



## Research article

## Isolation of haloalkaliphilic fungi from Lake Magadi in Kenya

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## ABSTRACT

In this study we explored the cultivable fungal diversity in Lake Magadi and their secondary metabolite production. Isolation was done on alkaline media (Potato dextrose agar, Malt extract agar, Oatmeal agar and Sabouraud dextrose agar). A total of 52 unique isolates were recovered from the lake and were characterized using different techniques. Growth was observed at pH, temperature and salinity ranges of between 6 - 10, 25 °C - 40 °C and 0%–20% respectively. Phylogenetically, the isolates were affiliated to 18 different genera with *Aspergillus*, *Penicillium*, *Cladosporium*, *Phoma* and *Acremonium* being dominant. A screen for the ability to produce extracellular enzymes showed that different isolates could produce proteases, chitinases, cellulases, amylases, pectinases and lipases. Production of antimicrobial metabolites was noted for isolate 11M affiliated to *Penicillium chrysogenum* (99%). Cell free extracts and crude extracts from this isolate had inhibitory effects on *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella spp.*, *Shigella spp.*, *Candida albicans* and fungal plant pathogens *Schizophyllum commune*, *Epicoccum sorghinum* strain JME-11, *Aspergillus fumigatus* strain EG11-4, *Cladosporium halotolerans* CBS 119416, *Phoma destructive* and *Didymella glomerata*). In this study we showed that different cultivation strategies can lead to recovery of more phylotypes from the extreme environments. Growth under different physiological characteristics typical of the soda lake environment (elevated temperature, pH and salts) confirmed the haloalkaliphilic nature of the fungal isolates. The use of suitable antimicrobial production media can also lead to discovery of more phylotypes producing diverse biocatalysts and bioactive metabolites.

## 1. Introduction

Fungi are eukaryotic organisms having either simple unicellular or multicellular cell structures. Within the environment, fungi are mainly found in soil and rock surfaces compared to other aquatic habitats. Most fungal species inhabit soil compared to other environments as they are organotrophs forming the major group involved in the breakdown of organic compounds including decaying matter and plant material (Hasan, 2015). The occurrence of fungi in water is subtle with only around 3,000 species known to be associated with aquatic habitats, whereas about 465 species are present in marine waters (Shearer et al., 2007). Diverse fungal groups have been documented from different extreme environments such as saline liquids, hot springs, surface of dried rocks, ocean pits, dry deserts, and very low pH as well as in the polar environments (Hassan et al., 2016). Filamentous fungi harbour alkalitolerant lineages similar to those of marine habitats (Grum-grzhimaylo et al., 2016). Most fungi that inhabit extreme environments are categorized as the imperfect stage of the Ascomycota (Ndwigah et al., 2016).

Different genera, including *Cladosporium*, *Aspergillus*, *Penicillium*, *Alternaria* and *Acremonium* have been reported to exist as either moderately or weakly alkali tolerant species in saline environments (Grum-Grzhimaylo et al., 2013). However, salinity directly affects fungal growth and sporulation. For example, at salinities above 5%, there is increased sporulation and more chlamydozoospores are formed while conidiogenesis is inhibited, and there are fewer hyphae (Mulder and El-Hendawy, 1999; Mandeel, 2006). High ambient salts and pH significantly stress the living organisms and, therefore, their overall biodiversity may be affected (Grum-grzhimaylo et al., 2016). To cope with the osmotic stress, fungi in extreme environments produce extremolytes and extremozymes (Rad-dadi et al., 2018). In cases of high osmolarity, fungi are able to counteract loss of water by the accumulation of K<sup>+</sup> ions into their cells (Plemenitaš et al., 2014) while others accumulate osmolytes (polysols, sugars and amino acids) as compatible organic solutes (Roberts, 2005).

Fungi from extreme environments such as the soda lakes have adapted to elevated temperatures and alkaline saline conditions, which may lead to evolution and modifications of various fungal pathways

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(Brakhage and Schroeckh, 2011). A lot of secondary metabolites including antimicrobial agents (antibiotics), pigments and toxins are produced from the modified fungal and actinomycetes pathways (Satyanarayana et al., 2005; Brakhage and Schroeckh, 2011; Liao et al.,

2015). Fungal genera that are known to produce secondary metabolites include *Fusarium*, *Aspergillus*, *Acremonium* and *Penicillium* (Wilson and Brimble, 2009). Studies on the physiology and genetics of fungi isolated from unusual habitats are important to foster the understanding of both

**Table 1.** Growth characteristics of different isolates under varied physiological conditions (temperature, pH and NaCl).

Isolate	Salt tolerance				Temperature tolerance				pH tolerance				
	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).

the ecological roles and potential industrial applications (Prakash and Sharma, 2016). Due to the few numbers of fungal isolates described from the soda lake ecosystems, their diversity, function and potential to produce secondary metabolites is still not well studied. In this study, we used different media and cultivation conditions in an effort to recover novel phylotypes with the potential to produce bioactive metabolites.

## 2. Results

### 2.1. Characterization of the isolates

A total of 52 isolates were obtained with most of them being from sampling site S3 (wet sediments). The other sites (S3 Dry sediments, S2 wet sediments, S5 wet sediments, microbial mats, grassland soil, and brine sediments) had a few isolates. After plating, growth of the isolates on the respective media was monitored and recorded after a period of between 7-21 days of incubation at 30 °C. Spore formation was observed after 14 days of growth for the spore-farmers. Malt extract agar, Sabourauds dextrose agar, potato dextrose agar and oatmeal agar supported growth of most of the fungi both in high and low nutrient composition. The isolates showed varied macroscopic and microscopic characteristics in terms of colony pigmentation, surface morphology and the hyphae under the microscope. The hyphae were either septate (hyaline) or aseptate in others.

### 2.2. Physiological characteristics

Moderate growth was observed in all the isolates at NaCl concentration of between 0% and 5%. When the salt concentration was increased to 10%, most of the isolates showed slow growth with isolate 9M, 10M, 11M, 24M, 32M, 36M, 40M, 64M, 82M and 100M growing the same as in the lower concentration. However, isolate 5M, 30M, 39M, 59M and 108M showed optimum growth at 10% while isolate 2M, 14M, 29M, 38M, 56M, 60M, 69M did not show any visible colonies on plates media (no growth). The highest salt concentration tested was 20% and only 13 isolates showed growth albeit slow while the rest did not grow (Table 1). Notable is that isolates 1M, 24M, 39M, 64M, 65M, 100M, 120M and 122M were able to grow across all the tested salt concentrations (Table 1). Different isolates showed varied growth patterns under different pH ranges. Except for pH 6 where quite a number of the 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 (Table 1).

It was observed that the optimum growth temperature for most of the isolates was 30 °C. At 25 °C, slow growth was observed in all isolates except isolate 38M which did not grow at all. When the temperature was raised to 40 °C, isolates M5, M14 and M122 exhibited fastest growth while four isolates (32M, 38M, 70M and 94M) did not grow at all at this temperature.

**Table 2.** Summary of enzymatic activity of some of the fungal isolates. Only isolates positive for at least one substrate are shown.

Isolate	Site	Sta.	CMC	Cas.	Pec.	Xan.	Lig.	T20	Gt.	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, **Gt**- Glyceryl Tributyrate, **Chit**-chitin, **Cel**-cellulose.

### 2.3. Screening for enzymatic activity

All the isolates were screened for their ability to produce extracellular proteases, cellulases, pectinases, chitinases, amylases, xylanases, lipases and esterases by growing them on a medium with the respective substrate. Production of cellulases was observed by positive enzymatic activity on cellulose and carboxymethylcellulose in 9 and 8 isolates respectively (Table 2). Utilization of the substrate was scored as positive if there was a halo around the colony after flooding the plate with iodine solution. Eight isolates were positive for amylase production. Positive protease activity was observed in 8 of the isolates by way of a clearance zone around colony on casein agar. Pectinase and chitinase production was observed after degradation of substrates pectin and chitin by 8 and 22 isolates respectively. However, 22 isolates did not show any enzymatic activity on any of the substrates. None of the isolates showed peroxidase/laccase activity. Some isolates were able to produce more than one enzyme with a few producing up to four enzymes. Interesting isolates in terms of polyenzymatic activity were 69M, 87M, 90M, 69M, 59M and 2M. Chitinase activity was observed in 22 of the isolates. This enzyme is important as a virulence factor in entomopathogenic fungi. It was observed that most fungi recovered in this study showed low enzymatic activity as indicated by the diameter of clearing zones around the colony. Those with a clearance zone measuring up to 3mm were 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 2).

### 2.4. Phylogenetic analysis

BLAST analysis was used to evaluate the phylogenetic affiliation or relatedness of the individual isolates to nearest neighbors and the results expressed as percentage similarity (Table 3). All the isolates clustered with 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) *Sarcocladium* (2) *Phaeosphaeria* (1) and *Juncaceicola* (1), *Biatrospora* (2). The constructed phylogenetic tree indicated the phylogenetic position of the isolates (Figure 1). The bootstrap values showed that different strains of isolates within a given genus were closely related (Figure 2). Based on bootstrap values, *Zasmidium* and *Aspergillus* are sister groups in the same clade and are therefore closely related. The genera *Juncaceicola*, *Septoriella* and *Phaeosphaeria* fall in the same clade and therefore have common evolutionary history. *Chaetomium*, *Aniptodera*, *Sarcocladium* fall in the same clade with *Acremonium* (commonly known fungal genera). Interestingly, isolate 69M, 87M and 90M had sequence similarity of 96 %, 97% and 95% respectively therefore they could represent novel species. However, isolates 72M, 80M, 95M, 108M, 113M and 100M had sequence similarity values below 94% which means they could potentially represent new genera (Table 3).

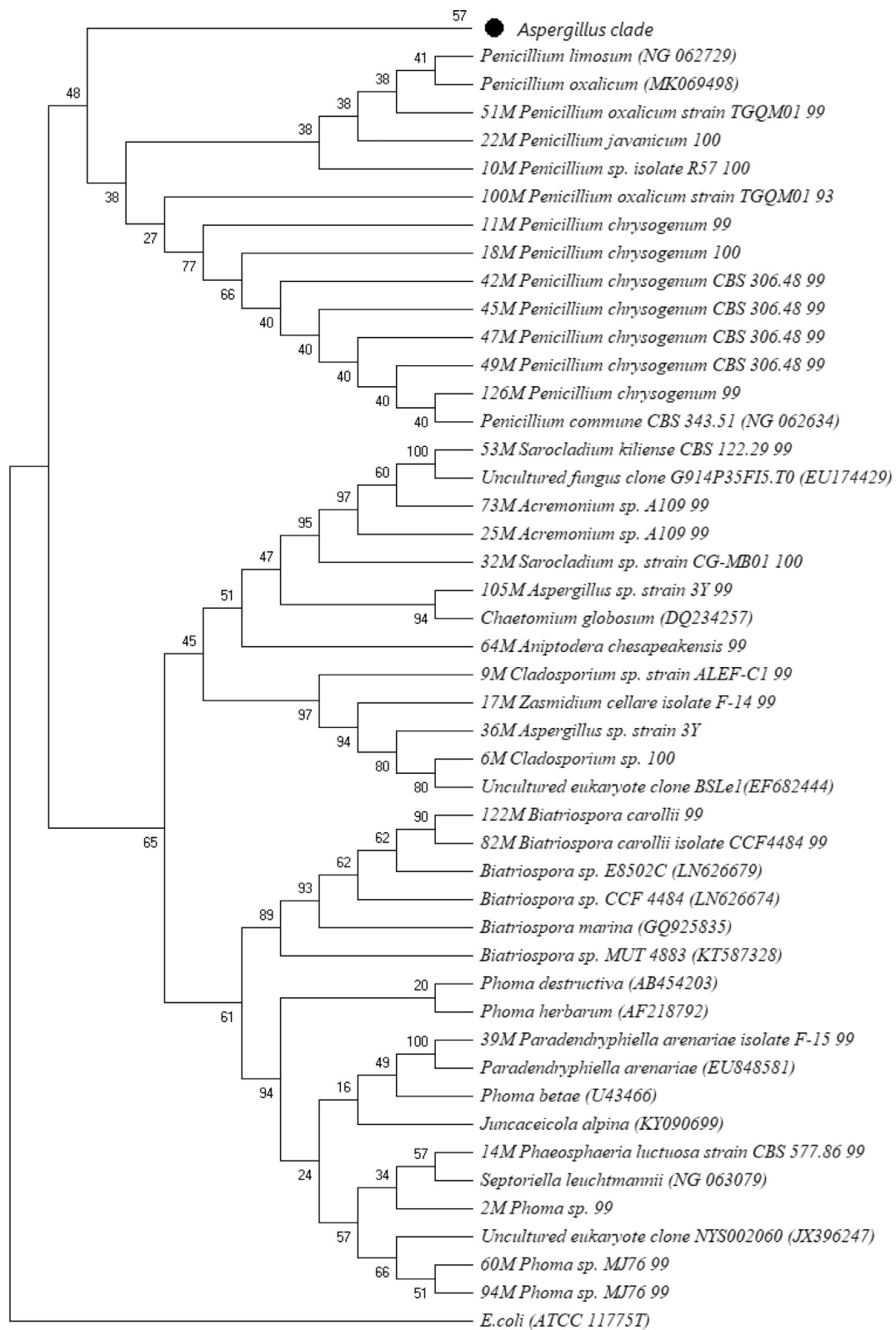
### 2.5. Antimicrobial screening

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

**Table 3.** BLAST analysis results of the fungal isolates from Lake Magadi and their close relative.

Isolate Code	Closest Relative (BLAST)	Identity
1M	<i>Aspergillus sp.</i> strain 3Y	100%
2M	<i>Phoma herbarum</i> strain BZYB-1	99%
5M	<i>Aspergillus fumigatus</i> strain EG11-4	98%
9M	<i>Cladosporium cladosporioides</i>	98%
10M	<i>Penicillium oxalicum</i> strain TGQM01	100%
11M	<i>Penicillium chrysogenum</i> CBS 306.48	99%
13M	<i>Cladosporium halotolerans</i> CBS 119416	99%
14M	<i>Phaeosphaeria luctuosa</i> strain CBS 577.8	99%
15M	<i>Aspergillus fumigatus</i> strain EG11-4	99%
16M	<i>Aspergillus versicolor</i> isolate F	100%
18M	<i>Penicillium commune</i> CBS 343.51	100%
22M	<i>Penicillium limosum</i> CBS 339.97	100%
24M	<i>Pyrenochaeta nobilis</i> CBS 407.76	96%
25M	<i>Acremonium roseolum</i> strain CBS 289.62	99%
29M	<i>Aspergillus versicolor</i> isolate CWJ2	100%
30M	Uncultured eukaryote clone NYS002060	98%
31M	<i>Aspergillus versicolor</i> strain MPE9	99%
32M	<i>Sarcocladium sp.</i> strain CG-MB01	100%
36M	<i>Zasmidium cellare</i> isolate F-14	100%
38M	<i>Chaetomium globosum</i> strain F0909	99%
39M	<i>Paradendryphiella arenariae</i> isolate F-15	99%
40M	<i>Acremonium sclerotigenum</i> CBS 124.42	99%
53M	<i>Sarcocladium kiliense</i> CBS 122.29	99%
56M	<i>Aspergillus keveii</i> strain CBS 209.92 28S	99%
57M	<i>Aspergillus glaucus</i> JCM 1575	98%
58M	<i>Penicillium citrinum</i> strain IITG_KP1	99%
59M	<i>Aspergillus flavipes</i> NRRL 302	98%
60M	<i>Phoma destructiva</i>	99%
64M	<i>Aniptodera chesapeakeensis</i>	99%
65M	<i>Phoma sp.</i> LF617	99%
67M	<i>Juncaceicola alpina</i> CBS 456.84	99%
68M	<i>Septoriella leuchtmanii</i> CBS 459.84	99%
69M	<i>Cladosporium velox</i>	96%
70M	<i>Aspergillus versicolor</i> strain MF557	99%
71M	<i>Aspergillus sp.</i> strain DX4H	99%
72M	<i>Penicillium viridicatum</i> CBS 390.48	81%
73M	<i>Acremonium roseolum</i> strain CBS 289.62	99%
80M	Uncultured fungus clone 42_Wound2L	80%
82M	<i>Biatrospora carollii</i>	99%
87M	<i>Aspergillus keveii</i> strain CBS 209.92 28S	97%
89M	<i>Talaromyces marneffeii</i> strain Tm-HIV	92%
90M	Uncultured eukaryote clone BSLe1	95%
94M	<i>Phoma destructiva</i> isolate: MUCC0064	99%
95M	<i>Aspergillus terreus</i> strain AZM03	84%
100M	<i>Penicillium oxalicum</i> strain TGQM01	93%
108M	<i>Aspergillus flavipes</i> NRRL 302	83%
111M	<i>Aspergillus sp.</i> strain AON1	98%
113M	<i>Talaromyces marneffeii</i> strain Tm-HIV	92%
114M	<i>Penicillium polonicum</i> CBS 222.28	98%
120M	<i>Septoria senecionis</i> CBS 102366	98%
122M	<i>Biatrospora carollii</i> CCF4484	99%
123M	<i>Penicillium citrinum</i> strain IITG_KP1	98%

Both 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 production media (PM3) showed positive results for inhibition against 13 different test pathogens. Production media

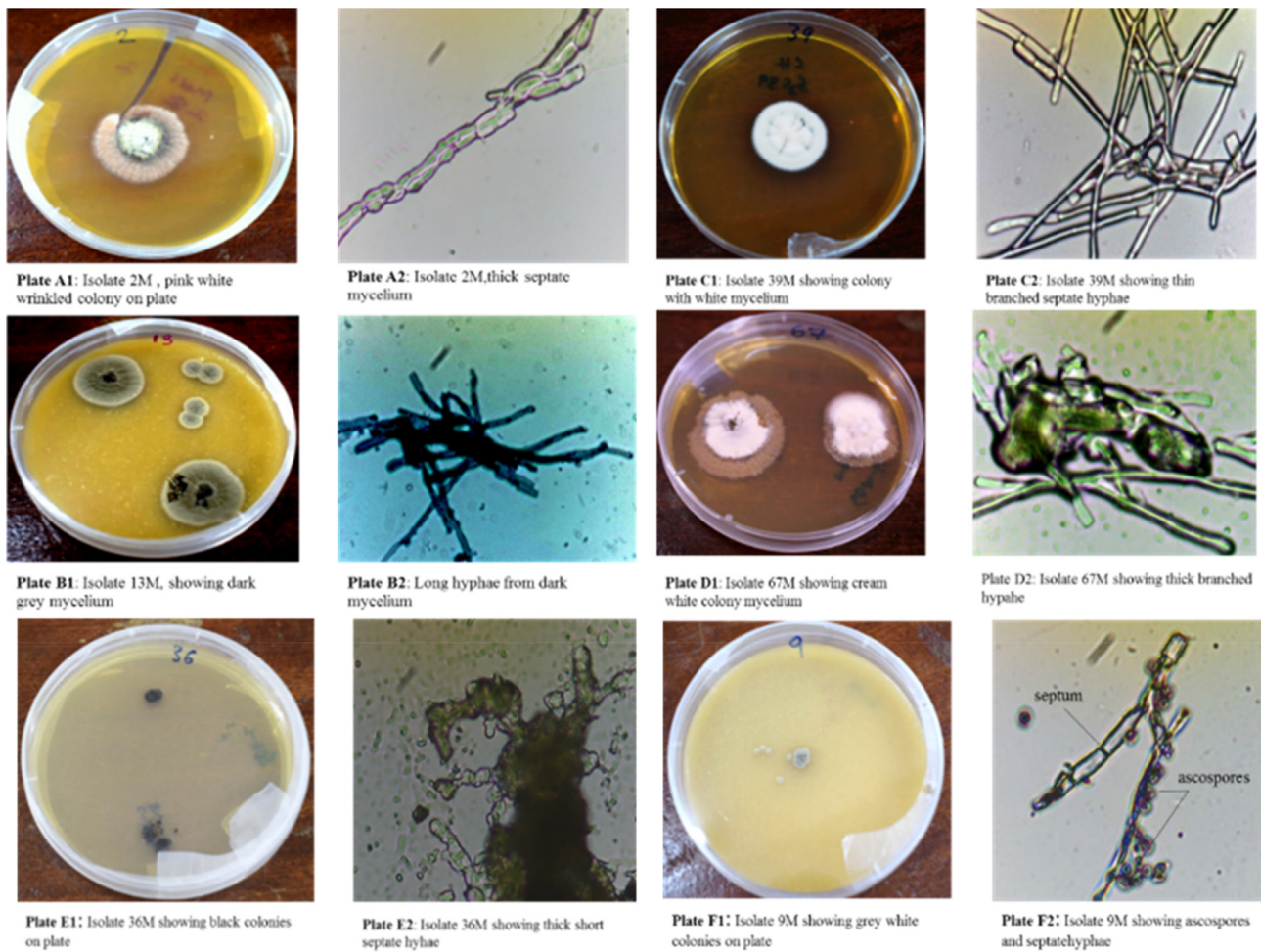


**Figure 1.** Unrooted Phylogenetic tree created using Neighbor-joining method based on a comparison of the 18S ribosomal DNA sequences of Lake Magadi isolates and their closest phylogenetic relatives. Percentages of bootstrap sampling derived from 1000 replications are indicated by the numbers on the tree.

YESD and YPSS however did not give good inhibition results as their extracts showed positive results against few pathogens (*Cladosporium halotolerans*, *Phoma destructiva*, *Dickeya dianthicola*, *Phoma destructiva*, *Schizophyllum commune* isolate ScGD28) pathogens. Crude extracts from PM3 media showed inhibition zones ranging between  $11.33 \pm$

$0.03\text{--}14.33 \pm 0.03$  and were 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. Crude extracts from YESD media showed activity against *Aspergillus fumigatus* strain EG11 ( $422.33 \pm 0.03$ ), *Cladosporium*





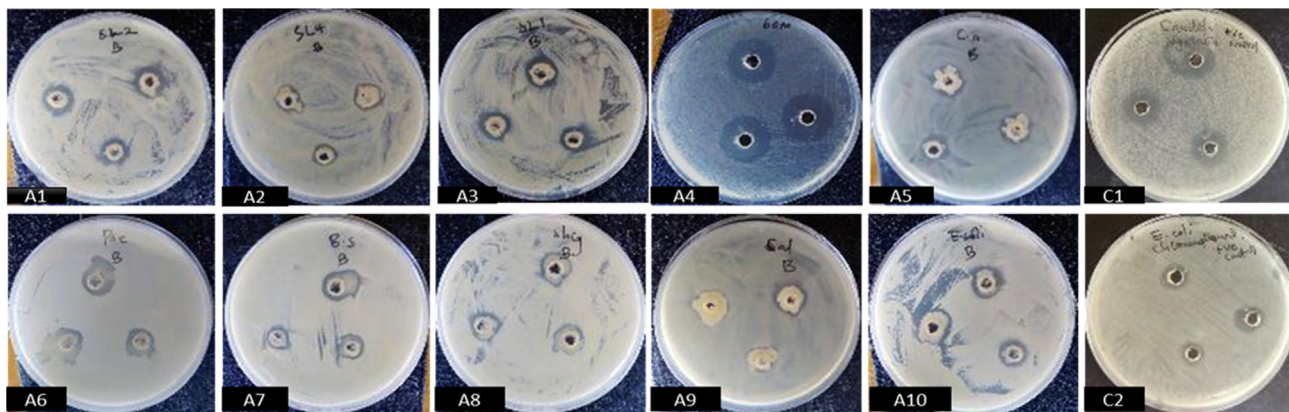
**Figure 2.** A representative sample of the 52 isolates based on colony and cell characteristics (Plate A1: colony on plates) and (Plate A2: under a compound microscope magnification x40).

*halotolerans* CBS 119416 ( $22.33 \pm 0.09$ ), *Phoma destructiva* ( $20.67 \pm 0.03$ ) while cell free extracts had inhibitory effect against *Schizophyllum commune* isolate ScGD28 ( $9.67 + 0.03$ ). Finally, crude extracts from YPSS media and cell free extracts showed inhibitory effects against *Cladosporium halotolerans* CBS 119416 ( $21.67 \pm 0.03$ ) and *Schizophyllum commune* isolate ScGD28 ( $9.33 \pm 0.03$ ) respectively. Positive controls using broad spectrum antimicrobial drugs chloramphenicol and nystatin showed

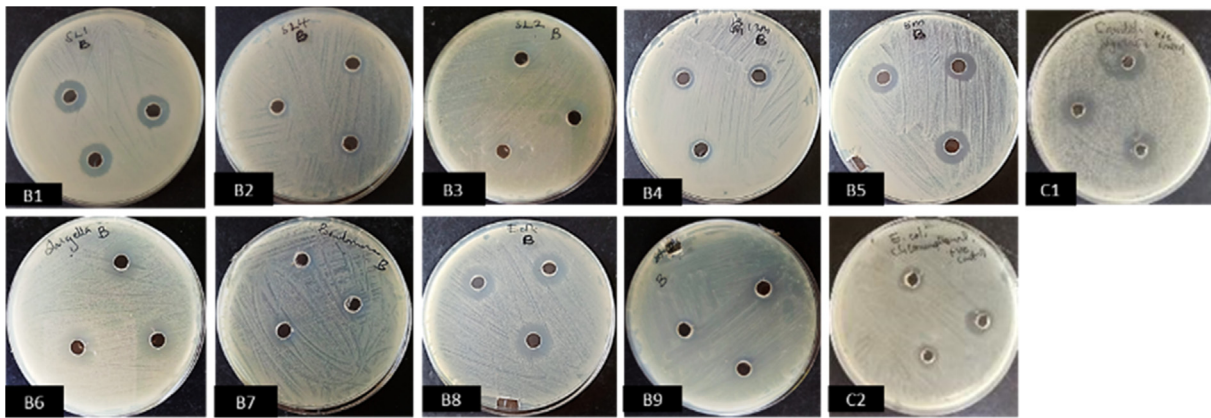
inhibition zones ranging closer to  $13.00 \pm 0.06$  and  $15.00 \pm 0.06$  respectively (see Figures 3 and 4).

### 3. Discussion

The present study focused on the isolation of fungi from Lake Magadi, and new fungal diversity was determined using various identification



**Figure 3.** Antimicrobial activity (inhibition zones) of crude extract from isolate 11M 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.** Antimicrobial activity (inhibition zones) of cell free extract from isolate 11M 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.

techniques. Modification of the culture dependent techniques in combination with molecular analysis using 18S rRNA gene aided in the search for new diversity in the soda lake. Fungal isolation using commercial media prepared both in high and low nutrient composition and diluted with soda lake water is a modification specific to this study. 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. For example, more isolates (14) were obtained using MEA-L than MEA-H whereby only five isolates were recovered. The dominant genera found in this study were present and includes: *Penicillium*, *Acremonium*, *Phoma*, and *Cladosporium*. The same scenario was observed for PDA and oat meal agar. It is therefore clear that nutrient fluctuations within different sites in the lake may have little contribution to the diversity of fungal communities.

Unique morphological characteristics specific to haloalkaliphilic fungi identified in this study was useful in the description of potential new species and genera isolated from Lake Magadi and other soda lakes. Morphologically based characterization was an important parameter in determining various cell features that are specific to the entire fungi kingdom. Specific cell structures for example the nature of hyphae/mycelium justified the occurrence of the isolates in group Ascomycota. Septate hyphae and oval spores (ascospores) is mainly attributed to phylum Ascomycota (Raja and Shearer, 2007). However, unique features including the thick mycelium observed in *Phoma herbarum* strain BZYB-1 –isolate 2M are important in stress tolerance. 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). 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 yeasts to thrive in the harsh environments, such as the surface of stone monuments (Liu et al., 2018). The hyphae produces melanin which brings about the black coloration in the fungi, an important feature to stress survival (Plemenitaš et al., 2008). The black coloration was also observed on most stone monuments, like Liu et al. (2018) reported. *Cladosporium* species for example is known to be a halophilic species dominant in black yeasts (Nazareth, 2014).

Slight differences of the sampling sites in terms of biotic and abiotic factors (pH, salts, moisture, vegetation cover and trace elements) indicated little difference in the fungal communities present. However, the highest level of fungal diversity was found in communities from S3 sediments, the common genera being *Penicillium*, *Acremonium*, *Phoma*, *Cladosporium*, *Zasmidium*, *Aniptodera*, *Pyrenochaeta*, *Septoria*, *Paraden-dryphiella* and *Biatriospora*. This may be attributed to the composition of

various physiochemical parameters especially the pH, Na<sup>+</sup>, K<sup>+</sup> ions and trace elements which differed from the other sampling sites. S3 sediments recorded the highest amounts of K<sup>+</sup> and Na<sup>+</sup> therefore the microorganisms present probably adopted the salt in strategy to survive the salt stress. The salt-tolerant yeast *D. hansenii* for example is known to maintain a relatively high internal sodium concentration to cope with salt stress (Prista et al., 1997, 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).

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 1). It was revealed in this study that the majority of fungi isolated from Lake Magadi are alkaliphiles since the pH of the lake had a range of pH 9–10. 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 chesapeakeensis*, *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 microorganism are known to grow optimally between 25 - 45 °C (Oren, 2016). 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 condition (Dhakar and Pandey, 2016). In these recent studies, such polyextremophiles have attracted great attention because of their possible application to biotechnology and also in aspects relevant to ecology (Dhakar and Pandey, 2016). Enzymatic activity of specific fungal isolates indicated their ability to produce at least more than one enzyme type. Interesting isolates for industrial enzymes included: 2M, 31M, 40M, 59M, 69M, 87M, 90M, 100M and 111M (Table 2). Their ability to produce proteases, amylase, lipases, esterase cellulases and chitinases makes them important extremophiles for industrial applications (Sheridan, 2004).

Lake Magadi has phylogenetic diversity composed of fungal communities with various dominant taxa in addition to species exhibiting spatial and temporal variations at low frequencies. Sequence analysis indicates a total of 18 different genera that were recovered in this study and all belong to the phylum Ascomycota except for 3 isolates which were grouped as unclassified fungi. These findings 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 proportions 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 that were dominant in this study were *Aspergillus*, *Penicillium*, *Cladosporium*, *Phoma*, and *Acremonium*. Most of these genera have been recovered from saline habitats, *Aspergillus* for example was found to be the dominant species in the sediments of Lake Magadi as well as 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. The above genera commonly found together in saline environments share ecological preferences to extreme conditions.

This study reports the presence of new diversity of fungi thriving in the sediments and soils of Lake Magadi which has not been previously reported. New fungal genera and different species have been recovered in this study including: *Septoriella leuchtmanii*, *Phoma* sp., *Zasmidium cellare*, *Chaetomium globosum*, *Aniptodera chesapeakeensis*, *Pyrenochaeta nobilis*, *Septoria senecionis*, *Paradendryphiella arenariae*, *Sarocladium kiliense*, *Juncaceicola alpina* and *Biatrispora carollii*. which has been isolated from other different saline environments (Georgieva et al., 2012; Bonugli-santos et al., 2015). This is the first report on the occurrence of this new genera isolated from Lake Magadi. Studies on Dead Sea, saline habitats of Wadi El-Natron, Egypt however was able to isolate *Chaetomium globosum* (Perl et al., 2018). Ndwigah (2017) isolated a fungal strain from Kenyan saline Lake Sonachi that had 100% alignment with *Sarocladium kiliense* (HQ232198). Grum-grzhimaylo et al. (2016) also recovered the same isolate in soda soil. There are limited reports on the recovery of *Biatrispora* sp. from saline environments however it is known to be a potential producer of potent antibiotics and a diverse set of metabolites (Kolarik et al., 2017). The marine fungus *Paradendryphiella arenariae* is a marine fungus and has been isolated from Thailand; investigations have indicated that it produces bioactive secondary metabolites (Yoiprommarat et al., 2015). *Septoriella leuchtmanii*, *Zasmidium cellare*, *Aniptodera chesapeakeensis*, *Pyrenochaeta nobilis*, *Septoria senecionis*, and *Juncaceicola alpina* are common causes of human and plant diseases.

The diverse groups of fungi present in the lake are 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 the form of spores or fungal hyphae. Such fungi develop effective strategies to cope with the extreme conditions of salts, alkaline pH and temperatures. Further studies on their metabolite production potential will be of interest because of their ability to adapt to extreme saline environments. 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. Appropriate strategies to control such pathogens can thus be developed.

Antimicrobial metabolite production 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. The use of modified production medium, in this case PM3, is an important consideration in the search for antimicrobial metabolites from haloalkaliphilic fungi. Since the discovery of *Penicillium chrysogenum* as the source for the first antibiotic penicillin, various studies on antimicrobial production have been done on various species of *Penicillium*. 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; Cantrell et al., 2006). Antimicrobial agents ( $\beta$ -lactam antibiotics) from *Penicillium chrysogenum* and other *Penicillium* species are known to be active against most of the Gram positive pathogenic bacteria (Salo, 2016). *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. Further purification and identification of the specific metabolites produced by *Penicillium chrysogenum* CBS 306 strain will be useful in agricultural and pharmaceutical systems especially when the active agents are formulated into products. 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 that will enhance activities to develop high metabolite producing strains.

## 4. Materials and methods

### 4.1. Isolation of fungal strains

Soil samples used in this study were collected from the hypersaline Lake Magadi located in the East African Rift valley (2 °S and 36 °E). The lake lies about 660 m above sea level, depth ranging between 1-5 m and covers an area estimated to be 90 km<sup>2</sup> (Behr and Röhrlich, 2000). Serially diluted samples (0.1 g in 1 ml of sterile lake water) were plated onto Potato dextrose agar (PDA), Malt extract agar (MEA), Oatmeal agar and Sabouraud dextrose agar (SDA) with chloramphenicol (100 mg/L) as an antibiotic to inhibit bacterial growth. Onto each medium, 100  $\mu$ l from each dilution was spread plated and the plates incubated at 28 °C until visible colonies appeared. The colonies were picked and transferred several times onto fresh medium until axenic cultures were obtained.

### 4.2. Physiological and biochemical characterization

Morphological features (color, pigmentation and colony surface morphology) together with cellular features (hyphae type) were used to select unique isolates from the different media. Physiological characterization was done to test the ability of the selected isolates to grow at elevated pH ranges (6, 7, 8, 9, and 10), different NaCl concentrations {(w/v) 0%, 5%, 10% and 20%} and varied temperature ranges (25 °C, 30 °C, 35 °C and 40 °C). Growth was scored in terms of the size of the colony after 48 h of growth. Growth was scored depending on the colony size. Ability of the different isolates to produce exoenzymes was tested on a basal medium (Tryptone 10g, Sodium chloride 10g, and Yeast Extract 5g) supplemented with the respective substrate as summarized in Table 4.

### 4.3. Molecular characterization

A sterile loop was used to aseptically scrape the mycelia/spores into 100 $\mu$ l sterilized resuspension buffer (50mM Tris pH 8.5, 50mM EDTA pH 8.0 and 25 % sucrose solution) in an Eppendorf tube. To it was added 400 $\mu$ l of the lysis buffer (10mM Tris pH 8.5, 5mM EDTA pH 8.0 and 1 % SDS), 10 $\mu$ l of Proteinase K (20 mg/l) followed by incubation at 65 °C for 60 min. Phase separation was achieved by addition of an equal volume of chloroform and centrifugation at 13,200 rpm for 10 min at 4 °C. The supernatant was carefully transferred to a new tube and recovered using the standard sodium acetate/isopropanol/ethanol method. An aliquot (3 $\mu$ l) of the recovered DNA was run on 1% agarose to confirm the presence and quality.



**Table 4.** Summary of enzymatic activity using different substrates.

Enzyme	Substrate	Duration	Temp.	Assay	Observation	Reference
Lipases	Tween 20	48 h	28 °C		Halo	Sierra (1957)
Esterase	Glyceryl Tributyrate	48 h	28 °C		Halo	Sierra (1957)
Proteases	Casein	24 h	28 °C		Halo	Vieira (1999)
Cellulases	Cellulose, Carboxymethylcellulose	24 h	28 °C	Congo red	Halo	Stamford et al., 1998
Pectinases	Pectin	24 h	28 °C	Lugol solution	Halo	Andro et al. (1984)
Peeroxidases/Laccases	Lignin	48 h	28 °C		Halo	Bonugli-santos et al., 2015
Amylases	Starch	24 h	28 °C	Lugol solution	Halo	Castro et al. (1993)
Chitinases	4-methylumbelliferylN-acetyl-b-D-glucosaminide solution	30minutes	37 °C	Phospahte buffer solution	Fluorecence under U.V light	(Chand et al., 2008)

The 18S rDNA gene was amplified using the primer pair Fungi 683f (5'-GCTCGTAGTTGAACCTTTGG-3') and Fungi1394r (5'-TCTGGACCTGGTGAGTTTC-3') on a Surecycler 8800 machine (Agilent Technologies). The PCR mix consisted 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 in a final volume of 30µl. Cycling was done as follows:36 cycles. Initial denaturation at 95 °C for 3 min followed by 30 cycles of denaturation at 95 °C for 1 min; Primer annealing at 58 °C for 45 s and extension at 72 °C for 1 min. A final extension step at 72 °C for 5 min was included. An aliquot of 5µl of the PCR products was run on a 1 % agarose gel stained with Cyber green in 1× TAE buffer and visualized under ultraviolet. The PCR products were purified using ExoSAP-IT™ (Applied Biosystems™) as described by the manufacturer. The cleaned amplicons were sent for Sequencing to Inqaba Biotech, South Africa.

#### 4.4. Screening for antimicrobial activity

Fungal isolates were grown for 14 days in broth at 30 °C in a shaker incubator at 100rpm. The crude extracts were tested for antimicrobial activity against Gram positive bacteria *Staphylococcus aureus* and *Bacillus subtilis*, Gram negative bacteria *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella spp.*, *Shigella spp.* and fungal human pathogen *Candida albicans*. to allow for a qualitative selection of bioactive isolates (Arora et al., 2016). The antimicrobial assay was done using the agar well diffusion method (Rajpal et al., 2016) on Muller Hinton agar (pH 8 and 5% NaCl). The fungal isolates were also tested for antagonistic effect against common fungal plant pathogens to determine their potential as biocontrol agents using agar well diffusion method. The test fungal pathogens used were *Epicoccum sorghinum* strain JME-11, *Alternaria tenuissima*, *Didymella glomerata*, *Schizophyllum commune* isolate ScGD28, *Phoma destructive*, *Cladosporium halotolerans* CBS 119416, *Aspergillus fumigatus* EG11-4 isolated from the lake and plant bacterial pathogen *Dickeya dianthicola* obtained from infected vegetable. Isolates that showed positive antimicrobial activity in primary screening were qualitatively selected for secondary screening using agar well diffusion method on Muller Hinton agar. However, for this test, the producer strain was grown on three production media (YESD, PM3 and YPSS, pH 9 and 5% NaCl) in a shaker incubator at 30 °C at 100rpm. Cell free extracts obtained after centrifugation of crude extracts at 10000 rpm for 10 min and crude extracts were used. Inhibition zones were measured as mean diameter of the wells 0.6cm plus the clearing zone in triplicates for every test organism used. Broad-spectrum antibiotic and antifungal (chloramphenicol 20 µg/ml/nystatin 5 µg/ml) were used as positive controls. 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 (190mg/10ml) and manganese chloride (12.58 mg/10ml) and tap water (Jose and Jebakumar, 2013).

**Media C** (YPSS)-g/L Yeast extract 4.0, starch 14.0, dibasic K2HPO4 1.0, MgSO4.7H2O 0.5, tap water (VanderMolen et al., 2013).

#### Declarations

##### Author contribution statement

Philemon Orwa, Romano Mwirichia: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

George Mugambi, Vitalis Wekesa: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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##### Competing interest statement

The authors declare no conflict of interest.

##### Additional information

No additional information is available for this paper.

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