CHAPTER 4

DISCUSSION

4.1 Media Preparation and Subculture

Many types of suitable media can be used to support the fungal growth and there is no specific medium ideally suited for the culture of species (Alexopoulos and Beneke, 1952). There are two main groups of culture media, classified according to their composition into natural and synthetic media. The natural media contain infusions of natural substances in which their chemical compositions varies from time to time while, the synthetic one containing ingredients of known chemical compositions (Alexopoulos and Beneke, 1952). Many types of natural media had been reported and were commonly using in the fungal researches such as potato dextrose agar, malt extract and sea water medium (Helmholz, Etoundi and Lindequist, 1998).

Difco™ Potato Dextrose Agar media was used to subculture Antarctic fungi. It is a recommended media to support the growth of soil fungi due to its constituent which include Potato starch and dextrose to support luxuriant growth of fungi and low pH of the medium to approximately 3.5 with sterile tartaric acid achieves the inhibition of bacterial growth (Patrick and Murray, 2003). It is important, to avoid heating the medium after it has been acidified because this action results in the hydrolysis of the agar and impairs its ability to solidify.

Moss (1984) stated that the culture medium is the best way to allow fungi to express their secondary metabolic capability. Potato dextrose agar/broth is a simple media that has been

proven to work well to produce novel active metabolites (Moss, 1984; Jennings, 1995). Therefore, in the present study all the fungi were cultured on PDA.

Mueller-Hinton agar is used for the disk diffusion testing; it's the recommended medium for this assay (NCCLS, 2000). This unsupplemented medium has been selected by national committee for clinical laboratory standard (NCCLS) for several reasons such as, it demonstrates good batch to batch reproducibility for susceptibility testing, and it is low in sulphonamide, trimmethoprim, and tetracycline inhibitors.

4.2 Preliminary screening for biological activity of Antarctic fungi

Generally, fungi produced secondary active metabolites to survive in extreme or competitive environment (Strongman et al, 1987; Oh et al, 2005), and these secondary metabolites will be produced after balanced growth has been achieved or entering the rest phase (Bu'lock, 1961; Moss, 1984). Preliminary screening for biological activity of Antarctic fungi was carried out using plug assay method since it is easy to perform, fast and inexpensive to screen large number of samples. However, the disadvantage of using this method is that the concentration of active metabolites present is unknown and it is difficult to determine whether active metabolites are secreted out to the medium or retained in the mycelia of the fungus.

In the assay, antimicrobial activity of twenty species of selected Antarctic fungi was screened against five species of test microorganisms namely, *Bacillus subtilis, Staphylococcus aureus, Escherichia coli*, *Pseudomonas aeruginosa,* and *Candida albicans*.

4.2.1 Antimicrobial activity of Antarctic fungi using plug assay method

In the present study, most of the investigated species did not posses any activity against test microorganisms. Only seven species showed antimicrobial activity against test organisms i.e.

35% out of the fungal species and these include: *Geomyces* sp.50-1/S_7, Unidentified sp.75-1/S_1, *Geomyces* sp.12-1/S_{21}, *Penecillium* sp. 75-1/S_{10}, *Geomyces* sp. 3-1/S_5, *Geomyces* sp. 3-4/S_5 and *Geomyces sp.146*/S_5.

The antibacterial activity is common against Gram-positive bacteria than Gram-negative bacteria, most of the strains showed activity against *S. auerus* and *B. subtilis*. Kumar *et a*l (2006) stated that, the antibiotic agents of fungal origin inhibited most of the Gram-positive microorganisms and this is also shown in this study. Nedialkova and Naidenova (2004) also reported the greater resistance of Gram-negative bacteria compared to Gram-positive bacteria, their study showed that many of fungal strains inhibited the growth of Gram-positive bacteria. The difference in resistance between Gram-positive and Gram- negative bacteria could be due to the difference in cell wall structure of these two types of bacteria, in which the cell wall of Gram-positive bacteria was less complex and lack the natural effect against large molecules (Hawkey, 1998; Gould and Booker, 2000), whereas the outer membrane and the periplasmic space are present in Gram-negative bacteria (Basile et al, 1998). No activity was detected against yeast and according to Brock and Madigan (1991), a good antibiotic is not always expected to have activities that act towards all test microorganisms.

4.3 Antimicrobial activity of extracts of selected Antarctic fungi using the disc diffusion assay method

Based on the results of the mentioned above screening, five strains with high inhibition activity were selected namely, *Geomyces* 50-1/S_7, *Geomyces* 12-1/S_{21}, *Geomyce*s 3-1/S_5, *Geomyces* 3-4/S_5 and *Geomyces* 146/S_5. Their activities were checked out against bacteria: *B. subtilis, S. aureus, E. coli*, *P. aeruginosa, B. cereus,* and yeasts: *C. albicans, S. cerevisiae* and *S. pombe* using the disk diffusion assay..

We have screened twenty species of selected soil fungi isolated from two islands of Antarctica, Dee and Barrientos islands for their ability to produce antimicrobial activity. Only seven strains exhibited good activity against test organisms this result is similar to Lyutskanova *et al*., (2009), they study ninety one strains of psychrotolerant streptomycetes for their antimicrobial activity and only five strains out of ninety one showed good activity.

There is no previous study that was specifically studied in particularly *Geomyces* species from Antarctic continent for their antimicrobial activity. In the present study, difference strains of *Geomyces* species were screened, not all the strains exhibited antimicrobial activity. This indicated that different strains may not produce the same antimicrobial activity, and this may be due to their habitat from where they were isolated.

The disk diffusion method was carried out to confirm the antimicrobial activity of ethyl acetate extracts of the five strains of Antarctic fungi. This method allows categorization of most isolates as susceptible, intermediate, or resistant to a variety of antimicrobial agents. In the bioassay, dimethyle sulphoxide (DMSO) was utilized as a negative control, because it was used as a solvent so, any activity detected will be from the fungal extract. The activity produced from the fungal extracts of 8mm diameter of zone inhibition was considered as positive. Chloramphenicol was used as positive control and it consider positive if it produced a diameter of the inhibition zone of more than 18mm at a concentration of 30ug ml^{-1}(Bauer *et al*., 1966).

The diffusion method has few limitations that might have influence the accuracy and precision of the test whereby the inhibition zone is not as well defined and may lead to difficulties in interpreting and measuring quantitatively (Jorgensen *et al*., 1999). However, this method has several advantages: it is technically simple to perform and very reproducible,

the reagents are relatively inexpensive; it does not require any special equipment (Patrick *et al*., 2003).

The production of active metabolites was assessed after harvesting the fungal mycelia after 15 days cultivation. No activity detected for any fungal extracts at 10 days incubation. *Geomyces* 3-1/S_5, *Geomyces* 3-4/S_5 and *Geomyces* 146/S_5, showed high activity at 15 days but the activity decrease after 21days. *Geomyces* 50-1/S_7, *Geomyces* 12-1/S_{21} lost their activity and this may be due to the type of the solvent that was used in this study it was better to used different types of the solvent because not all the solvent have the ability to extract the active metabolites, in addition some of the fungi restored their active metabolites in the mycelia and they did not released to the media. These may be the reasons for why the two strains exhibited antimicrobial activity in the preliminary screening and did not exhibited in disk diffusion. Nedialkova and Naidenova (2004) also reported that 30% of the strains have lost the inhibition activity in their study.

All the strains were cultivated on stationary phase. The suitable cultivation conditions are required for the continuous synthesis of biological active metabolites (Helmholz *et al*., 1998). The yield of bioactive compounds can be increased by the optimization of physical (pH value, temperature, salinity and light), and chemical factors (media components and inhibitors) for the growth of microbes (Miao *et al*., 2006). On the other hand, competitive cultivation between different microbial species may also boost the production of bioactive compounds (Miao *et al.,* 2006). In addition, the type of solvent used play an important role in the extraction of the bioactive compound, the correct selection of the solvent will increase the amount of extracted compound.

Recently, there are great efforts to investigate group of fungi isolated from different part of Antarctica, for their active metabolites production (Marinelli *et al*., 2004). Some investigators

indicated production of secondary antimicrobic compounds by some strains of Antarctic fungi and 29% of micro fungal species with antimicrobial activities were isolated from benthic mats of different Antarctic lakes (Marinelli *et al*., 2004).

According to our results, this study can be improved by many ways include;

a) Different types of solvents should be used in the extraction of bioactive metabolite; here just we used ethyl acetate as solvent but many types can be used such as methanol because we do not know which one will result in good extraction.

b) There are two phases to extract the bioactive metabolites, either mycelia or culture filtrate. In this study just we used culture filtrate so; the extraction from both mycelia and culture filtrate should be done.

c) The two culture conditions for the cultivation of fungi (stationary and shaken conditions) should be carried out. here just we used stationary conditions and also other physiological parameters must adjusted such as pH, temperature, salinity and using media with different nutrients supplements to increase the yield.

d) Different incubation periods should be required to evaluate the best period that will lead to increase the production of valuable metabolites.

4.4 Quantitative assay- Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The Minimum Inhibitory Concentration (MIC) was carried out against; *B. subtilis, S. aureus, B. cereus* and *P. aeruginosa,* the values were range between 6.25 mg ml^{-1}- 25 mg ml^{-1}. The resazurin assay utilising microtitre-plate described by Drummond and Waigh (2000), has been modified to achieve more accuracy in the determination of the minimum inhibitory concentration (MIC) values of natural products, including crude extracts, against various test

microorganisms. This modified resazurin method is simple, sensitive, rapid, and reliable, and could be used successfully to assess antimicrobial properties of natural products.

The Minimum Bactericidal concentration (MBC) was carried out against; *B. subtilis, S. aureus, B. cereus* and *P. aeruginosa,* values were range between 12.5 mg ml^{-1}- >25 mg ml^{-1}.