

CHAPTER 1

Introduction

1.1 Antarctic Continent

Antarctica is the fifth largest and most recently discovered continent on Earth. Some of the most popular attributes often associated with Antarctica, as a continent, has to do with it being the southernmost, coldest, windiest, highest and most remote part of the Earth planet. With an average temperature of -55°F and a wind speed of 57 mph, Antarctica has about 14 million square kilometers of cold habitat, which houses about 70% of the world's water in the form of ice and snow (Nicholas, 2006).

Basically, there are three main biogeographic zones under the Antarctic continent (Figure 1.1), namely: Continental Antarctic; Maritime Antarctic; and Sub-Antarctic (Convey, 2001). Firstly, the Sub-Antarctic zone comprises of a number of southern ocean isolated islands and archipelagos are representing the sub-Antarctic region. There is no seasonal pack or fast ice influences (except of South Georgia), with low air temperature and positive year-around in the most of the islands, in addition to the high precipitation (Convey, 2001).

The second zone is Maritime Antarctica which includes the western coastal regions of Antarctic Peninsula to Alexander Island, along with the Scotia Arc Island archipelagos, the isolated Bouvetøya and Peter I Øya. The maritime region have a positive mean air temperature for up to 4 month of the year, while both summer maxima and winter minima are buffered by the surrounding ocean, with possibility of thaws in all winter months. The South Sandwich archipelago and Bouvetøya mutually with

similar areas on Deception Island of South Shetland Islands and Victoria Land of continental Antarctic, are distinctive areas in Antarctica because of being geologically recent and active volcanic islands, with unique biological communities associated with the geothermal activity. However, precipitation is generally high (Convey, 2001).

Thirdly, the greater zone called Continental Antarctica, including East Antarctica, Balleny Islands and eastern side of the Antarctic Peninsula. This zone includes exposed coastal regions similar to those of the maritime Antarctic and inland nunataks with positive mean air temperatures was achieved for 1 month in these coastal regions. Nevertheless, terrestrial habitats are of limited extent and great isolation, except the Dry Valley region of Victoria Land. However, air temperatures rarely, if ever, become positive even for short periods in summer (Convey, 2001).

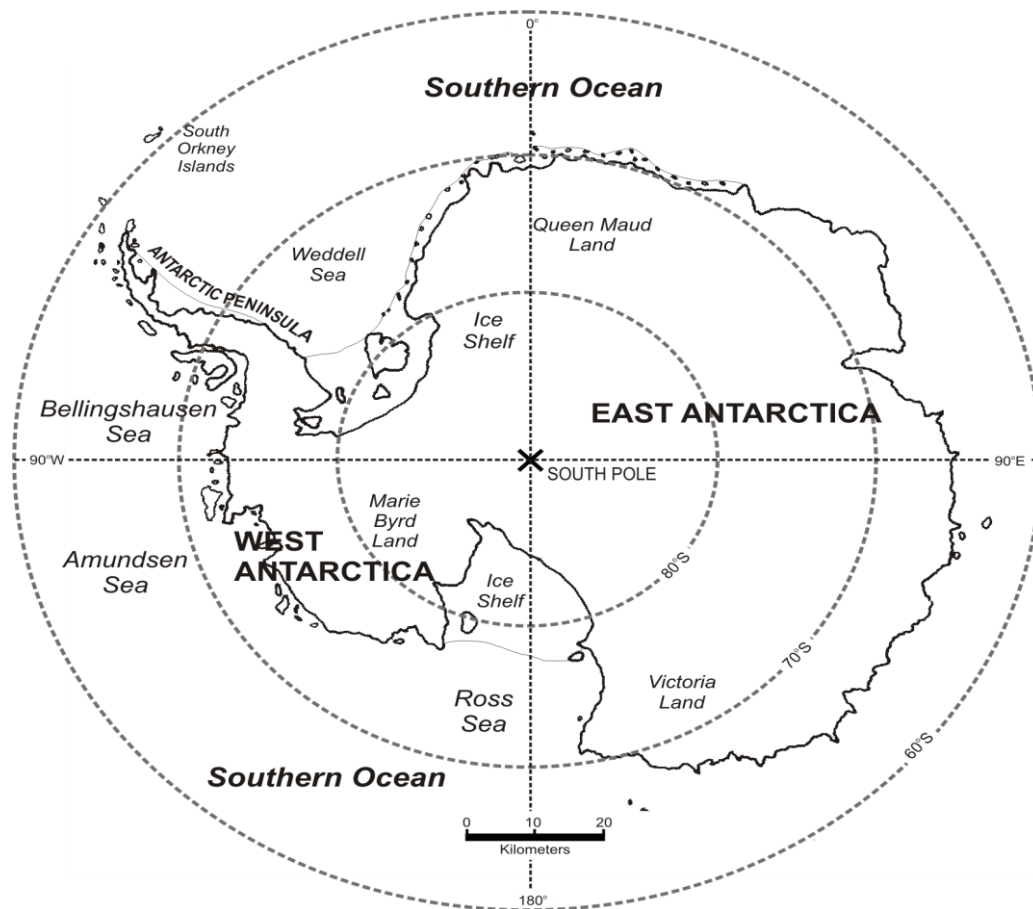


Figure 1.1 Geographic map of Antarctica.

1.2 Biodiversity and role of Antarctic fungi in polar ecosystem

Only a few species of fungi and bacteria have been described from the region in the recent past, most of them being from the marine environment, (sea water and sea ice). Past research has reported nine species of fungi from the Antarctica region. These include *Arthrobotrys ferox* Onofri & Tosi (Onofri & Tosi, 1992) on moss, *Torulopsis psychrophila* Goto, Sugiy. & Iizuka (Goto *et al*, 1969) and *Phoma herbarum* Westend on bird excreta, *P. herbarum* on skeletal remains, *Acremonium antarcticum* (Speg.) D.

Hawksw. (Hawksworth, 1974) and *A. psychrophilum* (Möller & Games, 1993) on lichens and species of *Torulopsis*, *Psychrophila* and *Cryptococcus* (Phaff and Fell, 1970) on ornithogenic soils. Besides, a few alien species of fungi, viz. *Hormoconis resinae* (Arx & de Vries, 1973) on oil spills and species of *Dacrymyces* and *Exidia* on wooden debris. Some of the tropical saprophytic fungi, viz. *Chaetomium globosum* Kunze, *Stemphylium* sp., *Curvularia lunata* Boendijn, *Memnoniella echinata* Galloway, *Aureobasidium pullulans* G. Arnand, *Aspergillus niger* Al-musallam, *Paecilomyces varioti* Bainier, *Penicillium funiculosum* G. Sm and *Cladosporium* sp., were exposed to Antarctic environment for a period of 14 months by Dayal *et al.* (1998) to study their viability, growth rate and virulence, but no major variation in activity was observed. In subsequent years, steady increase in summer temperatures and concomitant glaciological changes resulted in further exposure of soils in Antarctica and warranted continued studies on the life of the region (Singh, *et al.* 2006).

Microbiological studies in the southern cold temperature to Polar Regions have been concentrated largely on the sub-Antarctica Peninsula and the McMurdo Dry Valleys region of southern Victoria Land (Vishniac, 1993). Outside these area, mycological studies on the Antarctica continent are limited to reports from the Ongul Islands (Tubaki, 1961; Tubaki and Asano, 1965), Cape Hallett (Wicklow, 1968), Trans-Antarctica Mountain (Cameron, 1971), The Bunger Hills (Barker, 1977), The Vestfold Hills (Rounsevell, 1981; Kerry, 1990), Mac Roberton and Enderby Lands (Fletcher, Kerry and Weste, 1985; Kerry, 1990), Northern Victoria Land (Montermartini and Gestro, 1994) and other scattered localities such as the McMurdo sound region (Sugiyama and Iizuka, 1967; Latter and Heal, 1971; Greenfield, 1983). There have been few reports on fungi from Windmill Island (Azmi *et al.*, 1998; Cheryl and Seppelt.

1999). These studies suggest a low diversity of indigenous fungi continental Antarctica with around 150 taxa recognized in the literature.

The exceptional environmental harshness of terrestrial Antarctic generates simple structural ecosystem, dominated by the microbes (Yergeau *et al.*, 2006). Universally, fungi serve as one of the principal decomposers in ecosystems, returning various important elements such as carbon and nitrogen back into the environment, hence preventing them from becoming tied up in organic matter. In Antarctic ecosystems, the fungal taxonomic diversity is relatively poorly characterized. As a result of that, less is known about the function of fungal taxa in C and N cycling (Ludley and Robinson, 2008). Lack of knowledge on fungal contribution to ameliorating the Antarctic soil led Vishniac (1996) to describe it the simple way: “the prey of predators eats the predators themselves”. In other words, this implied that the population densities of Antarctic fungi represent the balance between reproduction and the activities of zymivorous and other fungivorous nematodes as well as death from other causes (Vishniac, 1996).

As indicated earlier, fungi are well-known for their biodegradable nature. One major role of filamentous fungi in the Antarctica terrestrial ecosystems is their ability to decompose litter, which has made these fungi popular as degraders of cellulose and lignin. However, since the soil ameliorating process in the temperate is jointly contributed by both fungi and bacteria, there are very little details on the exact details of the filamentous fungi’s direct role in this. For instance, detailed information on how parasymbiotic and mycosymbiotic fungi of the cryptoendolithic lichens produce oxalic and lichen acids to cause weathering of rocks appears to be unavailable, hence known as

just a hypothesis that they do have a role in the organic substance production to the soil (Vishniac, 1996). Conversely, yeasts are not usually credited with a major role in biodegradation in Antarctic terrestrial ecosystems (Vishniac, 1996). They reported to associate with bryophyte communities¹⁰ and are supposed to influence the bryophytes to release dissolved organic Carbon as result of freeze–thaw cycles damage (Ludley and Robinson, 2008). Basidiomycetous yeasts are typically known to release extracellular proteases, laccases, hemicellulases, and pectinases, which then use the hydrolyzed product as source of Carbon. However, while they are not lignin degraders, some *Cryptococcus* strains utilize aromatic compounds produced by lignin biodegradation. Moreover, there are phylloplane yeasts outcompeted microfungi on at least some occasions in sub-Antarctic, as reported the dominant microflora of some living grass leaves on South Georgia and Macquarie (Vishniac, 1996).

Again, the information on the role of Basidiomycetes yeast in Carbon cycling is fragmentary. Anamorphic Ascomycetes, particularly *Cadophora* sp., are seems to playing a predominant role in wood decomposition (Ludley and Robinson, 2008). Since the agents responsible for terrestrial biodegradation on the Antarctic continent are yet unknown, better understanding of fungal biodiversity and activity would help in adjusting the ecosystem functional models (Vishniac, 1996).

1.3 Thermal Classes of Antarctic Fungi

Fungi are recently shown not only able to sustain, but also to propagate successfully at different extreme environmental conditions such as hypersaline waters, dry rock surfaces, and ocean depths. Fungi have been isolated from extremely cold environments, such as vegetation, permafrost, water, snow and glacier ice

(Gundecimerman *et al.*, 2003). Fungal communities have been observed to be more abundant in Sub-Antarctic islands due to its strong humid and temperate nature, as compared to other Antarctic regions. Similarly to the bacteria, fungal abundance on the Sub-Antarctic Signy Island has been correlated to the organic matter, soil water content, pH and total Nitrogen (Yergeau *et al.*, 2006).

Cryptoendolithic communities constitute very simple communities comprising only a few species of Antarctic cryptoendolithic microorganisms. While, “lichen dominated community” is the most common and well-studied communities found in sandstone (Ruisi *et al.*, 2007). However, observation of several studies conducted on Antarctic fungal communities are dominated by psychrotolerant, rather than psychrophilic fungi, suggesting that tolerance of some fungal communities to the cold results in diverse fungal assembly (Yergeau *et al.*, 2006). Moreover, a recent molecular survey showed there is no increase in the eukaryotic diversity along the Antarctic regions as compared to the diversity of the lower eukaryotic in continental and maritime Antarctic (Yergeau *et al.*, 2006).

Most of the fungi in Antarctica can be classified as psychrophilic fungi, which are extremophile organisms that are capable of growth and reproduction in very low temperatures (–1 to –35°C during wintertime), and glacial ice habitats (–5°C) (Singh *et al.*, 2006). Cold adapted microorganisms comprised of two groups, psychrophilic and psychrotolerants (Margesin, *et.al.* 2007) which persist permanently cold habitats such as in Polar Regions, at high attitudes or in deep sea environment with periodic basis of their cardinal temperature. The psychrotolerants which grow faster in wide temperature 20°C the lower growth temperature is fixed by the physical properties of aqueous

solvent system of cell. On the other hand, psychrophiles are able to grow above 20°C but faster in below 15°C.

One type of psychrophiles is obligate psychrophiles, those organisms having a growth temperature optimum of 15°C or lower and cannot grow in a climate beyond a maximum temperature of 20°C (Reddy *et al.*, 2003). Another type of psychrophiles is facultative psychrophiles which can grow at 0°C up through approximately 40°C and exist in much larger numbers than obligate psychrophiles. They are generally not able to grow much below 0°C, though they may maintain basic functioning. They have evolved to tolerate cold but they are not as physiologically specialized as obligate psychrophiles and are usually not found in the very coldest of environments (David and Nichols, 2004).

Cold-adapted microorganisms are obviously unique because despite an internal temperature close, if not identical, to that of their surroundings, and despite the strong negative effect of low temperatures on biochemical reactions, they not only survive but breed and grow successfully, exhibiting metabolic fluxes more or less comparable to those exhibited by closely related mesophilic species living at moderate temperatures (Zecchinon *et al.*, 2001).

Mesophilic is an organism that grows best in moderate temperature, neither too hot nor too cold, typically between 15°C and 40°C (77°F and 104°F). The term is mainly applied to microorganisms. The habitats of these organisms include soil, the human body and animals. The optimal temperature of many pathogenic mesophiles is 37°C

(98°F), the normal human body temperature. Mesophilic organisms have important uses in food preparation especially in cheese, yogurt and beer (Madigan and Martino, 2006).

Thermophiles are an organism a type of extremophiles that thrives at relatively high temperature, between 45°C and 80°C (113°F and 176°F). Many thermophiles are archaea. Thermophiles are classified into obligate and facultative thermophiles (Madigan and Martino, 2006).

The physiological and ecological mechanisms that help fungi to overcome and survive cold environmental conditions are well explained by Robinson (2001), he revealed that there is a predominance of sterile mycelia in the Antarctic soils and this could be a physiological adaptation to overcome the harshness of sub-zero temperatures. He also attributed the production of melanin by these fungi as a protective mechanism for survival under extreme temperatures (Singh *et al.*, 2006).

1.4 Adaptation mechanisms of Antarctic Microfungi

Fungi are ubiquitous in nature, occupying ecological niches worldwide. In Antarctica, there is a long list of microfungi living in different Antarctic environments illustrating great adaptation to the harsh life condition such as extremely low temperatures, wide thermal fluctuations, high UV radiation, and low water and nutrients availability (Onofri *et al.*, 2004). Some of those biotic factors are long-lived or permanent feature of habitat, while others having short-lived effect.

Several physiological mechanisms of cold tolerance such as trehalose and cryoprotectant sugars, trehalose is a storage compound in fungal vegetative cells and

spores (Lewis and Smith, 1967), and is the most widely distributed disaccharide in fungi (Thevelein, 1984). Usually, in fungal vegetative structures trehalose exist together with sugar alcohols and glycogen. Also trehalose often present in much concentration than other storage carbohydrates (Thevelein, 1984). Trehalose appears to be a general stress protectant in the cytosol, and it is known to stabilize membranes during dehydration (Goodrich, *et al.*, 1988).

Another physiological mechanism of cold tolerance is polyols. Glycerol and mannitol may increase in concentration to maintain turgor pressure against heat-mediated decrease in external water potential (Cooke and Whipps, 1993). Mannitol is thought to be important in protection against water stress (Lewis and Smith, 1967).

On the other hands, lipids or fatty acids one of important adaptation of Antarctica micro-fungi, there is evidence to suggest that membrane composition can determine the ability of fungi to grow over specific temperature ranges (Cooke and Whipps, 1993). The structure and temperature at which their properties change from an inactive gel phase to an active, crystalline phase (Robinson, 2001).

Antifreeze proteins (AFPs) is another physiological mechanism of cold tolerance for fungi. Extracellular and intracellular AFPs may allow fungi to be active at subzero temperatures and they may slow the growth of ice if crystallization does occur. Fungi require the maintenance of an aqueous environment for growth to secrete enzymes and absorb carbon and nutrients. AFPs may be essential for inhibiting the re-crystallization of ice and promoting fungal survival through freeze-thaw cycles, in addition to preventing hyphae from freezing at temperatures just below zero. AFPs produced by

fungi may keep substrates from freezing since these compounds would be otherwise unavailable for use (Snider *et al.*, 2000). AFPs which are thought to contribute significantly to survival at subzero temperature by modifying the growth of ice, are found in fungi from cold Arctic and Antarctic environment (Newsted *et al.*, 1994).

1.5 Understanding the biological activity of microorganisms

The main kind of biological activity is substance's toxicity. Activity is generally dosage-dependent and it is uncommon to have effects ranging from beneficial to adverse for one substance when going from low to high doses. A material is considered bioactive if it has interaction with or effect on any cell tissue in the human body. Pharmacological activity is generally used to describe beneficial effects, i.e. the effect of drug candidates.

Antimicrobial activities of fungi from extreme environment were less investigated and only few antibacterial secondary metabolites from Antarctic fungi have been reported. For examples, a few of the *Penicillium* strains isolated from the sediments of ponds in continental Antarctica showed an antibacterial activity similar to β -lactam antibiotics (Montermartini *et al.*, 2000). Actinomycetes have also been isolated from Antarctica and screened for antimicrobial activity by Nedialkova *et al.* (2005). Some investigators indicated production of secondary antimicrobial compounds by some strains of Antarctic fungi and 29% of microfungal species with antimicrobial activities were isolated from benthic mats of different Antarctic lakes (Marinelli *et al.*, 2004).

Agar disk diffusion method which described by Bauer *et al.* (1966) has been used to confirm the bioactivity of fungi. This method is well documented and standard zones of inhibition have been determined for susceptible and resistant values. There is also a zone of intermediate resistance indicating that some inhibition occurs using this antimicrobial but it may not be sufficient inhibition to eradicate the organism from the body. Many conditions can affect a disc diffusion susceptibility test. When performing these tests certain things are held constant so only the size of the zone of inhibition is variable. Conditions that must be constant vary from test to test include the agar used, the amount of organism used, the concentration of chemical used, and incubation conditions (time, temperature, and atmosphere).

1.5.1 Biological activity of Antarctic fungi

Scanty works have been carried out so far on the bioactivity of Antarctic fungi. Su *et al.* (1995) have successfully isolated the Antibiotic C3368-A , produced by fungus from Antarctic soil sample. The antibiotic was found to significantly inhibit the mice carcinoma cells, and synergistically the human oral epidermoid carcinoma KB cells. The antibiotic C3368-A has also enhanced the inhibitory effect on the proliferation of human hepatoma BEL-7402 cells and potentiated the inhibitory effect of MMC against colon carcinoma in mice. With no significant augmentation of toxicity in treated mice, Su *et al.* (1995), suggested the use of the newly found nucleoside-transport inhibitor, in potentiating the effect of antitumor drugs. However, Selbmann *et al.* (2002) reported in the isolation of filamentous fungus *Phoma herbarum* CCFEE 5080, isolated from continental Antarctica soil, as a producer of exopolysaccharide (EPS). Selbmann *et al.* (2002) hypothesized the adaptation of *P. herbarum* CCFEE 5080 to the Antarctic

environment, (low temperature, high thermal fluctuations and freeze–thaw cycling) might be related to the EPS production ability.

Li *et al.* (2008) reported five novel asterric acid derivatives, as bioactive compounds, isolated from the Antarctic *Ascomycete* fungus *Geomyces* sp. They revealed that compound number 7 displayed antifungal activity against *Aspergillus fumigates* Fresen, whereas compound 8 showed only antibacterial activities against Gram-positive and Gram-negative bacteria. Also, Brunati *et al.* (2009) as a first report of new antibiotics produced by fungi from benthic microbial mats from Antarctic lakes, screened 160 filamentous fungi (from benthic mats of Antarctic lakes) for novel antimicrobial and cytotoxic compounds. Forty seven (29%) extracts were produce antimicrobial activity as fellow; Gram-positive *Staphylococcus aureus* (14%), Gram-negative *E. coli* (10%), and yeasts *Candida albicans* (11%) and *Cryptococcus neoformans* (8%). They reported the narrow of activity against representatives of enterobacteria and filamentous fungi. However, most of fungi with bioactivity were cold-tolerant cosmopolitan hyphomycetes such as *Penicillium*, *Aspergillus*, *Beauveria* and *Cladosporium*.

All in all, it can be concluded that these microbial assemblages represent an extremely rich source for the isolation of new strains producing novel bioactive metabolites with the potential to be developed as drugs. So this exploratory study, therefore, sought to investigate the bioactivity of soil microfungi isolated from different areas (Barrientos Island, Dee Island, Ampato Point-Greenwich Island and King George Island) in maritime, Antarctica. Optimistically these Antarctic fungi could be an

outstanding source of novel, biologically active natural products, which may be considered as potential new drug leads useful for the treatment of human diseases.

1.6 Research objectives

- To identify the Antarctic micro-fungal isolates.
- To investigate the bioactivity of fungi collected from Antarctic Peninsula in Antarctica, specifically from Greenwich Island (Barrientos Island; DEE Island; and Ampato Point-Greenwich Island) and King George Island, against selected human pathogens based on plug assay and disc diffusion methods.
- To determine quantitatively the bioactivity of selected species, by means of determining the Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC) and Minimum Fungicidal Concentration (MFC) values, to create a platform for future research.

CHAPTER 2

Materials and Methods

2.1 Materials

A total of 17 strains of micro-fungi from Greenwich and King George Islands of Antarctica were used for this research which comprised of 7 mesophilic and 10 psychrotrophic fungi (Table 2.1). The strains were kept in the National Antarctic Centre, University of Malaya. Soil samples were collected from Greenwich Island specifically Barrientos Island, DEE Island and Ampato Point-Greenwich Island and King George Island (Figure 2.1). These samples were collected during the expedition of January-February 2008 by scientist of National Antarctica Research Centre at the University of Malaya.

In this study, the 17 strains were initially isolated in Potato Dextrose Agar (PDA) medium. Hence, it was necessary to sub-culture all the 17 strains again in a fresh PDA to observe the growth and viability of the strains before being used in this study. All 17 isolates were in-vitro tested against Gram-positive, Gram negative bacteria and yeast human pathogens. Of these, five test microorganisms namely *Candida albicans* (Robin C.P.) Berkhout MTCC 3017 (ATCC 90028), *Staphylococcus aureus* Rosenbach MTCC 96 (ATCC 9144), *Bacillus subtilis* (Ehrenberg) Cohn (ATCC 6051), *Escherichia coli* (Migula) Castellani & Chalmers MTCC 443 (ATCC 25922) and *Pseudomonas aeruginosa* Reller (ATCC 27853) were used.

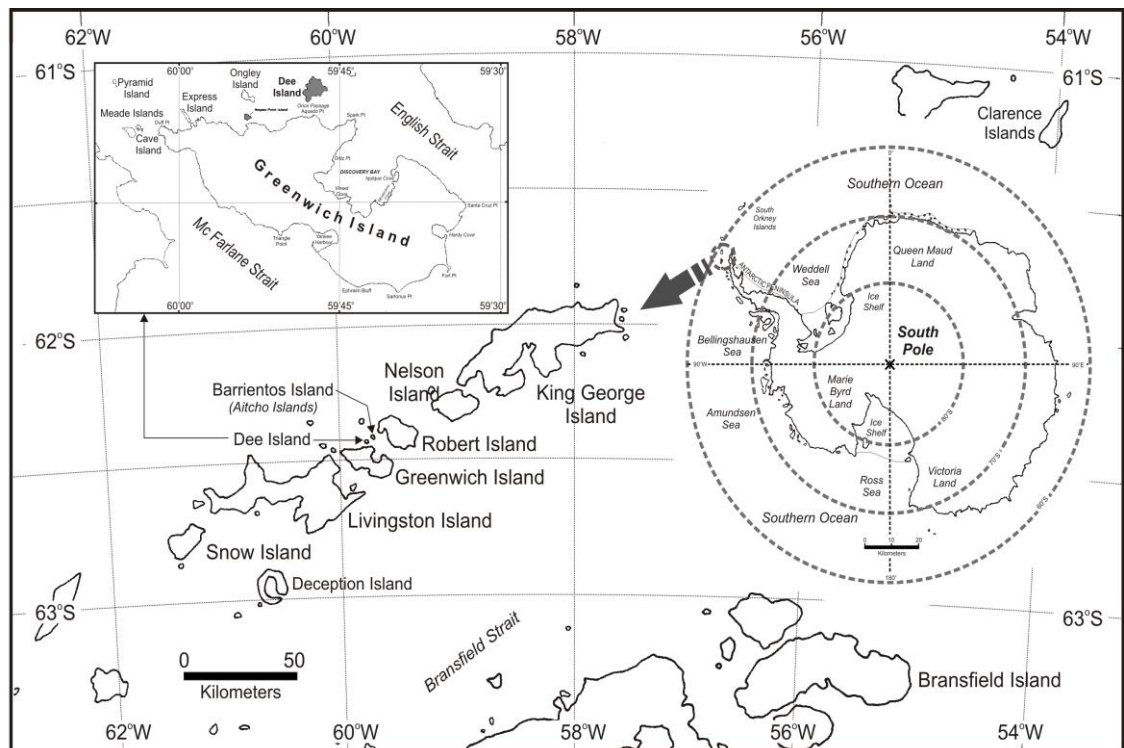


Figure 2.1: Map of collected area and Islands, namely: Greenwich Island (Barrientos Island; DEE Island; and Ampato Point-Greenwich Island) and King George Island.

Table 2.1: List of 17 strains of microfungi, collection site, GPS location (Global Positioning System), date of collection and their thermal classes.

Code	Description	Location	GPS	Date of Collection
AK07KGI 102 Sp.No.6	4°C, pH 5 Mesophilic	King George Island	62°02'S 58°21'W	Jan 2008
AK07KGI 102 R1-3 psco 1	4°C, pH 5 Psychrotrophic	King George Island	62°02'S 58°21'W	Jan 2008
AK07KGI 301 R1-2 Psco 7	4°C, pH 5 Psychrotrophic	King George Island	62°02'S 58°21'W	Jan 2008
AK07KGI 101 R3-1 Sp.2	4°C, pH 5 Psychrotrophic	King George Island	62°02'S 58°21'W	Jan 2008
AK07KGI 301 R3-3 Sp.17	4°C, pH 5 Psychrotrophic	King George Island	62°02'S 58°21'W	Jan 2008
AK07KGI 102 R1-2 Sp.2	4°C, pH 5 Psychrotrophic	King George Island	62°02'S 58°21'W	Jan 2008
AK07KGI 104 R1-2 Sp.18	4°C, pH 5 Psychrotrophic	King George Island	62°02'S 58°21'W	Jan 2008
AK07KGI 402 R2-1	4°C, pH 5 Psychrotrophic	King George Island	62°02'S 58°21'W	Jan 2008
AK07KGI 902 R2-1	4°C, pH 5 Psychrotrophic	King George Island	62°02'S 58°21'W	Jan 2008
AK07KGI 103 R2-1	4°C, pH 5 Psychrotrophic	King George Island	62°02'S 58°21'W	Jan 2008
SOEBI16-7S19	25°C, pH 5 Mesophilic	Barrientos Island	62°24'S, 59°47'W	Feb 2008
SOEBI4-7S20	25°C, pH 5 Mesophilic	Barrientos Island	62°24'S, 59°47'W	Feb 2008
SOEBI6-7S1	4°C, pH 5 Psychrotrophic	Barrientos Island	62°24'S, 59°47'W	Feb 2008
SOEBI214S2	25°C, pH 5 Mesophilic	Barrientos Island	62°24'S, 59°47'W	Feb 2008
Sp.1 D1T2 / 30-5-1 (a)	25°C, pH 5 Mesophilic	DEE Island	-	Feb 2008
Sp.2 D1T2 / 30-5-1 (a)	25°C, pH 5 Mesophilic	DEE Island	-	Feb 2008
Sp.2 AMP 8-8	25°C, pH 5 Mesophilic	Ampato Point- Greenwich Island	62°30'S, 59°30'W	Feb 2008

2.2 Methods

2.2.1 Potato dextrose agar (PDA) Media preparation

16g of Potato dextrose agar (PDA, Oxoid) powder, 3 mg of Chloramphenicol in 400ml of distilled water were mixed together and sterilised at 121°C at 15 psi for 20 minutes, the medium was left to cool and poured into petri dishes.

2.2.2 Reviving of Antarctic fungi

The surface of the table where the experiments were performed was wet with 70% of alcohol for disinfection and wiped with tissue papers. The Bunsen burner was switched on, and let it burn for several minute to sterilize the air around the experiment area. The stock culture was hold in one hand and the forceps were hold in other hand. The forceps were flame until red hot by holding it pointed down into the flame. The hot forceps was cooled down by touching them to the agar inside the sterile Petri dishes. Using the forceps fingers agar block from edge of the stock culture were transferred to the sterile plate and closed the plate immediately. Flame the fingers of the forceps. The plates were incubated according to their optimum growth temperature (25°C for Mesophilic and 4°C for Psychrotrophic).

2.2.3 Identification of Antarctic fungi

Fungal spores were cultivated on PDA medium on petri dish. The germination and growth of mycelium was observed daily under a light microscope. Fungi were identified into species based on morphological characteristic such as spore size, length, width, thickness, color, shape; hyphae (septate and aseptate); number (septum number of conidia); wall ornamentation (smooth, warty, presence or absence of hair) and measurement (Domsch *et al.*, 1980; Watanabe, 1994). Voucher of slides were deposited in the Institute of

Biological Science, University of Malaya, fungal collection which is located in National Antarctic Research Center, Kuala Lumpur.

2.2.4 Reviving of bacterial test microorganisms

To keep bacterial cultures growing, so that can be used in the various experiments, the test bacteria were undergo subculture regularly. Subcultures were carried-out on sterile medium of Muller Hinton Agar (MHA). Prior to looping out (streaking the plate) flame sterilization of the wire loop and neck of the growing culture bottle were preformed to ensure the aseptic conditions. The growing culture tube was hold on nearby the flam then inoculating loop was inserted into till touch the surfaces of the culture then carefully removed. Immediately, the loop was inserted into the sterile slant tube and a zig-zag motion on the surface of the slant was done to expose bacteria. Again, the mouths of the culture tubes were re-flamed by passing them through the flame 2-3 times then caps were placed. The new culture tube was incubated at 37°C for 18-24 hours.

2.2.5 Bioactivity screening

2.2.5.1 Preliminary screening-Plug assay

Preliminary screenings for biological activity of fungal isolates were tested using Plug assay method. Selected five human pathogens were provided by Department of Microbiology, University of Malaya. These human pathogens were pure cultures and well identified under the names *Staphylococcus aureus*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Candida albicans*. Out of these five human pathogens, the bacterial pathogens were inoculated by swabbing on Luria Base Agar (LBA) whilst yeast strains were done on Saboraud Dextrose Agar (SDA), in less than 30 minutes after

adjusted to provide an inocula equivalent to 0.5 McFarland standards. Preparations of inoculums were as in sections **2.2.5.2**. Then, a 6mm in diameter plug from the edge of actively grown fungal mycelium on Potato Dextrose Agar (PDA) medium was cut out with the aid of No.1 cork-borer and moved onto the surface of previously inoculated medium with the pathogen. Three replicates were produced and incubated under 37°C for 18-24 hours in the case of bacterial pathogens and 48hours for yeast. The bioactivities were determined by appearance of clear zones of inhibition. Only fungi with bioactivity were considered for next confirmation step.

2.2.5.2 Inoculums preparation-growth method

As all the test organisms used were non-fastidious, growth method for inoculums preparation were performed. Five colonies of same morphology colonies were transferred into Muller-Hinton broth (MHB) and incubated overnight at 37°C, and then density was matched to McFarland standard against white background with black lines by increasing the sterile Muller-Hinton broth (MHB). Suspensions expected to contain between 10^7 to 10^8 cfu/ml, were and made prepared ready to use within 30 minutes after preparation.

2.2.5.3 Preparation of 0.5 McFarland Standards

With regular and continual shaking, 0.5 ml of 0.048 M BaCl₂ (1.17% w/v BaCl₂.H₂O) was added to 99.5 ml of 0.18 M H₂SO₄ (1% v/v). The resulting solution was poured in to screw cap tubes, sealed tightly and kept in dark place at room temperature. This is approximately equal to 1.5×10^8 cfu/ml.

2.2.6 Qualitative Assay - Disc Diffusion Method

Agar disc diffusion method for antimicrobial susceptibility testing, as recommended by the U.S. Food and Drug Administration and by the National Committee for Clinical Laboratory standards (NCCLS) is slightly modified of that described by Bauer *et al.* (1966) research (Arthur and Thornsberry, 1991) which compares the spectrum bioactivity of fungal origin with those of chemical antimicrobial agents. The three yeast and five bacterial strains, namely *Candida albicans*, *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Staphylococcus aureus*, *Bacillus subtilis*, *Bacillus cereus*, *Escherichia coli* and *Pseudomonas aeruginosa*, respectively were used to check the antimicrobial activity of the viable microfungi that passed the test for the Plug Assay experiment which was initially conducted. Preparation of test microorganisms inocula and inoculation were done as described in Section 2.2.5.2. Muller-Hinton agar (MHA) was used as recommended by NCCLS. 20mg crude extract was dissolved in 1ml Dimethyl Sulfoxide (DMSO) to obtain a concentration of 20mg/ml, then 10µl was pipette on a sterile 6mm paper disc applied immediately on the medium surface.

Dimethyl Sulfoxide (DMSO) and Chloramphenicol (30µg commercial discs) were used as negative and positive control respectively. Incubation at 35-37°C was done in an incubator and growth inhibited zones were measured in mm by the aid of a transparent scale, after 18-24 hours for bacterial pathogens and for 48 hours for yeast.

2.2.6.1 Cultivation and Extraction

A block (about 1cm in diameter) of actively growing fungal mycelium of species with bioactivity was cultivated on Potato dextrose broth (PDB) using 500ml Erlenmeyer flasks containing 200ml of PDB. Five replicates were incubated at 25°C or

4°C upon the species optimum temperature for 3-4 weeks, under stationary phase. Firstly after the incubation period, cultures were centrifuged (to facilitate the filtration mechanisms) at 3000rpm and 4°C for 10 minutes, followed by filtration of the supernatant under vacuum using Whatman No.1 filter paper. Secondly, the filtrate was extracted twice with an equal volume (1:1) of ethyl acetate (EtOAc). with the aid of separating funnel and vigorously shaking. Finally, EtOAc layers were combined and evaporated to dryness using rotary evaporator after adjusted to 240/25mbar and 40°C and the crude extracts were store at 4°C prior to use.

2.2.7 Quantitative assays – MIC, MBC and MFC

2.2.7.1 Minimum inhibitory concentration (MIC)

There are several established methods to determine MIC but, the most common one are Broth Microdilution Method (Polanco *et al.*, 1995; Singh, *et al.*, 2000). In this study, minimum inhibitory concentrations (MIC) of fungal extracts were determined in U96 MicroWell plates (8×12 wells), with a range of concentrations (25.0 – 0.195 mg/ml) being prepared. An aliquot of 150 µl of PDB with the pathogen (after adjustment to turbidity of 0.5 McFarland standard) was placed in the well labeled A-1, and further aliquots of 100 µl into each of the remaining wells labeled from A-2 to A-8. Next, a 50 µl aliquot containing 100 mg/ml concentration of the fungal extract was pipetted into well A-1 and mixed thoroughly (to obtain a final concentration of 25 mg/ml). Then, 100 µl from well A-1 was transferred to well A-2 and mixed thoroughly. This process was repeated across the plate to well A-8, to give serial dilutions. Plates were sealed and incubated at 35 – 37°C overnight, or again up to 48h for tests involving yeast. Prior to assessing results, resazurin dye was added to all the wells, and further 20

min incubation completed to clarify the break-point (of completely inhibited growth) with color change.

2.2.7.2 Minimum Bactericidal Concentration (MBC) and Minimum Fungicidal Concentration (MFC)

Minimum Bactericidal and fungicidal concentration was carried out to determine lower concentration of crude extract that could kill the pathogen. During the Minimum inhibitory concentration (MIC) test, after the first incubation time, and before the addition of resazurin dye, a loop full of each well of the eight wells was transferred and streaked into Muller-Hinton Agar (MHA), and incubated overnight at 35-37°C. MBCs or MFCs were recorded as the lowest concentration completely inhibits the bacterial or fungal growth.

CHAPTER 3

Result

3.1 Identification of Antarctic fungi

The Antarctic fungi were identified as filamentous fungi belonging to the phyla Ascomycota (4 genera), Anamorphic (12 genera), Zygomycota (1 genera) are listed in Table 3.1. Most of isolates were identified to the phyla level while (4) were identified to the genus level and (12) to species level. The most frequently isolated species belong to the genera *Aspergillus* sp., *Antarctomyces* sp., *Aspergillus fumigatus*, *Aureobasidium* sp., *Cadophora malorum Traaen* sp., *Geomyces Cretaceous* sp., *Mortierella* sp., *Thelebolus microspores* sp., *Veriticillium* sp. and *Penicillium* sp.. All of these species have been reported as commonly isolated from Antarctic soil (Vishniac, 1996). A total of 17 different strains were identified to generic level and are listed in Table 3.1. Light micrographs of selected species are presented in Appendix 1.

Table 3.1: List of fungal strains.

No.	Code Name	Specific Name
1	AK07KGI 102 Sp.No.6	<i>Aspergillus</i> sp.
2	AK07KGI 102 R1-3	<i>Antarctomyces</i> sp. 1
3	AK07KGI 301 R1-2	<i>Antarctomyces</i> sp. 7
4	AK07KGI 101 R3-1 sp.2	<i>Aureobasidium</i> sp.2
5	AK07KGI 301 R3-3	<i>Veriticillium</i> sp.
6	AK07KGI 102 R1-2 sp.2	Deuteromycete sp. 25
7	AK07KGI 104 R1-2 sp.18	Deuteromycete sp. 18
8	AK07KGI 402 R2-1	<i>Geomyces Cretaceous</i> sp.
9	AK07KGI 902 R2-1	<i>Geomyces</i> sp. 2
10	AK07KGI 103 R2-1	<i>Mortierella</i> sp.
11	SOEBI16-7S19	<i>Aureobasidium</i> sp. 1
12	SOEBI4-7S20	<i>Penicillium</i> sp. 20
13	SOEBI6-7S1	<i>Cadophora malorum Traaen</i> sp.
14	SOEBI214S2	Deuteromycete sp. 3
15	sp.1 D1T2 / 30-5-1 (a)	<i>Thelebolus microspores</i> sp.

16	sp.2 D1T2 / 30-5-1 (a)	Ascomycota sp. 2
17	sp.2 AMP 8-8	Deuteromycete sp.2

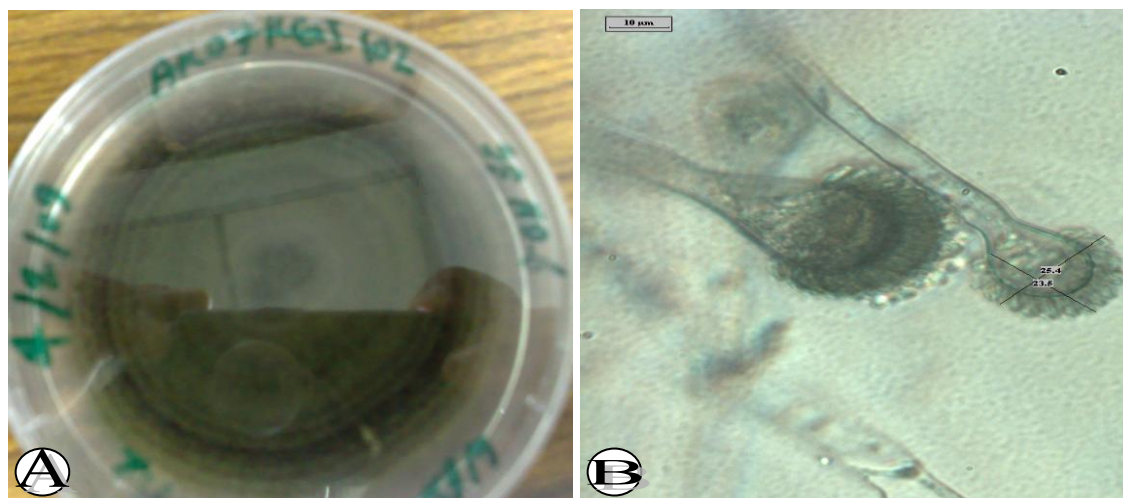


Figure 3.1a **A:** Photograph show the green colony of the Antarctic fungus (*Aspergillus* sp.) grown in PDA media. **B:** Light micrographs of Antarctic fungi under light microscopy (*Aspergillus* sp.).

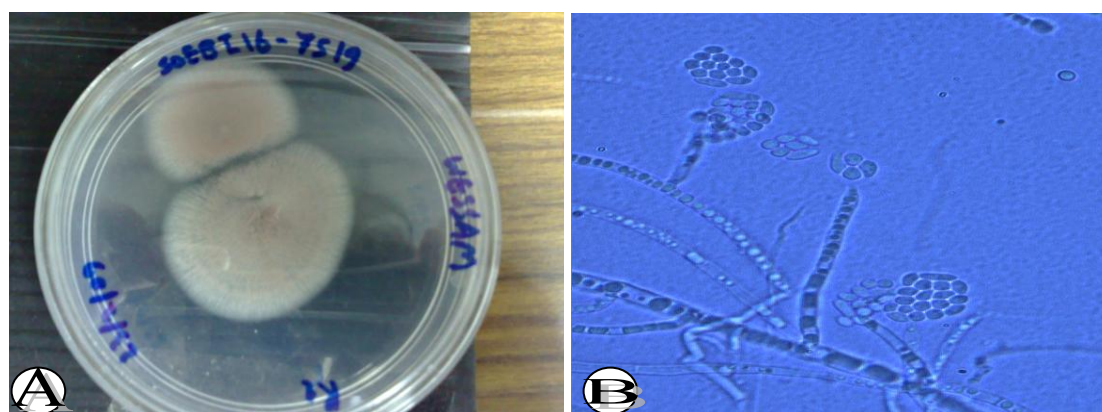


Figure 3.1b **A:** Photograph show the gray colony of the Antarctic fungus (*Aureobasidium* sp. 1) grown in PDA media. **B:** Light micrographs of Antarctic fungi under light microscopy (*Aureobasidium* sp. 1).

3.2. Bioactivity screening of fungi collected from King George Island and Greenwich Island based on plug assay

In preliminary screening using the plug assay method, 17 strains of Antarctic fungi were tested for their antimicrobial activity. Fungi with positive activity were indicated by P ($P > 6\text{mm}$), negative activity was indicated by NA (No visible zone). Results are shown in Figures (3.2 and 3.3) and Table 3.2.

Of the 10 isolates fungi from King George Island, only two isolates showed inhibition potential against the test-microorganisms (Table 3.2) and the rest of the pathogens were resistant. The two isolates were Deuteromycete sp. 18 and Deuteromycete sp. 25 which inhibited the growth of *C. albicans* and the growth of *B. subtilis* and *S. Aureus*, respectively.

Of the 7 isolates from Greenwich Island (4 from Barrientos Island, 2 from Dee Island and one from Ambato point), three strains showed positive activities. These are *Penicillium* sp., Deuteromycete sp. 2 and Deuteromycete sp. 25. Furthermore, *Penicillium* sp. isolate from Barrientos Island displayed excellent antibacterial and antifungal activities against all the pathogenic organisms. However, Deuteromycete sp. 2 from Dee Island, Deuteromycete sp. 25 from King George Island and Ascomycota sp. 2 from Ambato point show no antifungal activity, yet were active against some bacterial pathogens.

Table 3.2: Preliminary screening result of fungal against microorganisms in plug assay.

No	Code of fungi	Antibacterial activity			Antifungal activity	
		E.c	B.s	P.a	S.a	C.a
Strain from Ampato Point, Greenwich Island						
1	Deuteromycete sp. 2	P	P	NA	P	NA
Strains isolated from DEE Island, Greenwich Island						
2	Ascomycota sp. 2	NA	P	NA	P	NA
3	<i>Thelebolus microspores</i> sp.	NA	NA	NA	NA	NA
Strains isolated from Berrientos Island, Greenwich Island						
4	Deuteromycete sp. 3	NA	NA	NA	NA	NA
5	<i>Aureobasidium</i> sp. 1	NA	NA	NA	NA	NA
6	<i>Penicillium</i> sp.	P	P	P	P	P
7	<i>Cadophora malorum</i> Traaen sp.	NA	NA	NA	NA	NA
Strains isolated from King George Island						
8	<i>Aspergillus</i> sp.	NA	NA	NA	NA	NA
9	<i>Antarctomyces</i> sp. 1	NA	NA	NA	NA	NA
10	<i>Antarctomyces</i> sp. 7	NA	NA	NA	NA	NA
11	<i>Aureobasidium</i> sp. 2	NA	NA	NA	NA	NA
12	<i>Veriticillium</i> sp.	NA	NA	NA	NA	NA
13	<i>Geomyces Cretaceous</i> sp.	NA	NA	NA	NA	NA
14	<i>Geomyces</i> sp. 2	NA	NA	NA	NA	NA
15	Deuteromycete sp. 18	NA	NA	NA	NA	P
16	Deuteromycete sp. 25	NA	P	NA	P	NA
17	<i>Mortierella</i> sp.	NA	NA	NA	NA	NA

NA: No visible activity (<6mm), **P:** positive activity (>6mm), **E.c:** *E. coli*, **P.a:** *P. aeruginosa*, **B.s:** *B. subtilis*, **S.a:** *S. aureus*, **C.a:** *C. albicans*.

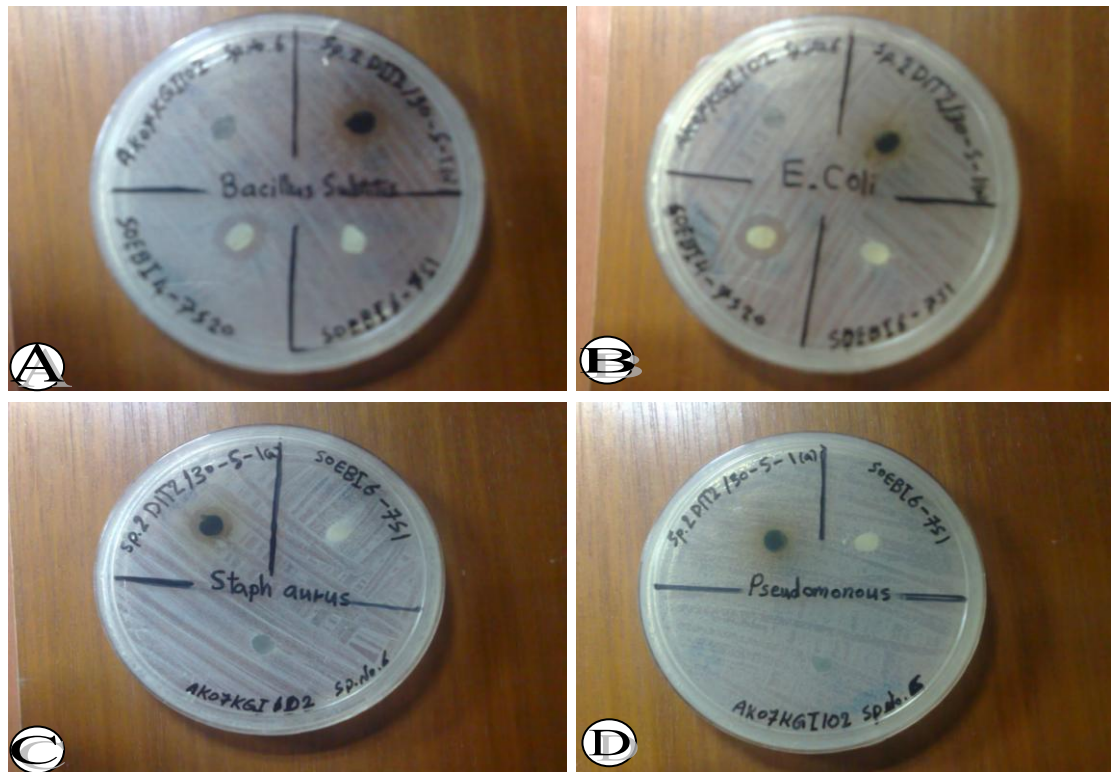


Figure 3.2 Photographs of antibacterial activity from plug assay results, **A-C**: showing intermediate to very good inhibition zones on *Bacillus subtilis*, *Escherichia coli* and *Staphylococcus aureus*. **D**: showing weak activity against *Pseudomonas aeruginosa*.



Figure 3.3 Photograph of antifungal activity from plug assay results.

3.3 Qualitative assay- Disc diffusion method

The five species, with good bioactivity on plug assay were selected to undergo the disc diffusion assay. Table 3.3 shows the strains 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 *Staphylococcus aureus*, *Bacillus subtilis*, *Bacillus cereus*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Candida albicans*, *Saccharomyces cerevisiae* and *Saccharomyces pombe*.

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

No	Fungal Code	Selected Fungal
1	SOEBI4-7S20	<i>Penicillium</i> sp.
2	Sp.2 D1T2/30-5-1(a)	Ascomycota sp. 2
3	Sp.2 AMP 8-8	Deuteromycete sp. 2
4	AK07KGI102 R1-2 Sp.2	Deuteromycete sp. 25
5	AK07KGI104 R1-2 Sp.18	Deuteromycete sp. 18

Dimethyl Sulfoxide (DMSO) (used as negative control) did not show any activity among the organisms. Although the fungal extract showed average from good to non activity against test microorganisms, Chloramphenicol (the positive control) showed the highest activity among the test microorganisms. Interestingly, extract of Deuteromycete sp. 25 demonstrate good inhibition zone against *Pseudomonas aeruginosa*, while Chloramphenicol did not.

Growth inhibitory zones were recorded as the mean of the 5 replicates in millimetres alongside the standard deviation (SD) showed in Table 3.4 as well as the positive and negative controls. Total of two filamentous fungi namely *Penicillium* sp. and Deuteromycete sp. 25 (out of 5) showed antimicrobial activity. Figure 3.4 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 Sulfoxide (DMSO) on test microorganisms in millimetres using disc diffusion method.

Fungal extract	Antibacterial activity (mm)					Antifungal activity (mm)		
	E.c	P.a	B.c	B.s	S.a	C.a	S.c	S.p
<i>Penicillium</i> sp.	NA	8 ±0.5	13 ±1.6	NA	NA	NA	NA	NA
<i>Ascomycota</i> sp. 2	NA	NA	NA	NA	NA	NA	NA	NA
<i>Deuteromycete</i> sp. 2	NA	NA	NA	NA	NA	NA	NA	NA
<i>Deuteromycete</i> sp. 25	NA	10 ±1.3	17 ±1.1	NA	NA	NA	NA	NA
<i>Deuteromycete</i> sp. 18	NA	NA	NA	NA	NA	NA	NA	NA
Chl.	30 ±0.5	16 ±0.7	30 ±0.8	24 ±1.0	32 ±0.8	NA	NA	NA
DMSO	NA	NA	NA	NA	NA	NA	NA	NA

DMSO: Dimethyl Sulfoxide, **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, **(±):** Standard Deviation of 5 replicates records.

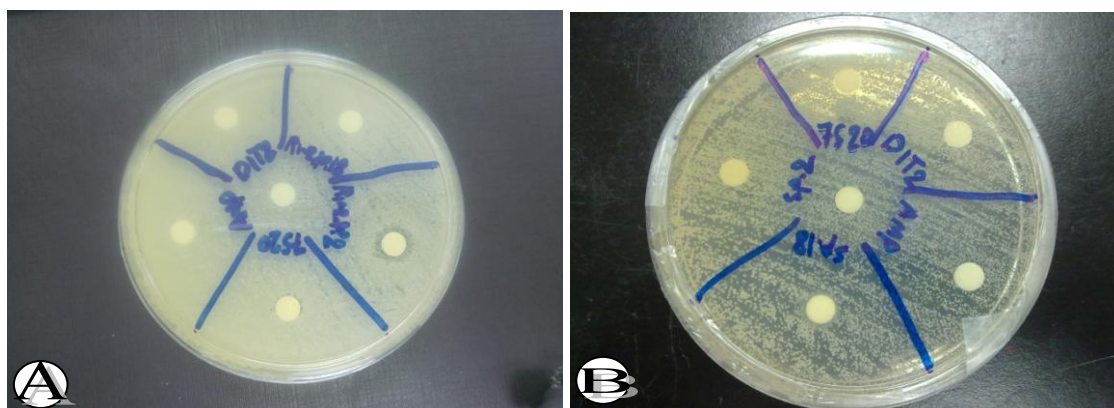


Figure 3.4 Photographs of antibacterial activity of fungal extracts in disc diffusion. **A:** only *Deuteromycete* sp. 25 extract showed good to intermediate activity against *Bacillus cereus*. **B:** showing No activity against antifungal activity (*Candida albicans*).

3.4 Quantitative assays – Broth microdilutions method

3.4.1 Minimum inhibitory concentrations (MIC)

Minimum concentrations of fungal extracts with bioactivity were determined using broth microdilution method. In Table 3.5 the Minimum Inhibitory Concentration (MIC) were recorded in mg/ml using the same test microorganisms that the extract was active on, which are *Penicillium* sp. and *Deuteromycete* sp. 25. The extract of *Penicillium* sp. recorded the highest MIC (25 mg/ml) on *B. cereus*. While the rest recorded MIC of 12.5 mg/ml. Figure 3.5 display the MIC break point determination according to the resazurine color change.

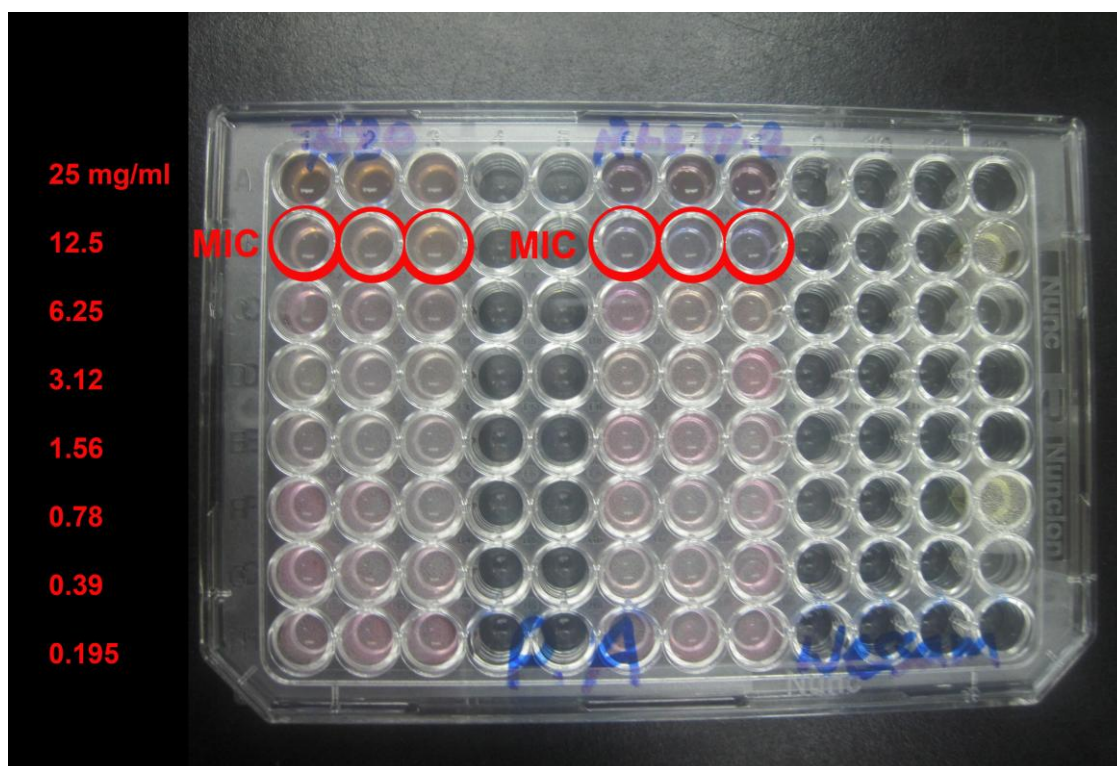


Figure 3.5 Photograph of U96 micro-well plate in broth microdilutions method. The red marked wells illustrate the MIC of *Penicillium* sp. and *Deuteromycete* sp. 25 extracts on *Pseudomonas aeruginosa*.

3.4.2 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 viewing a range of fungal extracts concentrations against the test microorganisms between 25 – 1.56 mg/ml. Figure 3.5 demonstrate how simply the Minimum bactericidal concentration (MBC) and Minimum fungicidal concentration (MFC) determined on the first plate lack the visible colonies.

Table 3.5: Quantified values of MICs and MBCs of fungal extracts against test microorganism in broth micro-dilutions method.

Fungal extract (mg/ml)	Pathogens			
	<i>B. cereus</i>		<i>P. aeruginosa</i>	
	MIC	MBC	MIC	MBC
<i>Penicillium</i> sp.	25	25	-	-
Deuteromycete sp. 25	12.5	12.5		
<i>Penicillium</i> sp.	-	-	12.5	25
Deuteromycete sp. 25	-	-	12.5	12.5

CHAPTER 4

Discussion

4.1 Occurrence of Antarctic fungi from King George Island and Greenwich Island

Greenwich Island and King George Island lies within the South Shetland Islands towards the north of the maritime Antarctic region. These habitats clearly experience a relatively mild macroclimate that is strongly buffered by the surrounding ocean with chronically low temperatures. Although, soil temperatures recorded during collection of the samples (range 5–10°C) was not unusual for northern maritime Antarctic soils during the austral summer (Krishnan *et al.* 2011). Therefore, our study focused on the mesophilic and psychrotolerant fungal strains.

Within the identification limitations imposed by morphological methods, many of the fungal taxa obtained in the current study have been reported in other studies of Antarctic fungi. However, detailed characterizations of these strains in taxonomic, molecular, ecophysiological or biochemical contexts are unavailable. Some representatives of several of these strains including *Mortierella* sp., *Antarctomyces* sp., *Geomyces* sp, undetermined Deuteromycete sp., *Veriticillium* sp., *Aureobasidium* sp. *Thelebolus* sp., *Cadophora* sp., undetermined Ascomycete sp., *Aspergillus* sp. and *Penicillium* sp. (Zucconi *et al.* 1996; Wicklow and Malloch 1971; Robinson 2001; Tosi *et al.* 2002; de Hoog *et al.* 2005; Vishniac 2006; Brunati *et al.* 2009; Gesheva 2009, 2010; Krishnan *et al.* 2011).

This report is possible to be the first report of the occurrence of filamentous microfungi from the Greenwich Island soil according to British Antarctic Survey (Bridge *et al.*, 2009). On the other hand, many of excellent extensive literature were reported on the King George Island fungi, for instants, Möller *et al.* (1995), Möller and Dreyfuss (1996) and Czarnecki and Bialasiewicz (1987). However, comparing to Rose *et al.* (2009) in Admiralty Bay, King George Island, South Shetland Islands, Antarctica reported a total of 26 fungal isolates obtained by a culture-based approach. *Alternaria* and *Phaeosphaeria* were the most frequent genera, in addition to, *Entrophospora* sp. and several undescribed *Ascomycete* species. While in this study only 10 species isolated from King George Island although the same isolation approach. Nevertheless, none of the strains reported by Ross *et al.* (2009) has been reported here.

Möller and Dreyfuss in (1996) succsesfully isolated large number of Antarctic microfungi associated with Antarctic lichens and/or moss i.e: *Penicillium* sp., *Geomyces* sp. and *Thelebolus* sp., which are similarly reported in this study. While a lot others not reported here i.e: *Acremonium butyric*, *Acremonium rutilum*, *Alternaria alternate*, *Arthroderma* sp., *Ascochyta* sp., *Tolypocladium nubicola*, *Paecilomyces variotti*, *Chaetomium globosum*, *Chalara* sp. and *Fusariums* sp.

Many other studies also together revealing considerable biodiversity and species richness of King George Island the such as Mel'nik (1995) reported *Volucrispora graminea*; Jumpponen *et al.* (2003) isolated the Helotiales (*Rhizoscyphus ericae*); Bial & Czarnecki (1999) isolated *Penicillium diversum* from air and *Thanatephorus cucumeris* from soil samples; Olech (1994) isolated the *Muellerella pygmaea*; Fenice *et al.* (1997) isolated the Anamorphic Capnodiales (*Cladosporium herbarum*); Czarnecki

& Bialasiewicz (1987) isolated the Anamorph of Botryotinia (*Botrytis cinerea*) from air, *Fusarium aqueductum* from water and Mucorales (*Rhizopus stolonifer*) from fruits; Stchigel *et al.* (2003) recorded the *Thielavia Antarctica* and *Apiosordaria Antarctica*.

4.2 Bioactivity of Antarctic fungi

The growing incidence of infectious diseases is becoming a worldwide problem (Oh *et al.*, 2006). Limited life spans of antimicrobial agent because of misuse demand the efforts to search for the antimicrobials agents effective against microorganisms (Abbas *et al.*, 2008). However, visible decline in the development of novel antimicrobial agent to combating pathogens resistance to antibiotics was reported (Williams, 2002). Microorganisms are being tie together with antimicrobial compounds as microbial assemblages represent an extremely rich source for the isolation of new strains producing novel bioactive metabolites with the potential to be developed as drugs (Rojas *et al.*, 2009; Brunati *et al.*, 2009). So this exploratory study, therefore, sought to investigate the bioactivity of soil microfungi isolated from different areas King George Island and Greenwich Island (Barrientos Island, Dee Island, Ampato Point) in maritime, Antarctica.

Comparing the obtained results with those of other scientists; examples of cold-tolerant fungi with bioactivity reported to date are reviewed here:

Brunati *et al.* (2009) screened 160 filamentous fungi (from benthic mats of Antarctic lakes) for novel antimicrobial and cytotoxic compounds. They used the PDA media, solid phase and liquid phase solvent extraction, then Microtiter liquid method applied. The frequency of antimicrobial activities was more than 29% in total. Forty

seven (29%) extracts were produce antimicrobial activity Gram-positive (14%), Gram-negative (10%), and yeasts *Candida albicans* (11%) and *Cryptococcus neoformans* (8%). They reported that most of fungi with bioactivity were cold-tolerant cosmopolitan hyphomycetes such as *Penicillium*, *Aspergillus*, *Beauveria* and *Cladosporium*.

Li *et al.* (2008) reported five novel asteric acid derivatives, as bioactive compounds, isolated from the Antarctic *Ascomycete* fungus *Geomyces* sp. They revealed that compound number 7 displayed antifungal activity against *Aspergillus fumigatus*, whereas compound 8 showed only antibacterial activities against Gram-positive and Gram-negative bacteria.

Su *et al.* (1995) have successfully isolated the Antibiotic C3368-A (CA), produced by fungus from Antarctic soil sample. CA found to significantly inhibit the mice carcinoma cells, and synergistically the human oral epidermoid carcinoma KB cells. CA also enhanced the inhibitory effect on the proliferation of human hepatoma BEL-7402 cells and potentiated the inhibitory effect of MMC against colon carcinoma in mice. With no significant augmentation of toxicity in treated mice, Su *et al.* (1995), suggested the use of the newly found nucleoside-transport inhibitor, in potentiating the effect of antitumor drugs.

Selbmann *et al.* (2002) reported the isolation of filamentous fungus *Phoma herbarum* CCFEE 5080, isolated from continental Antarctica soil, as a producer of exopolysaccharide (EPS). Selbmann *et al.* (2002) hypothesized the adaptation of *P. herbarum* CCFEE 5080 to the Antarctic environment, (low temperature, high thermal fluctuations and freeze–thaw cycling.) might be related to the EPS production ability.

Corte *et al.* (2000) isolated *Penicillium* sp. from the sediments of ponds in continental Antarctica. The preliminary screening of *Penicillium* sp. strains showed that strains *P. chrysogenum* and *P. melinii* inhibited the growth of *E. coli* as well as Gram-positive bacteria. The *P. spinulosum* strain has an excellent activity on Gram-positive bacteria, while the majority of the other strains show greater activity on Gram-positive bacteria even if not at high levels. MICs on liquid samples were also performed for some strains, it can be seen that their efficiency is greater on Gram-positive strains. Interestingly, they conclude that, except for two strains, there are no remarkable differences among the strains coming from a temperate environment and those coming from an extreme environment. Suggesting the reason might be that strains isolated from the Antarctic could have been brought there by birds or in foodstuffs, or they could be part of a spore bank which reached this continent from a place characterised by milder temperatures.

McClintocki and Gauthier (1992) used the methanol extracts of 17 common Antarctic marine sponges (isolated from shallow waters in McMurdo Sound) to test for bioactivity against Gram-positive and negative bacteria, yeasts and fungi. Weak to moderate levels of activity were occurred in all sponges. Antimicrobial activity was more common on Gram-negative bacteria; 47% of the sponge extracts caused growth inhibition in one or more gram-positive bacteria, while 100% of the extracts caused growth inhibition in Gram-negative bacteria. Antimicrobial responses against yeasts and fungi were generally non-existent or weak, with the exception of the yeast *Candida tropicalis*, which was strongly inhibited by extracts of the sponges *Homaxonella bayourensis*, *Dendrilla membranosa*, *Kirkpatrickia variolosa*, *Gellius benedeni*, *Cinachyra antarctica* and *Scolymastia joubinia*. They concluded that antimicrobial

activity in these polar sponges is widespread but generally weaker than that found in temperate and tropical sponges.

Xiano *et al.*, (2010) studied the antifreeze activities on various fungi and pseudofungi (Stramenopila) collected from various terrestrial materials (soils, mosses, algal mats, etc.) on King George Island and east Antarctica. The morphological characteristics of all isolates were examined on potato dextrose agar (PDA), corn meal agar (CMA) or peptone yeast glucose agar (PYG) plates. Obtained strains were 12 Oomycota, 2 Hyphochytridiomycota, 15 Zygomycota, 12 Chytridiomycota, 2 Blastocladiomycota, more than 50 Ascomycete and more than 50 Basidiomycete. Xiano *et al.*, (2010) have reported the presence of extracellular Antifreeze proteins (AFPs), suggesting that AFPs are widely distributed in fungal Kingdom with many differences in their biochemical characteristics.

The marin-derived fungus *Trichoderma asperellum* strain was isolated by Ren *et al.*, (2009) from the sediment of the Antarctic Penguin Island. Reporting 1-8 different peptaibols from at least 23 genera of ascomycetous fungi or their anamorphs. Peptaibols in general possess diverse bioactivities, as antibacterials, antifungals, and antiparasitics. However, chemical investigation of its fermentation broth led to the isolation of six novel peptaibols (1-6), all of them featuring a structurally unique prolinol residue. Compounds 1-6 were tested against fungi and bacteria, but they showed only weak inhibitory activity toward the early blight pathogen *Alternaria solani*, the rice blast *Pyricularia oryzae*, and the bacteria *Staphylococcus aureus* and *Escherichia coli* with $IC_{50} > 100 \mu\text{g/mL}$ and $IC_{90} > 500 \mu\text{g/mL}$. Ren *et al.*, (2009) study used to be the first report on the production of peptaibiotics by a psychrophilic fungus isolated from an

Antarctic habitat. In comparison to the present study; they used significantly sizeable sample culture but generally applied similar cultivation and extraction methods.

Since this research is an exploratory study, it could be said that, the results of the present study were relatively worthy comparing to those obtained by the other scientists, according to the 20 – 30 % isolates with bioactivity and the activity range obtained between 8 and 32 mm in diameter.

4.2.1 Preliminary bioactivity screening based on Agar block method

The preliminary screening was bioactivity-guided and based on the analysis of bioactivity profiles from the fungal isolates, which have put into evidence that they are metabolically versatile and they can produce a chemical variety of mycotoxins and antimicrobial activities. The reason for employing the agar blocks method or plug assay in this research was mainly based on its speedy and inexpensive nature, which enables it to be most suitable for examining a large amount of experimental samples within a relatively short time. It has been used by researchers like Ezra *et al.*, (2004) and Hoskisson *et al.*, (2001) for a similar purpose. In light of all the advantages of plug assay method; there are also some disadvantages such as the concentration of active metabolite present appears to be undeterminable with the use of only this method, and also whether or not the active metabolites are secreted out to the medium or retained in the mycelia of the fungus could not be determined. In the past, it was used by researchers like Ezra *et al.* (2004) and Hoskisson *et al.* (2001) for a similar purpose.

In the present study, out of the 17 micro-fungi used in this research, only 5 strains recorded positive ($P > 6\text{mm}$) antimicrobial activity in the plug assay. This meant

that only about 29.4% of the micro-fungi tested had antimicrobial activity. Within the 29.4% viable micro-fungi, the antibacterial activity is more pronounced on Gram-positive bacteria than Gram-negative bacteria. Most of the active fungal strains showed activity against Gram-positive bacteria of *B. subtilis* and *S. aureus*. Only 11.76% of all fungal strains used in this research exhibited an activity against Gram-negative bacteria of *E. coli* and *P. aeruginosa*. According to a research by Kumar *et al.* (2006) in most cases, penicillin and some of the other antibiotic agents of fungal origin inhibited most of the Gram-positive microorganisms. This fact was very eminent in this research. Other studies by Höller *et al.* (2000) and Suay *et al.* (2000) noted that many of fungal strains have the potential to inhibit the growth of Gram-positive bacteria as compared to that of Gram-negative bacteria. This inference of the difference in resistance of these two bacteria could be explained by difference in cell wall structure of Gram-positive bacteria and Gram-negative bacteria. The cell wall of Gram-positive bacteria is less complex and lacks the natural sieve effect against large molecules. Tortora, Funke and Case (2001) explained that the outer membrane of bacteria is the most important characteristic known to serve as a barrier for the penetration of numerous hazardous environmental substances, including antibiotic molecules.

In the present study, 80% of the fungal strains with antimicrobial activity under the Plug Assay exhibited antibacterial activity as compared to only 40% for antifungal activity. Gram-negative bacteria differ significantly from Gram-positive bacteria even though both are prokaryotes whereas fungi differ more because they are eukaryotes. Although, most of fungi showed neither antibacterial nor antifungal activities, one strain of fungi (*Penicillium* sp. from Barrientos Island) exhibited both antifungal and antibacterial activities. Therefore, it can be suggested that certain bioactive compounds

in *Penicillium* sp. samples may have wide spectrum of activities, whereas very few had narrow spectrum and most having no activity at all against the different test microorganisms.

The comparison between mechanisms of resistance to antifungal and antibacterial is a useful way of developing a perspective on antimicrobial resistance (Poeta *et al.*, 1999). Poeta *et al.* (1999) stated that the structures of fungi and bacteria are significantly different (such as the diploid nature of most fungi and the longer generation time of fungi compared to bacteria), and so is the available antibacterial and antifungal agents target structures and functions most relevant to the organisms to be inhibited. Most of antibacterial agents targets on the component of bacterial cell wall, which inhibit the formation of peptidoglycan compound. In contrast, most antifungal compounds target either the formation or the function of ergosterol, an important component of the fungal cell membrane.

4.2.2 Qualitative bioactivity assay based on disc diffusion method

Disc diffusion method was simply performed to confirm the antimicrobial activity of the ethyl acetate extract of the five success microfungi which proved to have antimicrobial activity by the agar block method. In the disc diffusion method, it does not require any expensive equipment and materials. The main limitations of this method which potentially could influence the results of the test are that the inhibition zone in the disc diffusion method may sometimes not be well defined, hence making it difficult to interpret results quantitatively (Jorgensen, Turnidge and Washington. 1999). Jorgensen *et al.* (1999) further added that the use of agar for this method could not recreate the in-vivo environment for most microorganisms.

However, fungal strain with at least 8 mm diameter of zone inhibition was considered as positive. In general, the positive controls utilized to evaluate the efficacy of the extracts are actually standard antibiotics as indicated for each microorganism. The activity of extracts is usually lower than positive control. However, there is no agreement on the level of acceptance for fungi when compared with standards. Therefore, some authors consider only activity comparable to antibiotics (Duarte *et al.*, 2005). Extract of fungi exhibited low activity against bacteria when compared to standard antibiotic, thus chloramphenicol (16-34 mm in diameter: *S. aureus* = 32mm, *B. subtilis* = 24mm, *E. coli* = 30mm, *P. aeruginosa* = 16mm, *B. cereus*=30mm). According to Bauer *et al.* (1966), Chloramphenicol was considered as sensitive if diameter of inhibition zone is more than 18 mm at concentration of 30 µg per disc.

Three species (out of five strains with good antimicrobial potential from the preliminary result) were observed to totally lose their inhibition potential. The production of active metabolites can be boosted in many ways such as by using other microbes to compete for space and nutrients, and the use of different media supplied with different nutrient sources with different physiological parameters such as different pH, temperature, salinity and light (Helmholz *et al.*, 1999).

The remaining two fungi (*Penicillium* sp. and Deuteromycete sp. 25) representing 40% of the fungi specimens used for this method showed positive inhibition activity with some test microorganism. It is worth to note that both *Penicillium* sp. and Deuteromycete sp. 25 showed inhibition of different degrees to *P. aeruginosa* and *B. cereus* only. Although they all showed Inhibition capabilities to both *P. aeruginosa* and *B. cereus* but, Deuteromycete sp. 25 exhibited advantage over

Penicillium sp.. Since different fungal extracts showed different antimicrobial activity against different microorganisms, this could suggest that the ability to produce these bioactive compounds was not distributed homogenously among fungi. Suay *et al.* (2000) demonstrated that only 45% of fungal extracts tested showed the presence of active antimicrobial activity. However it is crucial to note that the fungus with the highest inhibition capabilities was 17mm under the disc diffusion method. Also two extracts (Deuteromycete sp. 25 and *Penicillium* sp.) did not show the antifungal activity in this assay that had been apparent in the agar-block assay.

4.2.3 Quantitative bioactivity assay based on broth micro-dilutions method

Disc diffusion is known to be only qualitative assay with limitations of not able to determine the Minimum inhibitory concentration (MIC) and also not distinguish between the bacteriostatic and bactericidal effects (Ncube *et al.*, 2008). Therefore, Microtiter plate or broth microdilution method was performed as offer to determined MICs of large number of sample against variety of test microorganisms with reproducible results. Beside this method could be easily sub-cultured to determine the MBCs or MFCs values (Ncube *et al.*, 2008).

In the present study, the MICs and MBCs of fungal extracts were determined wherever they active on test microorganisms. Generally, MICs values were varied among the two extracts (*Penicillium* sp. and Deuteromycete sp. 25) which were considered for this analysis. Apart from *Penicillium* sp. on *B. cereus*, all other MIC recorded in this method was 12.5mg/ml for both on *P. aeruginosa*, and *B. cereus*. The MIC value for *Penicillium* sp. on *B. cereus* was recorded as 25mg/ml. However, low MIC value does not correlate to high activity on disc diffusion assay (Ncube *et al.*, 2008). Furthermore, 75% of MBC values were observed to have same values of MIC.

These results explain that, most of fungal extracts possessed relatively low lethal dose. Again comparing to others results, Paudel *et al.* (2008) studied the MICs values of 5 Antarctic lichens against gram-positive pathogens. The MIC against *B. subtilis* was recorded between 0.037 – 0.954 mg/ml whereas against *S. aureus* was recorded between 0.069 – 1 mg/ml.

In the end, the following recommendations might prove to improve to be helpful for success in future research which are either directly or indirectly related to the objectives of this project:

- Use of more than one isolation method, will probably provide more large and diverse fungi.
- Better if use another, not tedious, method to get the fungal extract. And consider the use of more than one solvent.
- When enough extract available, well diffusion method will be an advance to evaluate the bioactivity.
- Purify and identify the bioactive compound of *Penicillium* sp. and Deuteromycete sp. 25 extract, may provide good novel bioactive compound.
- Test the isolates for other biotechnological valuable metabolites that may produce, such as enzymes.
- Additional effort to complete the identification of the fungal isolates would be the next step, as the understanding of strains taxonomy that best inhibited growth.

CHAPTER 5

Conclusions

A total of 17 different strains were cultivated on PDA medium and identified into species based on morphological characteristic and belong to the phyla Ascomycota (4 genera), Anamorphic (12 genera), Zygomycota (1 genera). The most frequently isolated species belong to the genera *Aspergillus* sp., *Antarctomyces* sp., *Aspergillus fumigatus*, *Aureobasidium* sp., *Cadophora malorum* Traaen sp., *Geomyces Cretaceous* sp., *Mortierella* sp., *Thelebolus microspores* sp., *Veriticillium* sp. and *Penicillium* sp. Voucher of slides were deposited in the Institute of Biological Science, University of Malaya, fungal collection which is located in National Antarctic Research Center, Kuala Lumpur.

In the preliminary screening of antimicrobial activity, only five fungal species of the screened fungi (*Penicillium* sp., Ascomycota sp. 2, Deuteromycete sp. 2, Deuteromycete sp. 25 and Deuteromycete sp. 18) exhibited antibacterial potential against both Gram positive and Gram negative bacteria. Nevertheless, there was no antifungal activity among the test isolates. Agar block method were found to be good, fast and reliable method for qualitative study of antimicrobial activity.

Based on disc diffusion method, only two fungal strains *Penicillium* sp., showed weak to intermediate antibacterial activity against *P. aeruginosa* and *B. cereus*, while strain Deuteromycete sp. 25 exhibited weak to very good antibacterial activity against *P. aeruginosa* and *B. cereus*.

Quantification assays showed generally comparable MICs values among the test microorganisms from 12.5-25 mg/ml. However, low MICs values found to do not correlate to high activity on disc diffusion assay. This study also showed the relatively low lethal dose of fungal extracts, since 50% of MBCs values were observed to have the same values of MIC.

Appendices

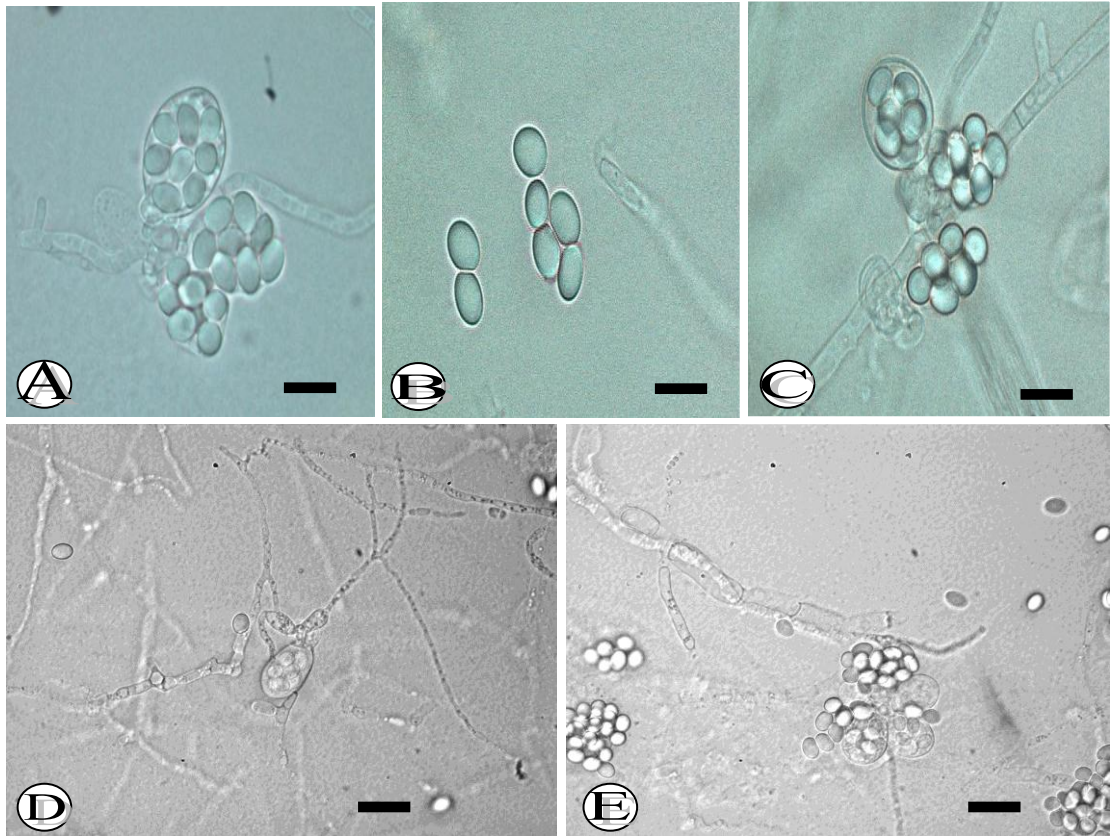


Figure 4: A-E: Micrographs of *Antarctomyces* sp. 1 (A-C) and *Antarctomyces* sp. 7 (D-E) **A.** Clusters of asci containing ascospores. **B.** Free ascospores **C.** Naked asci produced ascospores openly on septate hypha. **D.** Asci containing ascospores. **E.** Naked asci produced ascospores openly on septate hypha. Scale bar: 15 μ m.

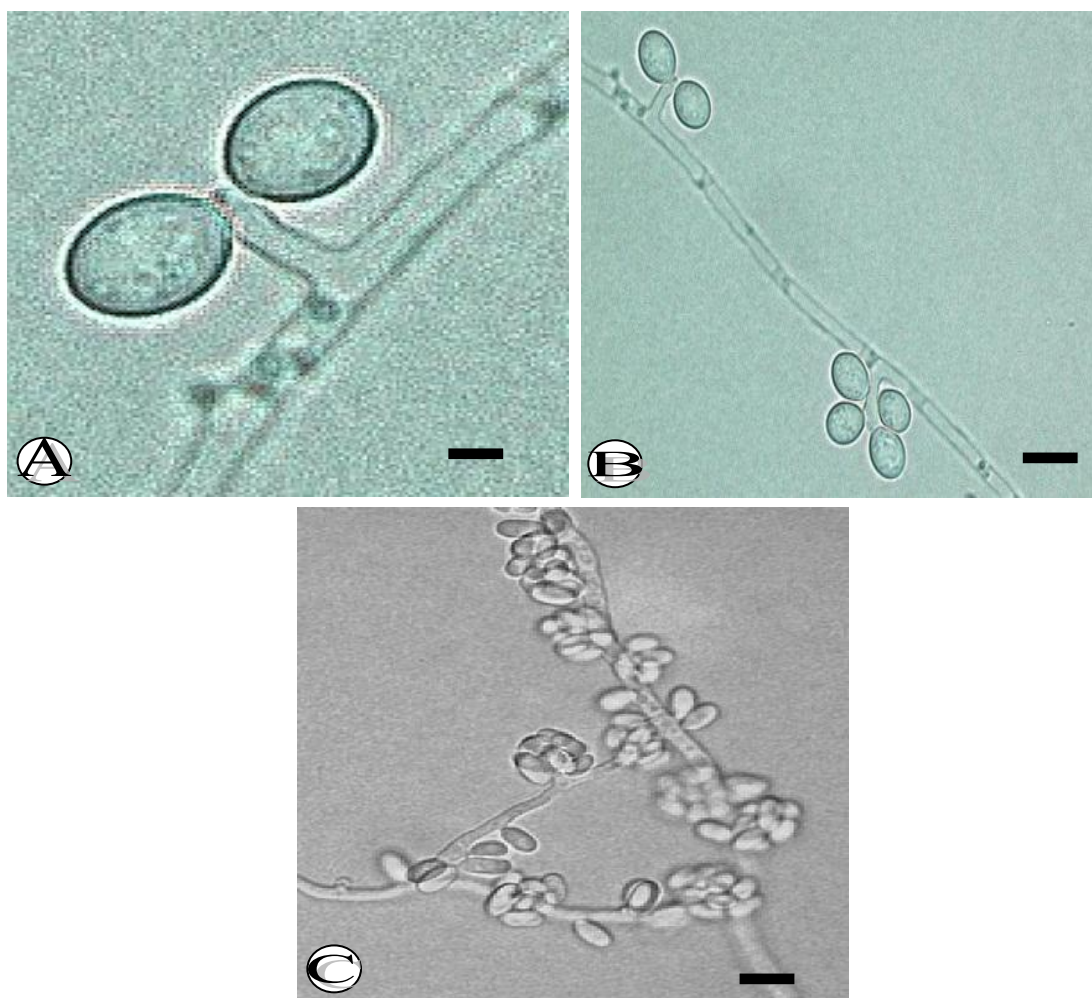


Figure 5: A-C: Micrographs A-B: *Aureobasidium* sp.2 C: *Aureobasidium* sp.1 (anamorphic fungi) Septate. The conidia are one-celled, hyaline and oval to cylindrical in shape. Scale bar: 15μm.

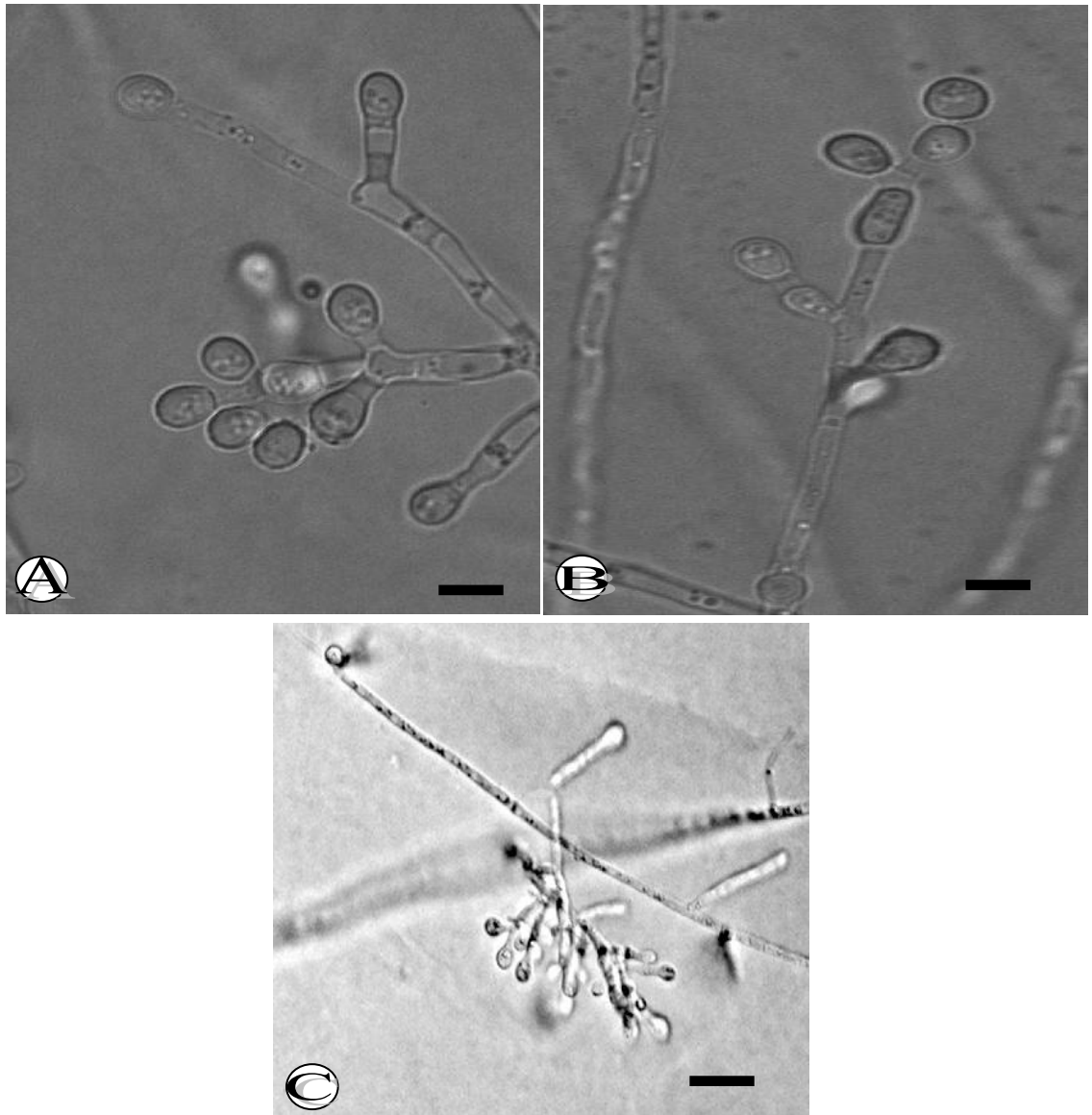


Figure 6: A-C: Micrographs of A-B: *Geomycescretaceus* sp., C: *Geomyces* sp. 2. Conidiophores branch verticillately at an acute angle (tree like branching of conidiophores with short chain of conidia). Conidia borne on the tips of conidiophores or on sides of the hyphae, are pyriform with truncate bases. Scale bar: 15 μ m.



Figure 7: Micrograph of *Veriticillium* sp. Conidiophores vertically branched over most of their length and phialides. Scale bar: 15 μ m.

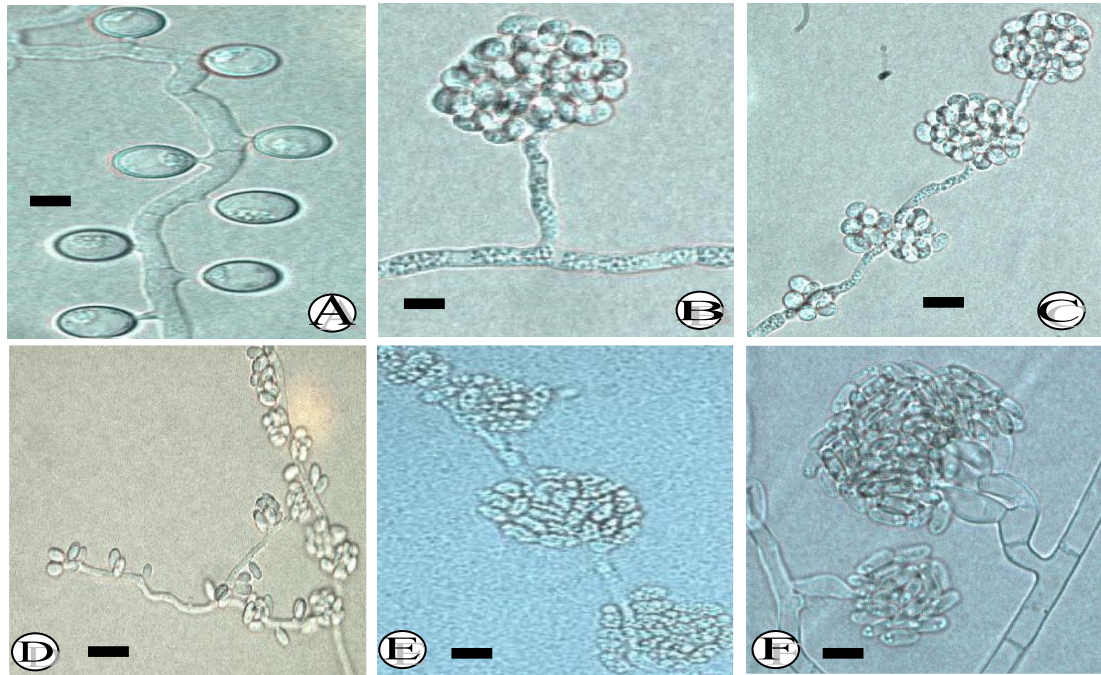


Figure 8: A-E: Micrographs of **A:** *Deuteromycets* sp. 18. Conidiophores are not clearly differentiated from the vegetative hyphae. Conidia are small, globose, smooth and hyaline. **B-C:** *Deuteromycete* sp. 2 conidia hyaline, subglobose. **D-E:** *Deuteromycete* sp. 25. **F:** *Deuteromycete* sp. 3. Scale bar: 10μm.

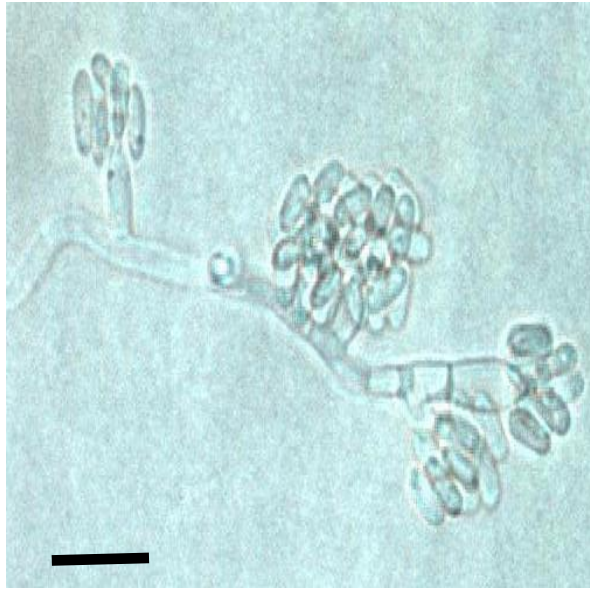


Figure 9: *Cadophora malorum*. Phialides are hyaline, develop laterally or terminally from conidiophores or directly along the hyphae itself. Conidia are elliptical and accumulate at the apices of the phialides in balls. Scale bar: 15 μ m.

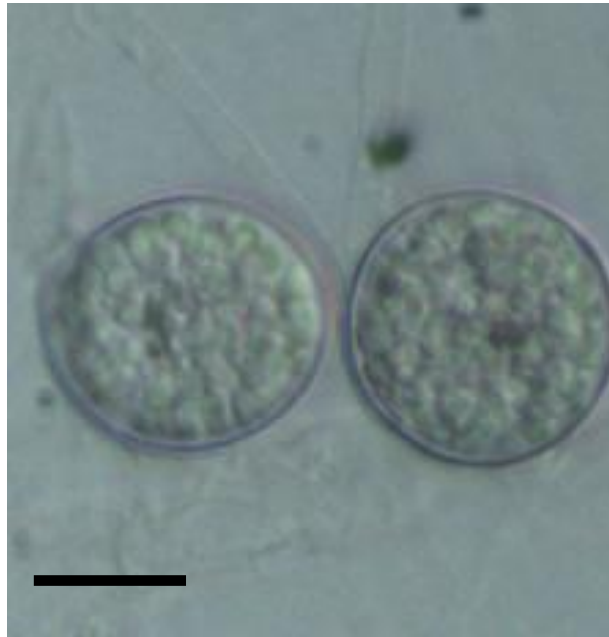


Figure 10: Micrograph of *Mortierella* sp. Conidia large and circular. Scale bar: 15 μ m.

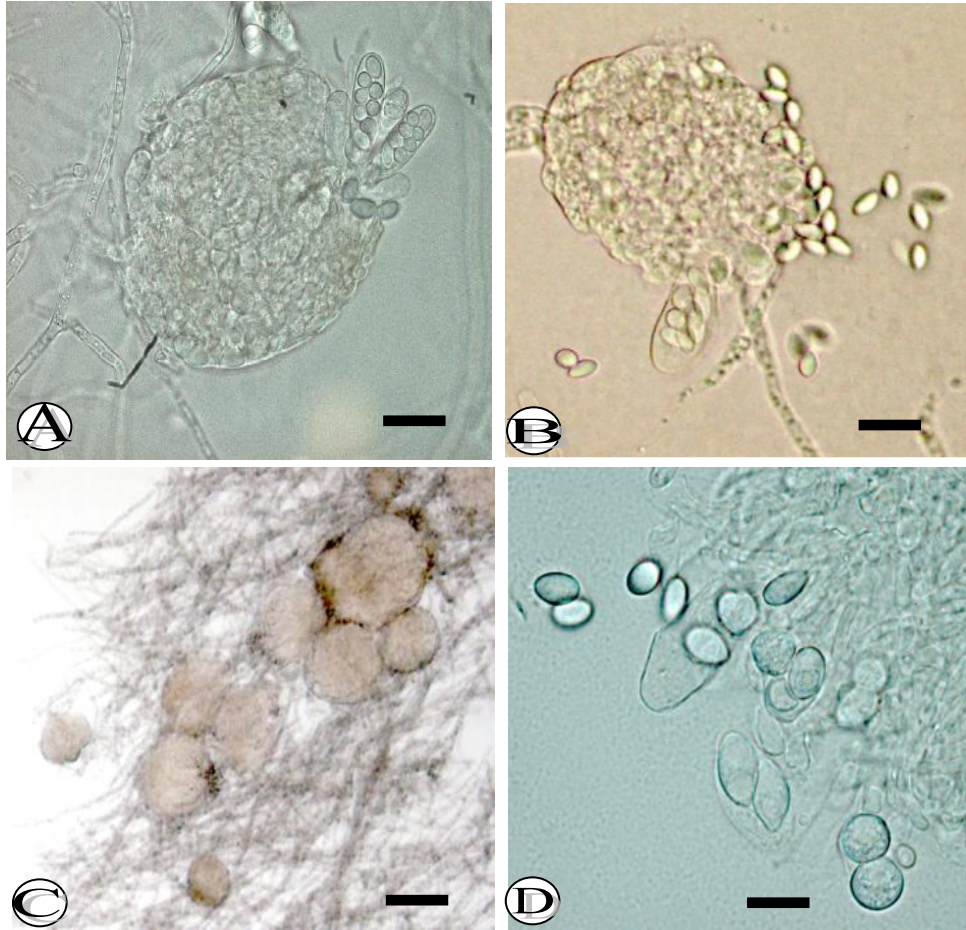


Figure 11: Micrographs of (Ascomata) **A-B:** *Thelebolus microsporus* show superficial, subglobose, hyaline. Asciclavate, 6-8 spored, apically rounded. Ascospores ellipsoidal, smooth walled. **C-D:** *Ascomycetes* sp. 2. Scale bar: 15μm.

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