# CHAPTER 3.0 MATERIALS AND METHODS

## 3.1 Soil samples

Soil samples were collected from various locations on Signy Island, Antarctica (Latitude 60 ºS, Longitude 45 ºW) (Figure 3.1). The soil samples which were selected for this study were collected from Backslope (BS-7), Elephant Flats (EF-1 and EF-2), Gourlay Peninsula (G-1 and G-2), Three Lakes Valley (LV-1) and Spindrift-Col (SD-1) respectively between the period of December 2005 to January 2006. The locations of soil collection can be found in Appendix A. The GPS location, date of collection and characteristics of soils are described in Table 3.1. Soil samples collected from Signy Island were kept in sterile screwed-capped containers and stored at -20˚C until use.

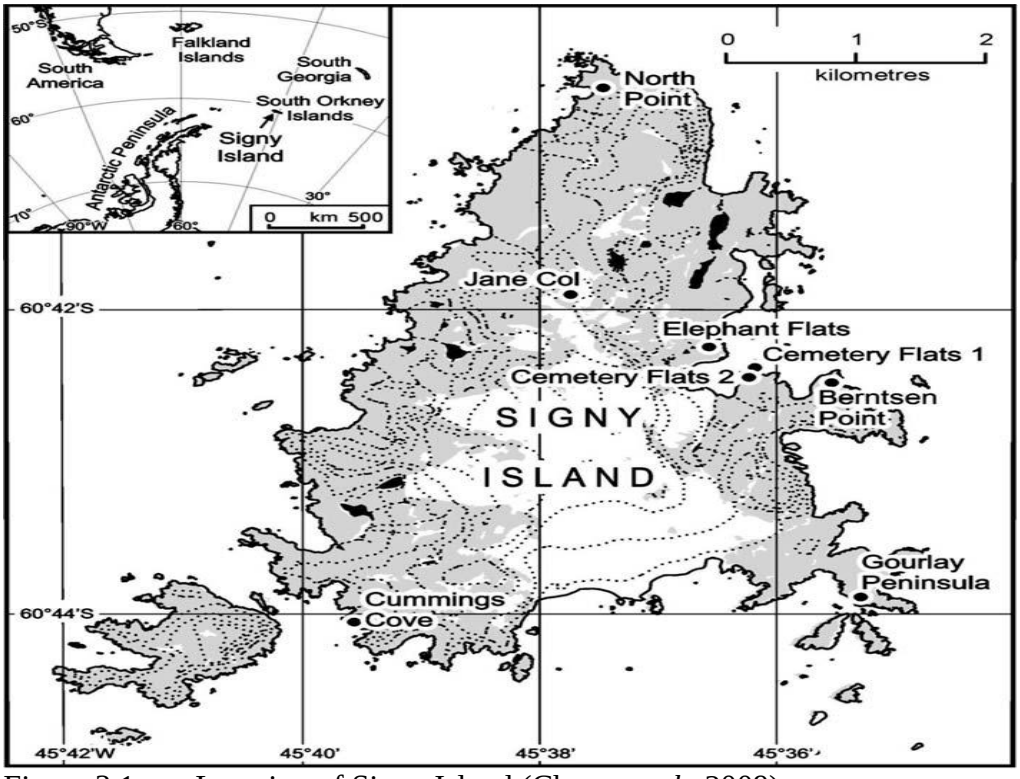

Figure 3.1 Location of Signy Island (Chong *et al.*, 2009).

Table 3.1 Details of the soil samples collected from Signy Island

| Site and GPS Location | Date of collection | Description of soil | Description of sites |
|---|---|---|---|
| BS-7<br>60 °42.578'S, 45 °35.594'W | 6/01/2006 | Clayish muddy soil | Lichens and mosses |
| EF-1<br>60 °42.233'S, 45 °36.469'W | 30/12/2005 | Coarse, rocky, grey sand | Seal wallows |
| EF-2<br>60 °42.339'S, 45°36.387'W | 30/12/2005 | Peaty brownish soil | Seal wallows |
| G-1<br>60 °43.852'S, 45 °35.296'W | 24/12/2005 | Dark brown soil | Penguin rookeries |
| G-2<br>60 °43.861'S, 45 °35.284'W | 24/12/2005 | Brownish, muddy soil | Penguin rookeries |
| LV-1<br>60 °41'914'S, 45 °36.698''W | 30/12/2005 | Dark grey muddy soil. | Inland lake |
| SD-1<br>60°41'053''S, 45 °38.341'W | 19/12/2005 | Sandy, brown soil | Barren soils |

## 3.2 Determination of soil pH

Soil samples (1g) were transferred into universal bottles containing 9ml of Ringer's solution (Appendix B). The mixture was vortexed to homogenize the samples. The pH was determined as average of three readings by measuring the soil suspensions with a Waterproof pH tester 2 (Eutech Instruments, Malaysia).

3.3 Culture Media

Various culture media in one-tenth strength were used in the study for isolation and purification of the actinobacterial isolates. The media formulations can be found in the Appendix B. Cycloheximide (25 µg/ml) and nystatin (25 µg/ml) were added to the isolation media to suppress the growth of fungi. The concentration of the antibiotics was half of what is commonly used for non-polar soils as the higher concentration of antibiotics might affect the growth of bacteria in Antarctica.

**Isolation media (one-tenth strength)**

i. Reasoner's 2A – R2A (Ronald, 1993)
ii. Reasoner's 2A – R2A with addition of 0.4% (w/v) sodium propionate (Ronald, 1993; Crook *et al*., 1950)
iii. Reasoner's 2A – R2A with addition of 50 µg/ml rose Bengal (Ronald, 1993; Ottow, 1972)
iv. SM3 medium (Gauze's medium 2) (Tan *et al*., 2006)
v. Starch Casein Nitrate (SCN) agar (Kuster and Williams, 1964)
vi. Starch Casein Nitrate (SCN) agar with addition of 2% NaCl (Kuster and Williams, 1964; Mackay, 1977)
vii. Tryptic soy agar (Difco laboratories)
viii. Tryptic soy agar with addition of 0.1% (w/v) starch (Difco laboratories)
ix. Tryptic soy agar with addition of 0.1% (w/v) colloidal chitin (Difco laboratories)

**Purification medium**

i. Yeast Malt Extract agar (ISP2) (Shirling and Gottlieb, 1966)

**Antimicrobial assay media**

i. Nutrient agar

## 3.4 Isolation and enumeration of actinobacteria from soil

1g of the soil sample was transferred into a universal bottle containing 9ml of Ringer's solution (Appendix B). 1ml of this $10^{-1}$ soil suspension was transferred to a second test tube containing 9ml of ringer's solution and this was repeated until the $10^{-4}$ dilution. 100 µl of the soil suspension from all dilutions were transferred by spread plate technique on various isolation media (Appendix B). In addition, for the isolation on tryptic soy agar using LV-1 soil, 1g of soil was enriched with one-tenth tryptic soy broth with addition of sterilized hairs as carbon and nitrogen source for 1 day, at 15 ℃ before inoculation onto the isolation medium to improve the growth of actinobacteria.

All plates were subjected to 15 ℃ incubation for a period of one month to four months. This incubation temperature was chosen because Antarctic bacterial soil isolates or psychrophiles have both their optimum and upper limit between 15 ℃-20 ℃ (Russell, 2006). Well separated bacterial colonies as well as actinobacterial colonies were counted for enumeration purposes.

## 3.5 Purification of actinobacteria

Dry, powdery and raised colonies were picked using inoculation needle and dilution streaked on ISP2 medium to purify the colonies. Pure cultures were incubated at 15 ℃ for 20 days. Aerial mycelia, substrate mycelia as well as absence or presence of diffusible pigment determined by the change of colour in the agar were recorded by referring to the National Bureau of Standards Colour Name Charts (http://swiss.csail.mit.edu/~jaffer/Color/nbs-iscc.pdf).

### 3.6 Morphological observations

Characteristics of actinobacteria colonies such as form, size, colour, elevation and margin were observed. The colours of aerial mycelia, substrate mycelia and diffusible pigments of the isolates were recorded after incubation at 15℃ for 20 days. Actinobacterial isolates were also incubated at 25℃ and their growth and morphology recorded.

Cell morphology of the isolates were observed using the Gram stain and coverslip methods. In Gram stain method, heat fixed cells on slides were flooded with crystal violet for 1 minute and then rinsed with water. Iodine, which acts as a mordant was added onto the smear for 1 minute before the decolourizing step with acetone. Cells were counterstained with safranin for 30 seconds and then rinsed with water. The slides were then examined under microscope under 100X, 400X and 1000X (Nikon SE, anti-mould, Japan).

The coverslip method (Kawato and Shinobu, 1959) was employed to observe the mycelial and spore formation of the isolates. Sterile coverslips were inserted at 45 $^{o}$into the agar medium and isolates were streaked on the agar near the coverslips. The agar plates were incubated at 15℃ for 20 days before the coverslips are removed, air-dried observed under microscope.

## 3.7 Analysis of Diaminopimelic acid isomers

Thin layer chromatography method was used to identify the diaminopimelic acid in the cell walls of actinobacteria (Staneck and Roberts, 1974). Actinobacteria can be distinguished to a genus level by determining LL-DAP and *meso*-DAP cell wall type. In this study, a modified method was applied to identify the diaminopimelic acid of the actinobacterial isolates.

Representatives of each group of actinobacteria from ARDRA method was selected and grown on ISP2 agar for 14 days. Two loopfuls of cells were scrapped from the strains and transferred into 250 µl of 6N HCL followed by vortex until homogenize. The mixtures were then wrapped with aluminium foil and autoclaved for 121 ℃, 15psi for 20 minutes.

The tubes were then centrifuged at 13,000 rpm for 15 minutes after autoclaving the strains. Then, the supernatant is transferred into a new eppendorf tube. The eppendorf tubes were then kept at 100 ℃ to evaporate and concentrate the mixture using a heat block (Eppendorf, Cambridge). The residue formed was resuspended with 30 µl sterile distilled water, followed by evaporating it at 100 ℃.

After evaporation of the strains for the second time, the tubes were resuspended with 10 µl of sterile distilled water for spotting purposes. The concentrates were spotted on the 20cm X 20cm TLC cellulose plate (Merck, Germany) for 15-20 times using a capillary tube. The standard D, L-α, ε Diaminopimelic acid (Sigma, Germany), which contains the *meso*- and LL-DAP isomers were used as a reference standard.

The spotted cellulose plates were then placed into the tank of mobile phase containing methanol-distilled water-6N HCL-pyridine (80:26:4:10 v/v) (Appendix C). The plate is developed in the tank for 3 hours. After 3 hours, the plate was sprayed with 0.2% (w/v) ninhydrin solution in acetone. The cellulose plate was then heated at 80 ℃ for 5 minutes and results were recorded.

## 3.8 Molecular characterization

### 3.8.1 DNA extraction from soil samples

DNA extractions were performed using a method modified from the procedure described by Kowalchuk *et al*. (1998) and Griffiths *et al*. (2000). DNA extraction from soil samples was performed by adding 0.5ml 5% (w/v) hexadecyltrimethylammonium bromide (CTAB) buffer (w/v) in 0.7M NaCL with 240mM phosphate buffer and 0.5ml phenol-chloroform iso-amyl alcohol (25:24:1, v/v) to 0.5g of soil and 0.25g of glass beads (<106µm; Sigma G-4649) in 2-ml-vials. The samples were lysed for 1 minute on a Mikro-dismembrator u (B.braun Biotech International, Germany) at 1000rpm. The aqueous phase that contained nucleic acids was separated by centrifugation at 12,000 rpm for 5 minutes. The aqueous phase was transferred to a new microfuge tube and the phenol is removed by mixing with an equal volume of chloroform-iso-amyl alcohol (24:1, v/v) followed by repeated centrifugation. Total nucleic acids were subsequently precipitated from the extracted upper aqueous phase with two volumes of 30% (w/v) polyethylene glycol 6000-1.6M NaCl for 2 hours at room temperature, followed by centrifugation at 14,000 rpm for 10 minutes. Pelleted nucleic acids were then washed with ice cold 70% ethanol and air dried prior to resuspension in 30 µl TE buffer (Appendix D). The quality of DNA preparations was checked by agarose gel electrophoresis (0.8%, w/v) run for 30 minutes. The nucleic acid preparations were visualized using UV imager (Cleaver Scientific, UK) and the products were stored at -20 ℃ until further use.

### 3.8.2 DNA extraction from pure cultures

Total genomic DNA was extracted using a modified method (Sambrook *et al*., 1989; Tan *et al*., 2006). A loopful of bacterial cells was picked from a freshly grown pure colony. The cells were suspended in 150 µl of TE buffer containing glass beads

(<106µm; Sigma G-4649), 2.5 µl of lysozyme (50mg/ml) and 5 µl of Proteinase K (20mg/ml). The suspension was mixed by vortexing, incubated at 37℃ for 2 hours and centrifuged at 14,000 rpm for 10 minutes. The supernatant was then transferred into a new tube, incubated at 75℃ for 15 minutes and centrifuged. The quality of DNA preparations was checked by agarose gel electrophoresis (0.8%, w/v) run for 30 minutes. The nucleic acid preparations were visualized using UV imager (Cleaver Scientific, UK) and the products were stored at -20℃ until further use.

### 3.8.3 16S rRNA gene amplification

The DNA extracts from soil samples and pure cultures were used as template DNA (*ca*. 50µg/µl) in 50 µl reactions containing 0.2mM of each of the four dNTPs, 0.2µM of primers 27f (5'AGAGTTTGATCMTGGCTCAG3') and 1525r (5'AAGGAGGTGATCCAGCC3') (Lane, 1991) for soil DNA or 0.2µM of primers 27f (5'AGAGTTTGATCMTGGCTCAG3') and 1492r (5' TACGGYTACCTTGTTACGA CTT3') (Lane, 1991) for pure cultures, 1.5mM $MgCl_2$ and 1.25 U Bio*Taq* DNA Polymerase (Fermentas, Lithuania) with the appropriate 1x reaction buffer. Controls without template DNA were included in each PCR experiment. Amplifications were performed in a Swift$^{TM}$ MAXI, ESCO thermal cycler, Singapore according to the following profile: initial denaturation of 5 minutes at 95℃ prior to the addition of *BioTaq*, followed by 30 cycles of one minute each at 94℃, 55℃ and 72℃, and a final 10 minute incubation at 72℃. A 1500bp length of the amplification products were analysed by agarose gel electrophoresis (1.0%, w/v) run for 45 minutes. The amplification products were visualized using UV imager (Cleaver Scientific, UK) and the products were stored at -20℃ until further use.

### 3.8.4 Amplification using Actinobacterial specific primers

DNA preparations from all pure culture isolates were used as template DNA for Bio*Taq* DNA Polymerase (Fermentas, Lithuania). However, for soil samples, the 16S rRNA genes were first amplified using PCR and were then diluted to $10^{-1}$ prior to being used as template (nested PCR amplification). The reactions were preformed in a final volume of 25µl containing 0.2mM each of the four dNTPS, 0.1µM of primers S-C-Act-235-a-S-20 (5'CGCGGCCTATCAGCTTGTTG3') and S-C-Act-878-a-A-19 (5'CCGTACTCCCCAGGCGGGG3') (Stach *et al*., 2003), 3mM $MgCl_2$ and 1.25 U Bio*Taq* DNA Polymerase (Fermentas, Lithuania) with the appropriate 1x reaction buffer.

Amplifications were performed using a Swift$^{TM}$ MAXI, ESCO thermal cycler according to the following profile: initial denaturation at 95℃ at 4 minutes prior to the addition of *Taq* DNA polymerase (Fermentas, Lithuania), followed by 35 cycles of 95℃ for 30seconds, 70℃ for 1 minute, 72℃ for 1 minute and a final 10 minutes incubation at 72℃. A 640bp length of the amplification products were analysed by agarose gel electrophoresis (1.0%, w/v) run for 45 minutes The amplification products were visualized using UV imager (Cleaver Scientific, UK) and the products were stored at -20℃ until further use.

### 3.8.5 Dereplication of isolated actinobacterial strains using ARDRA

In this study, ARDRA method was employed to dereplicate the isolated actinobacterial strains. Two restriction enzymes, *Bss*MI (Vivantis, Malaysia) and *Hha*I (Promega, USA) which recognition sequence were 5'-↓GATC-3' and 5'-GCG↓C-3' respectively, were used to digest the amplified 16S rRNA fragment. For dereplication using *BsSM*I, the reactions were preformed in final volumes of 20 µl containing 4 µl of 10X storage buffer, 15 µl of of 16S rRNA products and 1 µl of *BsSM*I restriction

enzyme. For dereplication using *Hha*I restriction enzymes, the reactions were preformed in a final volume of 20 µl containing 17.3 µl of 16S rRNA products, 0.2 µl acetylated BSA (10 µg/ µl), 2 µl of 10X reaction buffer C and 0.5 µl of *Hha*I restriction enzyme. The restriction digestions were incubated at 37 ℃ for 3 hours on a heat block. The digestions were analysed by agarose gel electrophoresis (1.5%, w/v) run for 1 hour at 110V. The banding patterns were visualized using UV imager (Cleaver Scientific, UK).

### 3.8.6 Partial sequencing of 16S rRNA genes

16S rRNA products were purified by using QIAGEN (QIA quick® PCR purification kit, USA) and sent for sequencing to 1st Base, which uses Applied Biosystems 3730xl DNA analyzer. The sequence results were analyzed by using BLAST search (Altschul *et al*., 1990) in the NCBI database (http://www.ncbi.nlm.nih.gov/blast).

### 3.8.7 Phylogenetic analysis of actinobacterial isolates

The sequences from each ARDRA group representatives were aligned using Clustal W and neighbour-joining (NJ) method (Saitou and Nei, 1987) were used to construct the phylogenetic tree with bootstrap analysis of 1000 replications. Phylogenetic dendrograms were constructed using Molecular Evolutionary Genectics Analysis (MEGA) version 4.1 (Tamura *et al*., 2007; Kumar *et al*., 2008).

## 3.9 Primary screening of actinobacterial isolates for antimicrobial activity

Actinobacteria isolates were tested for antimicrobial activity against *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* strain M16, *Proteus vulgaris* ATCC 13315, *Salmonella typhi* ATCC 14028, *Staphylococcus aureus* ATCC 25923 and

*Staphylococcus epidermidis* ATCC 12228. The bacterial cultures were incubated at 37℃ overnight. Well-grown colonies were picked and transferred to a test tube containing 4ml sterile saline. The bacterial suspension was then compared with 0.5M MacFarland standard. A sterile cotton swab was dipped into the inoculum suspension and inoculated on the surface of nutrient agar by horizontal streak plate method (Appendix B).

Pure isolates of actinobacterial strains were cultured on nutrient agar and incubated at 15℃ for 20 days. Plaques of the actinobacterial strains were obtained by sterile straws and placed on to the nutrient agar inoculated with the test bacteria. The cultures were incubated for an hour at 15℃ for metabolite diffusion followed by incubation at 37℃ for 24 hours modified from Bauer *et al*. (1966). The diameters of zone of inhibition were recorded in mm.

### 3.10 Detection of NRPS genes in actinobacterial isolates

All isolated actinobacterial strains were screened for NRPS genes. DNA templates (*ca*. 50 µg/µl) from isolates were used as template DNA for NRPS gene detection. The reactions were prepared in a final volume of 50µl containing 0.2mM each of the four dNTPS, 0.4µM of primers A3f (5'CSTACSYSATSTACACSTCSGG3') and A7r (5'SASGTCVCCSGTSCGGTAS3'), 10% DMSO and 1 U Bio*Taq* DNA Polymerase (Vivantis, Malaysia) with the appropriate 1x reaction buffer S (Ayuso-Sacido and Genilloud, 2005). A 700-800bp length of the amplification products were analysed by agarose gel electrophoresis (1.0%, w/v) run for 45 minutes. The amplification products were visualized using UV imager (Cleaver Scientific, UK) and the products were stored at -20℃ until further use.