Chapter 2

Materials and Methods

2.1 Materials

In this study, soil samples were collected from three different locations in within Maritime Antarctica namely: Deception Island, Wilhelmina Bay and Yankee bay (Figure 2.1). Deception Island it is an active volcano in the South Shetland Islands, off the Antarctic Peninsula, archipelago has 73 Km^2 with approximately 15 Km diameter situated at 62 58’ S and 60 39’W. It is unique landscape comprises barren volcanic slopes, steaming beaches and ash-layered glaciers. However, Wilhelmina Bay is a bay 15 miles wide between Reclus Peninsula and Cape Anna along the west coast of Graham Land. Yankee Bay (Yankee Harbor) is located within MacFarlane Strait, Greenwich Island, South Shetland Islands.

A total of 14 soil samples (Table 2.1) from Deception Island, Yankee Bay and Wilhelmina Bay, Maritime Antarctica, were collected (aseptically using sterile spoons and plastic containers and kept freezing at -20°C) during the expedition of January-February 2008 by scientist of National Antarctica Research Centre at University of Malaya. The exact place of collection was recorded with a Global Positioning System (GPS).

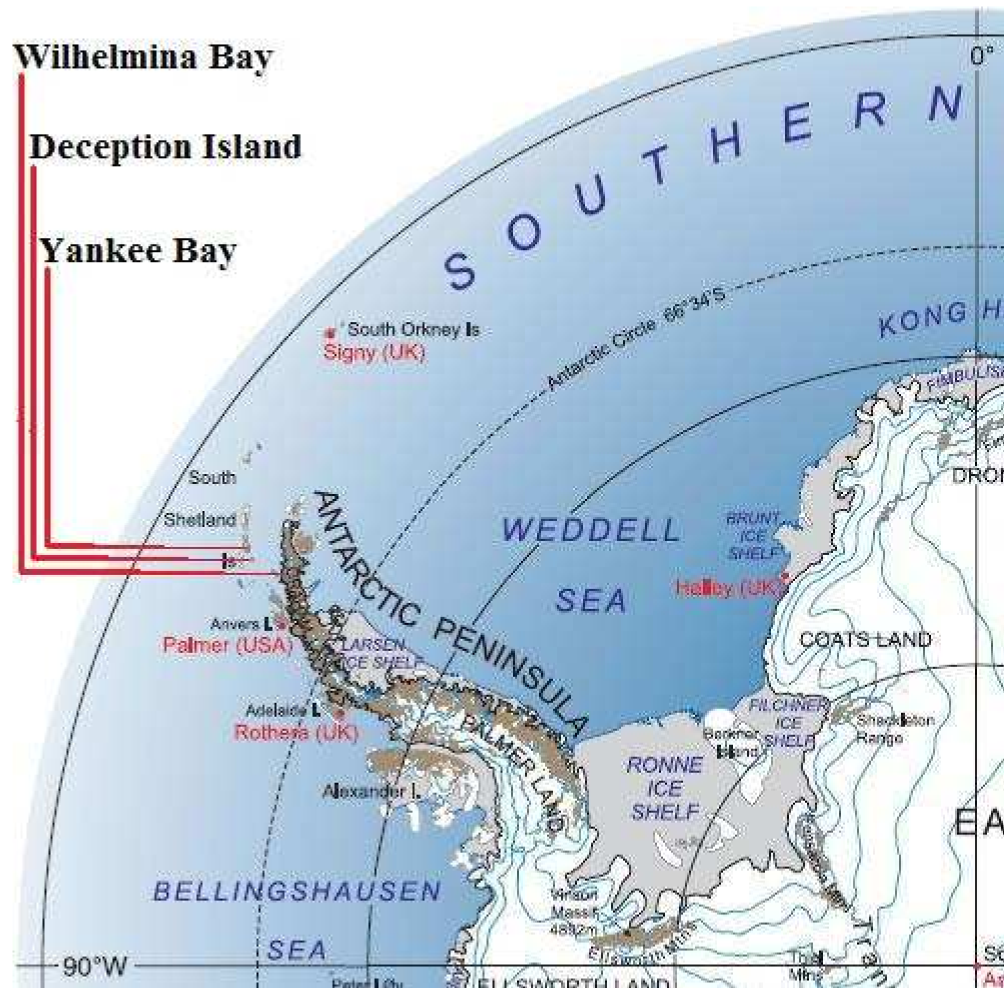

Figure 2.1 Map highlighting the locations of the three interested areas. Customized photograph adopted from [www.geology.com/worldantarctica-map/html].

Table 2.1 Soil samples exact location by GPS, date of collection and descriptions if any.

NO.	Code	Location	Description	GPS	Date of collection
1	DCP-1	Deception Island	98ºC, pH 5, volcanic soil	S 62° 58’ 42.2" W 60° 42’ 71.5"	26.1.2008
2	DCP-2	Deception Island	98ºC, pH 5, volcanic soil	S 62° 58’ 42.2" W 60° 42’ 71.5"	26.1.2008
3	DCP-3	Deception Island	98ºC, pH 5, volcanic soil	S 62° 58’ 42.2" W 60° 42’ 71.5"	26.1.2008
4	DCP-4	Deception Island	94ºC, pH 5, sea side	S 62° 58’ 25.0" W 60° 43’ 92.5"	26.1.2008
5	DCP-5	Deception Island	94ºC, pH 5, sea side	S 62° 58’ 25.0" W 60° 43’ 92.5"	26.1.2008
6	WHB-1	Wilhelmina Bay	Rudy area	S 64° 33’ 495" W 62° 11’ 279"	1.2.2008
7	WHB-2	Wilhelmina Bay	-	S 64° 33’ 25.0" W 62° 11’ 269"	1.2.2008
8	WHB-3	Wilhelmina Bay	Soil on hill with mosses	S 64º 33’ 474" W 62º 11’ 230"	1.2.2008
9	WHB-4	Wilhelmina Bay	Soil on hill	S 64º 33’ 520" W 62º 11’ 322"	1.2.2008
10	YKB-1	Yankee Bay	-	16	22.1.2008
11	YKB-2	Yankee Bay	-	19	22.1.2008
12	YKB-3	Yankee Bay	-	19	22.1.2008
13	YKB-4	Yankee Bay	-	2	22.1.2008
14	YKB-5	Yankee Bay	Rocky area	S 64º 20’ 074" W 64º 08’ 291"	2.2.2008

GPS: Global Positioning System.

2.2 Methods

2.2.1 Isolation of fungi from soil – Soil Plating Technique

Fourteen soil samples from focused sites were moved from -20°C to 4°C in the refrigerator for 1 week to allow the temperature to equilibrate and reduce the amount of condensation. Then, modified soil-plating technique described by Warcup (1950) was applied to isolate fungi from soil. Approximately 0.01 - 0.015g of soil was placed on the sterile Petri dish and 8-10ml of cooled medium was added then soil participles dispersed throughout the agar by gently rotating the plate before the agar solidified. Potato Dextrose agar (PDA) was the isolation medium. The plates were incubated at 4 and 25°C, however, for samples from Deception Island; the samples were also incubated at 50°C, as it is known a volcanic soil. Five replicates were prepared for each incubation temperature.

The plates were then examined and sub-cultured of any visible growth everyday (to avoid the overgrowth of fungal colonies over each other) for 2-4 weeks. Pure isolated colonies were undergo to identification to species level as possible. Identification method relies solely on the microscopic morphology features of conidia, plate colony features and pigment production, and optimum growth temperature. Lastly, the data was recorded and then calculated using the following formulas:

$$\text{Percentage colonization} = \frac{\text{Number of samples colonized by sporulating fungi}}{\text{Number of samples collected}} \times 100$$

$$\text{Number of fungi per sample} = \frac{\text{Total number of fungal collections}}{\text{Number of samples collected}} \times 100$$

2.2.2 Bioactivity screening

2.2.2.1 Preliminary screening-Plug assay

Preliminary screening for biological activity of fungal isolates was tested, using plug assay method, against five selected human pathogens, namely *Staphylococcus aureus*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Candida albicans.* Bacterial pathogens were inoculated by swabbing on Luria Base Agar (LBA) and Saboraud Dextrose Agar (SDA) for yeast pathogen, in less than 30 minutes after adjusted to provide an inocula equivalent to 0.5 McFarland standards. Preparations of inoculums were as in sections 2.2.2.1.1. Then, a 6mm in diameter plug from the edge of actively grown fungal mycelium on Potato Dextrose Agar (PDA) medium was cut out by the aid of No.1 cork-borer and moved into the surface of previously inoculated medium with the pathogen. Three replicate plates were incubated in 37°C for 18-24 hours in case of bacterial pathogens and 48 hours for yeast. The bioactivities were determined by appearance of clear zones of inhibition. Only fungi with good bioactivity were considered for qualitative assay by disc diffusion method.

2.2.2.1.1 Inoculums preparation-growth method

Growth method for inoculums preparation were performed by transfer of 4-5 colonies with same morphology into Muller-Hinton broth and incubated overnight. 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, and ready to use up to 30 minutes after preparation.

2.2.2.1.2 Preparation of 0.5 McFarland standard

With regular and continual shaking, 0.5 ml of 0.048 M $BaCl_2$ (1.17% w/v $BaCl_2 \cdot H_2O$) was added to 99.5 ml of 0.18 M H_2SO_4 (1% v/v). 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 (Hendrickson and Krenz, 1991).

2.2.2.2 Qualitative Assay - Disc Diffusion Method

Antimicrobial activity testing was applied by use of agar disc diffusion method (as recommended by the U.S. Food and Drug Administration and the National Committee for Clinical Laboratory standards (NCCLS)). However, it is slightly modified of that described by Kirby-Bauer *et al.* (1966). Disc diffusion method was performed to compare the spectrum bioactivity of fungal origin (natural origin) with those of chemically antimicrobial agents (Chloramphenicol). Therefore, three yeast and five bacterial strains were selected as test microorganisms, namely *Candida albicans*, *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Staphylococcus aureus*, *Bacillus subtilis*, *Bacillus cereus*, *Escherichia coli* and *Pseudomonas aeruginosa*, respectively.

Firstly, preparation of test microorganisms inocula and inoculation were done as described in Section 2.2.2.1.1. Muller-Hinton agar (MHA) was used as recommended by NCCLS. Crude extract (after extracted, section 2.2.2.2.1) was prepared by dissolved 20mg in 1ml of Dimethyl Sulfoxoid (DMSO) to obtain a concentration of 20mg/ml. Then 10µl was pipette on a sterile 6mm paper disc applied immediately on the medium of swabbed surface.

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

2.2.2.2.1 Fungal cultivation and extracellular metabolites 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) or 1-Butanol as last resort for extracting the very polar compounds, with the aid of separating funnel and vigorously shaking. Finally, EtOAc/1-Butanol layers were combined and evaporated to dryness using rotary evaporator after adjusted to 240/25mbar and 40°C and the crude extracts were stored at 4°C prior to use in both qualitative and quantitative assays.

2.2.2.3 Quantitative assays – MIC , MBC and MFC

2.2.2.3.1 Minimum inhibitory concentration (MIC)

Broth Microdilution method was performed to determine the minimum concentration of the fungal extract inhibit the pathogen growth, using Potato Dextrose Broth (PDB) as medium and dilution agent at the same time of inoculums preparation of bacterial and yeast pathogens. Quantification was carried out in U96 MicroWell plates (8x12 wells) where different concentrations (25.0 – 0.195 mg/ml) were prepared by placing of 150µl, previously inoculated Potato Dextrose broth (PDB) with pathogens after adjusted to turbidity of McFarland standard, into well labeled 1 and 100µl into each well of the rest seven wells labeled from 2 to 8. Further, another 50µl with concentration of 100mg/ml of the crude extract dissolved in Dimethyl Sulfoxoid (DMSO) as solubilizing agent were pipette to obtain a final concentration of 25mg/ml in the well number 1 after mixed thoroughly, and then a 100µl from well number 1 was transferred to the well number 2 and mixed thoroughly. Again 100µl from well number 2 was transferred to well number 3 and mixed thoroughly, repetition of transferring 100µl and mixing were made subsequently till 100µl from well number 8 was discharged. Furthermore, negative and positive controls were used to qualify the test using inoculated well without crude extract and another well of crude extract without pathogens, respectively.

Finally, plates were covered and sealed (to avoid evaporation) before incubated at 35 – 37°C overnight or 48 hours for yeast pathogens. Prior to result reading addition of resazurin dye was made and further incubation for 2 hours to clarify the break point with color change (from blue to pink). Beside, the test was carried out in triplicates and their mean recorded.

2.2.2.3.2 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.