AN ASPERGILLUS ISOLATE AND ITS SECONDARY METABOLITES FROM LAKE ELMENTAITA IN KENYA

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Abstract
The soda lakes provide a high biodiversity of halophilic and alkaliphilic fungi. The fungus in this study was isolated from sediments collected from Lake Elmentaita in Kenya. The effect of pH and sodium chloride on growth was done on Malt Extract Agar (MEA) and Potato Dextrose Agar (PDA) media. Fermentation of fungal isolate in 500 ml conical flasks was done in fourteen days' duration on a shaker. Extraction of crude fermentation solution was done using the solvents, Ethyl acetate and hexane in the ratio 2:1 respectively. Molecular characterization of the fungus was done using the18s rDNA Gas-chromatography-Mass spectrophotometry (GC-MS) analysis revealed seventeen secondary metabolites produced by the fungus. The fungus grew well at alkaline pH of 9 to12 and a temperature range of 26°C to 30°C. Growth was only observed on 0% to 10% sodium chloride. The culture-dependent assays and GC-MS analysis showed that the fungus produces bioactive compounds that can be applied in biotechnology research. Moreover, molecular analysis grouped the isolate as a close relative to the genus *Aspergillus.*

Key words: Aspergillus, alkaliphilic, halophilic, secondary metabolites, and growth

1.0 Introduction

1.1 Background

The alkaline saline, soda lakes of Kenyan Rift valley include Bogoria, Elmentaita, Magadi, Nakuru, Natron and Sonachi (formerly Naivasha Crater Lake). Their development is a consequence of geological and topological factors (Mwatha, 1991).

Soda Lakes are formed by unusual combination of environmental factors, which result in large amount of sodium carbonate and high concentration of Calcium and Magnesium ions. These elements are insoluble as carbonate minerals under alkaline conditions. The pH of the lakes range from 8 to12 (Grant and Mwatha, 1989, Jones *et al*., 1994), while the salinity of these lakes ranges from around 5% total salts (W/V) in lake Bogoria, Nakuru, Elmentaita and Sonachi, but saturated in Lake Magadi and Natron with roughly equal proportions of Na_2CO_3 and NaCl as major salt components (Mwatha, 1991).

The soda lakes also exhibit active volcanism with numerous hot springs on the shores of some of the lakes (Wilson and Brimble, 2009). Microorganisms from such regions have to endure extreme environmental conditions in terms of pH, salinity and temperature in the hot springs.
The soda lakes of East Africa provide an extremophilic environment.

Microorganisms that survive such extremely harsh environment have been reported to be probable source of novel secondary metabolites of biotechnological applications (Satyanarayana *et al.,* 2005). Moreover, some of the secondary metabolites are potential antimicrobial agents (Satyanarayana *et al.,* 2005; Brakhage and Schroeckh, 2011). Such microorganisms have adapted to alkaline saline conditions and relatively high temperatures (Brakhage and Schroeckh, 2011). These conditions can facilitate change and modification of specific fungal pathways to produce different metabolites, which may have antimicrobial activity.
Extremophilic microbes are adapted to high temperatures and are called thermophiles, acidophiles are adapted to low pH and alkaliphiles are adapted to conditions of high pH, however some microorganisms occur in one or more environmental conditions. They are called polyextremophiles (Tan *et al.,* 2008; Chela-Flores, 2013; Capece, 2013). An example is the archeabacterial species, *Sulfolobus acidocalderius*, which can survive at temperatures exceeding 80^{o}C and at acidities of pH less than 3 (Tan *et al.,* 2008). However, halophiles can survive in high salt concentration environments (Horikoshi, 1998), a characteristic of the Kenyan soda lakes. The major aim of this study was to isolate and characterize fungi from Lake Elementaita, a saline lake within the Rift Valley, Kenya. However, only one isolate was considered in this study.

1.2 Halophilic Fungi

Halophilic and halotolerant fungi use polyols such as glycerol, erythritol, arabitol, and mannitol as osmotic solutes and retain low salt concentrations in their cytoplasm. Molecular studies on osmotic adaptation of *Hortaea werneckii* and *Wallemia ichthyophaga* have been presented (Vaupotič *at al.,* 2007). An in-depth understanding has been obtained of the high osmolarity glycerol (HOG) pathway, and this understanding may be applied in the future to the development of improved salt-resistant crops. Glycerol-3-phosphate dehydrogenase is involved in glycerol synthesis by both *Wallemia* and *Hortaea.* (Gunde-Cimerman *et al.,* 2009; Lenassi *et al.,* 2011)

Most of the fungi that can be found in extreme environments belong to the imperfect stage of the Ascomycota, which inhabit mangroves, saline soils, marine sediments, sea water, salt marshes, and sand dunes (Hyde and Pointing, 2000). Function of fungi in these extreme conditions is still unclear due to limited information on hyper-saline environments (Gunde-Cimerman *et al.,* 2009).

Diversity of fungal fauna has been discovered in saline (15–32 %) environments (Takishita *et al.,* 2007), where only bacteria were known to colonize (Foti *et al.,* 2007). These fungi were first isolated in hypersaline waters of Secovlje salterns in Slovenia (Gunde-Cimerman *et al*., 2000). The majority of species isolated belonged to melanized meristematic and yeast-like fungi, and a few different genera of filamentous fungi were also identified (Méjanelle *et al.,* 2001).

Fungal metabolism results to essential or non-essential by-products called secondary metabolites (Brakhage and Schroeckh, 2011). The metabolites constitute a wide array of natural products (Brakhage and Schroeckh, 2011). They are derived from the primary products, such as amino acids or nucleotides through modifications, such as: methylation, hydroxylation, and glycosylation (Bentley and Bennet, 1988). Fungi have been surpassed by only actinomycetales as a source of biologically active metabolites (Brakhage and Schroeckh, 2011). The fungal biodiversity on land seems to be nearly exhausted, thus, nowadays; researchers throughout the world have paid increasing attention towards marine microorganisms as an alternative source for isolation of novel metabolites (Brakhage and Schroeckh, 2011).

Approximately, 3000 to 4000 known fungal secondary metabolites have been isolated (Brakhage and Schroeckh, 2011). However, not more than 5000 to 7000 taxonomic species have been studied in this respect (Brakhage and Schroeckh, 2011). *Aspergillus, Penicillium, Fusarium,* and *Acremonium* are among fungal genera capable of producing diverse secondary metabolites (Wilson and Brimble, 2009).

Acetyl-CoA is the most common precursor of fungal secondary metabolites, leading to the production of polyketides, terpenes, steroids, and other metabolites derived from fatty acids (Wilson and Brimble, 2009). Other secondary metabolites are derived from intermediates of the shikimic acid pathway, the tricarboxylic acid cycle, and from amino acids (Wilson and Brimble, 2009).

Current research in drug discovery from medicinal higher fungi, involves a multifaceted approach combining mycological, biochemical, pharmacological, metabolic, biosynthetic and molecular techniques. In recent years, many new secondary metabolites from higher fungi have been isolated and are more likely to provide novel compounds for new drug discovery (Brakhage and Schroeckh, 2011). The compounds may include chemo-preventive agents possessing the bioactivity of immune modulatory systems, anticancer, and other relevant compounds (Wilson and Brimble, 2009; Brakhage and Schroeckh, 2011). Conversely, the success of their productions is greatly challenged by various factors like bioseparation, identification, biosynthetic metabolism, and screening model issues (Zhong and Xiao. 2009).

2.0 Materials and Method

2.1 Study Site

Sediment samples were collected from Lake Elmentaita in the Kenyan rift valley (figure 1) at elevation 1774 m above sea level and position 37 19 54 73 E and 99 47 486 N. The lake is located in a basin whose water budget is maintained by recharge from hot springs located on the southern lakeshore, two in-flowing rivers, surface run off, direct rainfall and evapo-transpiration. The lake has no surface outlet or underground seepage for releasing its water to other aquifers (Mwaura, 1999).

2.2 Sampling

Samples were collected at different sites and then pooled together. The pool of samples was transported to the laboratory for fungal isolation and downstream analysis. Malt Extract Agar (MEA) and Potato Dextrose Agar media were used to isolate fungi from the sediment sample and the establishment of pure cultures from which morphological studies were conducted on the isolate. Effect of pH and Sodium Chloride (NaCl) concentration on growth was done by measuring the radial growth of the isolate in triplicate on malt extract medium in a petridish.

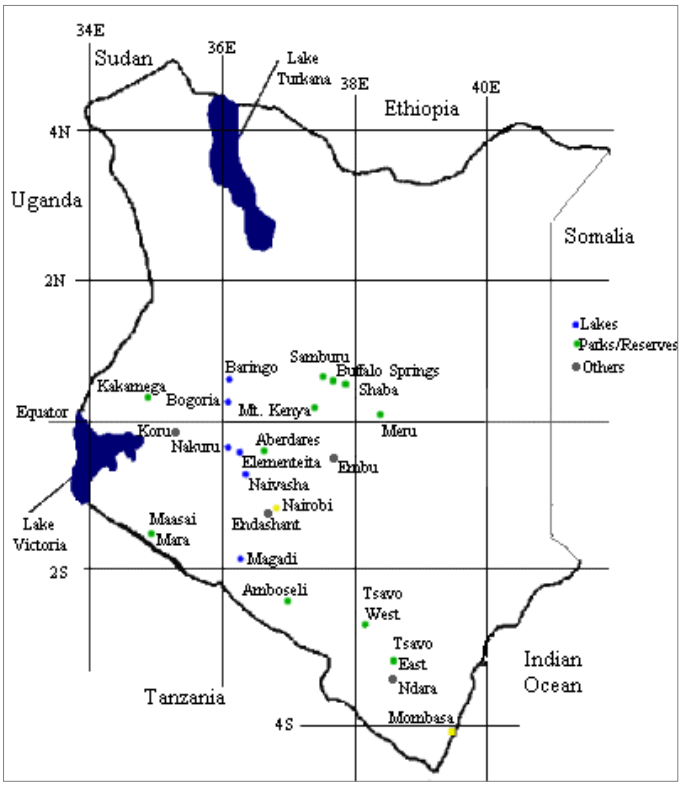

Figure 1: Study site Lake Elmentaita

2.3 Morphological and Physiological Studies

Morphological studies were conducted based on the anamorphic features of the isolate, whereas physiological analysis was based on the effect of NaCl concentration and pH. The effect of pH on culture growth was analyzed by measuring the radial growth of the isolate on MEA in millimeter.

2.4 DNA Extraction

DNA was extracted using the bead beater machine method and two lyses buffers as solution A (50mM Tris pH 8.5, 50mM EDTA pH 8.0 and 25 % sucrose solution) and solution B (10mM Tris pH 8.5, 5mM EDTA pH 8.0 and 1 % SDS). Total genomic DNA of the isolate was extracted from the cells in duplicate using two lysis buffers. The cells were scrapped aseptically using a sterile surgical blade taking care not to pick the media. These were put in separate tubes containing 100 µl solution A and beads. The bead beater machine protocol was used to remove the fungal cell wall. This was followed by addition of 30 µl of 20 mg/l Lysozyme and 15 µl of RNase, gently mixed and incubated at 37^{o}C for two hours to lyse the cell wall. Six hundred µl of Solution B was then added and gently mixed by inverting the tubes severally, followed by the addition of 10 µl of Proteinase K (20 mg/l) and the mixture incubated at 60^{o}C for 1 hour. The mixture was centrifuged at 10,000 g (13,000 rpm) for 15 minutes. The supernatant was discarded and DNA pellets rinsed with 200 µl 70% alcohol three times while discarding the supernatant. DNA pellets were dried in a dryer at 45^{o}C for 20 minutes. Dried pellets were re-suspended in 100 µl TE buffer. The presence of DNA was checked using 1% agarose in 1 x TAE buffer and visualized under ultraviolet after staining with ethidium bromide. The DNA was stored at -20 ^{o}C for further use (Sambrook *et al.,* 1989).

Total DNA from the isolate was used as a template for amplification of the 18S rDNA gene. Fungal primer pair Fung5 forward 5'-GTAAAAGTCCTGGTTCCCC-3' and Fung5390r reverse, 5'-CGATAACGA ACGAGA CCT-3' (Vainio and Hantula, 2000; Lueders *et al*., 2004) were used.

Amplification was performed using Peqlab primus 96 PCR machine. Amplification was carried out in a 40 µl mixture (5 µl of PCR buffer (×10), 3 µl dNTP's (2.5mM), 1 µl (5 pmol) of Fung5f forward primer, 1µl (5pmol) of FF390r reverse primer, 0.3 µl taq polymerase, 1.5 µl of the DNA template and 28.2 µl of water). The control contained all the above except the DNA template. Reaction mixtures were subjected to 36 cycles: Initial activation of the enzyme at 96^{o}C for five minutes, denaturation at 95^{o}C for 45 seconds, primer annealing at 48^{o}C for 45 seconds, chain extension at 72^{o}C for 1.30 minutes and a final extension at 72^{o}C for 5 minutes. Amplification product (5 µl) was separated on a 1 % agarose gel in 1× TBE buffer and visualized under ultraviolet after staining with ethidium bromide. PCR products of the isolate were purified using the QIAquick PCR purification Kit protocol (Qiagen, Germany) and then sent for sequencing at ILRI.

2.5 Fermentation of Fungi in Liquid Medium

The fungal isolate was grown in liquid medium composed of 15 g malt extract, 5 g Bacteriological peptone, 5 g Glucose, 2% NaCl in 1litre of distilled sterile water at pH 8.5. Two thousand five hundred milliliters of the sterile medium was dispensed into sterile 500 ml conical flask. The flask was inoculated with a four millimeter agar disc cut from two days fungal isolate culture and incubated at 28^{o}C on a shaker (1000 rpm) for fourteen days. The crude filtrate was recovered from the fungal isolate by filtration using muslin cloth. This filtrate was subjected to ethyl acetate/hexane extraction (ratio 2:1) three times. The precipitate was eluted with 1ml ethyl acetate. The solution was stored at 3°C awaiting Gas Chromatography-Mass Spectrophotometry (GC-MS) One ml of eluted solution was used for metabolite analysis.

3.0 Results

The morphology of the fungal isolate showed conidial heads with grey colour, septate hyphae and phialidic chains of conidia whish appeared green in colour

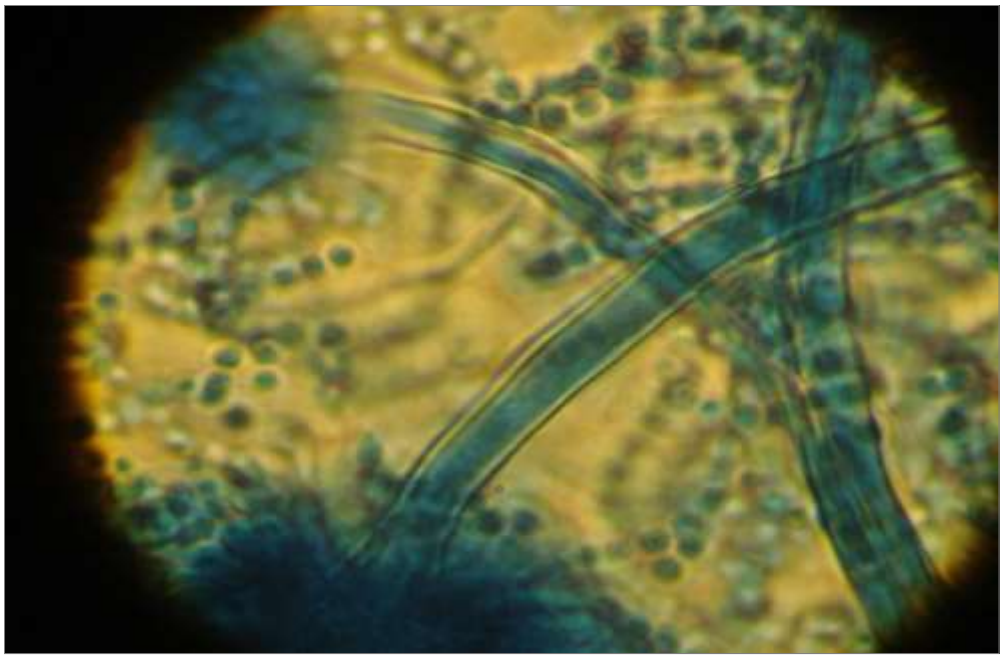

Figure 2: Conidial heads, Conidiophores and chains of conidia.

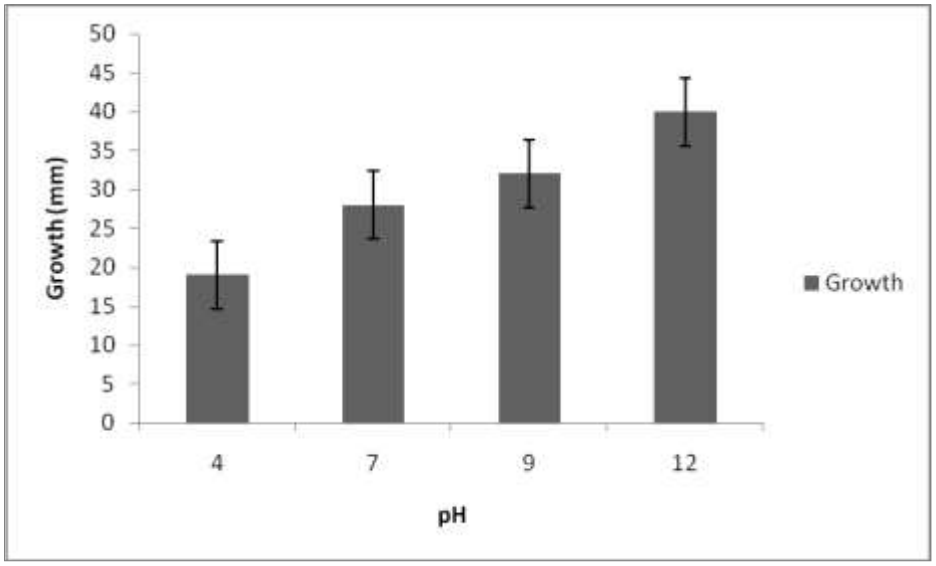

Figure 3: Effect of pH on the growth of isolate at 28°C for eight days

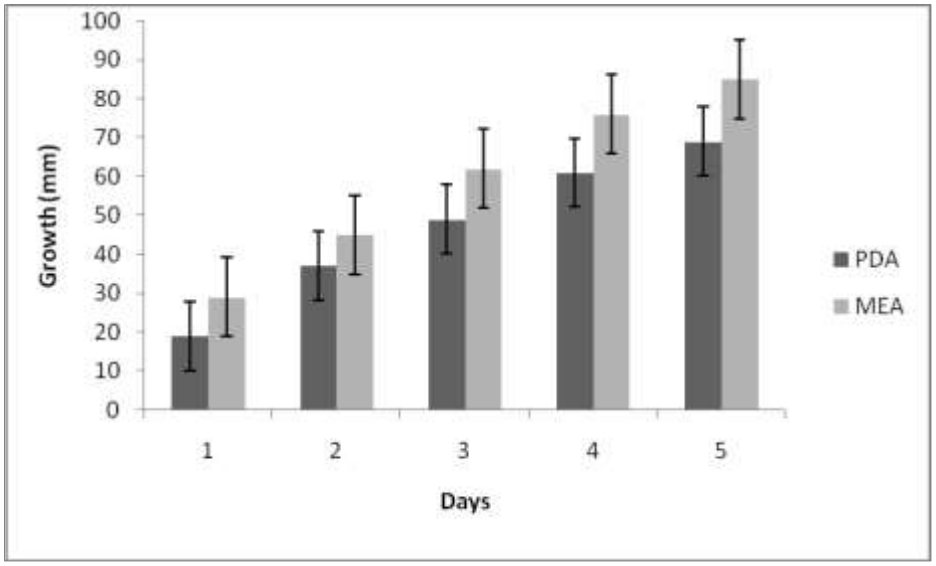

Figure 4: Fungal growth patterns on Malt Extract Agar (MEA) and Potato Dextrose Agar (PDA).

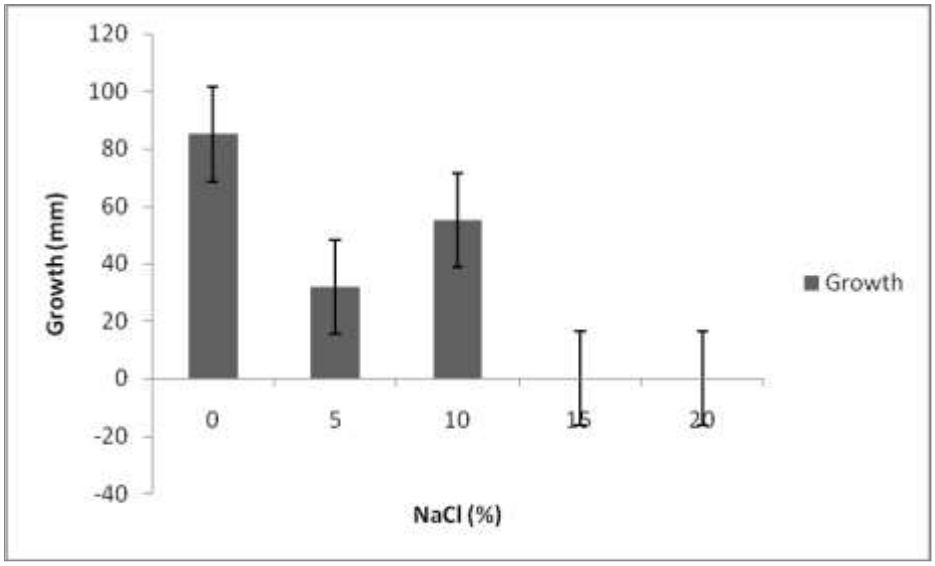

Figure 5: *The effect of NaCl concentration on the growth of the isolate on MEA*

3.1 Molecular Characterization

The 18s rDNA gene sequence for the isolate

TTTTACTGTGAAAAAATTAGAGTGTTCAAAGCAGGCCTTTGCTCGAATACATTAGCATGG
AATAATAGAATAGGACGTGCGGTTCTATTTTGTTGGTTTCTAGGACCGCCGTAATGATTA
ATAGGGATAGTCGGGGGCGTCAGTATTCAGCTGTCAGAGGTGAAATTCTTGGATTTGCTG
AAGACTAACTACTGCGAAAGCATTCGCCAAGGATGTTTTCATTAATCAGGGAACGAAAGT
TAGGGGATCGAAGACGATCAGATACCGTCGTAGTCTTAACCATAAACTATGCCGACTAGG
GATCGGGCGGTGTTTCTATGATGACCCGCTCGGCACCTTACGAGAAATCAAAGTTTTTGG
GTTCTGGGGGGAGTATGGTCGCAAGGCTGAAACTTAAAGAAATTGACGGAAGGGCACC
AC
AAGGCGTGGAGCCTGCGGCTTAATTTGACTCAACACGGGGAAACTCACCAGGTCCAGAC
A
AAATAAGGATTGACAGATTGAGAGCTCTTTCTTGATCTTTGGATGGTGGTGCATGGC

Figure 6: Nucleotide sequence of 18Sr DNA gene of the fungus.

Blast results showing the closest neighbors

FUNGUS	ACCESSION	CLASSIFICATION	% SIMILARITY
Aspergillus sp	KC120773	Ascomycota; Pezizomycotina; Eurotiomycetes;	99
Aspergillus terreus strain	JQ812052	Ascomycota; Pezizomycotina; Eurotiomycetes	99
Aspergillus fumigatus strain	HQ871898	Ascomycota; Pezizomycotina; Eurotiomycetes	99
Aspergillus flavus isolate	JF824683	Ascomycota; Pezizomycotina; Eurotiomycetes	99

Table 1: Closest neighbors and percentage similarity of isolated fungus to the haplotypes from the public data banks (NCBI)

3.2 Secondary Metabolites from the Isolate

Graph of mass spectrophotometer was used in compound identification (fig 7). A total of seventeen different compounds were identified in the fungal extract of the isolate (Table 2). The concentration of compounds for this isolate ranged between 0.1- 3.9%. Isopentyl alcohol (3.9%) was the most abundant compound followed by Cresol<meta-> (3.3%) and Propanoic acid, 2-methyl-(2.5%). The least two in abundance were 2,3-Butanediol (0.2%) and N-(1-Cyclopenten-1-yl)-morpholine (0.1%).

Peak no.	Rt (min)	Metabolite	% area
1	3.905	2-Butanone, 3-hydroxy-	0.940
2	4.699	Isopentyl alcohol	3.909
3	5.192	1,8-Nonadien-3-ol	0.197
4	5.617	Propanoic acid	0.540
5	7.611	2,3-Butanediol	0.178
6	8.820	Propanoic acid, 2-methyl-	2.508
7	9.335	Isovaleric acid	1.439
8	11.390	Trimethyl benzene<1,2,4->	1.015
9	12.426	Cyclopentene<3,5-dimethylene-1,4,4-trimethyl->	0.731
10	12.538	Cymene<ortho->	0.510
11	13.613	Pentanoic acid, 2-hydroxy-4-methyl-,	0.728
12	14.979	2-Coumaranone	0.647
13	18.272	4H-Pyran-4-one, 5-hydroxy-2-	1.875

		(hydroxymethyl)-	
14	19.772	Benzaldehyde, 2-hydroxy-5-methoxy-	0.614
15	19.862	N-(1-Cyclopenten-1-yl)-morpholine	0.147
16	22.751	Flopropione	2.463
17	28.082	Cresol<meta->	3.348

Table 2: Metabolite Profile for the isolate. (Rt=Retention time)

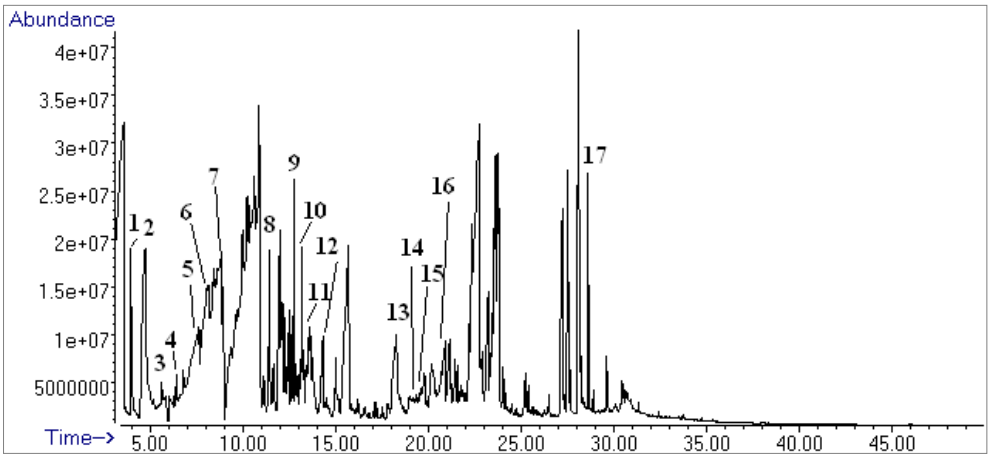

Figure 7: Mass spectrum for the isolate showing the seventeen metabolites.

4.0 Discussion

From a pool of sample a single culture was isolated, characterized and screened for production of secondary metabolites. Morphologically, the isolate had informative anamorphic structures. The conidial heads and chain of conidia from the sterigmata clearly indicated that the isolate is in the genus *Aspergillus*.

The isolate grew best on MEA at pH9 and lower growth on PDA at the same pH. Malt Extract was the most favoured medium for the isolate growth. The isolate grew well at 5% NaCl, on MEA at pH9. Growth of the isolate increased gradually with increasing pH but shot up at pH10 and 12 indicating that the isolate is well adapted for the alkaline environment. This growth at pH range of 5 to10 is in consistent with an earlier study by (Horikoshi, 1998) which showed that a low to high pH range of 5.7 to 9.0, favours growth of alkaliphiles and that a pH range 9.0 to 10 may serve as their selective optimum pH.

The effect of NaCl concentration showed that the isolate grew well at the range of 0-10% NaCl. An increase in salt concentration resulted in a drastic decrease in growth, which was arrested at 20% NaCl. Thus the fungal isolate thrives in low saline condition. Results have been shown elsewhere that fungi are not only able

to sustain, but also to propagate at different environmental extremes such as hyper-saline waters (Gunde-Cimerman *et al.,* 2000).

Molecular characterization showed that the isolate belongs to the phylum *Ascomycota*, sub phylum *Pezizomycotina*, and class *Eurotiomycetes* It was closely aligned to the haplotypes *Aspergillus sp*. (KC120773), *Aspergillus terreus* (JQ812052), *A.fumgatus* (HQ871898), and *A.flavus* (JF824683) all with 99% similarity. The morphological characteristics also indicated that the isolate belongs to the genus *Aspergillus.*

GC-MS analysis identified seventeen different metabolites from the extract of the isolate. The compounds are in the chemical groups of acid, alcohols, ketones, aldehydes and heterocyclic compounds among others, such chemical groups of compounds are known to have antimicrobial activity. Floropione extracted from roots of *Piper chaba* has been found to be active against Grm-negative and Gram-positive bacteria (Tarannum *et al.,* 2008). Faty acids such propionic acid, isovaleric acid and butyric acid extracted from plants have shown antimicrobial activity against Gram-negative oral bacteria (Huang *et al,.*2011). 2, 3-Butanediol extracted from the plant *Michalia champaca* has shown antimicrobial and anticancer properties (Lee *et al*., 2011). These compounds have been identified in the extract through GC-MS among others from the fungal isolate, an indication that some of the metabolites are kwon bioactive compounds.

Conclusions and Recommendations

The fugal isolate from the soda Lake Elmentaita belongs to the genus *Aspergillus* in the phylum *Ascomycota*. The fungus grew well at alkaline range of pH 9 to12 and at salt concentration of 0 to 5% on MEA medium. Results suggest that the isolate has high potential of producing metabolites that may be useful in biotechnological application. The isolate has high potential of producing a range of metabolites which can be explored for future application in industries and biotechnology.

There is need for extensive studies on soda lakes fungi and their secondary metabolites with an objective of finding Novel species and compounds of biotechnological applications.

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