

Chapter 3

Results

3.1 Occurrence of microfungi in soil samples

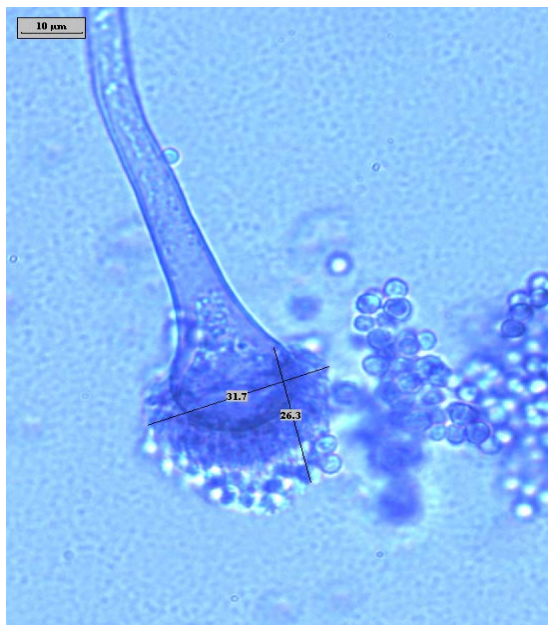
The frequency occurrence of Antarctic fungi in the soil samples from Deception Island, Wilhelmina Bay and Yankee Bay shown in Table 3.1. After soil plate applied, each pure colony was sub-cultured on new plate prior to identification. A total of 27 fungal isolates of Antarctic fungi were isolated from 14 soil samples. Of that, Deception Island harbored 13 fungal species out of 5 soil samples, while Wilhelmina Bay harbored 8 species out of 4 soil samples and 6 species found in Yankee Bay from 5 soil samples. The study recorded *Ascomycetes* sp.4a (60% of 5 samples) and *Geomyces* sp.1 (60% of 5 samples) as most frequently occurred species in Deception Island, Whereas, *Ascomycetes* sp.6a (75% of 4 samples) and *Hyphomycetes* sp.10 (80% of 4 samples) were abundant in Wilhelmina Bay and Yankee Bay respectively. Of all locations, *Ascomycetes* sp.6 and *Ascomycetes* sp.4 were the most frequently occurred species (42.85% of 5 samples). However, The majority of the genera were found in samples from only one collection sites, e.g. fungi of the genus *Mucor* sp.1 was exclusively isolated from one sample of Deception Island. The yellow yeast was only found in one sample of Wilhelmina Bay soil. Whereas few fungi were abundance among the samples of different sites such as *Ascomycetes* sp.4 as recorded in 3 samples of Deception Island and 3 samples from Yankee Bay. However, *Hyphomycetes* sp.10 was recorded from almost every collection within Yankee bay.

Isolated fungi were also categorized according to their thermal characteristics, of 27 isolates, there were 15 psychrophilic species, 9 mesophilic and 3 psychrotrophic species. Isolated antarctic fungi were found to represent three different taxa in which 10 species were Ascomycota, 4 Zygomycota, 12 undetermined filamentous fungi and 1 yeast species. Despite the incubation of volcanic soil of Deception Island in 50°C; no thermophiles fungi was isolated. However, only one fungus was completely identified, based on the microscopic features of the fruiting bodies, (*Aspergillus fumigatus*) and 3 species were partially identified (*Mucor* sp.1, *Mucor* sp.2 and *Geomyces* sp.1) and 23 unidentified species. Figure 3.1a-b showed a light micrographs of some fungal species (filamentous and yeast fungi) microscopic features under the light microscopy.

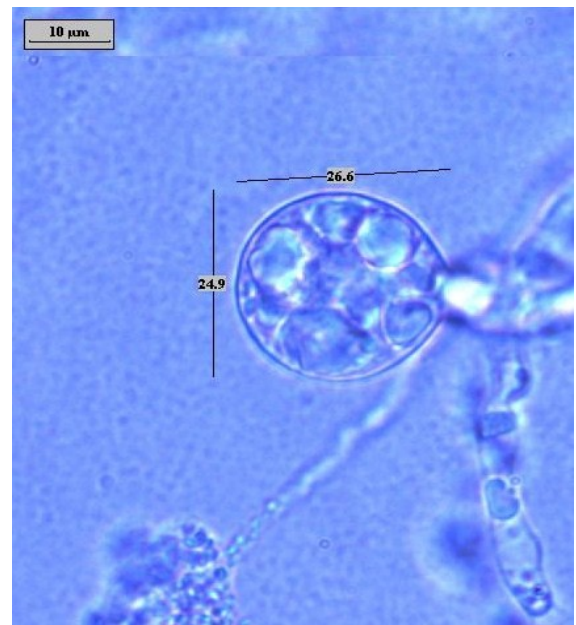
Table 3.1 Percentage of occurrence of successfully isolated fungi from soil of Deception Island, Wilhelmina Bay and Yankee Bay and their optimum growth temperatures.

Sp. No	Fungal isolate	DCP		WHB		YKB		Total		Temp.
		No	%	No	%	No	%	No	%	
1	<i>Aspergillus fumigatus</i>	2	40	0	0	0	0	2	14.28	25°C
2	Unidentified <i>Ascomycetes</i> sp.2	1	20	0	0	0	0	1	7.14	4°C
3	Unidentified <i>Ascomycetes</i> sp.3	2	40	0	0	0	0	1	14.28	25°C
4	<i>Mucor</i> sp.1	1	20	0	0	0	0	1	7.14	25°C & 4°C
5	Undetermined <i>Hyphomycetes</i> sp.1	1	20	0	0	0	0	1	7.14	25°C
6	Unidentified <i>Zygomycetes</i> sp.2	1	20	0	0	0	0	1	7.14	25°C
7	Undetermined <i>Hyphomycetes</i> sp.2	1	20	0	0	0	0	1	7.14	25°C
8	Undetermined <i>Hyphomycetes</i> sp.3	1	20	0	0	0	0	1	7.14	25°C
9	Unidentified <i>Ascomycetes</i> sp.4a	3	60	0	0	3	60	6	42.85	25°C & 4°C
10	Undetermined <i>Hyphomycetes</i> sp.4	1	20	0	0	0	0	1	7.14	4°C
11	Undetermined <i>Hyphomycetes</i> sp.5	1	20	0	0	0	0	1	7.14	4°C
12	<i>Geomyces</i> sp.1	3	60	0	0	0	0	3	21.42	4°C
13	Undetermined <i>Hyphomycetes</i> sp.6	1	20	0	0	0	0	3	7.14	4°C
14	<i>Mucor</i> sp.2	0	0	1	25	0	0	1	7.14	4°C
15	Unidentified yellow yeast	0	0	2	50	0	0	2	14.28	4°C
16	Unidentified <i>Ascomycetes</i> sp.6a	0	0	3	75	3	60	6	42.85	4°C
17	Unidentified <i>Ascomycetes</i> sp.7a	0	0	2	50	1	20	3	21.42	4°C
18	Unidentified <i>Zygomycetes</i> sp.4	0	0	1	25	0	0	1	7.14	25°C
19	Undetermined <i>Hyphomycetes</i> sp.7	0	0	1	25	0	0	1	7.14	4°C
20	Undetermined <i>Hyphomycetes</i> sp.8	0	0	1	25	0	0	1	7.14	25°C
21	Undetermined <i>Hyphomycetes</i> sp.9	0	0	1	25	0	0	1	7.14	4°C
22	Unidentified <i>Ascomycetes</i> sp.4b	3	60	0	0	3	60	6	42.85	25°C
23	Undetermined <i>Hyphomycetes</i> sp.10	0	0	0	0	4	80	4	28.57	25°C & 4°C
24	Undetermined <i>Hyphomycetes</i> sp.11	0	0	0	0	1	20	1	7.14	4°C
25	Unidentified <i>Ascomycetes</i> sp.7b	0	0	2	50	1	20	3	21.42	4°C
26	Unidentified <i>Ascomycetes</i> sp.6b	0	0	3	75	3	60	6	42.28	4°C
27	Undetermined <i>Hyphomycetes</i> sp.12	0	0	0	0	3	60	3	21.42	4°C

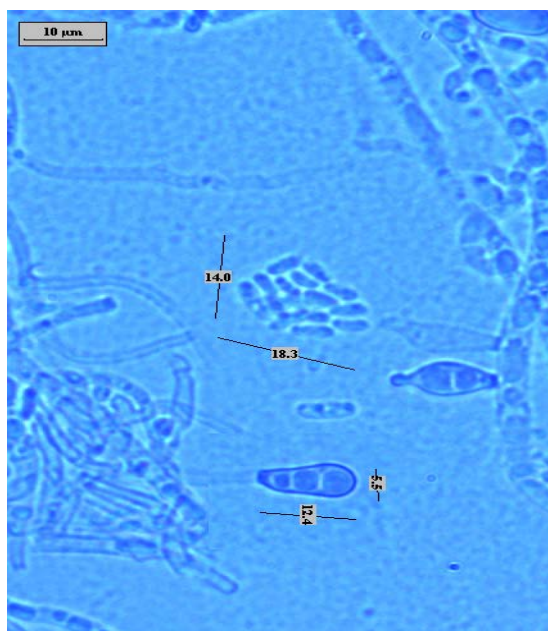
DCP: Deception Island, **WHB:** Wilhelmina Bay, **YKB:** Yankee Bay, **Temp.:** Optimum growth temperature, **No.:** Number of soil samples colonized by the fungi, **%:** Percentage colonization.



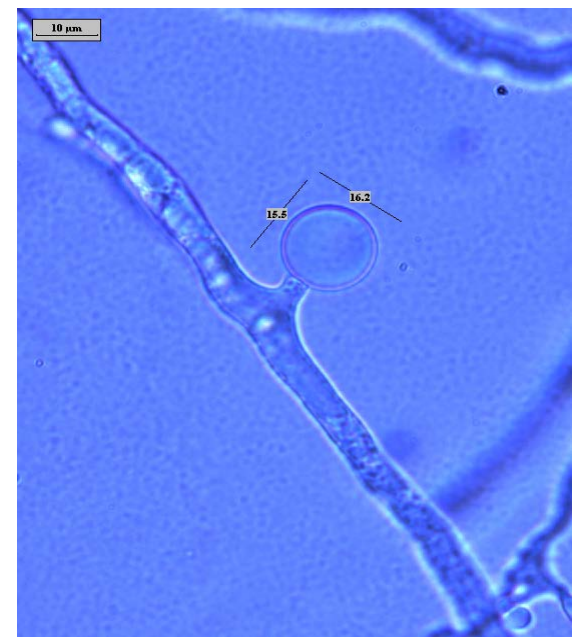
A.



B.

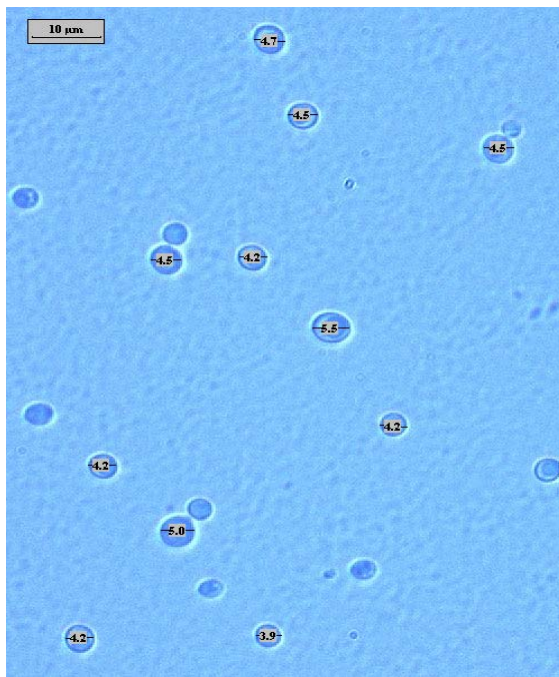


C.



D.

Figure 3.1.a Light micrographs of Antarctic fungi under light microscopy: A: *Aspergillus fumigatus*, B: Unidentified *Ascomycetes* sp.4a, C: Unidentified *Ascomycetes* sp.3 and D: *Mucor* sp.1.



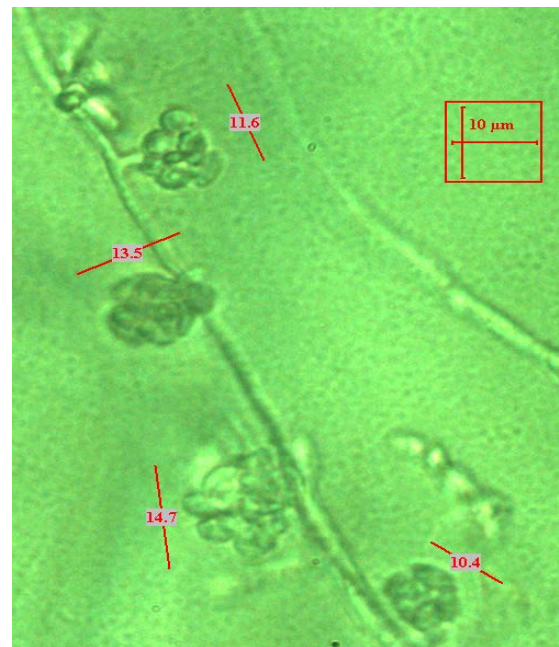
E.



F.



G.



H.

Figure 3.1.b Light micrographs of Antarctic fungi under light microscopy: E: Unidentified yellow yeasts, F: *Mucor* sp.2, G: Unidentified *Zygomycetes* sp.2, H: Undetermined *Hyphomycetes* sp.1.

3.2 Bioactivity Screening

3.2.1 Preliminary screening - Plug assay

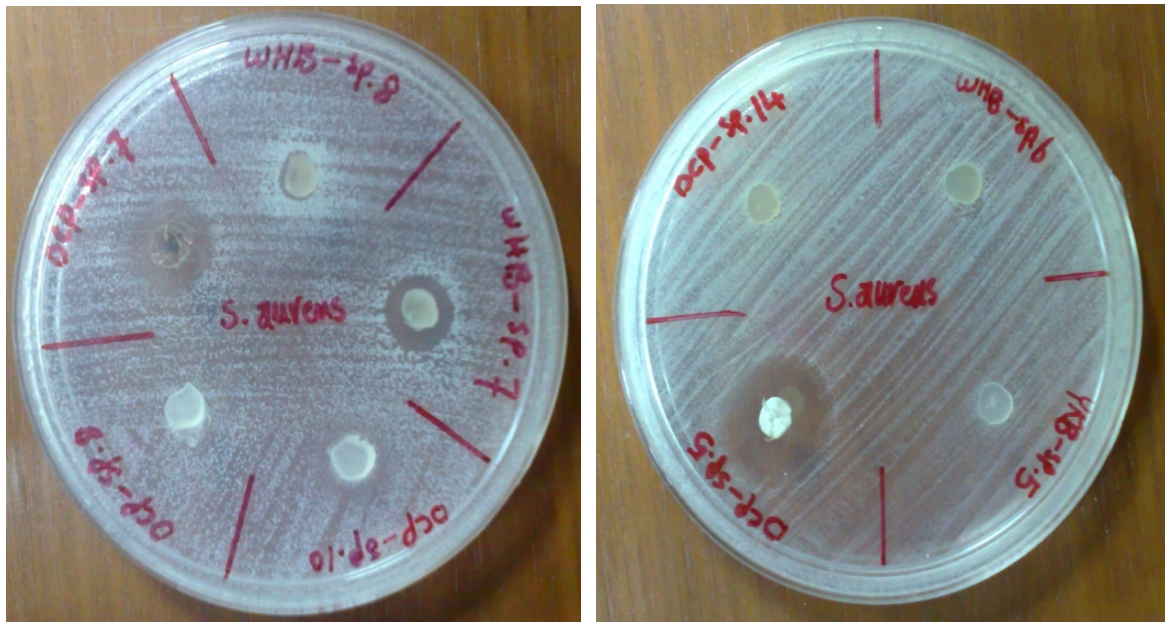
The twenty seven antarctic fungi isolates (15 psychrophilic, 9 mesophilic and 3 psychrotrophic) were screened against 5 Gram positive, Gram negative and yeast pathogens namely, *Staphylococcus aureus*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Candida albicans*. Total of 18 fungal species exhibited bioactivity potential against one or more Gram negative and Gram positive bacteria. The growth inhibited zone diameters were varied between 8-18 mm. Table 3.2 classified the degree of bioactivity based on the diameter of zone of inhibition into: weak (7-10 mm), intermediate (11-12 mm), good (13-15 mm) and very good activity (>15 mm).

The majority of the species showed weak to intermediate activity. But, *Hyphomycetes* sp.1 and *Hyphomycetes* sp.6 exhibit very good activity against *Staphylococcus aureus* and *Pseudomonas aeruginosa* respectively. Also *Aspergillus fumigatus* and *Hyphomycetes* sp.2 showed good inhibition of *Bacillus subtilis* and *Staphylococcus aureus* respectively. It should be highlighted that none of the studied isolates illustrated antifungal activity against the yeast pathogens (Table 3.2). Figure 3.2a-b showed a photograph of selected swabbed plates with the test microorganism after overnight incubation and the zone of growth inhibition surrounding the agar plug of the tested fungal species.

Table 3.2 Preliminary screening results of fungal bioactivity on the test microorganisms in plug assay.

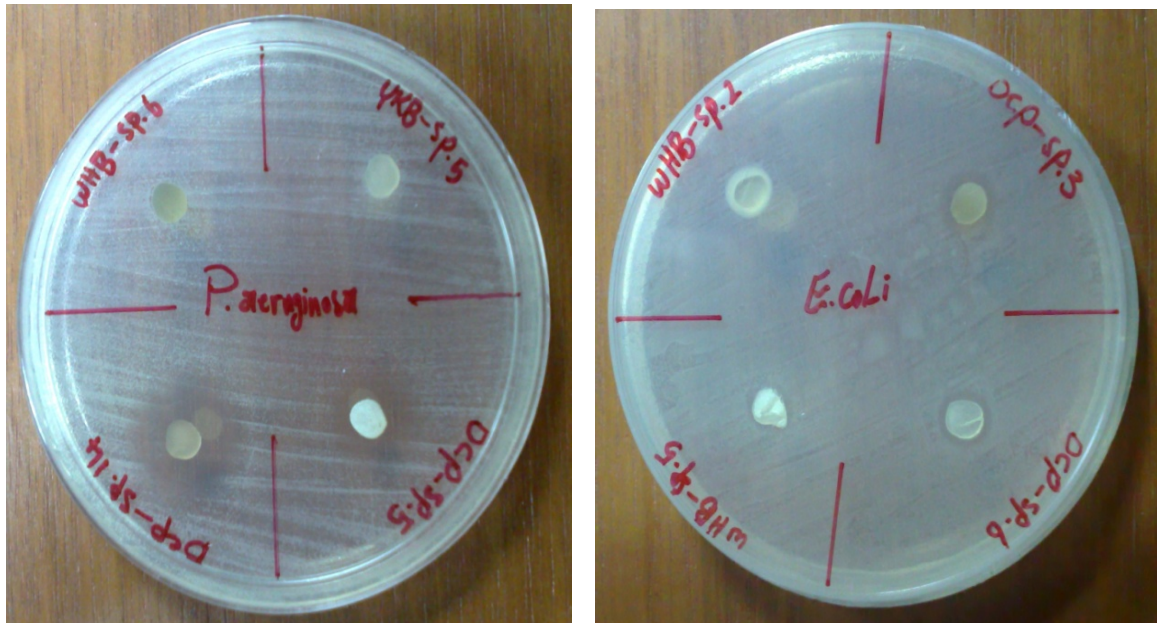
No	Code	Fungal isolate	Antibacterial activity				Antifungal activity
			E.c	P.a	B.s	S.a	C.a
1	DCP-sp.1	<i>Aspergillus fumigatus</i>	NA	NA	+++	NA	NA
2	DCP-sp.2	Unidentified <i>Ascomycetes</i> sp.2	NA	NA	+	++	NA
3	DCP-sp.3	Unidentified <i>Ascomycetes</i> sp.3	+	NA	NA	NA	NA
4	DCP-sp.4	<i>Mucor</i> sp.1	NA	NA	NA	+	NA
5	DCP-sp.5	Undetermined <i>Hyphomycetes</i> sp.1	+	+++	+	++++	NA
6	DCP-sp.6	Unidentified <i>Zygomycetes</i> sp.2	+	NA	NA	NA	NA
7	DCP-sp.7	Undetermined <i>Hyphomycetes</i> sp.2	NA	NA	+	+++	NA
8	DCP-sp.8	Undetermined <i>Hyphomycetes</i> sp.3	NA	NA	NA	NA	NA
9	DCP-sp.9	Unidentified <i>Ascomycetes</i> sp.4a	NA	NA	NA	NA	NA
10	DCP-sp.10	Undetermined <i>Hyphomycetes</i> sp.4	NA	NA	NA	NA	NA
11	DCP-sp.11	Undetermined <i>Hyphomycetes</i> sp.5	+	++	+	+	NA
12	DCP-sp.12	<i>Geomyces</i> sp.	NA	NA	NA	NA	NA
13	DCP-sp.14	Undetermined <i>Hyphomycetes</i> sp.6	+	++++	+	NA	NA
14	WHB-sp.1	<i>Mucor</i> sp.2	NA	NA	NA	NA	NA
15	WHB-sp.2	Unidentified yellow yeast	NA	NA	NA	NA	NA
16	WHB-sp.3	Unidentified <i>Ascomycetes</i> sp.5a	+	NA	NA	NA	NA
17	WHB-sp.4	Unidentified <i>Ascomycetes</i> sp.6a	+	NA	+	+	NA
18	WHB-sp.5	Unidentified <i>Zygomycetes</i> sp.4	+	NA	NA	NA	NA
19	WHB-sp.6	Undetermined <i>Hyphomycetes</i> sp.7	+	NA	NA	NA	NA
20	WHB-sp.7	Undetermined <i>Hyphomycetes</i> sp.8	NA	NA	+	++	NA
21	WHB-sp.8	Undetermined <i>Hyphomycetes</i> sp.9	NA	NA	+	+	NA
22	YKB-sp.1	Unidentified <i>Ascomycetes</i> sp.4b	NA	NA	NA	NA	NA
23	YKB-sp.2	Undetermined <i>Hyphomycetes</i> sp.10	NA	NA	NA	NA	NA
24	YKB-sp.3	Undetermined <i>Hyphomycetes</i> sp.11	NA	NA	NA	NA	NA
25	YKB-sp.4	Unidentified <i>Ascomycetes</i> sp.6b	+	NA	+	+	NA
26	YKB-sp.5	Unidentified <i>Ascomycetes</i> sp.5b	+	NA	NA	NA	NA
27	YKB-sp.7	Undetermined <i>Hyphomycetes</i> sp.12	+	NA	NA	NA	NA

NA: No activity, (+): weak activity, (++): intermediate activity, (+++): good activity, (++++): Very good, **E.c**: *Escherichia coli*, **P.a**: *Pseudomonas aeruginosa*, **B.s**: *Bacillus subtilis*, **S.a**: *Staphylococcus aureus*, **C.a**: *Candida albicans*.



A.

B.



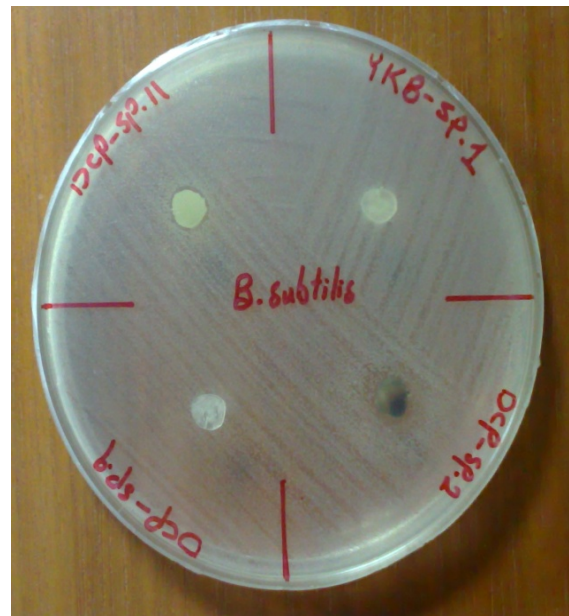
C.

D.

Figure 3.2a Photographs of antibacterial activity from plug assay results, **A-C**: showing intermediate to very good inhibition zones on *Staphylococcus aureus* and *Pseudomonas aeruginosa*. **D**: showing weak activity against *Escherichia coli*.



E.



F.

Figure 3.2b Photographs of bioactivity from plug assay results, **F**: wholly resistance of *Candida albicans* to all fungal species. **G**: DCP-sp.2 shown weak antibacterial activity against *Bacillus subtilis*.

3.2.2 Qualitative assay - Disc diffusion method

Ten species with good bioactivity (either produce >13mm or exhibiting activity in more the one test microorganism) on plug assay were selected to undergo the disc diffusion assay. Table 3.3 shows the name list of those selected fungal species and codes used in disc diffusion method. After enough crude extract were obtained successfully through extraction procedures, disc diffusion was proceeded against 8 test microorganisms, representing Gram positive, Gram negative bacteria and yeast namely *S. aureus*, *B. subtilis*, *B. cereus*, *P. aeruginosa*, *E. coli*, *C. albicans*, *S. cerevisiae* and *S. pombe*.

Table 3.3 List of the selected fungal species used in the disc diffusion method.

No.	Code	Code on preliminary screening	Fungal species
1	D1	DCP sp.1	<i>Aspergillus fumigatus</i>
2	D11	DCP sp.11	Undetermined <i>Hyphomycetes</i> sp.5
3	Y4	YKB sp.4	Unidentified <i>Ascomycetes</i> sp.6b
4	W7	WHB sp.7	Undetermined <i>Hyphomycetes</i> sp.8
5	W8	WHB sp.8	Undetermined <i>Hyphomycetes</i> sp.9
6	D2	DCP sp.2	Unidentified <i>Ascomycetes</i> sp.2
7	D7	DCP sp.7	Undetermined <i>Hyphomycetes</i> sp.2
8	D14	DCP sp.14	Undetermined <i>Hyphomycetes</i> sp.6
9	W4	WHB sp.4	Unidentified <i>Ascomycetes</i> sp.6a
10	D5	DCP sp.5	Undetermined <i>Hyphomycetes</i> sp.1

Dimethyl Sulfoxoid (DMSO) (used as negative control) did not show any activity among the organisms. Although the fungal extract showed average from good to nov activity against test microorganisms, chloramphenicol (the positive control) was recorded the highest activity among the test microorganisms. Interestingly, *Ascomycetes* sp.9 extract

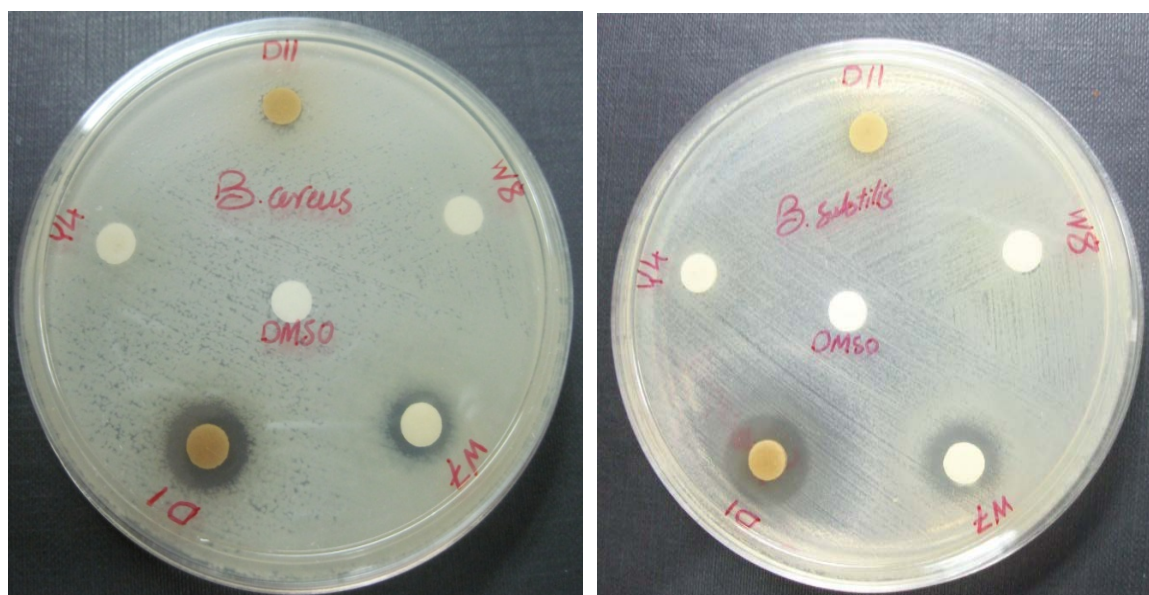
demonstrate good inhibition zone against *Saccharomyces cerevisiae*, while Chloramphenicol did not.

Growth inhibitory zones were recorded as the mean of the 5 replicates in millimeters showed in Table 3.4 as well as the positive and negative controls. Total of five extracts (out of 10) showed antimicrobial activity, and unexpectedly, five fungal extract were found to lose their activity in disc diffusion method. However, the selected photographs in Figure 3.3a-b shows filter paper disc containing the fungal extract applied on the swabbed test microorganism and surrounding with clear zone of inhibition.

Table 3.4 Biological activity of fungal extracts, Chloramphenicol and Dimethyl Sulfoxoid on test microorganisms in millimeters using disc diffusion method.

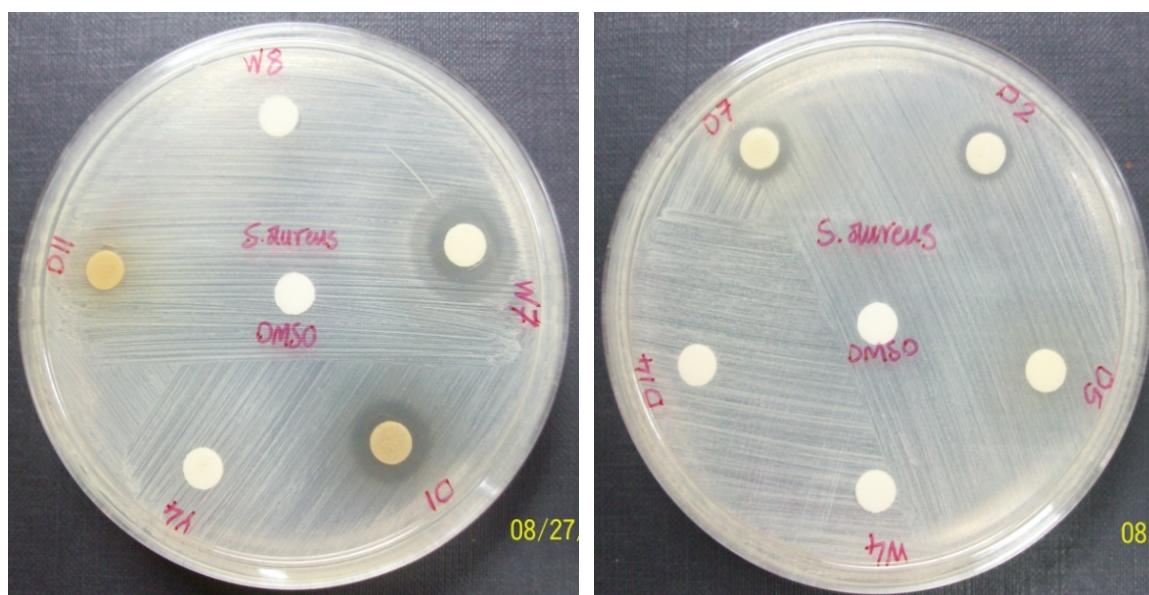
Fungal extract code	Antibacterial activity (mm)					Antifungal activity (mm)		
	E.c	P.a	B.c	B.s	S.a	C.a	S.c	S.p
<i>Aspergillus fumigatus</i>	12	13	14	15	14	NA	NA	NA
<i>Hyphomycetes</i> sp.5	8	15	NA	NA	NA	NA	NA	NA
<i>Ascomycetes</i> sp.6b	NA	NA	NA	NA	NA	NA	NA	NA
<i>Hyphomycetes</i> sp.8	12	28	12	13	13	NA	17	NA
<i>Hyphomycetes</i> sp.9	NA	NA	NA	NA	NA	NA	NA	NA
<i>Ascomycetes</i> sp.2	8	11	NA	NA	10	NA	NA	NA
<i>Hyphomycetes</i> sp.2	8	13	8	8	12	NA	NA	NA
<i>Hyphomycetes</i> sp.6	NA	NA	NA	NA	NA	NA	NA	NA
<i>Ascomycetes</i> sp.6a	NA	NA	NA	NA	NA	NA	NA	NA
<i>Hyphomycetes</i> sp.1	NA	NA	NA	NA	NA	NA	NA	NA
Chl.	30	16	30	24	32	NA	NA	NA
DMSO	NA	NA	NA	NA	NA	NA	NA	NA

DMSO: Dimethyl Sulfoxoid, **Chl.:** Chloramphenicol, **E.c:** *Escherichia coli*, **P.a:** *Pseudomonas aeruginosa*, **B.c:** *Bacillus cereus*, **B.s:** *Bacillus subtilis*, **S.a:** *Staphylococcus aureus*, **C.a:** *Candida albicans*, **S.c:** *Saccharomyces cerevisiae*, **S.p:** *Saccharomyces pombe*, **NA:** No activity, **(8-10mm):** weak activity, **(11-12mm):** intermediate, **(13-15):** good, **(>15):** very good activity.



A.

B.



C.

D.

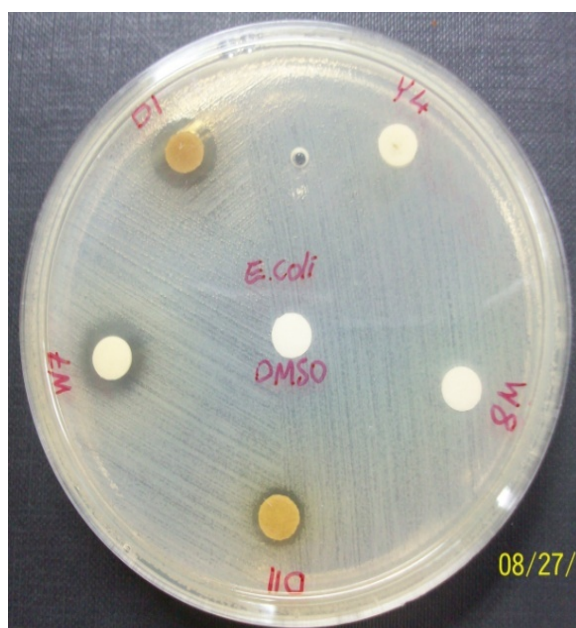
Figure 3.3a Photographs of antibacterial activity of fungal extracts in disc diffusion. **A-C:** only W7 and D1 extract showed good to intermediate activity against *Staphylococcus aureus* and *Bacillus subtilis*. **D:** showing weak activity of D7 and D2 against *Staphylococcus aureus*.



E.



F.



G.



H.

Figure 3.3b Photographs of bioactivity of fungal extracts in disc diffusion method. **E-F:** *Pseudomonas aeruginosa* showed very good susceptibility to W7, good to D11, intermediate to D1 and weak susceptibility against D2, D5 and D7. **G:** also W7 and D1 were showed intermediate activity against *Escherichia coli*, and weak susceptibility to D11. **H:** W7 was the only extract exhibited very good antifungal activity against *Saccharomyces cerevisiae*.

3.2.3 Quantitative assays – Broth microdilutions method

3.2.3.1 Minimum inhibitory concentrations (MIC)

Minimum inhibitory concentrations of *Aspergillus fumigatus*, *Hyphomycetes* sp.8 and *Hyphomycetes* sp.2 extracts (extracts with good bioactivity) were determined by broth microdilution method. In Table 3.5 the minimum inhibitory concentration (MIC) recorded in mg/ml using the same test microorganisms that the extract was active on. The extract of *Hyphomycetes* sp.8 recorded the lowest inhibitory concentration among the tested extract. Otherwise the rest arranged between 0.78-12.5 mg/ml. Figure 3.4 display the MIC break point determination according to the resazurine color change.



Figure 3.4 Photograph of U96 micro-well plate in broth microdilutions method. The pink colored wells due to actively growing *S. aureus*, while the non-pink wells were growth inhibited due to the extracts antibacterial activity. *Hyphomycetes* sp.8 (**W7**): showed the highest MIC with 0.39mg/ml followed by *Hyphomycetes* sp.2 (**D7**) with 0.78mg/ml, and then *Aspergillus fumigatus* (**D1**) with 1.56mg/ml.

3.2.3.2 Minimum bactericidal concentration (MBC) and Minimum fungicidal concentration (MFC)

Minimum bactericidal concentration (MBC) and Minimum fungicidal concentration (MFC) were tested and recorded in mg/ml in Table 3.5. Table 3.5 show range of extracts concentrations against the test microorganisms between 25 – 1.56 mg/ml. Extract of *Aspergillus fumigates* (D1) recorded the lowest MBC against *E. coli*, *S. aureus* and *B. subtilis*. While, *Hyphomyces* sp.8 (W7) was the lowest against *B. cereus*, *P. aeruginosa* and share the lowest concentration with *Hyphomyces* sp.2 (D7) against *B. subtilis* and *S. aureus*. *Hyphomyces* sp.5 (D11) recorded relatively high MICs against test pathogens. Figure 3.5 demonstrate how clearly the MBC and MFC determined on the first plate lack the visible colonies.

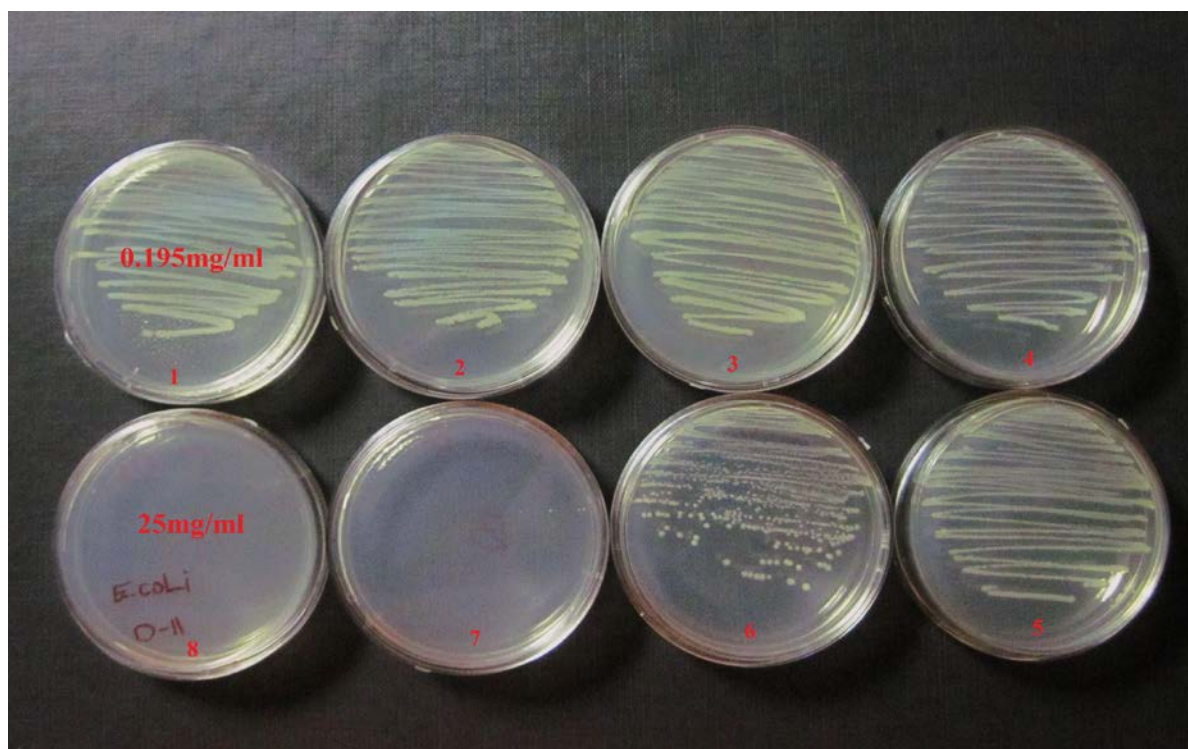


Figure 3.5 Photography of cultured plates in MBC determination. **1-6:** showed visible growth of *Escherichia coli*. Plate number **7** showed clearly the MBC of **D11** extract was 12.5mg/ml.

Table 3.5 Quantified values of MICs and MBCs of fungal extracts against test microorganism in broth microdilutions method.

Fungal extract	Test microorganism											
	<i>E.coli</i>		<i>B.subtilis</i>		<i>B.cereus</i>		<i>P.aeruginosa</i>		<i>S.aureus</i>		<i>S.cerevisiae</i>	
	MIC (mg/ml)	MBC (mg/ml)	MIC (mg/ml)	MBC (mg/ml)	MIC (mg/ml)	MBC (mg/ml)	MIC (mg/ml)	MBC (mg/ml)	MIC (mg/ml)	MBC (mg/ml)	MIC (mg/ml)	MFC (mg/ml)
D1	3.13	3.13	1.56	1.56	3.13	6.25	6.25	6.25	1.56	1.56	-	-
D11	12.5	12.5	6.25	6.25	-	-	6.25	12.5	-	-	-	-
W7	12.5	12.5	1.56	12.5	0.78	3.13	1.56	1.56	0.78	12.5	6.25	6.25
D7	-	-	-	-	3.13	6.25	-	-	0.78	25	-	-

MIC: Minimum inhibitory concentration.

MBC: Minimum Bactericidal concentration.

MFC: Minimum fungicidal concentration.