CHAPTER 2

MATERILAS AND METHODS

Twenty species of pychrotrophic fungi were screened for their antimicrobial activity; all were isolated from the soil of Dee and Barrientos Islands, Antarctica on 22 of January 2007, using soil plating technique and then microscopically identified to see the morphology, all this work was done by Suhaila Omar.

2.1 Media preparation and subculture of selected Antarctic fungi

Potato Dextrose Agar (PDA) media was used in sub culturing of fungi, 39gm of PDA powder (weighting using digital weighting machine) were added to 1L of distilled water and mixed thoroughly. Then, the autoclave indicator tape was pasted on the cap of the bottles which will reveal dark bands if the autoclave had been done successfully. The bottles were then placed into the autoclave baskets and the media were then autoclaved at 121^{o}C for 15mins. The sterilized media were poured on to the Petri dishes in a laminar flow chamber and before working on laminar flow the UV light is switch on and left for about 15mintes to kill any microbes on it and the Petri dishes were left cooled down in laminar flow chamber.

Luria agar (LA) media was also prepared by adding 37g of LA powder to 1L distilled water and mix thoroughly, and then we followed the same steps that were used in the preparation of potato dextrose agar media.

In addition, Mueller Hinton agar, Mueller Hinton broth and Sabouraud Dextrose Agar were also prepared. All these types of media were prepared just at the time of needs to be used in the method followed the same steps.

Subcultures were made from the fungal strains (table 2.1) and the fungal growth was observed.

Table 2.1: Fungal strains and their locations

Code	**Species**	**Location**
SOEDIT2/ 75-1	Unidentified sp S_1	Dee Island
SOEDIT2/ 50-1	*Geomyce*s S_7	Dee Island
SOEDIT2/ 0-1	*Antarctomyces* S_{10}	Dee Island
SOEDIT2/ 75-4	*Geomyces* S_5	Dee Island
SOEDIT2/ 30-2	*Geomyces* S_{7a}	Dee Island
SOEDIT2/ 75-1	*Penecillium* S_{10}	Dee Island
SOEDIT2/ 80-1	Unidentified sp S_8	Dee Island
SOEBI/ 1-2	*Antarctomyces* S_{14}	Barrientos Island
SOEBI/ 12-1	*Geomyces* S_{21}	Barrientos Island
SOEBI/ 3-4	*Geomyces* S_5	Barrientos Island
SOEBI/ 4-3	*Antarctomyce*s S_{14}	Barrientos Island
SOEBI/ 6-7	*Phoma* S_1	Barrientos Island
SOEBI/ 3-1	*Geomyces* S_5	Barrientos Island
SOEBI/ 214	*Mortierella* S_2	Barrientos Island
SOEBI/ 146	*Geomyces* S_5	Barrientos Island
SOEBI/16-7	*Thelebolus* S_{19}	Barrientos Island
SOEBI/ 6-7	*Penecillium* S_{20}	Barrientos Island
SOEBI/ 12-1	*Geomyces* S_5	Barrientos Island

SOEBI/ 20-7	*Deutromycete* S_7	Barrientos Island
SOEBI/ 186	*Geomyces* S_5	Barrientos Island

2.2 Preliminary screening for biological activity of Antarctic fungi

Plug assay method was carried out to screen the biological activity of Antarctic fungi. Cultures of Antarctic fungi were grown on potato dextrose agar (PDA) without antibiotic supplements. A 6mm plug of fungal mycelium was taken from the edge of actively growing mycelia using no. 1 cork-borer and transferred into the medium containing pathogen organisms' namely *Candida albicans, Staphylococcus aurous, Bacillus subtilis, Escherichia coli* and *Pseudomonous aeruginosa.* Each were made in triplicate and incubated at 35^{o}C for 24hrs for bacteria and 48hrs for yeast. Antimicrobial activity was recorded as the clear zone of inhibition surrounding the plug of mycelia. Fungi show positive results only was selected and cultivated for further test.

2.2.1 Preparation of the test bacteria agents

Before bioassay was performed, the bacteria were prepared and cultured on the Luria agar medium and the yeast, *Candida albicans* was cultured on sabouraud Dextrose Agar (SDA). The inoculation of each test organisms were done by streaking onto Luria agar then incubated overnight at 35^{o}C. The incubation was 48hrs for yeast. After incubation the colonies appeared and by using sterile loop, growth was transferred into a universal bottle of sterile normal saline and shaken well.

The microorganisms' suspension was similar concentration of the 0.5 McFarland standard. The 0.5 McFarland standard was prepared by adding 0.5 ml of a 1.175%

(wt/vol) barium chloride dehydrate ($BaCl_2 2H_2O$) solution to 99.5 ml of 1% (vol/vol) sulphuric acid. It was shacked and poured onto the sealing tube in a dark place, it formed approximately 1.5x 10^8cfu/ml. The absorbance of 0.5 McFarland standards was checked by using a spectrophotometer at a wave length of 625 nm.

After adjusting the turbidity of the inoculums suspension, a sterile cotton swab was dipped into the suspension and slightly pressed against the inside wall of the tube just above the fluid level to remove excess liquid. Then the entire surface of the medium was swabbed and the plate rotated approximately 60 degrees after each application to ensure an even distribution of the inoculums. Finally, all around the edge of the agar surface was swabbed.

2.3 Qualitative assay- The disc diffusion method

Agar disk diffusion method described by Kirby-Bauer et al. (1966) and recommended by national committee for clinical laboratory standard (NCCLC) has been used to confirm the bioactivity of fungi from preliminary study. The disc diffusion method was a qualitative method based on the effect of antibiotics on bacterial growth on a solid medium. An inhibition zone around the antibiotic disc occurred if the organism was susceptible to the antibiotic.

This method is well documented and standard zones of inhibition have been determined for susceptible value. Many conditions can affect a disc diffusion susceptibility test. When performing these tests certain parameters were held at constant so only the size of the zone of inhibition was variable. Conditions that must be constant 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).

2.3.1 Extraction of fungal culture filtrate

The fungi were cultivated on sterile potato dextrose agar (PDA) in Petri dishes for 15 days at 4ºC. The mycelium of the fungi was removed from the petri dishes and added into 250 ml conical flasks containing100 ml potato dextrose broth. Then, the conical flasks were incubated at 4°C, under stationary phase for 10, 15 and 21 days.

After incubation, the fungal biomass was separated by centrifuge at 3000 rpm for 10 minutes at 4ºC to make a better separation. As a result, two layers were observed. Then, cultures were harvested by filtering the mycelium under vacuum using vacuum pump and the filtrates were extracting twice with an equal volume of ethyl acetate (EtOAc) (1:1). The EtOAc layers were combined and evaporated to dryness using rotary evaporator 240 mbr at 100 rpm at 40^{o}C. At last, the weight of the extract will measure and store at 4º C prior to use.

2.3.2 Bioassays against test microorganisms

Inhibition of bacteria and yeast growth by EtOAc extracts was measured by the disc-diffusion method. Bacterial strains used were *Bacillus subtilis, Escherichia coli, Pseudomonous aeruginosa, Staphylococcus aureus and Bacillus cereus.* The yeast cultures used were *Candida albicans, Saccharomyces cerevisiae* and *Schizosaccharomyces pombe.* The concentrations of 20 mg ml^{-1} of crude fungi extract were prepared.

Preparation of test microorganisms was carried out same as in Section 2.2.1 and swab inoculation apply onto the surface of the Mueller Hinton agar plate. A sterile 6 mm paper discs were placed on the plat and 10µl of each crude extract was pipetted on the sterile discs. Dimethyle sulphpxide (DMSO) solvent was used as negative control and Chloramphenicol was used as positive control. The plates were then incubated at 35º C in an incubator and the formation of inhibition zones was observed and measure after 24 hours for bacteria and 48hours for yeast.

Finally, the degree of sensitivity of the extracts towards the bacteria and fungi was determined by measuring the zone of inhibition in mm by using a ruler. A strain was considered having an activity when at least one of its cultures extracts caused a zone of growth inhibition. All experiments were carried out in five replicates under aseptic conditions.

2.3.3 Quantitative assay – Minimum Inhibitory Concentration (MIC)

The minimal inhibitory concentration (MIC) is defined as the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation (French, 2006). The test was performed using a microdilution method using a 96 wells (12x8) microtitre plates. Four bacteria were used as test microorganisms in this quantitative assay include: *Bacillus subtilis, Pseudomonous aeroginosa, Staphylococcus aureus and Bacillus cereus.* Muller Hinton broth was used as suspension media for the preparation of test organisms.

To perform the assay, a 50 ul of sample stock solution with original concentration of 100 mg ml^{-1} and 150 ul of bacterial inoculums was pippeted to the first well which was labelled A. Then, 100 ul of inoculums were pippeted into the rest of the wells which

label from B-H. The extract and inoculums in well A were mixed carefully and 100 ul of the result mixtures were transferred to well B. The same procedure was repeated for inoculums mixtures in well B until H. This process resulting in serial dilutions of the test samples. Dimethyl sulfoxide (DMSO, Sigma) was used as negative control.

Then, the microtitre plates were incubated at 35^{o}C for 24hours. After overnight incubation 10 ul of resazurin dye were added to all wells and incubated for 2 hours. Resazurin dye was used as growth indicator in which the blue colour changed to pink in presence of growth and it was prepared by adding 11mg of resazurin powder into 200ml of warm distilled water.

2.3.4 Quantitative assay- Minimum Bactericidal Concentration (MBC)

The minimum bactericidal concentration (MBC) is the lowest concentration of antimicrobial that will prevent the growth of an organism after subculture on to an antibiotic-free media (French, 2006). Antimicrobials are usually regarded as bactericidal if the MBC is no more than four times the MIC (French, 2006).

To do the test, after overnight incubation of MIC microtitre plates and before adding the resazurin dye, the subculture were done from each well (from A-H) to 8 Luria agar plates and incubated overnight at 35^{o}C.