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DIVERSITY AND BIOLOGICAL CONTROL POTENTIAL OF HALOALKALIPHILIC FUNGI FROM LAKE MAGADI, KENYA

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DECLARATION

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

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DEDICATION

I dedicate this work to Almighty God, my family and my mentor Dr. Romano Mwirichia.

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LIST OF ABBREVIATIONS AND ACRONYMS

Bp	Base pairs
CMC	Carboxy methyl cellulose
DNA	Deoxyribonucleic acid
dNTP's	Deoxynucleotide triphosphates
EDTA	Ethylene diamine tetra-acetic acid
EPF	Entomopathogenic fungi
EtBr	Ethidium bromide
MEA	Malt extract agar
OA	Oat meal agar
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
rDNA	ribosomal deoxyribonucleic acid
rRNA	ribosomal ribonucleic acid
SDA	Sabouraud dextrose agar
SDS	Sodium dodecyl sulphate
TBE	Tris boric acid ethylene diamine tetra-acetic acid
TE	Tris ethylene diamine tetra-acetic acid

ABSTRACT

Fungi are eukaryotic microorganisms that have simple multicellular or unicellular cell structures. They are distributed in terrestrial soils, forests, aquatic habitats and in extreme environments with high ambient salts, temperature, pH and pressure. Fungi from extreme environments are potential sources of novel biocatalysts for example antimicrobial agents that can help solve the rising cases of drug resistance. However, the diversity of fungi recovered and described from less studied environments such as the soda lakes is quite low as compared to the soil ecosystem. Very few haloalkaliphic fungi have been recovered from hypersaline environments. In this study, we explored the cultivable fungal diversity in Lake Magadi and their ability to produce secondary metabolites. Dilution plate technique was used to isolate fungi from soda lake soils using alkaline media (Potato dextrose agar, Malt extract agar, Oatmeal agar and Sabouraud dextrose agar) prepared using sterilized lake water from the lake. Unique isolates were selected based on morphological features and subjected to physiological tests to determine their ability to grow at different pH, temperature and salts ranges. Molecular characterization was done by analysis (BLAST) of the amplified conserved regions of 18SrDNA followed by phylogeny. Ability of the isolates to utilize different carbon sources was tested on media supplemented with different substrates (starch, cellulose, casein, lignin, pectin, xanthan, chitin, Tween 20). Antimicrobial screening was done using both crude and cell free extracts to determine the ability of the isolates to produce metabolites (comparison of mean diameter of inhibition zones) that can be used to control both human pathogens and agricultural pathogens. The test pathogens included human pathogens *Staphylococcus aureus, Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa, Salmonella spp., Shigella spp.* and *Candida albicans* which were laboratory isolates and plant pathogens *Epicoccum sorghinum* strain JME-11, *Alternaria tenuissima, Didymella glomerata, Schizophyllum commune* isolate ScGD28, *Phoma destructiva*, *Cladosporium halotolerans* CBS 119416, *Aspergillus fumigatus* EG11-4 and *Dickeya dianthicola* (field isolates). In total, 52 isolates were recovered from the study having different pigmentations. Their physiology depicted actual characteristic of haloalkaliphilic environments as the fungi tolerated extreme pH of up to 10 and salts (15-20%) and some grew at 35-40ºC. Sequence analysis indicated that the isolates were affiliated to 18 different genera with *Aspergillus*, *Penicillium*, *Phoma*, *Cladosporium,* and *Acremonium*. Substrate utilization tests showed that the different isolates produced proteases, chitinases, cellulases, amylases, pectinases and lipases. Isolates 2M, 59M, 69M, 87M, 100M, 111M and 113M were outstanding as they produced more than four enzymes. Results from the antimicrobial screening showed one isolate (11M from Site 3 soil) with 99% affiliation to *Penicillium chrysogenum* CBS 306.48 was able to inhibit major enteric bacteria and plant pathogenic fungi. Similar studies on saline environments have reported the phylum *Ascomycota* as the dominant fungal group and all the genera recovered in this study are classified in the same phylum. Antimicrobial from the fungal isolates provide a basis for pharmacological research since they can further be purified to obtain compounds that can be used as antibiotics or biopesticide against human and plant pathogens respectively. This can provide a solution to the rising problem of antibiotic resistance and pesticide resistance.

CHAPTER ONE

INTRODUCTION

1.1 Background information

Fungi are ubiquitous microscopic eukaryotes having simple multicellular or unicellular structures. Majority are saprophytic (living on decayed and dead organic matter) whereas other are parasitic (live in or on a host organism to obtain nutrients). The fact that fungi are heterotrophic makes them important in breakdown of organic and inorganic compounds hence enhance nutrient cycling and6 biodegradation processes respectively (Musa and Subash, 2018). Specific fungal groups such as yeasts are useful in fermentation in industries (bread, fermented drinks), detergents and alcohol. Most fungal species inhabit soil compared to other environments as they are organotrophic organisms forming the major group involved in the breakdown of organic compounds (Hasan, 2015). Occurrence of fungi in water is subtle with only around 3,000 species known to be associated with aquatic habitats while 465 species are estimated to be present in marine waters (Shearer *et al*., 2007). Aquatic "true fungi" (*Eumycota*) absorb nutrients across their cell wall and are considered to be osmoorganotrophs (Christian *et al*. 1998).

Fungi not only survive but also propagate and grow in unusual environments. Diverse fungal groups have been documented from environments such as saline liquids, hot springs, surface of dried rocks, ocean pits, dry deserts, and very low pH as well as in the coldest polar environments (Hassan *et al*., 2016). In fact, they are adapted to environmental conditions such as fresh water, high temperatures, alkaline- saline environments and some can tolerate more than one extreme condition (polyextremophiles). Studies have shown the existence of some thermophilic fungi such as *Rhizumucor miehei, Chaetomium thermophile* from the hot springs that tolerate temperature of above 45 ºC (Ndwigah *et al*., 2016; Vázquez-Campos et al., 2014; Hawksworth, 2004;). Psychrophilic fungi that survive temperatures below 10 ºC have been reported from glaciers, ice sheets and icebergs (Tojo and Newsham, 2012). Moreover, there are limited fungal communities present in highly acidic sites and only a

few dematiaceous fungal species dominate facilitating decomposition in acidophilic food webs. Acidophilic biofilms also have fungal hyphae as the dominant eukaryotic element serving as prokaryotic surfaces as well as anchor system into the sediments (Das *et al*., 2009). Extreme saline environments that are of interest to this study are particularly the athalassohaline type of hypersaline lakes whose composition is similar to that of the surrounding environmental conditions, geology and topography. They include, the alkaline lakes of the Kenyan rift valley e.g. lakes Magadi, Nakuru, Elementaita, Sonachi, Natron and Wadi-El-Natrun lakes of Egypt (Moubasher *et al.*, 2016).

For many years, Lake Magadi has been of great interest due to its unique composition of vast sodium hydrogen carbonate ($NaHCO_3.Na_2CO_3.2H_2O$) deposits. The physicochemical conditions are a limiting factor to diversity of microbial life because of the high salinity up to saturation and high pH (Nielsen, 1999). The alkalinity of Lake Magadi ranges from 9 to 12 making it an extreme alkaline environment of interest for biotechnological research (Salano, 2011). The high pH is because of carbonate salts, which are highly concentrated in the lake, particularly sodium carbonate, and sodium chloride, which is a related salt complex (Salano, 2011). Unlimited HCO^-_3 reserves, high light intensity and surface temperatures of between 30-45°C make Lake Magadi very productive (Salano, 2011). These enhance all biological processes taking place within the soda lakes.

Fungi within the hypersaline habitats have various adaptive strategies to either tolerate the presence of salts or use salts for survival. They withstand water loss and toxicity of sodium. Fungi have adapted to the extreme environments by production of extremolytes and extremozymes which are protective organic for them to survive the harsh conditions (Raddadi *et al*., 2018). In cases of high osmolarity, such fungi are able to counteract loss of water by the accumulation of K+ ions into their cells (Madern *et al*., 2000). This 'salt-in' strategy then creates an osmotic balance as the high concentration of inorganic salts in the cells exclude as many Na^+ ions away from cells (Madern *et al*., 2000). Both halolerant and halophilic fungi also employ an alternative strategy whereby they accumulate osmolytes (polysols, sugars and amino acids) as compatible organic solutes which serve as osmotic regulators (Roberts, 2005). The compatible solutes also help in maintaining stability of fungi proteins so as they can withstand extreme conditions of temperature or

pressure (Roberts, 2005).

Microorganisms that are able to tolerate extreme environments are known to be potential sources of biotechnologically important novel secondary metabolites (Ndwigah *et al*., 2016). This is because of their adaptive response to tolerate the extreme abiotic environments (Brakhage and Schroeckh, 2011). Such conditions enhance various modifications and changes of particular pathways of fungi to enable for production of different metabolites. Fungi from soda lakes for example have adapted to relatively high temperatures and alkaline saline conditions which enhances changes and modifications of particular fungal pathways (Brakhage and Schroeckh, 2011). Extremophilic fungi therefore produce organic molecules with specific biological activities such as antimicrobial agents, cryoprotectant molecules such as sugars and polyols to stabilize membranes, osmotically active compounds such as polyols in xerotolerant fungi, and fungal melanin for protection against freezing and UV radiation (Chavez *et al*., 2015). Reports indicate that various metabolites of industrial potential are produced by halophilic organisms and includes the compatible solutes, biopolymers, peptides, terpenes, polyketides, proteins, antibiotics and amino acid derivatives (Musa and Subash, 2018). Among the already identified fungal metabolites, include antimicrobial metabolites, bioactive compounds, pigments and toxins. Specifically, a collection of fungal genera that are known to be able to produce diverse secondary metabolites includes *Acremonium*, *Fusarium*, *Aspergillus* and *Penicillium* (Ndwigah *et al*., 2016).

Immense applications of extremophilic metabolites in biotechnology have put a lot of interest from microbiologists to study the microbial diversity of the extreme habitats. Fungi from unusual habitats when cultivated and characterized enhance the study of their physiology and genetics which are important in ecological and industrial applications (Prakash and Sharma, 2016a; Prakash and Sharma, 2016b). An In-depth understanding of the diversity and function of majority of haloalkaliphilic fungi would help in the identification of new secondary metabolites from the larger unidentified group of fungi. The aim of this study was to isolate novel fungi from Lake Magadi and screening them for bioactive metabolites.

1.2 Statement of the problem

The search for natural product has focused on microbial groups with known potential to produce secondary metabolites. This is driven by the desire to discover new antimicrobial compounds to counter the increased antibiotic resistance and as well as pesticide resistance in insect pests. Fungi comprises of one of the largest group of organisms on globe known for their diverse existence and metabolic complexity (Hawksworth, 2002; Moore *et al*., 2011). The already identified species implies that only a few secondary metabolites are known from the fungal species and just a smaller number have been isolated from the extreme environments. The unknown diversity especially from the extreme environments has enormous potential for new metabolites and bio-control agents. Existing metabolites obtained from non-extreme environments are unreliable, less stable in industrial products and processes, and are not prone to genetic manipulations as compared to metabolites obtained from polyextreme environments of high salinity and temperature (Chinnathambi, 2016). Moreover, the use of chemical pest control in agricultural systems is also ineffective due to elevated levels of insecticide resistance besides causing environmental pollution. Therefore, there is an urgent need for new biological control agents and strategies. Fungal diversity particularly from less studied habitats for example saline lakes holds enormous potential.

1.3 Justification

Fungi are known as the most prolific producers of bioactive compounds secreting about 42% of the total bioactive compounds (enzymes, toxins, bioactive chemicals, antimicrobial agents among others (Chavez *et al*., 2015). Only five fungal genera have been documented to be able to produce about 75% of the industrial enzymes (Prakash and Sharma, 2016a). This implies that a large fraction of fungi still remains industrially un-explored (Østergaard and Olsen, 2011). Halophilic and halotolerant fungal species having been adapted to toxic, extreme environments consequently produce metabolites that enable them habit the harsh conditions. Halophiles and the metabolites they produce provide tremendous opportunity for biotechnological applications such as in agriculture, food industry, nanotechnology, genetic engineering and bioremediation. Antimicrobial agents, toxins and pigments from haloalkaliphilic fungi are useful in pharmaceutical formulations (Musa and Subash, 2018; Uratani *et al*., 2014). Study on diversity and

function of fungi from Lake Magadi will enhance the need to bio prospect for metabolites and antimicrobial compounds of pharmaceutical and biotechnological applications.

Natural products from microorganisms have proved to be a preferred source of new drugs because of the specific advantages they have over synthetic drugs/chemicals. They have the best structural diversity than chemically synthesized compounds and are therefore considered to present a wide range of biological activities (Demain, 2014). Haloalkaliphilic metabolites are of large use because they remain stable for a long period in formulated products, save energy and are more reliable and quick to produce (Chinnathambi, 2016). The linkages existing between primary and secondary metabolic pathways imply that even more secondary metabolites could be obtained from such fungi. In addition, resistance to the existing antimicrobial compounds can be lowered by exploiting the various antibacterial and antifungal compounds (metabolites) produced by various halophilic fungi and this will satisfy the increasing demand for new and active antibiotics.

1.4 Hypothesis

Fungi isolated from Lake Magadi are diverse and are potential biological control agents against plant and human pathogens.

1.5 Objectives

1.5.1 General objective

To isolate, characterize and identify new fungi diversity from Lake Magadi and determine their biological control potential.

1.5.2 Specific objectives

1. To isolate, characterize and identify the fungal isolates from Lake Magadi using morphological, biochemical and molecular techniques.

2. To screen the fungal isolates for biological activity against human and plant pathogens.

3. To determine the enzymatic activities of haloalkaliphilic fungi from Lake Magadi.

CHAPTER TWO

LITERATURE REVIEW

2.1 Soda lake environments

Hypersaline environments are present in various continents including Utah, Great Salt Lake, Wadi El-Natrun (Egypt's alkaline lake), Antarctica soda lakes, Dead Sea, Tanzania's lake Natron, Mono Lake (California) and Big Soda Lake (Cantrell *et al*., 2006); Grant and Sorokin, 2011). Lakes including Bogoria, Magadi, Natron, Elementaita, Sonachi and Nakuru are the major representatives of highly alkaline environments that occur naturally along the East African Rift Valley (Kempe and Degens, 1985). Analysis of the chemical composition of the lakes indicates that Na^+, CO_3^{2-} and Cl^-, ions are the main causes of salinity in the lakes (Kempe and Degens, 1985). However, CO_3^{2-} concentration is exceeded by that of Cl^- in brine (Kis-papo *et al*., 2003). For many years, Lake Magadi has been of great interest due to its unique composition of vast sodium hydrogen carbonate ($NaHCO_3.Na_2CO_3.2H_2O$) deposits (stable crystalline Na_2CO_3 product, Sodium hydrogen carbonate and CO_2-equilibrium). Harvested deposits when kilned aid in the production of Na_2CO_3 (anhydrous), soda ash that is useful in the manufacturing of glass. Interestingly, the most studied alkaline Soda Lake of East Africa remains to be Lake Magadi since its niche is a limiting factor to diversity of microbial life because of the high salinity up to saturation and high pH (Nielsen, 1999). Researchers have thus been attracted to microbial life specificity in the lake's niche.

2.2 Microbial biodiversity and productivity of soda lakes

Soda lakes when compared to all other aquatic environments are exceptional in terms of their high pH (9.0–12.0), salinity (up to saturation) and high productivity rates (Grant, 2006). Although occurring in areas of soda soils, they have high productivity than the freshwater lakes (Grum-grzhimaylo *et al*., 2016). Studies on microbial biodiversity in soda lakes indicate primary producers are mainly the cyanobacteria species (Antony *et al.*, 2013). The occurrence of bacteria and archaeal (red haloalkaliphilic Archaea) blooms in water basins and soda soils is responsible for the coloration of soda lakes e.g. as it has been observed in Lake Magadi (Ndwigah *et al*., 2016). Surveys on biodiversity have

confirmed the presence of eukaryotes which includes haptophytes, green algae, cryptophytes and plankton diatoms in the soda lakes (Nagy *et al.*, 2006). Other dominant species of eukaryotes found in soda lakes are for example, crustaceans (brine shrimp) and various copepod species (Schneider *et al*., 2012). In addition, halophytic grasses (Chenopodiaceae) are the dorminant vegetation in various soda soils (Grum-grzhimaylo, *et al*., 2015).

In natural environments, fungi form major biodiversity component and drives crucial ecological processes (Hawksworth, 2002). They are involved in the maintenance of ecosystem through nutrient cycling (Pan *et al.*, 2008). Melanized polymorphic black yeast dominates in the hypersaline waters as the main fungal group (Gostinčar *et al*., 2009). The imperfect forms of *Ascomycota species* has been found severally and in large numbers in extremes of salts as they thrive in saline soils, salt marshes, marine sediments, seawater, mangroves and sand dunes (Salano, 2011). The genus *Cladosporium* and species; *Aureobasidium pullulans, Hortaea werneckii, Trimmatostroma salinum* and *Phaeotheca triangularis* have also been identified as major group of melanized yeasts (Musa and Subash, 2018). Several species of non melanized yeasts for example *Curvularia, Aspergillus, Alternaria, Rhizopus, Cladosporium, Phoma, Fusarium, Penicillium* and *Drechslera* have been found to dominate marine sediments (Saravanan and Sivakumar, 2013). Recent studies employing the use of illumina sequencing also indicates that phyla *Basidiomycota* and *Ascomycota* dominate the diversity in water and sediments of Lake Magadi and Little Magadi (Kambura, 2016). This was further confirmed in a study carried out by Ndwigah (2017), who isolated anamorphic fungi in the genera; *Rhodotorula, Fusarium, Acremonium, Aspergillus* and *Scopulariopsis* which belonged to anamorphs of the phylum *Ascomycota* and *Basidiomycota*.

2.3 Fungal diversity in extreme ecosystems

Fungi are heterotrophs and are adapted to various environment types including fresh water, low and high temperatures in addition to alkaline- saline habitats (Alexopoalos and Mims, 1979). Studies have confirmed the occurrence of psychrotrophic and psychrophilic fungi in cold environments including permafrost, glacial ice, cold water and off-shore polar habitats (Golubev, 1998; Tosi *et al*., 2000; Ma *et al.*, 1999; Broady and Weinstein,

1998). Filamentous Ascomycota, Helotiales/Pleosporales and basidiomycetous yeasts are among the cultured fungal groups from niches present in cold glaciers (Hassan *et al*., 2016). In addition, Basidiomycota and Ascomycota have also been recovered as the major phyla from deep marine ecosystems and have psychrotrophic nature (Hassan *et al*., 2016).

Studies from thermophilic environments indicate that fungal species including *Chaetomium thermophile, Melanocarpus albomyces* and, *Rhizumucor miehei*, exist from soils, hot springs and other sources (Ndwigah *et al.*, 2016). Halophilic and halotolerant strains of fungi are able to survive in hydrothermal vents having elevated temperatures as well as in high ultraviolet irradiation (Musa and Subash, 2018). Fungal lineages branching from *Chytridiomycota* have been isolated from deep sea hydrothermal ecosystems (Calvez *et al.*, 2009). About 77 species of fungi have been isolated from Dead Sea water and includes endemic groups which have not previously identified but are known to be rare or sporadic (Kis-papo *et al.*, 2003). Spatiotemporal study of samples from Dead Sea revealed that *Aspergillus versicolor* and *Eurotium herbariorum* were present as one of the core steady species (Kis-papo *et al.*, 2003). Some Acidophilic/acidotolerant fungi identified so far cannot survive in less acidic habitats and are therefore known to exhibit community specificity in acid drainages, uranium mines and highly acidic hot springs (Vázquez-Campos *et al.*, 2014). Fungal acidophiles identified include *Acidiella uranophila, Acidomyces acidophilus, Coniochaeta fodinicola, Hortaea acidophila* and *Teratosphaeria acidotherma* (Vázquez-Campos *et al*., 2014; Hawksworth, 2004;Yamazaki, *et al*., 2010). The protein structure in acidophilic fungi are adapted to keep them active and stable in high concentration of acids as their enzymes have optimum acidic pH range (Dhakar and Pandey, 2016).

2.4 Fungal adaptation to extreme salinity

Halotolerant organisms and most halophilic microbes prevents protein segregation by expending energy to aid in the removal of salt from their cytoplasm, a process called 'salting out' (Santos and Da Costa, 2002). Strict halophilic microorganisms for example in cases of high osmolarity counteract loss of water through the accumulation of K^+ ions within their cells. Halophilic filamentous fungi being polyphyletic employ various strategies combating salt stress for example, accumulation of compatible organic solutes

mainly glycerol in their cell such that there is osmotic pressure balance between the cells and the surroundings (Kis-Papo *et al.*, 2003). Fungi are thus able to maintain intracellular toxic salt (Na^+ ions) concentrations at low levels. High osmolarity glycerol (HOG) pathway is the major signaling fungi pathway that ensures responses in cases of cellular stress (Kis-Papo *et al.*, 2003). Extensive studies on *S. cerevisiae* based on osmotic stress has revealed that the compatible solute (glycerol) is produced via homeostatic process, the control of this signaling pathway employs glycerol as the main target (Hohmann *et al*., 2007). Cellular osmotic balance is restored after the production of glycerol via the major effect of HOG pathway. The HOG pathway has for example been identified from the Dead Sea Species *Aspergillus glaucus* and is responsible for the synthesis of glycerol (Zajc *et al*., 2014). Both halotolerant and halophilic fungi use polyols (mannitol, erythritol, glycerol and arabitol) as osmotic solutes and are therefore able to prevent salt entry into their cytoplasm (Zajc *et al*., 2014).

2.5 Production of secondary metabolites by haloalkaliphilic/halotolerant fungi

Recently, the discovery of novel metabolites having potential use in pharmacology have increased especially from marine extremophiles. Secondary metabolites for example proteins, glycosides, alkaloids, polyketides, peptides, lipids and isoprenoids exhibiting biological use as antibiotic, anticancer, antitumor, antimicrotubule, and photo protective agents have been identified (Rateb and Ebel, 2011). Fungal secondary metabolites are produced in form of organic compounds under restricted growth. Various primary metabolic pathways are precursors to secondary metabolites like Entner-Doudoroff pathway (EDP), Embden- Myerhof-Panas pathway (EMP) and (Hexose monophosphate pathway (Liu *et al*., 2013).

Modifications like carboxylation, glycosylation, methylation, condensation and hydroxylation of the primary products (nucleotides and amino acids) gives the secondary metabolites. The main precursor of fungal secondary metabolites is Acetyl-CoA leading to terpenes, polyketides, steroids, and fatty acid metabolites (Dreyfuss and Chapela, 1994). Recently, an estimate of about 5% of the already described fungal species exist therefore there is a very huge potential in discovering novel metabolites from fungi (Chávez *et al.,* 2015). About 3000 to 4000 secondary metabolites from fungi are known

and have been isolated (Brakhage and Schroeckh, 2011). However, only 5000 to 7000 taxonomically known species of fungi have been studied for their secondary metabolites (Brakhage and Schroeckh, 2011).

Biochemicals are the major fungal secondary metabolites that have been used for industrial applications (Ndwigah *et al*., 2015). Reports indicate that specific fungal species that are able to produce industrially important metabolites such as *Candida magnolia*, a halophilic yeast for example are known to possess various applications of compatible solutes e.g. trehalose glycerol and proline (Raspor and Zupan, 2006). Both halophilic and halotolerant *Penicillium* and *Aspergillus* species are able to produce various polyketides (penicillic acids, patulins, antafumicins and anthraqunones), antimicrobials (e.g. penicillins, griseofulvin and cephalosporins), extrolites derived from amino acids (e.g. dipodazin, asperloxins, chrysogines and asperphenamate) important in pharmaceutical industry (Frisvad, 2005). Such antimicrobial agents are formulated into drugs that are active against bacterial pathogens (Rajpal *et al*., 2016). For example, extracts from fungal species (*Aspergillus terreus, Aspergillus versicolor, E. amstelodami, A. flavus* and *P. purpurogenum*) have been found to have a broad spectrum bactericidal activity against *B. cereus, K. pneumonia, S. aureus* and *E. coli* (Rajpal *et al*., 2016). Quite a number of filamentous fungi especially *Aspergillus, Penicillium,* and *Trichoderma* have been reported to synthesize silver nano- particles that are biological materials for nanomaterial synthesis and design of nano-devices and nano-systems (Srivastava and Kowshik, 2015). Melanin production in the whole cell of halophilic fungus *H. werneckii* at increased salt concentration (more than 1%) has been reported and have protective functions, their quinoid groups makes them have antioxidant properties (Kogej *et al*., 2007). Species of *T. purpurogenus* and *Talaromyces amestolkiae,* have also been documented as producers of MPA pigments (Yilmaz *et al*., 2012).

Great attention has been attracted to alkaliphilic enzymes that are available for industrial uses and also in enhancing fungal bio-control activity (Souza *et al.*, 2015). These include lipases, xylanases, proteases, pullulanases and cellulases. Fungal proteases have been isolated from genera *Mucor, Penicillium, Humicola, Thermomyces, Aspergillus* among others present in saline habitats (Souza *et al*., 2015). Halotolerant isolates of fungi

exhibiting various enzymatic activities (phosphatase, amylase, protease and β-galactosidase) have also been obtained from solar salterns Gulf of Cambay, India. *Aspergillus spp.* showed positive results for the production of β-galactosidase (Raol, 2014). Other fungal species including *Chaetomium globosum, Thermomyces lanuginosus* and *Aspergillus terreus* are known to produce lipids (Subramaniam *et al.,* 2010). Various halophilic (obligate) fungal species of *Sterigmatomyces halophilus, Aspergillus flavus,* have been used in heavy metals bio-sorption like copper, cadmium, zinc, manganeese, ferrous and lead (Bano *et al.,* 2018). This is due to the action of bioactive compounds they produce.

2.6 Fungi as biological control agents

Efforts on the use biological control agents have been intensified because of the rising concerns on the environmental effects of pesticides, which are toxic chemicals, used to control pests. Moreover, increasing cases of antibiotic and pesticide resistance of the existing antimicrobial agents have led to devoted research in the development of new drugs and pesticides. Fungi have been formulated into different products mycoinsecticides, mycofungicides and mycoherbicides to control insects, fungal pathogens and weeds respectively (de Faria and Wraight, 2007). Entomopathogenic fungi including *Beauveria, Metarhizium, Lecanicillium and Isaria* have been developed and mass produced in various formulations as active biological control agents against mites, ticks and insects (Rai *et al.,* 2014). However, the negative effects of abiotic stresses for example UV radiations, temperature and humidity have greatly affected the efficacy of entomopathogens in the field (Rangel *et al*., 2005). The potential of fungi endophytes to synthesize metabolites that are bioactive to be used as therapeutic agents in controlling diseases have also been confirmed (Strobel *et al.*, 2004); Staniek *et al*., 2008); Aly *et al.,* 2010). Secondary metabolites for example camptothecin and structural analogs, emodin, paclitaxel podophyllo- toxin and hypericin have been discovered from host plant endophytes and are of therapeutic value (Kusari *et al.,* 2012). However, it is still difficult to practically prove that the metabolites from endophytic fungi can be commercial produced. The famous antimicrobial agent cephalosporin C was obtained from marine derived *Cephalosporium* sp. (Jin *et al*., 2016). Moreover, *Penicillium chrysogenum* gave the first commercial antibiotic penicillin and since then, various species of *Penicillium*

have been studied and identified as major producers of bioactive secondary metabolites (Frisvad, 2005). Marine derived *Penicillum commune* for example is known to be able to produce secondary metabolites called comazaphilones which proved to have positive inhibition against *Pseudomonas fluorescens*, *Bacillus subtilis* and methicillin- resistant *Staphylococcus aureus* (Shang, 2012). Alkaloids from marine *Talaromyces* fungal species have also proved to be active against *Staphylococcus aureus*, other compounds from *Talaromyces thermophilus* have also shown to be toxic against plant free living nematodes (Jie *et al.*, 2016).

Qualitative and quantitative methods have been used for antimicrobial susceptibility screening and are based on diffusion assays on agar or broth (Pereira *et al.*, 2013). Co-culturing has however, been used in filamentous fungi as the most common way to detect antimicrobial activity whereby fungal extracts or two filamentous fungal strains are used to test against single-cell microorganisms (Zheng *et al.*, 2011). In most cases, the assay methods for antimicrobial susceptibility assays require the purification of the antimicrobial substance or one to use of an enriched fraction of extract (Othman *et al*., 2011). The pure compound/fraction are thus tested by the use of diffusion or dilution methods (Wiegand, *et al.*, 2008). The detection of the antimicrobial compounds via in vivo production can be done by modifying diffusion assay whereby instead of using paper disc, cells of the microorganism (yeasts or bacteria) are used. In filamentous fungi, other methods like dual cultures on solid culture medium (Rehman *et al.*, 2011; Zheng *et al*. 2011) and growth in a liquid media previously utilized by the fungal antagonist can be employed. Dual cultures are efficient when used to test fungal antagonism against other filamentous strains. However, this method has the limitation of being dependent on the slow fungal growth and requires time to detect antagonism. The use of liquid media where the fungus to be tested for antimicrobial potential has grown is usually exploited for testing antimicrobial activity against bacteria or yeasts, it depends on how stable the antimicrobial compound(s) is and is also much time consuming (Pereira *et al.*, 2013).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study site

Samples were obtained from Lake Magadi, a hypersaline alkaline East African Rift valley lake. It is found in Southern Kenya almost to the Kenya-Tanzania border lying to the south of Lake Natron and north of Lake Naivasha. The lake is approximated to lie 2° S and 36° E of the Equator at about 600 m elevation above sea level. It has a depth ranging between 1-5 m and covers an area estimated to be 90 km^2 (Behr and Röhricht, 2000). Lake Magadi lies on the lowest part of trough occurring in a closed lake basin. The lake is described as an "alkaline saline pan" with salinity being greater above 35 % w/v, pH ranging from between 9.0-12.5 and temperature range of between 22 $^{\circ}$C-34 $^{\circ}$C. The lake and surrounding area receive about 500 mm annual rainfall (Baumgarte, 2003).

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

3.2 Sample collection

Samples were collected from seven sites around the lakeshore exhibiting different alkalinity levels. The sites included: Site 3 sediments (S3S), Site 3 dry (S3Dry), Site 2 sediments (S2S), site 5 sediments (S5S), Microbial mats, Magadi Grass Sediments (MGS), Site 2 brine (Br2S). Purposive sampling technique was used in the identification of sampling sites and it depended on nature of different microbial habitats (dry soil, sediments, microbial mats and brine). This was done by scooping about 50 g of wet sediments, dry soil, microbial mats and brine with sterilized hand shovel into sterile 50 ml falcon tubes. The collected samples were placed in cooler box, transported to the University of Embu microbiology laboratory and stored at 4 ºC. The physicochemical parameters of the sample sites including organic carbon, pH and dissolved minerals were measured and recorded. The samples were labeled according to the site from which they are collected. Lake water was collected from different sites in sterile 500 ml containers and placed in a cool box.

3.3 Media preparation

Lake water was used to prepare culture medium to mimic similar salt concentration and pH of the lake in the laboratory. Artificial high and low nutrient medium was prepared in the laboratory from potato dextrose agar (PDA), malt extract agar (MEA), oatmeal agar and Sabouraud dextrose agar (SDA). High nutrient media agar (PDA-H, MEA-H, OA-H, SDA-H) were prepared by measuring out the ingredients as per the manufacturer's instructions while for the low nutrient media (PDA-L, MEA-L, OA-L, SDA-L), the ingredients were reduced to 10% of the recommended weight. Lake water (brine) was diluted with tap water and autoclaved separately from the other ingredients for media preparation. The media were sterilized by autoclaving at 121 ºC for 15 minutes. The media was left to cool to 50 ºC and an antibiotic (chloramphenicol, penicillin and streptomycin) (100mg/L) added to the media to inhibit growth of bacteria. Sterile lake water was mixed with the rest of the media in a ratio of 60 % to 40 % respectively.

3.4 Fungal isolation

3.4.1 Dilution plate technique

From each of the samples (dry soil, sediments, brine and mats samples), 1g was suspended separately in 1ml sterile lake water, vortexed and allowed to settle for 10 min. Serial dilution was performed up to 10^{-2} using sterile lake water. Aliquots (100 µl) from all dilutions was plated on sterile agar medium aseptically. Each sample was plated onto all the media (PDA, MEA, SDA and Oatmeal agar) in duplicates (high and low nutrient). Sterile spreader was used to spread the inoculum onto the agar plate surface. The plates were incubated in duplicates at 28 ºC for 4 to 8 weeks until visible colonies appeared. Several rounds of sub-culturing were done into fresh agar plates to obtain pure colonies. This was done by picking mycelium of each isolate and spotting onto fresh agar plates every 7-14 days and at 28 ºC until a pure culture is obtained.

3.5 Morphological characterization of the isolates

3.5.1 Colonial morphology

Colony color, shape, size, margin, pigmentation, elevation and spore production of the pure cultures was determined for each fungal isolate. This was done by observing the colonies under an inverted microscope.

3.5.2 Cellular morphology

Preliminary characterization by simple staining of each of the isolates was carried out by observing the fungal mycelia cells under a light microscope at ×100 magnification (Keast *et al.,* 1984). Mycelium of each isolate was picked using a sterile loop and placed on a drop of water on a microscope slide. A drop of methylene blue stain was added before visualization using compound light microscope. Morphological features for identification included hyphae, conidia and conidiophores structures. The fungal isolate code, morphological description of fungus and medium were recorded. Micrographs of fungal isolates were made from a camera mounted on a microscope at ×100 magnification.

3.6 Physiological characterization of the isolates

The ability of each fungal isolate to grow at different ranges of temperature, pH and salt concentration was tested by growing the isolates in malt extract Agar (20g/L) prepared using lake water for 7 days at 28 ºC. Physiological characteristics recorded were effect of

pH at 6, 7, 8, 9, and 10; NaCl at 0%, 5%, 10% and 20%), and temperature at 25 ºC, 30 ºC, 35 ºC and 40 ºC.

3.6.1 Tolerance to different salt concentration

This was tested as the first physiological parameter whereby all the isolates were spot inoculated on petri dishes having malt extract agar (pH 9) and with different concentrations of NaCl (0%, 5%, 10% and 20%). The petri dishes were incubated at 28 ºC for 48 hours. Growth was scored by estimating the size of the colony. The optimum salt concentration for the fungal growth was noted.

3.6.2 Tolerance to different pH

The pH was tested as the second physiological characteristic whereby malt extract agar (5% NaCl) was prepared having different pH levels (6, 7, 8, 9, and 10). All the isolates were spot inoculated on petri dishes and incubated at 28 ºC for 48 hours after which colony size estimated and recorded. The optimum pH was noted.

3.6.3 Tolerance to different temperatures

Tolerance to different temperatures was the last parameter to be tested. Malt extract agar (5% NaCl and pH 9) was prepared. All the isolates were spot inoculated on to the petri dishes and incubated at different temperatures (25 ºC, 30 ºC, 35 ºC and 40 ºC) for 48 hours Growth was scored depending on the colony size.

3.7 Biochemical characterization of the isolates

The fungal isolates were screened for their ability to produce proteases, amylases, lipases, xylanases, cellulases and esterases, exoenzymes. Basal medium composed of (Tryptone 10 g, Sodium chloride 10 g, and Yeast Extract 5 g) was supplemented with different substrates as summarized on Table 3.1. The results for enzymatic activity were recorded based on the size of the halos or level of fluorescence for the chitin test.

Table 3. 1 Summary of enzymatic screening using different substrates

Enzyme	Substrate	Duration	Temperature	Assay	Observation	Reference
Lipases	Tween 20	48 hours	28 ºC		Halo	Sierra, 1957
Esterase	Glyceryl Tributyrate	48 hours	28 ºC		Halo	Sierra, 1957
Proteases	Casein	24 hours	28 ºC		Halo	Vieira, 1999
Cellulases	Cellulose, Carboxymethylcellulose	24 hours	28 ºC	Flood with Congo red	Halo	Stamford *et al*., 1998
Pectinases	Pectin	24 hours	28 ºC	Lugol solution	Halo	Andro *et al.*, 1984
Peeroxidases/Laccases	Lignin	48 hours	28 ºC	syringaldazine	Halo	Bonugli-santos *et al*., 2015
Amylases	Starch	24 hours	28 ºC	Lugol solution		Castro, *et al*., 1993
Chitinases	4-methylumbelliferylN-acetyl-b-D-glucosaminide solution	30minutes	37 ºC	Phosphate buffer	Fluorecence under U.V light	Ali *et al*., 2010

3.7.1 Determination of esterase and lipase activity

For lipases, alkaline substrate media was constituted using lake water and contained (gL^{-1}): LB broth 10.0, Tween 20 5.0 ml, agar 15.0, and pH 9. Isolates grown in broth were spotted on the agar surface and incubated at 28 ºC for 2 days. To determine esterase activity, Tween 20 was replaced with Glyceryl Tributyrate in the medium. Occurrence of a halo indicated positive enzymatic activity (Sierra, 1957).

3.7.2 Determination of proteinase activity

For proteinases, alkaline substrate media was constituted using lake water and contained (gL^{-1}): LB broth 10.0, casein 2.0, and agar 15.0, pH 9. Isolates grown in broth were spotted on the agar surface and incubated at 28 ºC for 1 day. Clear halo zones around the fungal colony indicated a positive result (Vieira, 1999).

3.7.3 Determination of pectinase activity

For pectinases, alkaline substrate media was constituted using lake water and contained (gL^{-1}): LB broth 10.0, pectin 1.0, and agar 15.0, pH 9. The isolates grown in broth were spotted on the agar surface and incubated at 28 ºC for 1 day. Positive results were indicated by presence of a clear zone around the fungal colony after flooding the plate with iodine solution (Andro *et al*., 1984).

3.7.4 Determination of Amylolytic activity

Isolates were screened for their ability to degrade starch. The alkaline medium used composed of (gL^{-1}): LB broth 10.0, starch 1.0, agar 15.0, pH 9. Isolates were grown in broth and spotted on the agar surface. Incubation was done at 28 ºC for 1 day and the plates were flooded with 0.6% KI (Lugol's) solution. Clear zone around a colony showed positive amylolytic activity (Castro, *et al.*, 1993).

3.7.5 Determination of the cellulolytic activity

Alkaline substrate media used constituted (gL^{-1}): LB broth 10.0, cellulose/ carboxymethylcellulose 2.0, agar 15.0, pH 9. Isolates were grown in broth and spotted on the agar surface. Incubation was done at 28 ºC for 1 day and the carboxymethylcellulose and cellulose plates media were flooded with Congo red dye and rinsed with 1M NaCl followed by distilled water. The plates were observed for halos around the isolates to show positive polymer utilization (Stamford *et al*., 1998).

3.7.6 Determination of peroxidase/laccase activity

The medium was constituted with lake water and composed of (gL^{-1}): LB broth 10.0, lignin 1.0, and agar 15.0, pH 9. The fungal isolates were grown in broth and spotted on the agar surface. Incubation was done for two days. The plates were flooded with syringaldazine solution (0.1g dissolved in 50 ml of 95% ethanol) and occurrence of dark pink halo indicated positive activity (Bonugli-santos *et al.*, 2015).

3.7.7 Determination of chitinase activity

Fungal isolates from broth were spotted on Whatman filter paper placed in petri dishes to prevent them from drying. Exactly 40 µl of 4-methylumbelliferyl N-acetyl-b-D-glucosaminide solution was added on to the spots and incubated at 37 ºC for 30 minutes. The plates were flooded with phosphate buffer solution after incubation. Positive isolates for chitinase activity were detected by observing for fluorescence around the isolates under UV light (Ali *et al.*, 2010).

3.8 Molecular characterization

3.8.1 DNA Extraction

Fungal isolates were grown for 7 days on media similar to the one used in isolation. Extraction of the total genomic DNA of the isolates was done using lysis buffers, solution A (50 mM Tris pH 8.5, 50 mM EDTA pH 8.0 and 25 % sucrose solution) and solution B (10mM Tris pH 8.5, 5 mM EDTA pH 8.0 and 1 % SDS). Sterile wooden toothpick was used to aseptically scrap the mycelia/spores into 100 µl solution A in an eppendorf tube. Solution B (400 µl) was added to the mixture and the tube was inverted severally. Exactly 10µl of Proteinase K (20 mg/l) was added and the mixture incubated at 65 ºC for 60 minutes. Extraction followed the phenol/chloroform method whereby equal volume of chloroform was added and the resultant mixture was centrifuged at 13200 rpm for 5 minutes at 4 ºC (Sambrook *et al.*, 1989). The supernatant was transferred to a new tube and the volume was noted. To the supernatant, 150 µl of sodium acetate (pH 5.2) was added. Equal volume (to the supernatant + sodium acetate) of isopropyl alcohol was added to the mixture and the tube mixed by inversion gently. The tube was spun at 13,200 rpm for 10 minutes and the supernatant discarded. The resulting DNA pellet was washed by adding 500 µl of 70% ethanol followed by centrifugation for 1 min at 10,000 rpm. The supernatant was discarded, the DNA pellet air dried in a clean bench for not less than 5

hours and dissolved in 30 µl of PCR water. DNA was checked on 1% agarose gel stained with Sybr green and visualized under ultraviolet.

3.8.2 PCR amplification of 18S rRNA

Total genomic DNA (template) extracted from each isolate was used to amplify the 18S rDNA genes using fungal primer pair Fungi683f forward 5'-GCTCGTAGTTGAACCTTTGG-3' and Fungi1394r reverse 5'-TCTGGACCTGGTGAGTTTC-3'. Amplification was carried out using Surecycler 8800 machine (Agilent Technologies). Initial step of the amplification was PCR optimization to determine the best annealing temperature for the fungal isolates. For optimization, gradient PCR was prepared in a 50 µl volume. The gradient PCR mixture consisted of 1.0 µl dNTP's, 10 µl of PCR buffer (×10), 2.5 µl (5pmol) of Fungi1394r reverse primer, 2.5 µl (5pmol) of Fungi683f forward primer, 1.2µl of template DNA, 0.5µl taq polymerase and 32.3 µl of water. The control had all the above reagents except the DNA template. The temperature cycling profiles was repeated for 35 cycles. Initial activation of the enzyme was done for three minutes at 95 ºC. Denaturation was done at 95 ºC for one minute to separate the double-stranded DNA into two single strands. Annealing step temperature was lowered to a range of between 50 ºC – 60 ºC for 45 seconds. Initial extension occurred at 72 ºC for 1 minute and final extension at 72 ºC for 5 minutes. Exactly 5 µl of PCR products was run on a 1 % agarose gel in 1× TAE buffer. Visualization was done under ultraviolet by staining with Cyber green (Sambrook *et al*., 1989). The best annealing temperature was noted to be 58 ºC.

The actual PCR amplification followed. This was done in a 30µl volume consisting of 0.6µl dNTP's, 6µl of PCR buffer (×10), 1.5µl (5pmol) of FF390r reverse primer, 1.5µl (5pmol) of Fung5f forward primer, 0.5µl of template DNA, 0.1µl taq polymerase and 18.3µl of water. The control had all the above reagents except the DNA template. The temperature cycling profiles of the reaction mixtures was done for 36 cycles. Initial activation of the enzyme was done for three minutes at 95ºC. Denaturation was done at 95ºC for one minute to separate the double-stranded DNA into two single strands. Initial extension occurred at 72 ºC for 1 minute and final extension at 72 ºC for 5 minutes. Primer annealing occurred at 58 ºC for 45 seconds so as the primers to attach to the DNA template.

Initial extension occurred at 72 ºC for 1 minute and final extension at 72 ºC for 5 minutes to allow for the formation of new strands. After complete PCR reaction, 5µl of PCR products was run on a 1 % agarose gel in 1× TAE buffer. Visualization was done under ultraviolet light by staining with Cyber green (Sambrook *et al*., 1989).

3.8.3 Purification of PCR products and sequencing

Purification of the PCR products for each isolate was done using ExoSAP-IT™ PCR product cleanup reagent (Applied Biosystems™). The procedure was performed on ice whereby 12.5 µL of a post-PCR reaction product was mixed with 2.5 µL of ExoSAP-IT™ reagent. The mixture was incubated at 37 °C for 15 minutes to degrade remaining primers and nucleotides. Incubation was done at 80 °C for 15 minutes to inactivate ExoSAP-IT™ reagent. The amplicons were sequenced at Inqaba Biotech, South Africa.

3.8.4 Phylogenetic data analysis

Raw sequences were quality checked and edited using CHROMAS-LITE. Basic Local Alignment Search Tool (BLAST) was used to compare the 18S rRNA gene sequences obtained with known sequences present in public database in the NCBI (National Center for biotechnology Information website) using the link (http://www.ncbi.nih.gov). Similar sequences with those in the Genebank database were noted (Altschul *et al*., 1990). Based on BLAST results, alignment of 18S rRNA gene sequences having high similarities to the ones obtained from the study was done using Mega 7 (Tamura *et al*., 2013). Evolutionary history was inferred using MRBAYES program (Huelsenbeck and Ronquist, 2001). Phylogenetic tree was drawn to scale with similar units of branch lengths with the inferred evolutionary distances. In order to compute the evolutionary distances, Maximum Composite Likelihood method was used. Phylogenetic analyses were performed using MEGA7 (Tamura *et al*., 2013).

3.9 Screening for antimicrobial activity

3.9.1 Primary antimicrobial screening

Broth media Potato dextrose broth, oatmeal broth, and malt extract broth media were prepared using lake water and 15 ml was dispensed into sterile 50 ml falcon tubes. A lump of mycelia or spores from fungal isolates were used to inoculate the broth media. The tubes were tightly capped and placed in a shaker incubator for 14 days at 30 °C and 100

rpm. Optimization of a standard pH, temperature and NaCl concentration for the growth of test organisms on Muller Hinton broth was done. This was to determine optimum growth conditions for the isolates that could enhance production of secondary metabolites. Each test organism was grown in Muller Hinton broth (pH 8 and 5% salts) prepared in sterile tubes and incubated at 30 °C for 48 hours. The test organisms used included: Gram positive bacteria *Staphylococcus aureus* and *Bacillus subtilis*, Gram negative bacteria *Escherichia coli, Pseudomonas aeruginosa, Salmonella spp., Shigella spp.*, fungal human pathogen *Candida albicans* (all human pathogens were laboratory cultures) and fungal plant pathogens *Epicoccum sorghinum* strain JME-11, *Alternaria tenuissima, Didymella glomerata, Aspergillus fumigatus* EG11-4, *Schizophyllum commune* isolate ScGD28, *Phoma destructiva, Cladosporium halotolerans* CBS 119416 and plant bacterial pathogen *Dickeya dianthicola* (all were field cultures). Antimicrobial assay was done using the agar well diffusion method (Rajpal *et al.,* 2016), on Muller Hinton agar (pH 8 and 5% NaCl). Turbidity of the test organisms from broth was adjusted to 0.5 McFarland standard. Bioassay petri plates first plated with agar on the bottom layer and on overlay of Mueller Hinton agar. Aliquots of 100 µL inoculum from each test organism were spread on Muller Hinton agar plates and wells (6 mm) made on the plates. Crude extracts (50 µL) of each fungal isolate obtained after 14 days from shaker incubator was used to inoculate the wells. Positive controls used were 5 µL of chloramphenicol (20 µg/ml) and nystatin (5 µg/ml) which were inoculated into the wells. The plates were incubated at 30 °C for 24 hours to allow for a qualitative selection of bioactive isolates showing inhibition zones *(*Arora *et al.*, 2016).

3.9.2 Secondary antimicrobial screening

Fungal isolates whose crude extracts showed positive antimicrobial activity against the test pathogens in primary test were subjected to secondary screening. In this antimicrobial assay, the producer isolate was grown for 14 days in a shaker incubator at 30 °C at 100 rpm using different production media (pH 8 and 5% NaCl) and (pH 7.4 and 0% NaCl). The media used are:

Media A (YESD)-g/L Tryptone soya broth 30.0, yeast extract 5.0, tap water (VanderMolen *et al.,* 2013).

Media B (PM3)-g/L glucose 0.5, glycerol- 2.5ml, oat meal 5.0, soy bean 5.0, casamino acids 2.0, yeast extract 0.5, 1ml from solutions of calcium chloride (156mg/10ml), magnesium chloride (190 mg/10 ml) and manganese chloride (12.58 mg/10 ml) and tap water (Jose and Jebakumar, 2013).

Media C (YPSS)-g/L Yeast extract 4.0, starch 14.0, dibasic K_2HPO_4 1.0, $MgSO_4.7H_2O$ 0.5, tap water (VanderMolen *et al.,* 2013).

Cell free extracts (supernatant) was obtained by centrifuging the cultures at 10,000 rpm for 10 minutes. Cell free extracts and crude extracts from cultures grown in shaker incubator were used as active agents to inoculate into the wells. Each test organism was grown in Muller Hinton broth (pH 8 and 5% salts) prepared in sterile tubes and incubated at 30 °C for 48 hours. The test organisms used included: Gram positive bacteria *Staphylococcus aureus* and *Bacillus subtilis*, Gram negative bacteria *Escherichia coli*, *Pseudomonas aeruginosa, Salmonella spp., Shigella spp.,* fungal human pathogen *Candida albicans* and fungal plant pathogens *Epicoccum sorghinum* strain JME-11, *Alternaria tenuissima, Didymella glomerata*, *Aspergillus fumigatus* EG11-4, *Schizophyllum commune* isolate ScGD28, *Phoma destructiva*, *Cladosporium halotolerans* CBS 119416 and plant bacterial pathogen *Dickeya dianthicola*. Antimicrobial assay was done using the agar well diffusion method (Rajpal *et al*., 2016), on Muller Hinton agar (pH 8 and 5% NaCl and pH 7 and 0% NaCl). Turbidity of the test organisms from broth was adjusted to 0.5 McFarland standard. Bioassay screening plates were made by first plating agar on the bottom layer and on overlay of Mueller Hinton agar. Aliquots of 100 µL inoculum from each test organism were spread on Muller Hinton agar plates and wells (6 mm) made on the plates. Cell free extracts and 50 µL crude extracts of each fungal isolate obtained after 14 days from shaker incubator was used to inoculate the wells. Positive controls used were 5 µL of chloramphenicol (20 µg/ml) and nystatin (5 µg/ml) were to inoculate the wells (6 mm diameter). The assay was done in triplicates for each of the test organism. The plates were incubated at 30 °C for 24 hours. Positive antimicrobial activity was indicated by the occurrence of inhibition zones. Size of inhibition zones were recorded in triplicates as mean diameter of the wells 0.6 cm plus the clearing zone for each test organism.

CHAPTER FOUR

RESULTS

4.1 Sampling

4.1.1 Physicochemical characteristics

The physicochemical parameters including; pH, organic carbon, minerals, trace elements, was recorded at the site. The data was recorded for selected sampling sites including site 3 sediments (S3S), Site 2 sediments (S2S), site 5 sediments (S5S), Site 2 brine (Br2S) as they were good representatives of the entire sampling location. Analysis of the geochemical properties of the sediment samples indicated the physicochemical features of the microbial habitats which were the sample collection sites. There were slight differences in the levels of organic carbon, nitrates ammmonium and trace metals among the sites. Br2S (brine) recorded the highest concentration of salts but pH had minimal variations among the sites. The results of the geochemical properties of the sites are summarised in Table 4.1.

Table 4. 1: Summary of physiochemical results for different sampling sites

Parameter	Measurement	S2S June	S3S June	S5S June	Br2S June
pH	uS/cm	10.1	9.9	10.18	10.1
Phosphorus	ppm	6.3	27.7	55.7	6.98
Potassium	ppm	1780	3450	1790	2420
Calcium	ppm	9660	6690	2530	793
Magnesium	ppm	41	43	80.5	21.3
Manganese	ppm	98.9	191	150	58.4
Sulphur	ppm	2.5	<0.50	224	<0.50
Copper	ppm	0.87	1.8	1.85	0.97
Boron	ppm	53.4	50.3	28	40.1
Zinc	ppm	5.16	12.4	7.51	5.99
Sodium	ppm	26200	34200	15500	21400
Iron	ppm	102	171	389	98
Nitrates	ppm	4.11	2.27	5.75	2.19
Ammonium	ppm	0.32	<0.01	2.63	<0.01
Organic Carbon	%(percentage)	1.28	0.89	1.04	0.35

KEY

ppm -parts per million; %- percentage; uS/cm - microsiemens per centimeter

4.2 Fungal isolation

A total of 52 isolates were obtained from various selected sampling sites in Lake Magadi. Most of the fungal isolates (36) were obtained from site 3 sediments (S3S). However, other sites including Site 3 dry (S3Dry), Site 2 sediments (S2S), site 5 sediments (S5S), Microbial mats, Magadi Grass Sediments (MGS), Site 2 brine (Br2S) averagely produced few number of isolates 4, 0, 1, 3, 5 and 2 respectively. The isolates grew after a period of between 7-21 days of incubation at 30 °C whereby visible fungal colonies were observed on petri dishes. The spore forming fungi were able to produce spores after 14 days of growth. Malt extract agar (MEA), Sabourads dextrose agar (SDA), potato dextrose agar (PDA) and oat meal agar (OA) averagely supported most of the fungi growth during the isolation. The number of isolates recovered from each category of media (high, H and low nutrient, L) is summarised as: MEA-H 5 and MEA-L 14; SDA-H 12 and SDA-L 1; PDA-H 1 and PDA-L 5; OA-H 6 and OA-L 7.

4.3 Morphological characterization of the isolated fungi

Morphological characteristics were used to presumptively identify the isolates based on the observable features including colony morphology and cell characteristics.

4.3.1 Colony morphology

On the basis of macroscopic characteristics, colony color and surface of pure fungal isolates were distinct. The mycelia colour of isolates on different media types ranged from green-light green, cottony, brown-white, grey-white, yellow-white, dark grey, cream-white and colorless. Some of the isolates showed variations in color between the mycelial and sporualtion stage.

4.3.2 Cell morphology

Microscopic analysis indicated the hyphae type of mycelium of the fungal isolates and most had septate/hyaline and a few others had aseptate hyphae. In addition, microspores and macrospores were visible in some fungal isolates with majority being round/oval in shape. The spores were clustered while and others were scattered. Microscopic identification also indicated the presence of branched mycelium either as thick or thin in some fungal isolates. The plate images below are true representatives of the 52 isolates

based on the actual colony morphology on plates and cell morphology as visualised under the motic compound light microscope magnification x 40.

Plate 4. 1: A representative sample of the 52 isolates based on colony and cell characteristics.

The colony and cell characteristics recorded for the various isolates are summarised in the table below.

Plate 4.1a (i):: Isolate 2M , pink white wrinkled colony on plate

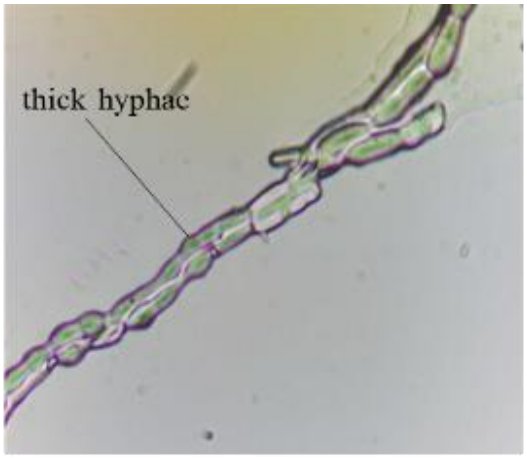


Plate 4.1a (ii): Isolate 2M,thick septate mycelium

Plate 4.1b (i): Isolate 13M, showing dark grey mycelium

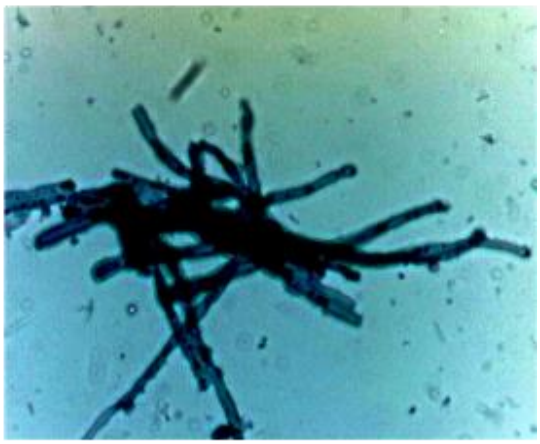


Plate 4.1b (ii): Long hyphae from dark mycelium

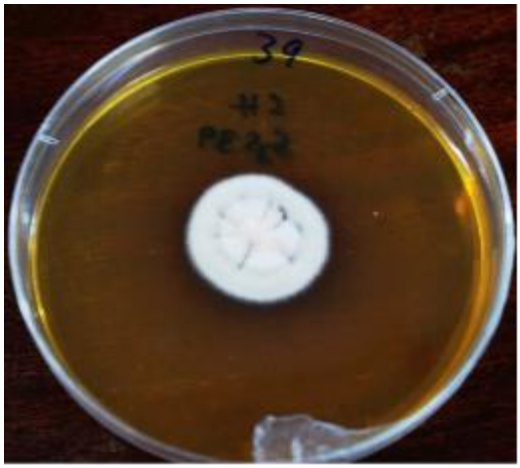


Plate 4.1c (i): Isolate 39M showing colony with white mycelium

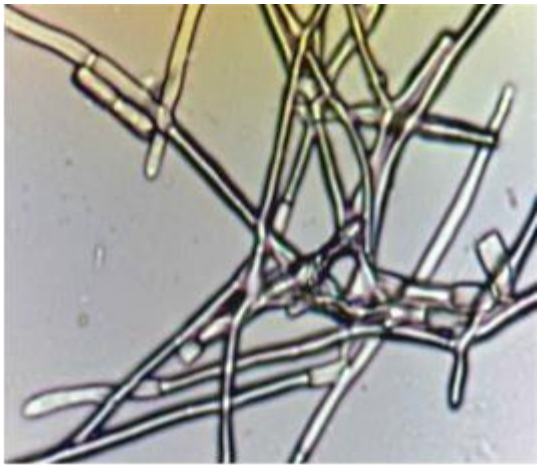


Plate 4.1c (ii): Isolate 39M showing thin branched septate hyphae

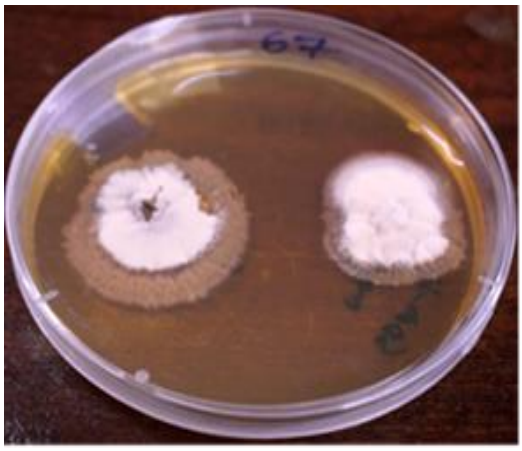


Plate 4.1d (i): Isolate 67M showing cream white colony mycelium

Plate 4.1d (ii): Isolate 67M showing thick branched hypahe

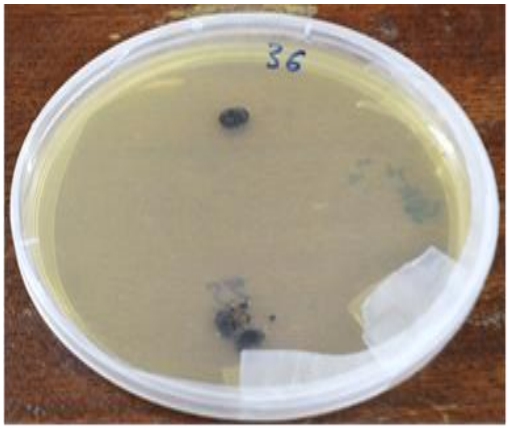


Plate 4.1e (i): Isolate 36M showing black colonies on plate

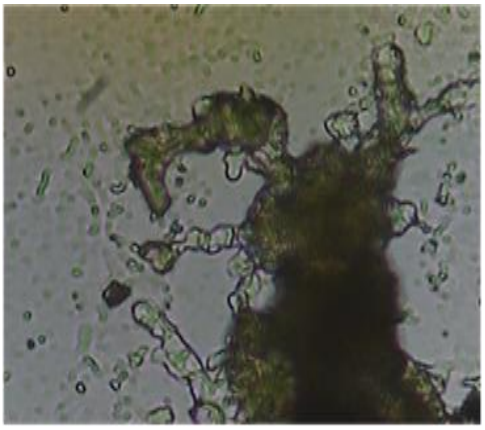


Plate 4.1e (ii): Isolate 36M showing thick short septate hyhae

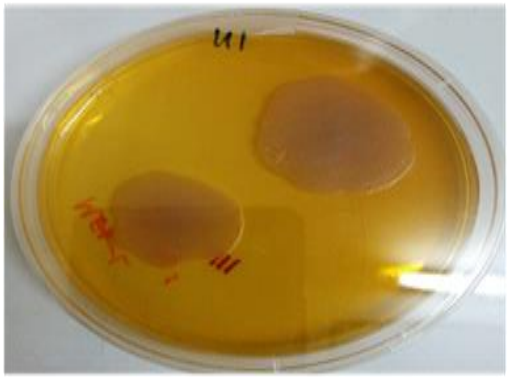


Plate 4.1f (i): Isolate 111M showing mucoid colonies on plate

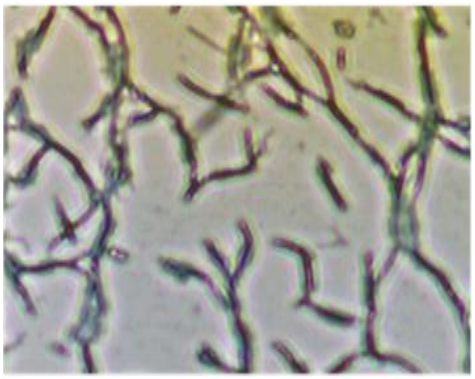


Plate 4.1f (ii): Isolate 111M showing thin septate hyhae

Plate 4.1g (i): Isolate 9M showing grey white colonies on plate

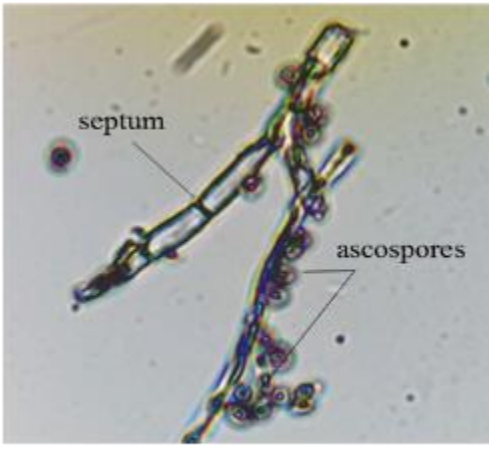


Plate 4.1g (ii): Isolate 9M showing ascospores and septatehyphae

Table 4. 2: Morphological characteristics of the fungi isolates from Lake Magadi

The table highlights isolation media/sampling and also colony and cell features identified.

Isolate	Site/Media	Colony Color	Colony surface	Cellular features
1M	Mats-SDA-H	Pale pink	Wrinkled powdery	Septate/hyaline hyphae
2M	Mats-SDA-H	Pink white	Wrinkled smooth	Septate hyphae
5M	S3S-PDA-H	Dark green	Powdery rough	Septate hyphae/ micro conidia
9M	S3S-OA-H	Grey white	Smooth powdery	Septate hyphae/ round spores
10M	MGS-SDA-H	White-green	Wrinkled powdery	Aseptate hyphae/ round spores
11M	S3S-MEA-L	Light green	Powdery	Short aseptate hyphae
13M	S3S-OA-H	Dark grey	Wrinkled powdery	Septate hyphae
14M	S3S-OA-L	Cream	Smooth	Hyaline hyphae/microspores
15M	S3S-MEA-L	Dull white	Cottony	Aseptate hyphae/ microspores
16M	S3S-OA-H	Brown-white	Powdery	Septate hyphae/sporangium
18M	S3S-MEA-L	Grey white	Rough	Aseptate hyphae/clustered spores
22M	S3S-MEA-H	Grey white	Cottony	Thin aseptate hyphae/oval spores
24M	S3S-OA-H	Dark grey	Wrinkled	Septate hyphae/sporangium
25M	S3S-SDA-H	White	Cottony smooth	Septate/hyaline hyphae
29M	S3S-PDA-L	Brown white	Powdery	Septate/hyaline hyphae
30M	Br2S-PDA-H	Dark brown	Wrinkled rough	Septate hyphae
31M	MGS-PDA-L	Pale pink	Smooth	Septate hyphae
32M	S3S-MEA-H	Brown	Hairy	Aseptate hyphae/microspores
36M	S3S-SDA-L	Dark	Rough	Aseptate hyphae
38M	MGS-SDA-H	White	Smooth	Septate hyphae/sporangium
39M	S3S-SDA-H	Grey-white	Cottony	Septate hyphae/sporangium
40M	S3S-SDA-H	Cream white	Rough	Hyaline hyphae/macrospores
53M	S3dry-PDA-L	Pale yellow	Powdery dry	Short aseptate hyphae
56M	S3S-MEA-L	Dark grey	Powdery	Septate hyphae/clustered spores
57M	S3S-OA-L	Cream white	Wrinkled	Septate hyphae
58M	S3S-OA-L	Cream white	Dry	Aseptate hyphae/micro conidia
59M	MGS-MEA-H	Dark grey	Smooth	Aseptate hyphae
60M	S3S-MEA-H	Red brown	Rough	Aseptate hyphae
64M	S3S-OA-H	Orange	Smooth	Septate hyphae
65M	S3S-SDA-H	White	Smooth	Branched septate hyphae
67M	Mats-SDA-H	Cream white	Powdery rough	Aseptate hyphae
68M	S3S-OA-L	Cream white	Dry	Septate hyphae/micro conidia
69M	Mats-SDA-H	Dark green	Powdery	Septate hyphae/round conidia
70M	S3S-OA-L	Grey white	Rough	Aseptate branched hyphae
71M	S3S-PDA-L	Dark green	Rough	Septate hyphae/macrospores

72M	S3S-MEA-L	Cream	Wrinkled mucoid	short septate hyphae
73M	S3S-MEA-L	White	Dry	short septate hyphae
80M	S3dry-PDA-L	Pale Yellow	Mucoid	Septate hyphae
82M	S3S-MEA-L	Dark	Powdery	septate hyphae/microspores
87M	S5S-SDA-H	Brown White	Powdery rough	septate hyphae/round spores
89M	S3dry-OA-L	Cream White	Mucoid	long septate hyphae
90M	S3S-MEA-L	Yellow	Mucoid	septate short hypahe
94M	S3S-OA-H	Cream	Mucoid	septate hyphae
95M	S3S-MEA-L	Colorless	Dry	round clustered cells
100M	Br2S-SDA-H	Pale Green	Powdery	septate hyphae
108M	S3S-MEA-L	Cream	Mucoid	septate hyphae/microspores
111M	S3S-MEA-L	Pale Pink	Mucoid	branched septate hyphae
113M	S3dry-MEA-L	Cream White	Mucoid	septate short hyphae
114M	MGS-MEA-L	Cream	Wrinkled	thin septate hyphae
120M	S3S-MEA-H	Dark Grey	Powdery	septate hyphae
122M	S3S-MEA-L	Dark Grey	Smooth	septate hyphae
123M	S3S-OA-L	Grey	Powdery	short septate hyphae

OA-Oatmeal agar; MEA-Malt extract agar; PDA-Potato dextrose agar; SDA-Sabourauds dextrose agar; L-low nutrient medium; H- high nutrient medium; S3S-Site 3 sediment; S5S- Site 5 sediment; S3dry-Site 3 dry sediments; MGS-Magadi grass sediments; Br2S-Brine site 2

4.4 Physiological characterization of the isolates

4.4.1 Salt Tolerance

In this physiological test, at least every fungal isolate was able to grow in more than one concentration of salts tested. All the fungal isolates were able to grow moderately at both 0% and 5% NaCl concentration. A few isolates also showed abundant growth at 0% salts and these included isolates 9M, 30M, 39M, 108M and 123M while the rest exhibited slight growth. Optimum growth was observed at 5% salt concentration for most of the fungal isolates however a few isolates for example 2M, 13M, 29M, 38M, 56M, 69M, 71M, 80M,95M and114M showed slight growth. At 10% salts, most of the isolates showed slow growth and a few exhibited optimum growth and includes 5M, 30M, 39M, 59M and 89M. Some of isolates 2M, 13M, 29M, 38M, 56M, 60M and 69M did not grow completely at 10% salts. The highest salt concentration that was used to grow the isolates was 20% and only 13 isolates showed slow growth while the rest did not grow. The isolates include 1M, 24M, 39M, 40M, 53M, 57M, 64M, 65M, 87M, 94M, 100M, 120M and 122M. Fungal

isolates 1M, 24M, 39M, 64M, 65M, 100M, 120M and 122M were able to grow across all the tested salt concentrations. (Table 4.3).

4.4.2 pH Tolerance

Fungal isolates showed different growth patterns after they were subjected to different pH ranges. Except for pH 6 where 9 isolates did not grow completely, most of the fungal isolates grew in pH 7, 8, 9 and 10. Optimum growth for majority of the fungal isolates was recorded at pH 8-10. All the isolates were able to grow in at least more than two different pH range. Most fungi recovered grew slightly in both pH 7 and pH 9 with only a few isolates showing moderate to abundant growth measured in terms of colony size. At pH 10, one isolate (14M) did not grow completely while the rest showed slight to abundant growth. Isolates 9M, 10M, 39M, 70M, 89M, 100M and 108M indicated abundant growth at pH 10 (Table 4.3)

4.4.3 Temperature Tolerance

At 25 °C, all the isolates grew except for isolate M38 though majority exhibited slow growth. There was optimum growth for most of the isolates at 30 °C and all of them grew at this temperature from moderate to abundant growth. At 35 °C, only two isolates 38M and 70M did not grow, although the rest showed average to optimum growth in terms of the colony size. Isolates 5M, 11M, 15M, 18M, 40M, 65M, 90M, 100M and 120M showed their best growth temperature to be 35 °C. At 40 °C most of the isolates showed slight growth while a few including M5, M14 and M122 exhibited abundant growth. Four isolates 32M, 38M, 70M and 94M did not grow at 40 °C (Table 4.3).

Table 4. 3 : Summary of physiological characteristics of fungal isolates

	Salt tolerance				Temperature tolerance				pH tolerance				
Isolate	**0%**	**5%**	**10%**	**20%**	**25°C**	**30°C**	**35°C**	**40°C**	**pH6**	**pH7**	**pH8**	**pH9**	**pH10**
1M	++	+++	+	+	+	++	++	++	+	+	+	+	+
2M	++	+	-	-	++	+++	+	+	-	++	++	+	++
5M	++	+++	+++	-	++	++	+++	+++	++	++	+++	+	++
9M	+++	++	++	-	++	+++	+	+	-	+	+++	+	+++
10M	+	+++	++	-	+	+	+	+	-	+	+	+	+++
11M	++	+++	++	-	++	+++	+++	++	+	++	+++	+	++
13M	++	+	+	-	++	++	++	++	+	+	++	+	+
14M	++	++	-	-	+	+++	++	+++	-	+	++	+	-
15M	+	+++	+	-	++	++	+++	+	+	+	+++	++	+
16M	++	++	+	-	++	+++	++	+	++	+	++	+	++
18M	++	++	+	-	+	+	+++	+	-	+	++	+++	+
22M	++	++	+	-	+	++	++	+	++	++	+	++	++
24M	++	++	++	+	+	++	+	+	++	++	++	++	++
25M	+	++	+	-	++	++	++	+	++	+	+++	++	+
29M	++	+	-	-	+	++	+	++	+	+	++	+	+
30M	+++	+++	+++	-	++	+++	++	++	++	+	+++	++	++
31M	+	++	+	-	++	++	++	++	+	+	+	+	+
32M	+	++	++	-	+	++	+	-	++	++	+++	+	+
36M	+	++	++	-	+	++	++	+	++	+	++	+	+
38M	+	+	-	-	-	++	-	-	-	-	+	+	+
39M	+++	+++	+++	+	+	+	+	+	++	++	++	++	+++
40M	+	++	++	+	+	++	+++	++	-	++	+++	+	+
53M	++	++	+	+	+	++	++	++	+	+	+++	+	++
56M	++	+	-	-	+	++	++	++	+	+	++	+	+
57M	+	++	+	+	+	+	++	+	++	+	++	+	+
58M	+	++	+	-	+	++	++	+	-	-	++	++	+
59M	++	+++	+++	-	++	+++	++	++	-	++	++	+	++

60M	+	++	-	-	++	++	++	+	+	++	++	+	+
64M	+	+++	++	+	+	++	+	+	+	++	++	+	++
65M	+	++	+	+	++	+++	+++	+	-	+	++	+	+
67M	+	+++	+	-	++	++	++	++	-	+	++	+	+
68M	++	++	+	-	+	++	++	++	-	+	+++	+++	+
69M	++	+	-	-	++	+++	+	+	-	++	++	+	++
70M	++	++	+	-	++	+	-	-	+++	+	+++	-	+++
71M	+	+	+	-	+	+++	++	++	++	+	++	+	+
72M	+	++	+	-	++	++	+	++	++	+	++	+	++
73M	+	+++	+	-	++	+++	++	++	-	++	++	++	++
80M	++	+	+	-	+	++	++	++	-	+	++	+	+
82M	+	++	++	-	+	++	++	+	++	+	++	+	+
87M	++	+++	+	+	+	++	+	+	-	+	++	+	++
89M	++	+++	+++	-	+	++	+	+	+++	+++	+++	++	+++
90M	+	++	+	-	+	++	+++	+	-	+	+	+++	+
94M	+	++	+	+	++	+	+	-	-	+	+	-	+
95M	++	+	+	-	+	+++	++	+	-	+	++	+	+
100M	+	+++	++	+	+	+++	+++	+	-	-	++	+	+++
108M	+++	+++	+++	-	+	++	+	+	++	+	+++	++	+++
111M	+	+++	+	-	+	++	+	+	+	+	+++	+++	+
113M	+	++	+	-	+	+++	+	+	++	+	++	+	+
114M	+	+	++	-	+	++	++	+	+	+	++	+	++
120M	+	++	++	+	+	++	+++	+	++	++	++	++	++
122M	++	++	++	+	+	++	+	+++	-	+	+	+++	++
123M	+++	++	+	-	+	++	++	+	++	+	++	++	+

KEY: - (no growth), + (0 – 2 mm, slight growth), ++ (2.1 – 4mm, moderate growth), +++ (> 5 mm, abundant growth)

4.5 Biochemical Characterization

4.5.1 Screening for enzymatic activity

All the isolates were screened for their ability to produce proteinases, celullases, pectinases, chitinases, amylases, lipases and esterases. Different isolates were able to utilize specific enzyme substrates as the sole carbon souce. Production of cellulases was observed by the degradation of substrates cellulose and carboxymethylcellulose. Only 9 isolates degraded cellulose whereas 8 isolates degraded CMC. Eight fungal isolates degraded starch and were therefore positive for amylase enzyme. Degradation of casein as sole carbon source was observed in 8 isolates and were thus positive for proteinases. Pectinase and chitinase production was observed after degradation of substrates pectin and chitin by 8 and 22 isolates respectively. However, 22 isolates did not produce any enzyme after they were grown in all the substrates. None of the isolates was able to peroxidases/laccases since they were not able to degrade lignin. Isolates that produced more than one enzyme with a few producing upto four enzymes showed polyenzymatic activity and included: 69M, 87M, 90M, 69M, 59M and 2M. Among them, 22 produced chitinase which is an important enzyme as one of the virulence factors for entomopathogenic fungi. Six isolates were positive for amylases. There was generally low enzymatic activity from the fungi recovered from the lake. This was based on the diameter of clearing zones with a mesurement of up to 3mm scored as (+). Moderate enzymatic activity was observed in isolates 1M, 2M, 39M, 69M, 87M, 94M, 100M, 123M whereby they had clearing zones of between 3.1-6mm and was recorded as (++). A few isolates 38M and 90M showed high enzymatic activity for chitinase and cellulase respectively, they recorded diameter of halo zones above 6mm which was scored as (+++) (Table 4.4).

Table 4.4: Enzymatic activity of the fungal isolates

Isolate	Site	Sta	CMC	Cas	Pec	Xan	Lig	T20	Chit	Cel
1M	mats	-	-	-	-	-	-	-	++	-
2M	mats	+	+	-	+	+	-	-	++	-
5M	S3S	+	-	-	-	-	-	-	-	-
13M	S3S	-	-	-	-	-	-	-	+	-
14M	S3S	-	-	-	-	-	-	-	+	-
29M	S3S	-	-	+	-	-	-	+	-	+
31M	mgs	-	-	+	-	-	-	+	++	-
32M	S3S	-	-	+	-	-	-	+	-	-
38M	mgs	-	-	-	-	-	-	-	+++	-
39M	S3S	-	-	-	-	-	-	-	++	-
40M	S3S	-	-	+	-	-	-	+	+	-
57M	S3S	-	-	-	-	-	-	-	+	-
59M	mgs	+	+	-	+	+	-	-	-	+
64M	S3S	-	-	-	-	-	-	-	+	-
68M	S3S	-	-	-	-	-	-	-	+	-
69M	mats	+	+	+	+	+	-	+	-	++
70M	S3S	-	-	-	-	-	-	-	+	-
71M	S3S	-	-	-	-	-	-	-	+	-
73M	S3S	-	-	-	-	-	-	-	++	-
87M	S5S	+	++	-	+	+	-	-	+	+
90M	S3S	+	++	-	++	+++	-	-	-	+++
94M	S3S	-	-	-	-	-	-	-	++	-
95M	S3S	-	+	-	-	-	-	-	+	-
100M	br2	+	-	+	+	+	-	+	++	++
108M	S3S	-	-	-	-	-	-	-	+	-
111M	S3S	+	+	+	+	+	-	-	-	+
113M	S3dry	+	+	+	+	+	-	-	-	+
120M	S3S	-	-	-	-	-	-	-	+	+
122M	S3S	-	-	-	-	-	-	-	+	-
123M	S3S	-	-	-	-	-	-	-	++	-

KEY: - (no activity); + (0 – 3 mm); ++ (3.1 – 6 mm); +++ (> 6 mm); Iso- isolate; Sta- starch; CMC- carboxymethylcellulose; Cas- casein; Pec- pectin; Xan- xanthan; Lig- lignin; T20- tween 20; Chit- chitin; Cel- cellulose; S3S-Site 3 sediment; S5S- Site 5 sediment; S3dry-Site 3 dry sediments; MGS-Magadi grass sediments; Br2S-Brine site 2.

4.6 Molecular Characterization

Fungal genomic DNA extraction for all the 52 isolates was confirmed on agarose gel electrophoresis visualised upon staining with cyber green. The amplification of the conserved regions in 18S rRNA gene using fungal primers 683f and 394r via PCR produced amplicons approximately 500bp that were purified and sequenced (Figure 4.1).

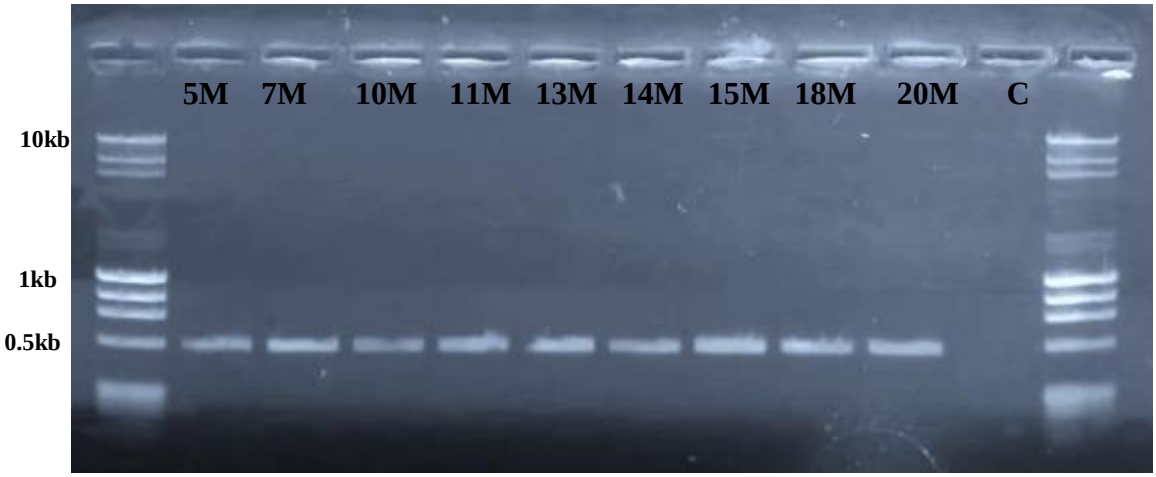


Figure 4.1: A 1% agarose gel showing 18S rDNA PCR amplification of the isolates visualized bands after cyber green staining. C = Control

4.7 Phylogenetic analysis results after sequencing

BLAST results were used in the identification of the highest homology of sequences (http://www.ncbi.nih.gov). The isolates were blasted and their nearest neighbors in the data bank were determined based on percentage similarity (Table 4.5). All the isolates clustered within the phylum Ascomycota and were distributed in 18 genera. Among the isolates, 15 were affiliated to the genus *Aspergillus* with most of them exhibiting between 99%-100% similarities. The other genera were also represented in small numbers and had close affiliation with known genera in the phylum Ascomycota. They included: *Penicillium* (9 isolates), *Acremonium* (3 isolates), *Phoma* (4 isolates), *Cladosporium* (3), *Septoriella* (1), *Talaromyces* (2), *Zasmidium* (1), *Chaetomium (1), Aniptodera* (1), *Pyrenochaeta* (1), *Septoria* (1), *Juncaceicola (1), Paradendryphiella (1) Sarocladium* (2) *Phaeosphaeria* (1) and *Juncaceicola* (1), *Biatriospora* (2). The constructed phylogenetic tree indicated the phylogenetic position of the isolates (Figure 4.2). The bootstrap values supported that different strains of isolates within a given genus are closely related with known strains from Genebank (Table 4.5). However, the bootstrap values were linked

with morphological features to confirm the close relationship between the isolates. For example, known strain *Penicillium oxalicum* (MK069498) are sister groups in the same clade with isolate 51M (F45_*Penicillium oxalicum* strain TGQM01) having 93% similarity level. The isolated genera *Juncaceicola*, *Septoriella* and *Phaeosphaeria* have a common origin in the phylogenetic tree and may be having a common evolutionary history. Interestingly, isolate 69M, 87M and 90M had sequence similarity of 96%, 97% and 95% respectively therefore they could be representing novel species within known affiliate genera. However, isolates 72M, 80M, 95M, 108M, 113M and 100M had sequence similarity of below 94% and could therefore represent new genera (Table 4.5).

Table 4. 5: Results obtained from BLAST showing close relatives of the fungal isolates from Lake Magadi.

Sequence Code	**Isolate Code**	**Closest Relative (BLAST)**	**Identity**	**Accession No.**
F1	1M	*Aspergillus sp.* strain 3Y	100%	MH071384.1
F2	2M	*Phoma herbarum* strain BZYB-1	99%	MG757513.1
F4	5M	*Aspergillus fumigatus* strain EG11-4	98%	KX457677.1
F7	9M	*Cladosporium cladosporioides*	98%	KJ614394.1
F8	10M	*Penicillium oxalicum* strain TGQM01	100%	MK069498.1
F9	11M	*Penicillium chrysogenum* CBS 306.48	99%	NG_062803.1
F10	13M	*Cladosporium halotolerans* CBS 119416	99%	NG_062721.1
F11	14M	*Phaeosphaeria luctuosa* strain CBS 577.8	99%	NG_063079.1
F12	15M	*Aspergillus fumigatus* strain EG11-4	99%	KX457677.1
F13	16M	*Aspergillus versicolor* isolate F	100%	KX272754.1
F15	18M	*Penicillium commune* CBS 343.51	100%	NG_062634.1
F18	22M	*Penicillium limosum* CBS 339.97	100%	NG_062729.1
F20	24M	*Pyrenochaeta nobilis* CBS 407.76	96%	NG_062727.1
F21	25M	*Acremonium roseolum* strain CBS 289.62	99%	HQ232207.1
F26	29M	*Aspergillus versicolor* isolate CWJ2	100%	KR233971.1
F27	30M	Uncultured eukaryote clone NYS002060	98%	JX396247.1
F28	31M	*Aspergillus versicolor* strain MPE9	99%	MH145425.1
F29	32M	*Sarocladium sp.* strain CG-MB01	100%	AB920177.1
F32	36M	*Zasmidium cellare* isolate F-14	100%	MK123328.1
F34	38M	*Chaetomium globosum* strain F0909	99%	MH045499.1
F35	39M	*Paradendryphiella arenariae* isolate F-15	99%	MK123330.1
F36	40M	*Acremonium sclerotigenum* CBS 124.42	99%	NG_062818.1
F47	53M	*Sarocladium kiliense* CBS 122.29	99%	EU174429.1
F50	56M	*Aspergillus keveii* strain CBS 209.92 28S	99%	MF004311.1
F51	57M	*Aspergillus glaucus* JCM 1575	98%	NG_063391.1

F52	58M	*Penicillium citrinum* strain IITG_KP1	99%	MH392275.1
F53	59M	*Aspergillus flavipes* NRRL 302	98%	NG_063230.1
F54	60M	*Phoma destructiva*	99%	AB454203.1
F58	64M	*Aniptodera chesapeakensis*	99%	AF279373.1
F59	65M	*Phoma sp*. LF617	99%	KM096195.1
F61	67M	*Juncaceicola alpina* CBS 456.84	99%	NG_063078.1
F62	68M	*Septoriella leuchtmannii* CBS 459.84	99%	NG_063079.1
F63	69M	*Cladosporium velox*	96%	NG_062725.1
F64	70M	*Aspergillus versicolor* strain MF557	99%	KM096354.1
F65	71M	*Aspergillus sp*. strain DX4H	99%	MF185177.1
F66	72M	*Penicillium viridicatum* CBS 390.48	81%	NG_062833.1
F67	73M	*Acremonium roseolum* strain CBS 289.62	99%	HQ232207.1
F74	80M	Uncultured fungus clone 42_Wound2L	80%	MF980830.1
F76	82M	*Biatriospora carollii*	99%	LN626679.1
F81	87M	*Aspergillus keveii* strain CBS 209.92 28S	97%	MF004311.1
F83	89M	*Talaromyces marneffei* strain Tm-HIV	92%	CP015870.1
F84	90M	Uncultured eukaryote clone BSLe1	95%	EF682444.1
F88	94M	*Phoma destructiva* isolate: MUCC0064	99%	AB454203.1
F89	95M	*Aspergillus terreus* strain AZM03	84%	MH550051.1
F94	100M	*Penicillium oxalicum* strain TGQM01	93%	MK069498.1
F99	108M	*Aspergillus flavipes* NRRL 302	83%	NG_063230.1
F102	111M	*Aspergillus sp*. strain AON1	98%	MH427203.1
F103	113M	*Talaromyces marneffei* strain Tm-HIV	92%	CP015870.1
F104	114M	*Penicillium polonicum* CBS 222.28	98%	NG_062834.1
F108	120M	*Septoria senecionis* CBS 102366	98%	NG_062783.1
F110	122M	*Biatriospora carollii* isolate CCF4484	99%	LN626674.2
F111	123M	*Penicillium citrinum* strain IITG_KP1	98%	MH392275.1

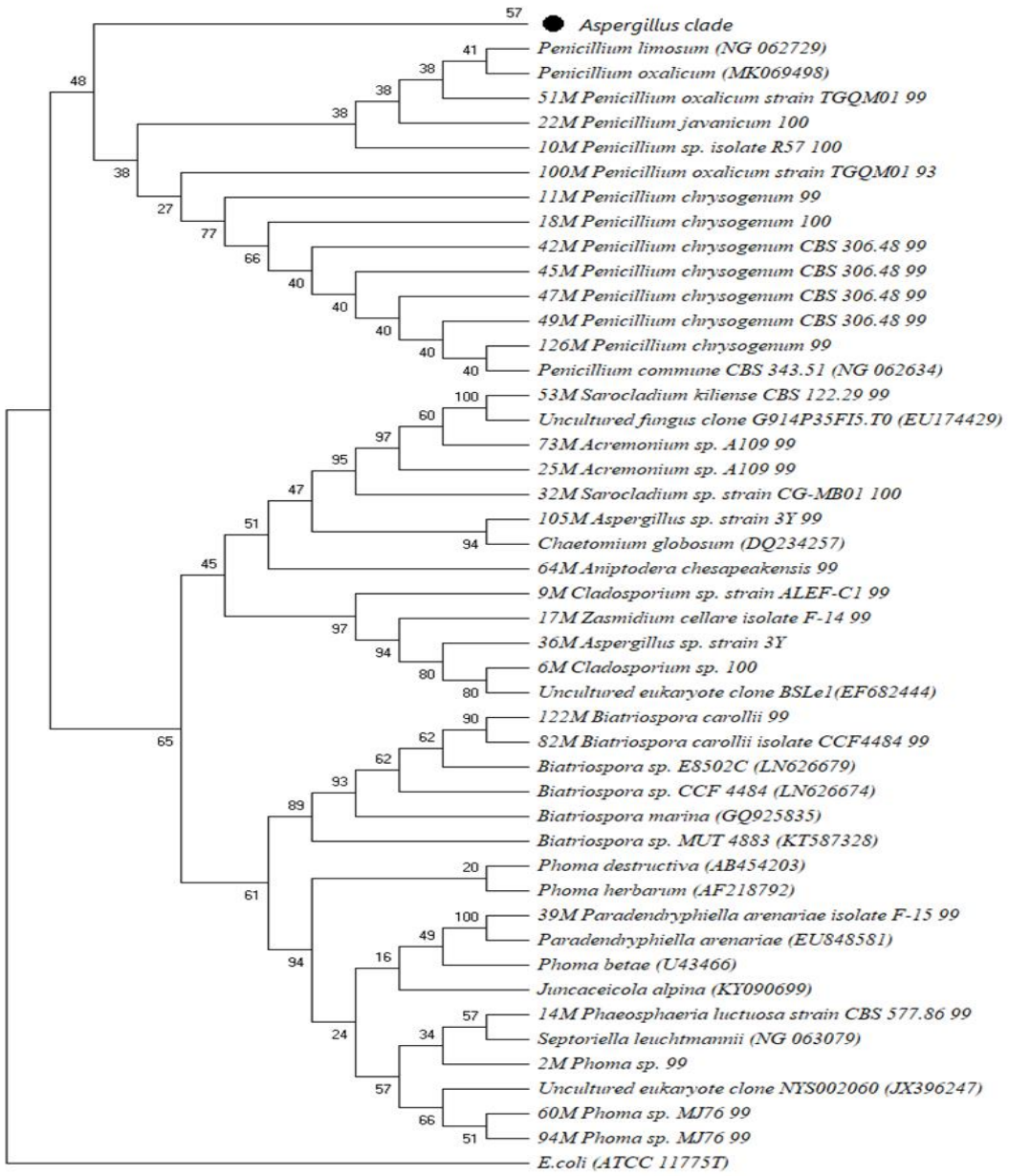
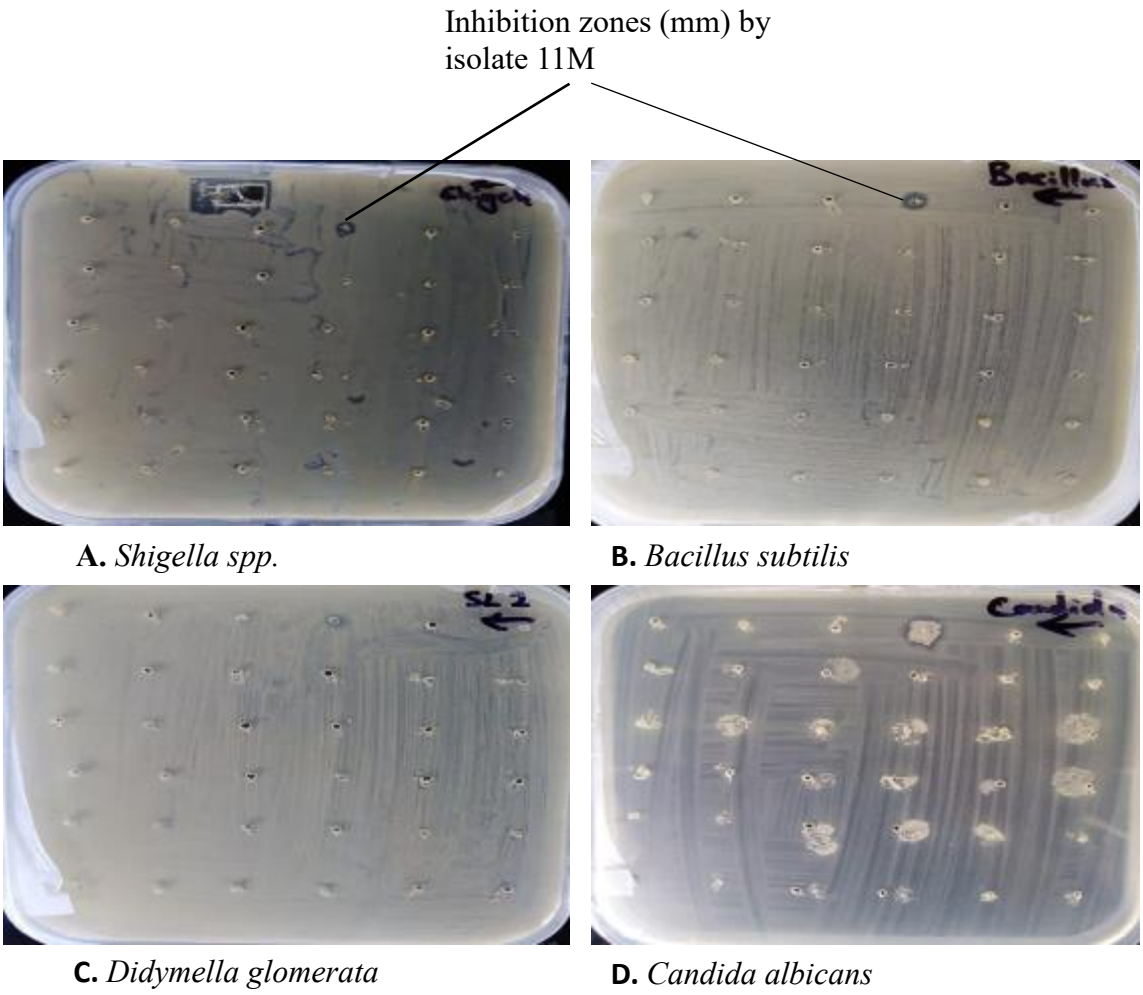

Aspergillus clade
Penicillium limosum (NG 062729)
Penicillium oxalicum (MK069498)
51M Penicillium oxalicum strain TGQM01 99
22M Penicillium javanicum 100
10M Penicillium sp. isolate R57 100
100M Penicillium oxalicum strain TGQM01 93
11M Penicillium chrysogenum 99
18M Penicillium chrysogenum 100
42M Penicillium chrysogenum CBS 306.48 99
45M Penicillium chrysogenum CBS 306.48 99
47M Penicillium chrysogenum CBS 306.48 99
49M Penicillium chrysogenum CBS 306.48 99
126M Penicillium chrysogenum 99
Penicillium commune CBS 343.51 (NG 062634)
53M Sarocladium kiliense CBS 122.29 99
Uncultured fungus clone G914P35FI5.T0 (EU174429)
73M Acremonium sp. A109 99
25M Acremonium sp. A109 99
32M Sarocladium sp. strain CG-MB01 100
105M Aspergillus sp. strain 3Y 99
Chaetomium globosum (DQ234257)
64M Aniptodera chesapeakensis 99
9M Cladosporium sp. strain ALEF-C1 99
17M Zasmidium cellare isolate F-14 99
36M Aspergillus sp. strain 3Y
6M Cladosporium sp. 100
Uncultured eukaryote clone BSLe1(EF682444)
122M Biatriospora carollii 99
82M Biatriospora carollii isolate CCF4484 99
Biatriospora sp. E8502C (LN626679)
Biatriospora sp. CCF 4484 (LN626674)
Biatriospora marina (GQ925835)
Biatriospora sp. MUT 4883 (KT587328)
Phoma destructiva (AB454203)
Phoma herbarum (AF218792)
39M Paradendryphiella arenariae isolate F-15 99
Paradendryphiella arenariae (EU848581)
Phoma betae (U43466)
Juncaceicola alpina (KY090699)
14M Phaeosphaeria luctuosa strain CBS 577.86 99
Septoriella leuchtmannii (NG 063079)
2M Phoma sp. 99
Uncultured eukaryote clone NYS002060 (JX396247)
60M Phoma sp. MJ76 99
94M Phoma sp. MJ76 99
E.coli (ATCC 11775T)

Figure 4.2: Rooted Phylogenetic tree created using Neighbor-joining method and is based on a comparison of the 18S ribosomal DNA sequences of Lake Magadi isolates and their closest phylogenetic relatives. *E coli* was used as the outgroup. Percentages of bootstrap sampling derived from 1000 replications are indicated by the numbers on the tree. Sequences from this study are strains with M codes.

4.8 Screening for secondary metabolites

4.8.1 Primary antimicrobial screening

In primary antimicrobial screening, only crude extracts from isolate 11M showed positive activity against enteric pathogens *Escherichia coli, Bacillus subtilis, Salmonella spp., Pseudomonas aeruginosa, Shigella spp.* and human fungal pathogen *Candida albicans*. Two fungal plant pathogens *Alternaria tenuissima* and *Didymella glomerata* were also inhibited by crude extracts from isolate 11M (plate4.2). Positive antimicrobial activity was qualitatively indicated by presence of inhibition zones.



A. *Shigella spp.* **B.** *Bacillus subtilis*

C. *Didymella glomerata* **D.** *Candida albicans*

Plate 4.2: Crude extracts from isolate 11M inhibiting four of the test organisms in primary antimicrobial screening.

4.8.2 Secondary antimicrobial screening

Crude filtrates and cell free extracts from isolate 11M showed antimicrobial effects against several test pathogens as compared to the results observed in primary screening. Cell free extracts gave larger inhibition zones as compared to zones observed from inhibition by crude extracts. Extracts from the three production media grown at pH 7.0 and 0% salts showed better results than extracts from production media grown at pH 8.0 and 5% salts.

4.8.2.1 Production media at pH 7.0 and 0% salts

The best production media in this case was PM3, which showed positive results for inhibition against 13 different test pathogens. Production media YESD and YPSS however did not give desired inhibition results as their extracts showed positive results against 4, 2

pathogens respectively. Crude extracts from PM3 media showed inhibition zones ranging between 11.33±0.03-14.33± 0.03 and were active against all pathogenic human enteric bacteria except *Staphylococcus aureus.* Three plant pathogenic fungi *Epicoccum sorghinum* strain JME-11, *Phoma destructiva, Didymella glomerata,* and *Schizophyllum commune* isolate ScGD28 were also inhibited by crude extract from PM3. There was slight difference on the effect of crude extract from PM3 on different test pathogens (Figure 4.3). Cell free extracts from PM3 media showed inhibition range between 10.00± 0.00-19.67± 0.03 and was active against *Shigella sp., Pseudomonas aeruginosa, Escherichia coli* and *Staphylococcus aureus* as the only human pathogens. However, five plant pathogenic fungi were inhibited by cell free extracts from PM3 (figure 4.4). YESD media produced crude extracts against plant pathogenic fungi *Aspergillus fumigatus* strain EG11 (42.33±0.03), *Cladosporium halotolerans* CBS 119416 (22.33± 0.09), *Phoma destructiva* (20.67±0.03) and cell free extracts that inhibited *Schizophyllum commune* isolate ScGD28 (9.67±0.03). YPSS media also gave out crude extracts and cell free extract active against *Cladosporium halotolerans* CBS 119416 (21.67± 0.03) and *Schizophyllum commune* isolate ScGD28 (9.33± 0.03) respectively (figure 4.4). Positive controls using broad spectrum antimicrobial drugs chloramphenicol and nystatin showed inhibition zones size ranging between 13.00± 0.06 and 15.00± 0.06 respectively.

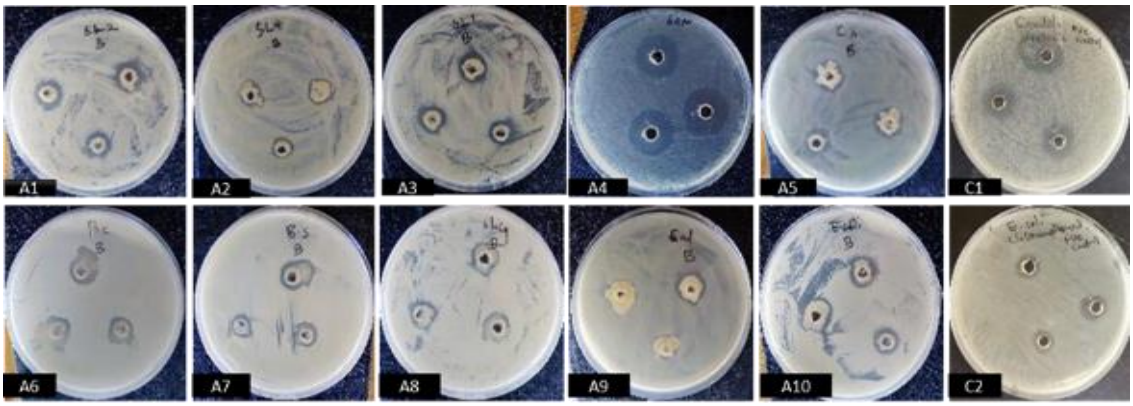


Plate 4.3: Antimicrobial activity (inhibition zones, mm) of crude extract from isolate 11M (grown at pH 7 and 0% salts) screened against test organisms using agar well diffusion method on plates. A1- *Didymella glomerata*; A2- *Schizophyllum commune* isolate ScGD28; A3- *Epicoccum sorghinum* strain JME-11; A4- *Phoma destructiva*; A5- *Candida albicans*; A6- *Pseudomonas aeruginosa*; A7- *Bacillus subtilis*; A8- *Shigella spp*.; A9- *Salmonella typhi*; A10- *Escherichia coli*; C1- Positive control with Nystatin; C2- Positive control with Chloramphenicol

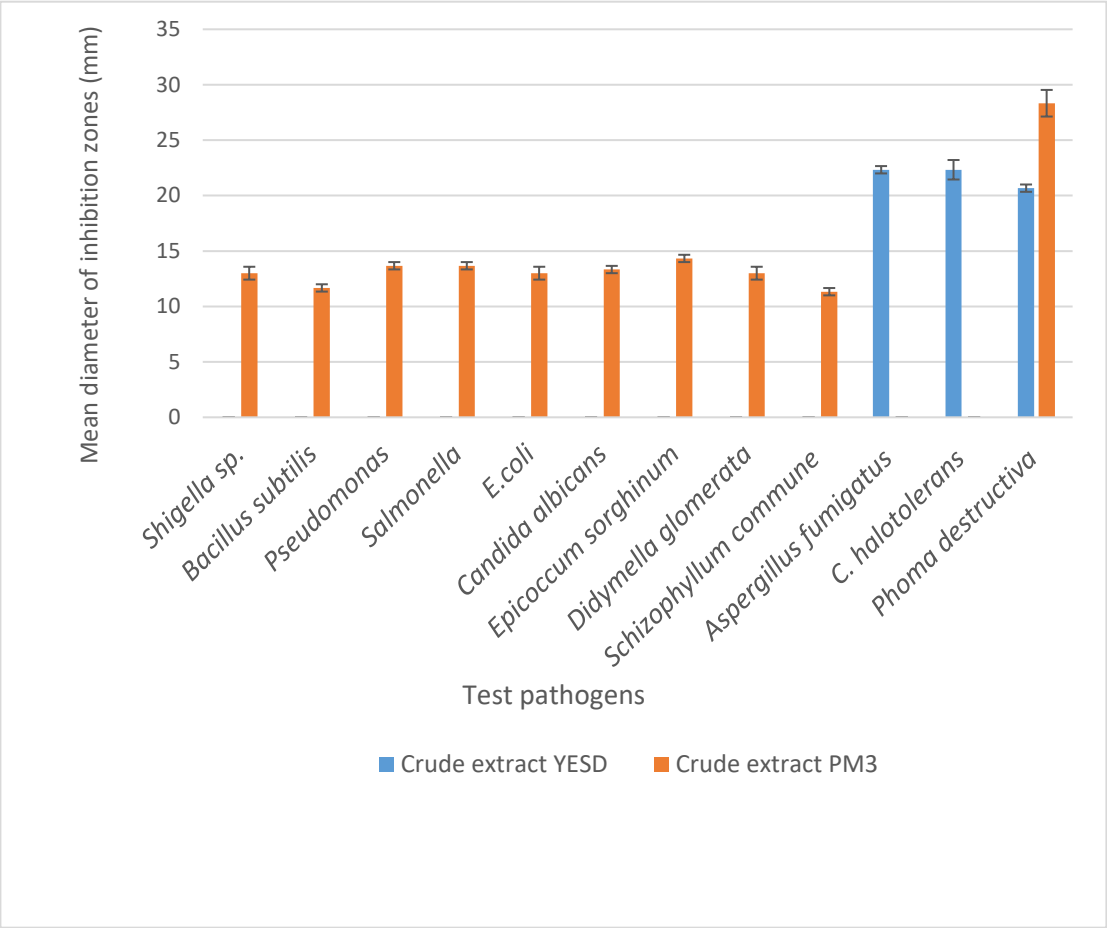


Figure 4.3: Graph showing standard error of mean of inhibition zones by crude extract from isolate 11M (grown at pH 7 and 0% salts) screened against test organisms.

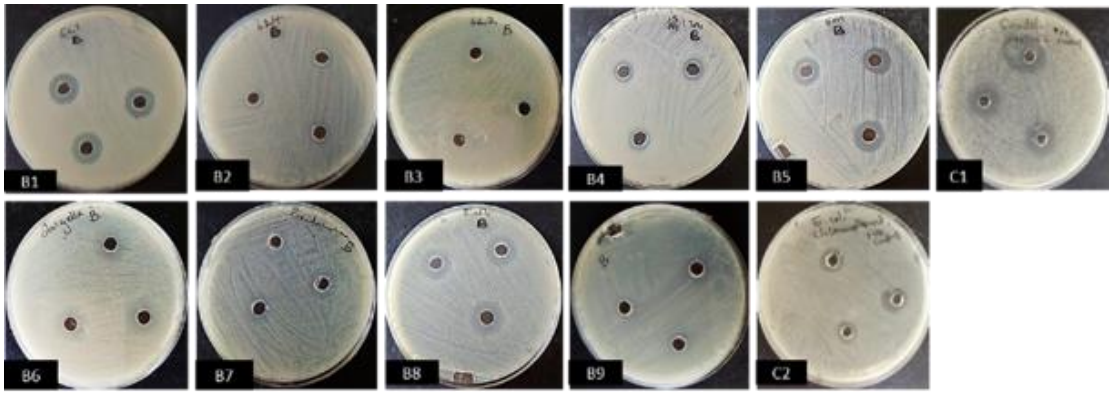


Plate 4. 4: Antimicrobial activity (inhibition zones, mm) of cell free extract from isolate 11M (grown at pH 7 and 0% salts) screened against test organisms using agar well diffusion method on plates. B1- *Epicoccum sorghinum*; B2- *Schizophyllum commune* isolate ScGD28; B3- *Didymella glomerata*; B4- *Cladosporium halotolerans* CBS 119416; B5- *Aspergillus fumigatus* strain EG11-4; B6- *Shigella spp*.; B7- *Pseudomonas aeruginosa*; B8- *Escherichia coli*; B9- *Staphylococcus aureus*; C1- Positive control with Nystatin; C2- Positive control with Chloramphenicol.

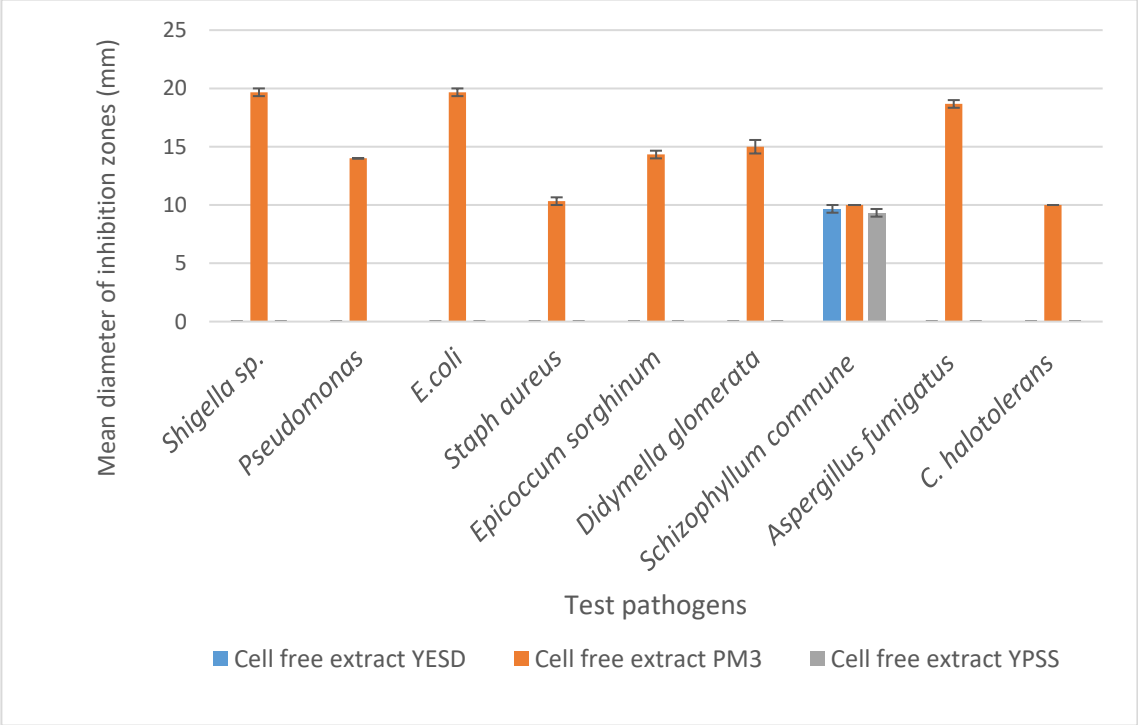


Figure 4.4: Graph showing standard error of mean of inhibition zones by cell free extract from isolate 11M (grown at pH 7 and 0% salts) screened against test organisms

4.8.2.1 Production media with pH 8.0 and 5% salts

Out of the 3 production media, YPSS gave the best results because extracts from this media had antimicrobial effects against 11 test pathogens. The other two production media (PM3, YESD) did not give better results. Extracts from YESD inhibited one plant fungal pathogen *Schizophyllum commune* isolate ScGD28 (21.00±0.58) whereas crude extracts from PM3 did not inhibit any test pathogen. Crude extracts from YPSS media inhibited 4 human bacterial pathogens with the diameter of inhibition zone ranging between 10.33±5.17 to 4.33±0.88. Human fungal pathogen, *Candida albicans*, was inhibited (10.67±5.37) by crude extract from YPSS media. Inhibition zones ranging from 12.67±0.88 to 17.67±0.33 was also observed when extracts from YPSS media was used against plant fungal pathogens and plant bacterial pathogen *Dickeya dianthicola*. Cell free extracts from PM3 inhibited 4 plant fungal pathogens. Based on the standard error mean (SEM) graph (Figure 4.5), there was slight differences on the antimicrobial activity of cell free extract from YPSS on the four plant pathogens (*Cladosporium halotolerans* CBS 119416, *Phoma destructive*, *Aspergillus fumigatus* and *Schizophyllum commune* isolate ScGD28) as the error bars did not overlap (Cumming *et al.*, 2007). Crude extract from YESD showed the highest inhibition zone against *Schizophyllum commune* isolate ScGD28.

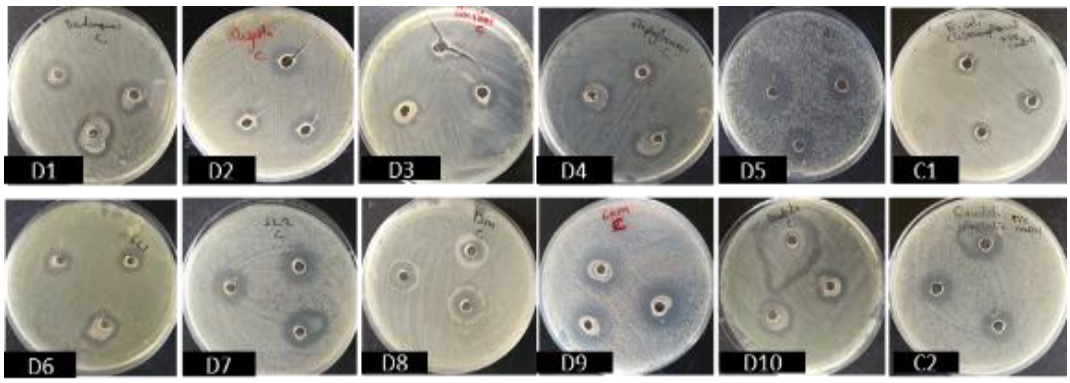


Plate 4. 5: Antimicrobial activity (inhibition zones, mm) of crude extract from isolate 11M (grown at pH 8 and 5% salts) screened against test organisms using agar well diffusion method on plates. D1- *Pseudomonas aeruginosa* D2- *Shigella spp*.; D3- *Bacillus subtilis*; D4- *Staphylococcus aureus* D5- *Dickeya dianthicola*; D6-*Epicoccum sorghinum*; D7- *Didymella glomerata* D8- *Cladosporium halotolerans* CBS 119416;

D6-*Epicoccum sorghinum*; D7- *Didymella glomerata* D8- *Cladosporium halotolerans* CBS 119416; D9- *Phoma destructiva;* D10- *Candida albicans*; C1- Positive control with Nystatin; C2- Positive control with Chloramphenicol.

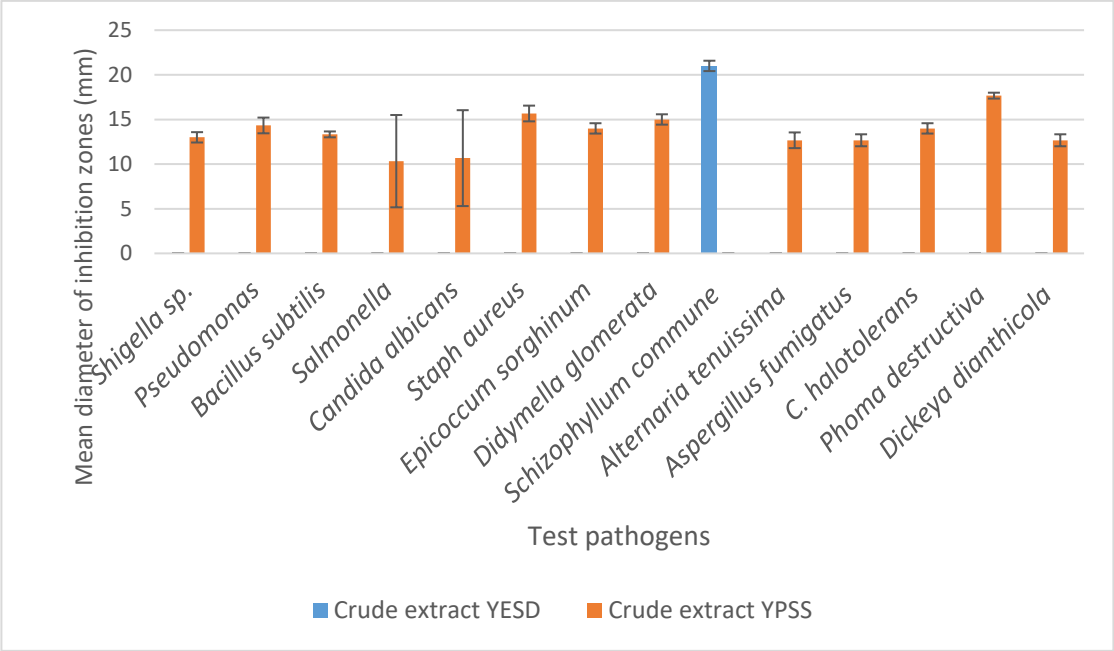


Figure 4.5: Graph showing standard error of mean of inhibition zones by crude extract from isolate 11M (grown at pH 8 and 5% salts) screened against test organisms

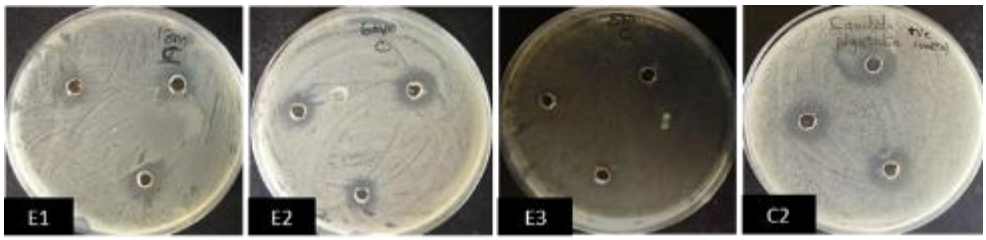


Plate 4. 6: Antimicrobial activity (inhibition zones, mm) of cell free extract from isolate 11M (grown at pH 8 and 54% salts) screened against test organisms using agar well diffusion method on plates. E1- *Cladosporium halotolerans* CBS 119416; E2- *Phoma destructiva*; E3- *Aspergillus fumigatus* strain EG11-4; C2- Positive control with Chloramphenicol

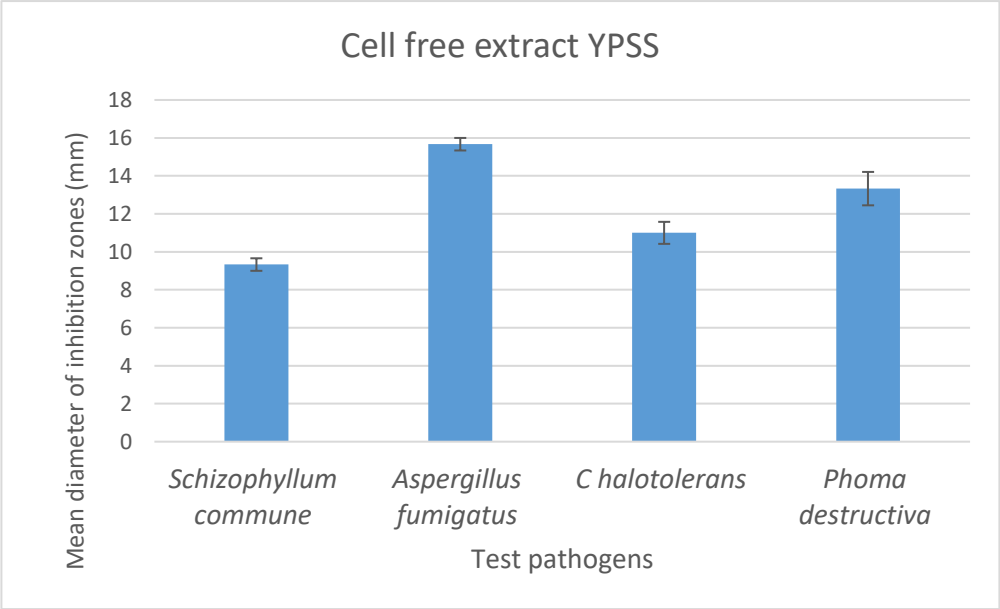


Figure 4.6: Graph showing standard error of mean of inhibition zones by cell free extract from isolate 11M (grown at pH 8 and 5% salts) screened against test organisms

CHAPTER FIVE

DISCUSSION

5.1 Fungi isolation

The aim of the study was to isolate fungi from Lake Magadi and identify them using various approaches. The lake sediments are chemically concentrated and have variable nutritional aspects which offers a unique environment that favors the occurrence of many different fungi (Woolard and Irvine, 1995). This explains why majority of the fungi were recovered from site 3 sediments as one of the sampling sites (S3S). There are higher values soluble phosphates, hydrogen sulfide and ammonia (physicochemical parameters) in the sediments and also varying pH levels within the lake (see table 4.1). Such variation suggest that biological processes contributes to the physical mixing surface and deep waters. High level of nutrients, availability of trace elements and unlimited CO_2 access makes soda lake environment very productive to enhance diverse fungal growth. Various organic compounds for example sugars, complex polysaccharides and hydrocarbons found in the lake are utilized by the biochemically active microbes (Hedi *et al.*, 2009). Soda lake fungi were characterized on the basis of the cell morphology, biochemical (enzymatic) features and molecular analysis. The study recovered 52 different fungi based on the different identification techniques. Modification of the culture dependent techniques in combination with molecular analysis using 18S rDNA gene aided in the search for new diversity in the soda lake. All the fungi isolated were anamorphs of the widely known phylum *Ascomycota*.

5.2 Modification of isolation media

Fungal isolation using commercial media prepared both in high and low nutrient composition and diluted with soda lake water was a strategy we used to test whether nutrients have an effect on the recoverable diversity. High and low nutrient media prepared from Malt extract agar, Oatmeal agar, Sabouraud dextrose agar and Potato dextrose agar showed varied results in terms of the number of isolates obtained. Fourteen isolates were obtained using MEA-L than MEA-H whereby only five isolates were

recovered. The same scenario was observed for PDA and Oat meal agar. It is therefore clear that most fungal groups thrive well in a low nutrient environment, which is consistent with the low levels observed in the chemical analysis of the environmental samples. Probably once the carbon source is depleted, pathways for secondary metabolites are activated to counteract salt and pH stress (Chavez *et al*., 2015). A study elsewhere indicated that soil silts provides nutrients that enhances both microbial growth and growth of aquatic plants (Schallenberg *et al*., 2003).

5.3 Identification using morphological features

Morphologically based characterization was an important parameter in preliminary identification of the isolated fungi. Specific cell structures for example the nature of hyphae/mycelium is one of the morphological characteristics used to distinguish the isolates. Majority of the isolates were anarmorphic fungi with asexual reproductive structures. Species within the group Ascomycota are asexual fungi (anamorphs) and produces conidia (spores) on coniodiophre stalks as asexual reproductive structures (Minter, *et al*., 1983). Sexual organs (ascomata) for example open cup-shaped structures (apothecia)and flask-shaped structures (perithecia) also exist in phylum *Ascomycota* and releases spores (Money, 2016). Such sexual structures were observed among some of isolates and were useful in their identification (See table 4.2). Sexual spores known as ascospores were identified under the microscope and are formed from asci, ascospores are specifically produced by ascomycetes. However, unique features including the thick mycelium observed in *Phoma herbarum* strain BZYB-1 –isolate 2M are important in stress tolerance (Gostinčar et al., 2009; Gunde-Cimerman, 2009). Thick mycelium has been studied in *Wallemia ichthyophaga,* the most halotolerant fungi known and it is known to have 3-fold cell wall thickening as a specific feature to withstand high NaCl concentrations (Gostinčar *et al*., 2009; Palková and Váchová, 2006). Pigmentation in fungi is greatly influenced by unfavorable lake conditions, culture medium and other stress factors (Aberoumand, 2011). Some of the fungal pigments are important biological compounds with antioxidant and antimicrobial activity, others are also immune moderators (Aberomand, 2011). The pigmented fungi isolated for example *Zasmidium cellare* isolate F-14 (isolate 36M) and others that are dark pigmented (*Aspergillus keveii*-56M, *Cladosporium velox*-69M) is a feature that enable such molds to thrive in the harsh

environments. The hyphae produce melanin which brings about the black coloration in the fungi, an important feature to stress survival (Plemenitaš *et al.*, 2008). *Cladosporium* species for example is known to be a halophilic species dominant in black molds (Nazareth, 2014).

5.4 Geological parameters of the sites in the lake

Slight differences of the sampling sites in terms of biotic and abiotic factors (pH, salts, moisture, vegetation cover and trace elements) indicated no much difference in the fungal communities present. However, there was highest diversity of fungal communities in S3 sediments, the common genera being *Penicillium, Acremonium, Phoma, Cladosporium, Zasmidium, Aniptodera, Pyrenochaeta, Septoria, Paradendryphiella* and *Biatriospora*. This may be attributed to the composition of various physiochemical parameters especially the pH, $Na^{+,}$ K^{+} ions and trace elements which differed from the other sampling sites. S3 sediments recorded the highest amounts of K^{+} and Na^{+} therefore the microorganisms present probably adopted the salt in strategy to survive the salt stress. The salt-tolerant yeast *Debaryomyces hansenii* for example is known to maintain a relatively high internal sodium concentration to cope with salt stress (Prista *et al.*, 1997; (Prista *et al.*, 2005). In addition, sediments in parts of the lake are considered to have high nutrient levels because of the continuous erosion of the surroundings which eventually are deposited as silts (Blomqvist *et al.*, 2004). Soda lakes have different chemical conditions rendering them as unique habitats that encourage microbial element cycling. Autotrophs also benefits from high soluble carbonates or from the highly concentrated inorganic carbon (Paul and Mormile, 2018; Paul *et al.*, 2016). Moreover, occurrence of less toxic free sulfide that lacks the hydrogen sulfide ions and chemically stable polymeric sulfur (polysulfides) makes soda lakes a zone for microbial nutrient cycling. Volatile non ionized forms ammonium compounds also adds to the nitrogen cycling (Sorokin *et al.*, 2015a).

5.5 Physiological characteristics

Different fungi recovered from the lake are able to thrive well in wide range of physiological conditions. Most of these fungi are known to live in other different environments including non-extreme habitats. The genus *Aspergillus* and *Penicillium* for example are known to be ubiquitous within the environment, other fungal genera are

animal parasites as well as plant pathogens. Occurrence of such fungi in the soda environment indicates their continuous adaptations to tolerate the hypersaline stress conditions. A similar phenomenon has been observed in the two 'salt loving' fungi, *Wallemia ichthyophaga* and *Hortaea werneckii*. Exposure of such fungi to high osmolarity makes them to activate pathways synthesizing glycerol and osmoregulatory solutes (Petelenz-Kurdziel *et al.*, 2011; Kis-Papo *et al*., 2001).

Soda lakes provides a unique and permanent haloalkaline ecosystem. The complex effects of physiological parameters (pH, salt concentration and temperature) are also vital in shaping the composition of fungal species within Lake Magadi (Table 4.3). Majority of the fungi isolated from Lake Magadi are alkaliphiles since the pH of the lake had a range of pH 9-10. Other diversity studies on yeasts and filamentous fungi for example, in muds from hypersaline alkaline lakes of Wadi El-Natrun also indicated a mean pH 9.21 to favor growth of alkaliphiles (Moubasher *et al.*, 2018). It was established from the pH range that isolates recovered from lake Magadi are alkaliphiles and not acidophiles because of the minimal fungal growth at pH4. This is similar to the work of (Neifar *et al*., 2015) on the study of alkaliphiles in Saharan Salt Flat in Southern Tunisia. Abundant fungal growth between pH8 to pH10 is a pH factor that classifies the fungi as alkaliphiles. Saline fungi are therefore classified as true alkaliphiles (Seckbach and Oren, 2000). The signal pathways for pH are activated by fungal alkaliphiles to enable them cope with external pH extreme (Calcagno-Pizarelli *et al*., 2007).

It is however interesting that there are moderately halophilic fungal species which grow well in salt concentrations of up to 5%. In addition, halotolerant strains which grew well in up to 20% salts were also reported from the lake. The specific species that were recovered from 20% salts included *Aniptodera chesapeakensis, Phoma sp*. and *Aspergillus sp*. Their ability to tolerate salt stress is a unique characteristic for such species especially in biotechnology. There was no specific temperature requirement for most of the fungi recovered from Lake Magadi because good growth was observed to range between 25–45°C. Most halophilic microorganisms are known to grow optimally between 25–45°C (Oren, 2016). The temporal difference in growth of the isolates recovered from Lake Magadi may have been attributed to sediments and soil siltation intensity. It is known

that sediments are quite stable and have no exposure to alternating heat from various physiological saline activities (Zahran, 1997). It is evident from this study that some of the fungal isolates recovered are polyextremophiles because they are able to colonize environments having more than one extreme conditions (Dhakar and Pandey, 2016). In the current studies, such polyextremophiles have attracted great attention among researchers because of their possible applications in biotechnology and also in aspects relevant to ecological studies (Dhakar and Pandey, 2016).

5.6 Production of enzymes by fungi isolated from Lake Magadi

Complex sources of nitrogen, phosphorus and carbon when present in soda lakes controls the enzymatic activity in halophiles (Demain, 2014). It is the enzymatic pathways in fungi that lead to secondary metabolite production (Pfeifer and Khosla, 2001). In this study, a number of the fungal isolates indicated their ability to produce enzymes capable of degrading different complex compounds. The enzymatic activity was low in the producer isolates and this could have been due to the nutrient cycling and pH ranges in the lake. Enzymatic activity of specific fungal isolates indicated their ability to produce at least more than one enzyme type. The ability of the isolates to produce proteases, amylase, lipases, esterase cellulases and chitinases makes them important extremophiles for applications in detergent and food industries (Sheridan, 2004). Chitinase was produced by several isolates. Chitinolytic enzymes are important biological control processes because they have antagonistic activity against agricultural pests which have chitin in their cuticle. The activity of the fungi on lipids may have been influenced by bicarbonate and sodium ions (Greichus *et al*., 1978). Lipolytic activity involve acidic reactions so enhance cleaving of complex bonds (McCoy *et al.*, 2002). Amylase enzyme activity was also low and might have been regulated by the elements phosphorus, carbon, and nitrogen cycling by lake microbiota (Sutton, 1996). Microorganisms that are involved in enzyme production are of great importance because of high production capability and at low cost. Such fungi are also susceptible to gene manipulation. Actually, enzymes that are obtained from microbes are of interest in biotechnology for example in food processing, detergent manufacturing, medical therapy, molecular biology and pharmaceutical products (Pilnik and Rombouts, 1985). Reports on various enzymes produced by fungi from marine origin are present in literature, they are involved in industrial production of: proteases, have been

used to produce anti-inflammatory and digestive drugs (Zhang and Kim, 2010); lipases, medicine components (clinical reagents) and cosmetics (Zhang and Kim, 2010; Murray *et al*., 2013). Improved detergent products in laundry have also been produced by alkali-stable cellulases, proteases and lipases (Horikoshi, 2007).

5.7 Phylogenetic diversity of fungi isolated from Lake Magadi

Phylogenetic diversity of Lake Magadi composed of fungal communities having various dominant taxa in addition to species exhibiting spatial and temporal variations at low frequencies. Further illustrations by the tree analysis and topology indicated species diversity in the soda lake. The observed relatives from the gene bank have been identified to inhabit soil and aquatic environments, in both fresh and saline habitats. Most phylogenetic studies involved in screening for environmental biodiversity have focused on 18S rRNA gene as an important marker for random target PCR (Meyer *et al*., 2010). In addition, it is easy to access rDNA gene sequences because they have highly conserved flanking regions that enables the use of universal primers (Meyer *et al.*, 2010). The findings on phylogenetic analysis are similar to the results from Salano *et al.* (2017) who found the phylum Ascomycota as the dominant fungal group from Lake Magadi. Members of the same phylum were also found in large proportion in Tundra soils after sequencing 125 cloned fungi (Schadt *et al.*, 2003). Fungal communities from other hypersaline environments studied by Santini, *et al* (2015) indicated that phylum Ascomycota was dominant with a score of 73% of the total OTUs. In contrary, reports from other hypersaline environments indicated that phylum Basidiomycota was the dominant fungal group from deep-sea environments (Singh *et al*., 2011; Bass *et al*., 2007).

The different fungal genera recovered in this study were *Aspergillus, Penicillium, Cladosporium, Phoma,* and *Acremonium*. Most of these genera have been recovered saline habitats, *Aspergillus* for example was found to be the abundant species in the sediments of Lake Magadi and few species of *Phaeosphaeria* (Kambura, 2016). Hypersaline waters of salterns have also been previously studied and *Penicillium* and *Aspergillus* species were present in diverse levels (Gunde-Cimerman *et al.*, 2005). Several strains of genus *Cladosporium* have also been isolated from Caspian Sea waters (Sadati *et al*., 2015). Moreover, a similar study by (Salano,2011) indicated the presence of genera *Aspergillus,*

Penicillium, Cladosporium, Talaromyces, and *Acremonium* from Lake Magadi. The presence of dominant species from different fungal genera is a suggestion that they are highly adaptable to the extreme conditions of the soda lake.

It is interesting to report the presence of new cultivable diversity of fungi thriving in the sediments and soils of Lake Magadi previously not reported that include: *Septoriella leuchtmannii, Phoma sp., Zasmidium cellare, Chaetomium globosum, Aniptodera chesapeakensis, Pyrenochaeta nobilis, Septoria senecionis, Paradendryphiella arenariae, Sarocladium kiliense, Juncaceicola alpina* and *Biatriospora carollii.* There are no reports on the occurrence of such fungi in Lake Magadi, however, they have close affiliation to known terrestrial fungi. Fungus *Chaetomium globosum* which has 99 percentage similarity with known species is mesophilic group of molds. The saprophytic fungus lives in soil, plants and dung. As an endophyte, *C. globosum* is important in cellulose decomposition found in plant debris. Research done on Dead Sea, saline habitats of Wadi El-Natrun, Egypt however was able to isolate *Chaetomium globosum* (Perl *et al*., 2018). An halotolerant isolate with 99% affliation to *Sarocladium kiliense* is known to survive up to 30 % NaCl has been recovered from hypersaline lakes of Wadi-El-Natrun, Egypt (Assiut and Mohamed, 2017). Ndwigah *et al.* (2017) isolated a fungal strain from saline Lake Sonachi, Kenya that had 100% alignment with *Sarocladium kiliense* (HQ232198). Grum-grzhimaylo *et al*. (2016) also recovered the same fungus in soda soil. Fungi in the genus *Biatriospora* consists of endophytic species of angiosperms that thrive in tropical, temperate and also marine ecosystem (Kolařík *et al*., 2017). There are limited reports on the recovery of *Biatriospora spp*. from saline environments however it is known to be a potential producer of potent antibiotics and diverse set of metabolites (Kolařík *et al*., 2017). The isolate affiliated to *Paradendryphiella arenariae* with 99% identity is a marine fungus and has been isolated from Thailand, investigations have indicated that it produces bioactive secondary metabolites (Yoiprommarat *et al.*, 2015). Isolates with similarity levels to known species for example *Septoriella leuchtmannii, Zasmidium cellare, Aniptodera chesapeakensis, Pyrenochaeta nobilis, Septoria senecionis* and *Juncaceicola alpine* are common causes of human and plant diseases. There are no reports on the occurrence of these terrestrial species in other saline environments. Their recovery from Lake Magadi implies that surface run off and erosion of the nearby vegetation probably

introduced the terrestrial species into the lake. Such terrestrial species thus device strategies to survive the extreme saline conditions.

5.8 Antimicrobial activity of fungal isolates from Lake Magadi

Fungal diversity from the Kenya soda lakes is an indication of availability diverse fungal metabolites. Antimicrobial activity noted for isolate 11M affiliated to *Penicillium chrysogenum* CBS 306.48 (99%) is of interest because it produces active agents against both human pathogenic bacteria and plant pathogenic fungi. Such antimicrobial activity can be initiated by known fungal secondary metabolites, phenols which are among the largest group. Phenolic compounds are composed of bromide, halogens and iodine occurring in large numbers in soda lakes and or marine environment (Rashid, 2012). Such compounds for example bromophenol are known to exhibit antimicrobial activity (Matanic and Castilla, 2004). A few polyketide compounds which are alkaloids have also been identified as strong antiviral and antimicrobial agents (Santos-Gandelman *et al*., 2014)(F Santos-Gandelman et al., 2014). Bioassay-guided fractionation has been used to extract these alkaloids from marine sponge and fungi (Laport *et al*., 2009; Santos-Gandelman, *et al.,* 2014). Fungal isolates from soda lakes have also been identified as producers of acetylenic fatty acids derivatives (Ndwigah *et al.*, 2017)(F I Ndwigah et al., 2017). Acetylenic fatty acids from calcareous marine sponge showed antimicrobial activity against *Escherichia coli* and *Staphylococcus aureus* (Carballeira, 2008). Other fungal secondary metabolites for example Flopropione, β-thujaplicin, α-thujaplicin, and γ-thujaplicin, Thujaplicins are known to be potent anti-bacterial and anti-fungal and agents (Chedgy *et al*., 2009; Ndwigah *et al*., 2015).

5.9 Production media for antimicrobial activity

Many of the laboratories in search for new natural products employ standard culturing procedures for all microbial strains. Microbial culturing in the laboratory is therefore considered a preliminary step towards the entire process of natural product discovery. Growth nutrients and environment has to be manipulated so that there can be substantial and quantitative impacts on the diversity of secondary metabolites produced by microorganisms (Xu *et al*., 2008; Shang, 2012; Kossuga *et al*., 2012). The three production media used in this study PM3, YPSS and YESD had different composition of

nutrients and elements. This can possibly explain why PM3 media was the best as it gave out extracts that showed good inhibition zones (antimicrobial activity). The output and success of natural product screening is enhanced with the optimization of the initial culturing step. Studies have shown that secondary metabolites production is affected by growth media conditions (Bode *et al.* 2002; Miao *et al*., 2006). The use of modified production medium in this case PM3, YPSS and YESD is an important consideration in the search for antimicrobial metabolites from haloalkaliphilic fungi. Incubating conditions and growth media have an impact on the production of secondary metabolite. Often, culture conditions are varied in order to optimize the yielding of specific compounds. For example, active metabolites production by medicine/drug fungus (Xu *et al*., 2008; Pu *et al*., 2013). A lot of factors including nitrogen sources, carbon sources, phosphate levels, physiological parameters (pH, temperature, NaCl levels), incubating time intervals and trace elements are involved in secondary metabolism (Betina, 1994). It is these regulatory factors that balances yielding and biosynthesis of antimicrobial agents. Adaptation of microorganisms to extreme environments determines their ability to produce bioactive metabolites (Anuhya *et al*., 2017). Extremophiles possess cell proteins that enhances their ability to adapt to extreme conditions making them functionally stable at conditions required to produce metabolites (Garcia-Moreno, 2009). To respond to the effects of external pH, such microorganisms have a system that switch on and off, at molecular level.

5.10 Antimicrobial activity and biological control potential

Findings from this study elaborates on how fungi from extreme soda lake environments are capable of producing more antimicrobial agents. Extracts from the one active isolate 11M affiliated to *Penicillium chrysogenum* CBS 306.48 showed different zones of inhibition on different test organisms. This may be attributed to the varying concentration of antimicrobial compounds present in both cell free and crude extracts. The inhibition zones from isolate 11M was averagely similar to zones obtained when positive controls were used except for zones observed against a few test pathogens (see plate 4.3-plate 4.6). Studies done elsewhere by Rodrigues *et al*. (2005) also found that extracts from *Guignardia* species was active against other fungi, enteric pathogens *Staphylococcus aureus* and *Escherichia coli*. Moreover, similar results have also been obtained by Castillo-Machalskis *et al*. (2007). In their study, analysis of extracts from *Penicillium*

citrinum and *Aspergillus ochraceus* showed different antibacterial spectra against *Escherichia coli* and *Pseudomonas aeruginosa*. Antimicrobial agents (β-lactam antibiotics) from *Penicillium chrysogenum* and other *Penicillium species* are known to be active against most of the Gram positive pathogenic bacteria (Salo, 2016). Penicillins discovered as the first β-lactam antibiotics have been in use therapeutically and commercially important for about six decades. The β-lactam antibiotics have been use to control Gram-positive bacteria for example *Staphylococcus* and *Streptococcus* strains that cause human diseases. Penicillin antibiotics bind to DD-transpeptidase enzyme that crosslinks the different bundles of peptidoglycan bundles in the cell wall. Sorbicillactone A isolated from *Penicillium spp.* has shown anti-HIV properties with low toxicity but is strongly cytotoxic against L5178y leukemic cells (Bringmann *et al*., 2005). Remarkable pharmaceutical properties of sorbicillactone A is encouraging the improvement of large scale culture techniques of *P. chrysogenum* (Bringmann *et al.,* 2007). *Penicillium chrysogenum* CBS 306 strain recovered in this study proved to be active against Gram negative, Gram positive, *Candida albicans* and also inhibited various plant pathogenic fungi (*Schizophyllum commune, Epicoccum sorghinum* strain JME-11, *Aspergillus fumigatus* strain EG11-4, *Cladosporium halotolerans* CBS 119416, *Phoma destructive* and *Didymella glomerata*). Similar findings on the same strain of *Penicillium* has not been reported in previous studies.

Biological control potential is realized from several fungi from Lake Magadi that produce chitinases. The enzyme chitinase is associated with degradation of insect cuticles making such fungi important for use in pest management. Fungal virulence is aided by the action of a complex chitinases and proteases in conjunction with multifactorial insect host/pathogen association phenomenon (Abdelaziz *et al*., 2018). A combination of hydrolytic enzymes mainly lipase, protease and chitinase facilitate penetration of fungal mycelia through barriers present in insect integument (Ali *et al*., 2010). Phytopathogenic efficiency of secondary metabolites (alkaline extracellular protease) from *Verticillium alfalfa, Cladosporium cladosporioides* and *Beauveria bassiana* were studied on locust (*Schisto-cerca gregaria*) and all the fungal species showed substantial toxic effects (Abdelaziz *et al*., 2018). Approximately 171 commercially prepared products based on

entomopathogenic fungi are already in use and further development of more products is still in progress (Mora *et al*., 2016).

Filamentous fungus *Penicillium chrysogenum* is known to be the major β-lactam antibiotic (penicillin) producer. Recently, because of the wide use of penicillin, programs that involves classical strain improvement have led to high β-lactam producing strains. Penicillin compounds consists of molecules that carries β-lactam ring nucleus in their chemical structure. Antibiotics with β-lactam ring are active against many Gram-positive bacteria as they inhibit the biosynthesis of the bacterial cell wall. Different bioactive metabolites have been isolated from *Penicillium chrysogenum* cultured in broth in the last few decades. The genes involved in the biosynthesis of such compounds have been identified. After *Penicillium chrysogenum* was discovered as the producer of first antibiotic penicillin, different studies have been done on antimicrobial metabolite production from this *Penicillium* species. The fungus *Penicillium chrysogenum* is known to thrive in diverse environments such as soil, air, spoiled fruits and in extreme conditions in oceans, soda lakes and Antarctic ice core (Foreman *et al.*, 2011; Henk *et al*., 2011). Some of the secondary metabolites like penicillins have been known to co-purify together (Cram and Tishler, 1948). On the other hand, Sorbicillins which are part of the largest group of polyketides are also produced by *P. chrysogenum.* Their purification and analysis have been done in detail (Salo, 2016). Sorrentanone which is also produced by *P. chrysogenum* and is a sobilicin derivative has been shown to have moderate antimicrobial activity against Enterococcus species and *Staphylococcus spp.* (Miller and Huang, 1995). Halophilic and halophilic species of *Penicillium* are known to be producers of various polyketides including penicillic acids, antibiotics (penicillins) and amino acid derived extrolites (Frisvad, 2005). In particular, different strains of *Penicillium chrysogenum* have been isolated from saline environments (Nayak *et al*., 2012; Gunde-cimerman and Zalar, 2014).

Further purification and identification of the specific metabolites produced by *Penicillium chrysogenum* CBS 306 strain will be useful in agricultural and pharmaceutical sys-tems especially when the active agents are formulated into products. Immediate remedy for test microorganisms used in this study including *Salmonella typhimurium*, *Candida albicans*,

Staphylococcus pneumoniae and the plant pathogens *Epicoccum sorghinum, Alternaria tenuissima, Didymella glomerata, Aspergillus fumigatus, Schizophyllum commune* may be obtained from isolate 11M affiliated to *Penicillium chrysogenum*. Reports on the bioactive compounds inhibiting the above pathogens isolated from fungal endophytes *Spondias mombin* exists (Rodrigues *et al*., 2005). Other known antimicrobial producer endophytes such as *Phomopsis species, Pestalotiopsis guepinii* and *Guignardia* species (Wikee *et al*., 2011). Exploration of extreme environments therefore enhances the isolation of new strains of producer fungi mainly with the modification of growth and production medium. This will ignite industrial improvement programs which work the development of high metabolite producing strains.

5.11 Conclusion

The results of this study show that there are diverse fungal groups existing in extreme environments particularly in saline ecosystems which are yet to be identified. These groups are closely related to terrestrial fungi and may have originated from the surrounding agricultural/vegetation soils. They are carried by surface run offs and deposited into various sediments in the lake either in form of spores or fungal hyphae. Such fungi develop effective strategies to cope with the extreme conditions of salts, alkaline pH and temperatures. The study has shown the capability of halophilic fungi to produce different exo-enzymes for example lipases, proteases, cellulases, pectinases among others. Exploitation of such enzymes is important in biotechnology especially for future applications in industries. In addition, fungi from the soda lake showed antimicrobial activity against human pathogenic bacteria (enterococci), human pathogenic fungi (C*andida albicans*) and plant pathogenic fungi. This may be due to production of bioactive compounds by halophilic fungi from the soda lake provides an additional ecosystem that boost the search for biotechnological tools important in proteomics. The ability of a soda lake fungi to produce active metabolites that inhibits important human pathogens for example *Escherichia coli, Candida albicans, Salmonella typhimurium* and *Staphylococcus aureus*, is a crucial step in disease infection mitigation process. The discovery of fungi that are common causes of plant and animal diseases from the soda lake will ignite more research on their genome structures so as to determine their toxin pathways.

5.12 Recommendations

- Appropriate strategies to control different human and plant pathogens can be developed by purification of antimicrobial extracts from the active fungi isolated from Lake Magadi.
- Findings from this study should be a motivation that open further research on bioprospecting for antimicrobial compounds and fungal enzymes from other soda lakes or extreme environments.
- Further studies on fungal metabolite production potential will be of interest because of their ability to adapt to extreme saline environments.
- Screening and identification of bioactive metabolites from isolate 11M and its further analysis for potential formulation of antibiotic or biological control product.

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