybotrys* and *Ulocladium*).

Seven out of the thirty (30) isolates from Lake Magadi namely LM4, LM8, LM10, LM20, LM21, LM22 and LM27 were closely clustered with members of the genus *Aspergillus* (**Table 4.10**) with a score of between 99 % and 100 %. This included *Aspergillus versicolor* strain UPSC 1532, *Aspergillus oryzae* strain SEMCC-3.248, *Aspergillus parasiticus*, *Aspergillus fumigatus* strain FS160, *Aspergillus nomius*, *Aspergillus ochraceus* strain UPSC 1983, *Aspergillus silvaticus* strain ALI 234 and *Aspergillus sparsus*. LM4 and LM24 were isolated from water, LM10 and LM22 from foam on the water surface, LM20 and LM8 were isolated from microbial mat at the hot springs, while LM21 was from microbial mat near the hot springs and LM27 was isolated from the salt. According to studies done by (Pitt, 1979; Christensen *et al.*, 2000; Klich, 2002; Asan, 2004), the species of *Aspergillus*, *Penicillium* and *Paecilomyces Bainier* are among the most abundant and widely distributed microfungi in nature. In another study, *Aspergillus versicolor*, *Chaetomium globosum*, *Eurotium herbariorum*, *E. amstelodami* and *E. rubrum* were isolated from Dead Sea waters and together with *Gymnascella marismortui* were tested for survival of spores and mycelia in Dead Sea water for prolonged time (Kis-Papo *et al.*, 2001 and 2003a). After the first

record of fungi, Kis-Papo et al. (2003b) performed a study of genomic diversity of *Aspergillus versicolor* and their results suggested that genomic diversity was positively correlated with stress. Traditional fermentations could take several months but this has been reduced to 2-3 days in a modern plant. The principal fungus involved, *Aspergillus oryzae*, is now also used to produce a range of commercially important enzymes.

LM 11 clustered closely with *Polyozellus multiplex* isolate AFTOL-ID 677 with a score of 98 %. The genus *Polyozellus* is monotypic and belongs to the *Thelephoraceae* family, also known as leathery earth fans. This genus only contains a single species, called the *Polyozellus multiplex*, also known as the clustered blue chanterelle, blue chanterelle, or the black chanterelle. It is an edible species that has been harvested for commercial purposes. According to research conducted in 2003, *Polyozellus multiplex* contains the bioactive compound polyozellin, shown to have various physiological properties, including suppressive effects on stomach cancer (Lee and Nishikawa, 2003; Lull *et al.*, 2005).

In this study, isolate LM 3 had members of various genera as the nearest neighbors in BLAST with 95 % similarity (**Table 4.9**). Some of these include; *Acremonium*, *Talaromyces*, *Penicillium*, *Sagenomella*, *Aspergillus*, *Paecilomyces* and *Aphanoascus*. This isolate could represent novel species and should be further investigated on in order to fully identify the organism. It hydrolyzed Skim milk, Tween 20, Tween 80, Xylan and Starch, indicating that it was able to produce extracellular proteases, esterases, lipases, xylanases and amylases. It had inhibition activity against Gram-

positive bacteria; hence it could have an ability to produce broad spectrum antimicrobial compounds.

Isolate LM 12 and LM 17 had *Pycnoporus sp.* and *Teratosphaeria mexicana* strain CBS 110502, as their nearest neighbors in BLAST with 89 % and 82 % similarity respectively (**Table 4.9**). Isolate LM12 hydrolyzed Skim milk, Tween 20, Tween 80, Xylan and Starch. Isolate LM17 hydrolyzed Tween 80, Xylan and Starch. This indicated that the isolates were able to produce extracellular proteases, esterases, lipases, xylanases and amylases. The isolates had inhibition activity against Gram-positive and Gram negative test organisms; hence their ability to produce broad spectrum antimicrobial compounds. This isolates could represent novel genera within the lake's ecosystem and requires further research.

The BLAST search results showed that all the isolates belong to the fungal domain and were clustered within two phyla; Basidiomycota and Ascomycota. Basidiomycota together with the Ascomycota, comprise the subkingdom Dikarya (often referred to as the "higher fungi") within the Kingdom Fungi. The Ascomycota are the largest phylum of Fungi, with over 64,000 species while Basidiomycota contains about 30,000 described species, which is 37 % of the described species of true Fungi (Kirk *et al.*, 2001). Basidiomycetes constitute the most conspicuous group of fungi in the environment and comprise very different ecological groups such as white rot, brown rot, and leaf litter fungi. Some of them are edible and/or medicinal fungi; some have important biotechnological and environmental applications (Songulashvili *et al.*, 2007).

5.2. CONCLUSIONS

- The study has demonstrated that L. Magadi harbours Alkaliphilic fungal species. Thirty isolates were obtained, characterized and identified.
- The isolated fungi grow well at pH ranging from 5 – 10, temperature range of 25 – 35 °C, and NaCl range of 0- 30 %. The above conditions are therefore to be adopted if the isolates are to be exploited industrially.
- The study has also demonstrated that, Alkaliphilic fungi from L. Magadi are a potential source of extracellular enzymes that include lipase, amylase, esterase and proteases at alkaline pH.
- The isolates showed antimicrobial activity against Gram positive and Gram negative test bacteria.
- Molecular characterization of isolates indicates that all of them belong to the fungal domain and clustered within two phyla; Basidiomycota and Ascomycota. Basidiomycota together with the Ascomycota, comprise the sub-Kingdom Dikarya (often referred to as the "higher fungi") within the Kingdom Fungi.
- One (1) isolate, LM3 showed identity of 95 % similarity with the previously known sequences in the GenBank database. These represent a novel species within the lake ecosystem.
- Two (2) isolates, LM12 and LM17 showed identity of 89 % and 82 % similarity respectively, representing novel genera of organisms within the lake ecosystem.

5.3. RECOMMENDATIONS

- Extensive research on the specific secondary metabolites released by these microorganisms is of great importance.
- More research is required to design studies that would compare the diversity of fungi in different seasons of the year such as the rainy and dry seasons of the year.
- Further analysis of fungi is necessary for total characterization and identification of more alkaliphilic strains from Lake Magadi.
- The novel organisms need to be further confirmed by methods such as fatty acid analyses and DNA-DNA hybridization.
- Different protocols should be designed so as to allow the isolation of more diverse genera.
- Extensive research on the specific enzymes and antimicrobial compounds produced by these microorganisms is of great importance. This will help to elucidate the structures and biochemical characteristics of any novel enzymes and bioactive metabolites detected.

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APPENDICES

Appendix 1: DNA Extraction Reagents

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

- Solution 2
 - 10 mM Tris pH 8.5
 - 5 mM EDTA pH 8.0
 - 1 % SDS

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

Appendix 2: Electrophoresis buffer Working Concentrated stock

TBE buffer 10×

Chemical	Volume
Tris	108g
Boric Acid	55g
Na ₂ EDTA.2H ₂ O	9.3g

Adjust the volume to 1 liter with ddH₂O and divide into 500ml bottles

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

Appendix 3: EDTA 0.5 M pH 8.0

Dissolve 186.1 g of disodium ethylenediaminetetra-acetate (EDTA.2H₂O Sigma ED2SS mw 372.2) in 800 ml of ddH₂O. Stir vigorously and adjust the pH to 8.0 with NaOH pellets (EDTA will not go into solution until the pH is near 8.0, so add some of the pellets before trying to adjust the pH. Bring it to a final volume of 1000 ml. Divide into 100 ml aliquots and autoclave.

Appendix 4: Ethidium Bromide 10 ×

Dissolve 1.0 g of EtBr in a final volume of 100 ml ddH₂O. Wrap the bottle in aluminum foil and stir several hours to get a true solution. Store at 4 °C.

To make the 1× stock used to stain gels take 10 ml of the 10× stock and bring to a final volume of 100 ml using ddH₂O. Wrap bottle in aluminum foil and store at room temperature.

Appendix 5: Proteinase K

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

Appendix 6: SDS 10 %

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

Appendix 7: TE pH 7.4 or pH 8.0

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

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

Appendix 8: Tris 1 M p H 7.4

Dissolve 121.1 g of Tris base in 800 ml of ddH₂O and adjust the pH to 7.4 with concentrated HCL. Bring the final volume to 1000 ml with ddH₂O. Divide into 100 ml bottles and autoclave.