CHAPTER 5.0 DISCUSSION

5.1 Isolation of actinobacterial strains

The presence of actinobacteria in soils are greatly influenced by the soil types, pH, location, soil temperature, organic matter content, aeration and vegetation (Basilio *et al*., 2003**;** Labeda, 1990). Soil samples collected from the Backslope site with lichens and mosses, Spindrift Col site with barren soil and Gourlay Peninsula site with penguin rookeries, Elephant flats site with seal wallows were slightly acidic whereas soils from the Three Lakes Valley site with inland lake were slightly alkaline (Table 4.1). Soils from the Gourlay site and Elephant Flats site were slightly acidic were probably due to the presence of guano from penguins and seals. Higher organic content loading was also associated with lower pH values (Mayer and Xing, 2001).

Fifty three actinobacterial strains were isolated from the Three Lakes Valley site which was slightly alkaline. All the strains showed high homology to *Streptomyces beijiangensis* (Table 5.1). Previous reports showed that soil actinomycetes favour neutral and slightly alkaline conditions especially *Streptomyces* spp. have better growth condition in neutral or alkaline soils (Basilio *et al*., 2003; Waksman, 1967). The soils from Backslope which contain lichens and mosses were slightly acidic and this might be due to the microbial decomposition of plant materials (Cho *et al*., 2006). Six actinobacterial strains isolated from this site showed high homology to *Mycobacterium* spp. (Table 5.1). This result is similar to those from other studies that *Mycobacterium* spp. were more frequently isolated from acidic soils (Niva *et al*., 2006; Iivanainen *et al*., 1993).

Actinobacteria enumeration from all six soil samples collected from Signy Island ranged from (5.00 ± 0.71) x 10 cfu/g to 1.00 x 10^5 cfu/g. The enumeration of

actinobacteria was much lower than that of Lim (2008), who had 0.98 x 10^7 cfu/g to 8.0 x 10^7 cfu/g of actinobacteria in tropical soil samples obtained from Sarawak. Actinobacteria counts from Gandhimathi *et al*. (2008) who isolated actinobacteria from marine sponges were 2 x 10^5 cfu/g to 10 x 10^5 cfu/g which were also higher than our counts from Signy Island. The reason of lower actinobacterial counts might be due to the extreme and harsh conditions in Antarctica.

Table 5.1 Summary of characteristics of actinobacterial isolates from Antarctic Signy samples. Strain labels in bold are isolates with partial sequence of 16S rRNA gene.

Strain label	**Soil**	**ARDRA Group (Closest match in BLAST results)**	**Colour group**	**Cell wall DAP**	**Primary screening**	**NRPS**
PSY001- PSY008, PSY010, PSY011, **PSY013**, PSY014, PSY041- PSY043, PSY046- PSY051, PSY053, PSY054, PSY055, PSY057, PSY058, **PSY059**, PSY060- PSY064, PSY067 -PSY072, PSY077, PSY080, **PSY081**, PSY082, PSY088, PSY089, PSY090, **PSY097**	LV-1	1 (*Streptomyces beijiangensis*) [AB249973]	1	LL-DAP	Positive	Positive
PSY009, PSY015, PSY017, PSY018, PSY022, **PSY056**	LV-1				Negative	
PSY020	LV-1		2			
PSY012, PSY033, PSY040, **PSY092**	EF-1	2 (*Rhodococcus* sp.) [FJ195998]	3	*meso*-DAP	Negative	Positive
PSY019	EF-2					
PSY023	LV-1					
PSY038, **PSY039**	SD-1					
PSY065, PSY073	BS-7	3 (*Mycobacterium* sp.) [EU167989]	5	*meso*-DAP	Negative	Positive
PSY074, PSY075, PSY076, **PSY078**	BS-7					Negative
PSY044	BS-7	4 (*Demetria terragena*) [Y1452]	3	None	Negative	Negative
PSY045	G-1		3			
PSY066	G-1		6			

'Table 5.1, continued'

Strain label	Soil	ARDRA Group (Closest match in BLAST results)	Colour group	Cell wall DAP	Primary screening	NRPS
PSY026, **PSY027, PSY028**, PSY029, PSY034, PSY035, PSY036, **PSY037, PSY052**	BS-7	5 (*Rhodococcus Corynebacterioi-des*) [X80615]	7	*meso*-DAP	Negative	Positive
PSY025	BS-7					Negative
PSY085	G-2	6 (*Kocuria* sp.) [FJ357623]	6	None	Negative	Negative
PSY087, PSY091	BS-7					Positive
PSY095	G-1	7 (*Glaciibacter superstes*) [AB378302]	8	None	Negative	Negative
PSY079	G-2	8 (*Humicoccus* sp.) [EU939310]	15	*meso*-DAP	Negative	Negative
PSY096	BS-7	9 (Actinobacterium P23) [D1351736]	9	*meso*-DAP	Negative	Negative
PSY086	G-2	10 (*Microbacterium* sp.) [AB461113]	10	None	Negative	Negative
PSY016	EF-1	11 (*Streptomyces argenteolus*) [EU570529]	11	LL-DAP	Negative	Positive
PSY084, **PSY094**	SD-1	12 (*Marmoricola aequoreus*) [AM295338]	13	LL-DAP	Negative	Positive
PSY093						Negative
PSY024	G-1	13 (*Tsukamurella* sp.) [EF514880]	14	*meso*-DAP	Negative	Positive
PSY031	BS-7	14 (*Micrococcus luteus*) [FN984531]	8	None	Negative	Negative
PSY032	SD-1	15 (Actinobacterium kmd_307) [EU723162]	4	LL-DAP	Negative	Negative
PSY021	BS-7	16 (*Nocardia ninae*) [DQ235678]	12	*meso*-DAP	Negative	Positive

Nine different culture media were used to isolate as many actinobacterial strains as possible. Media used for this study for the isolation of actinobacteria ranged from R2A, R2A with addition of 50µg/ml rose Bengal, R2A with addition of 0.4% (w/v) sodium propionate, SCN, SCN supplemented with 2% NaCl, SM3, TSA, TSA with addition of 0.1% (w/v) starch and TSA with addition of 0.1% (w/v) colloidal chitin. One-tenth strength of isolation media were employed because the nutrient level in Antarctica is low and the soil microbes would prefer lower concentration of nutrients (Russell and Cowan, 2006). A total of ninety five actinobacterial strains were isolated from the soil samples collected from various locations on Signy Island, Antarctica (Figure 5.1). Most of the strains were isolated from the Three Lakes Valley (LV-1) site where low numbers of isolates were obtained from Elephant Flats, Spindrift Col and Gourlay sites. However, not all types of media were employed for all sites (Table 4.2 and Table 4.3) to isolate actinobacteria due to time constraints.

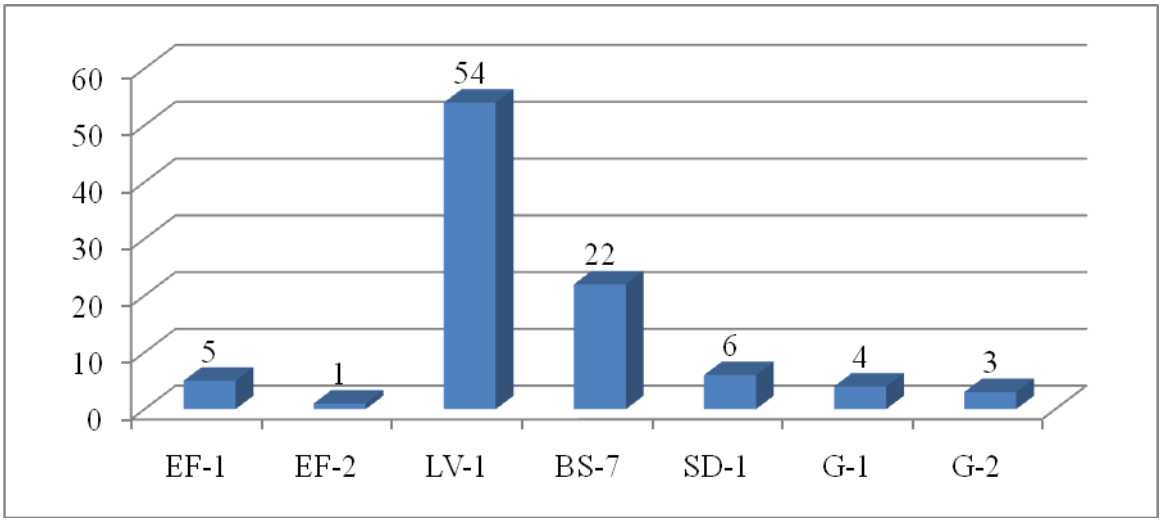

Figure 5.1 Number of actinobacterial strains isolated from Signy Island, Antarctica. EF-1 and EF-2, Elephant Flats; LV-1, Three Lakes Valley; BS-7, Backslope; SD-1, Spindrift Col; G-1and G-2, Gourlay Peninsula.

All ninety five actinobacteria strains have distinct colony appearances on the isolation media which were dry, powdery, irregular, convex with aerial mycelia ranging from white, yellow white, yellowish pink, orange, yellow and red on the isolation medium (Table 4.4). The morphology of actinobacteria colonies may be raised, flat and

sometimes covered with a leathery layer with colours ranging from white, yellow, orange, rose, red, purple, blue, green, brown and black (Midayoh *et al.*, 1997). The actinobacterial strains were then purified on to one-tenth ISP2 agar to obtain pure cultures because ISP2 characterization gives the best descriptions of actinobacteria (Shirling and Gottlieb, 1966) (Table 4.5).

A total of fifty four actinobacterial strains were isolated from Three Lakes Valley (LV-1) site using various isolation medium (Figure 5.2). Fifty three actinobacterial strains isolated from the Three Lakes Valley site showed high homology to *Streptomyces beijiangensis* where one strain showed high homology to *Rhodococcus* sp. (Table 5.1 and Table 5.2). Hence, different media formulations used did not show difference in actinobacterial diversity. This indicates that *Streptomyces beijiangensis* may be the dominant bacteria in Three Lakes Valley site. The highest numbers of actinobacteria were isolated using TSA with addition of 0.1% (w/v) starch (Table 5.2). Previous reports indicated that media containing starch or glucose as the carbon source promoted the growth of actinobacteria (Sivakumar *et al.*, 2007).

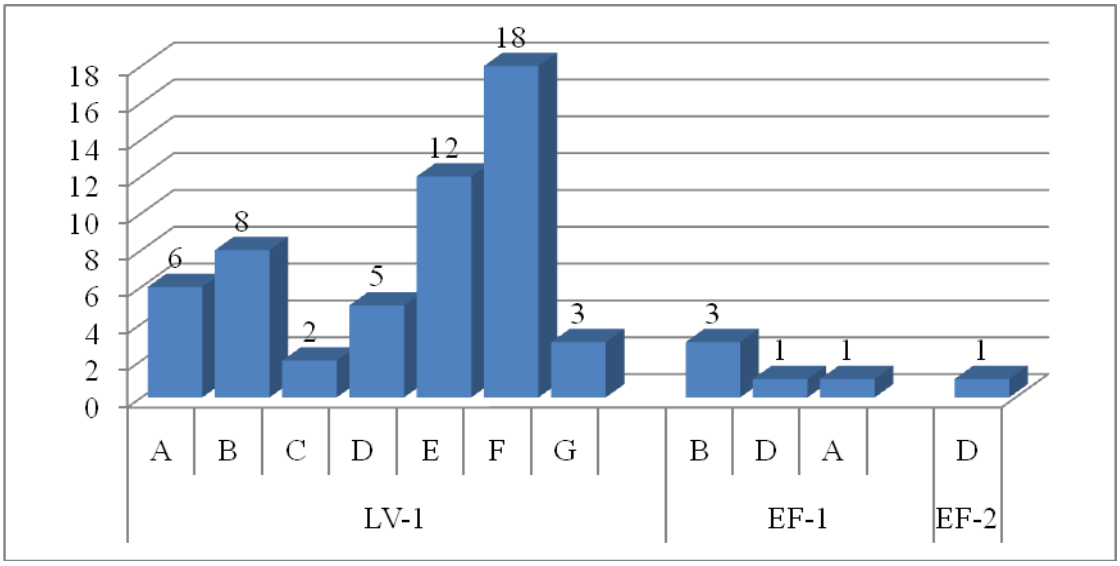

Figure 5.2 Number of actinobacterial strains isolated from soil sample EF-1, EF-2 and LV-1. A: R2A, B: R2A with addition of 50 µg/ml rose Bengal, C: R2A with addition of 0.4% (w/v) sodium propionate, D: SM3, E: Tryptic soy agar, F: TSA with addition of 0.1% (w/v) starch, G: TSA with addition of 0.1% (w/v) colloidal chitin.

Table 5.2 Summary of actinobacterial isolates from seven Antarctic Signy soil samples

Soil sample	Media	Number of isolates	Closest match to BLAST results
BS-7	SCN	1	*Nocardia ninae*
		1	*Rhodococcus corynebacterioides*
		6	*Mycobacterium* sp.
		1	*Micrococcus luteus*
		2	*Kocuria* sp.
	SCN with addition of 2% NaCl	9	*Rhodococcus corynebacterioides*
		1	Actinobacterium P23
		1	*Demetria terragena*
EF-1	R2A	1	*Rhodococcus* sp.
	R2A with addition of 50 µg/ml rose Bengal	1	*Streptomyces argenteolus*
		2	*Rhodococcus* sp.
	SM3	1	*Rhodococcus* sp.
EF-2	SM3	1	*Rhodococcus* sp.
G-1	SCN	1	*Demetria terragena*
		1	*Tsukamurella* sp.
	SCN with addition of 2% NaCl	1	*Demetria terragena*
		1	*Glaciibacter superstes*
G-2	SCN	1	*Microbacterium* sp.
	SCN with addition of 2% NaCl	1	*Humicoccus* sp.
		1	*Kocuria* sp.
LV-1	R2A	1	*Rhodococcus* sp.
		5	*Streptomyces beijiangensis*
	SM3	5	*Streptomyces beijiangensis*
	R2A with addition of 50 µg/ml rose Bengal	8	*Streptomyces beijiangensis*
	R2A with addition of 0.4% (w/v) sodium propionate	2	*Streptomyces beijiangensis*
	TSA with addition of 0.1% (w/v) starch	18	*Streptomyces beijiangensis*
	TSA with addition of 0.1% (w/v) colloidal chitin	3	*Streptomyces beijiangensis*
	TSA	12	*Streptomyces beijiangensis*
SD-1	SCN	2	*Marmoricola aequoreus*
	SCN with addition of 2% NaCl	1	Actinobacterium kmd_307
		2	*Rhodococcus* sp.
		1	*Marmoricola aequoreus*

Supplementation of a bacteriostatic dye, rose Bengal and sodium propionate were employed for the isolation of actinobacteria in the Three Lakes Valley (LV-1) site. The isolation media which were supplemented with rose Bengal and sodium propionate did not exhibit any fungus growth. Eight actinobacteria strains were isolated using R2A with addition of 50 µg/ml rose Bengal media while six actinobacteria strains were isolated using R2A media. This suggests that addition of rose Bengal may aid in isolating actinobacteria. However, only two actinobacterial strains were isolated using R2A with addition of 0.4% (w/v) sodium propionate and this suggests that sodium propionate may suppress the growth of actinobacteria. Rose Bengal (Ottow, 1972; Alef and Nannipieri, 1995) and sodium propionate (Crook *et al.*, 1950) were known to suppress bacteria and inhibit the growth and spreading of fungal colonies.

SCN agar and SCN supplemented with 2% NaCl were used to isolate actinobacteria from the Backslope (BS-7), Spindrift Col (SD-1) and Gourlay (G-1 and G-2) sites (Table 4.2 and Figure 5.3). Soil sample from the Backslope (BS-7) site yielded the highest number of actinobacteria in which twenty two strains were isolated (Figure 5.3). Nineteen actinobacterial strains were isolated from SCN supplemented with 2% NaCl compared to sixteen strains from SCN media. It was observed that bacterial enumeration using SCN supplemented with 2% NaCl ranged from (1.18 ± 0.93) x 10^4 cfu/g - (1.16 ± 0.17) x 10^6 cfu/g was lower as compared to SCN with (1.17 ± 0.55) x 10^5 cfu/g to (2.44 ± 0.61) x 10^7 cfu/g. SCN serves as a selective medium in isolating soil bacteria, especially actinobacteria while the addition of NaCl to the media aids the growth of actinobacteria thus suppressing the growth of bacteria (Mackay, 1977). NaCl may serve as a significant role in selecting actinobacteria in Spindrift Col (SD-1) soil samples in which different types of actinobacteria ranging from Actinobacterium kmd_307, *Rhodococcus* sp. and *Marmoricola aequoreus* were isolated and only *Marmoricola aequoreus* was isolated using SCN media (Table 5.2). In this

study, the number of actinobacteria genera isolated in SCN media was higher than using R2A media (Table 5.2). Thus, it is suggested that SCN serves as the better media for the actinobacteria isolation and the media could be used for isolation of actinobacteria in Three Lakes Valley (LV-1) site.

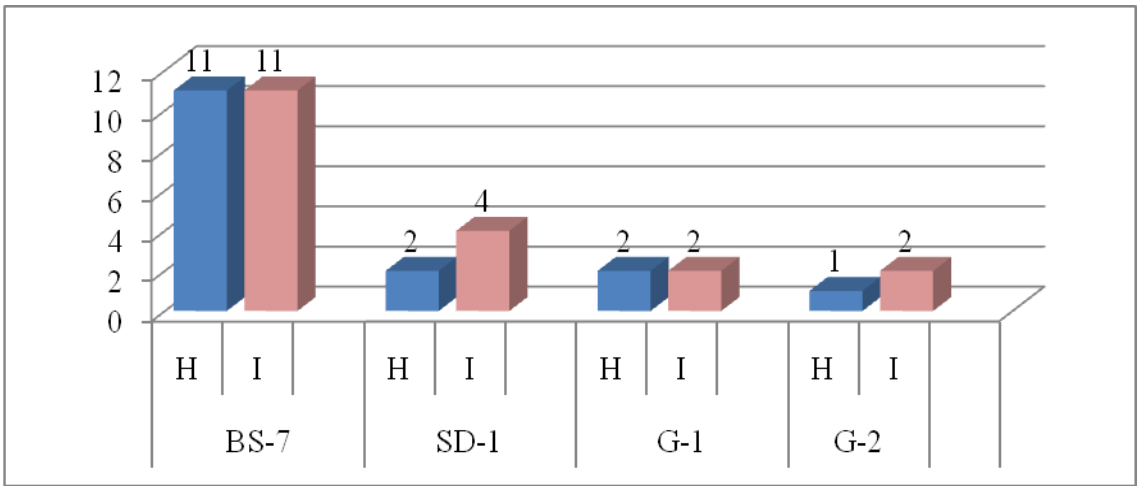

Figure 5.3 Number of actinobacterial strains isolated from soil sample BS-7, SD-1, G-1 and G-2. H: SCN, I: SCN supplemented with 2% NaCl.

Actinobacteria diversity in Backslope (BS-7) site was the highest in which Actinobacterium P23, *Demetria terragena*, *Kocuria* sp., *Micrococcus luteus*, *Mycobacterium* sp., *Nocardia ninae* and *Rhodococcus corynebacterioides* were isolated (Table 5.2). Different actinobacteria diversity was isolated in Gourlay (G-1 and G-2) site. *Demetria terragena*, *Glaciibacter superstes* and *Tsukamurella* sp. were isolated from Gourlay (G-1) site while *Humicoccus* sp., *Kocuria* sp. and *Microbacterium* sp. were isolated from Gourlay (G-2) site (Table 5.2). Presence of *Rhodococcus* sp. was also detected in Elephant Flats (EF-1 and EF-2) soil samples.

Supplementation of antifungal compounds (cycloheximide and nystatin) in the isolation media is important in inhibiting or suppressing fungal growth (Imada *et al*., 2007; Hirsch, 1983). In this study, SM3 medium gave rise to mostly fungal growth and not bacterial growth because it was not supplemented with antifungal compounds. Other isolation media supplemented with cycloheximide and nystatin showed decrease in

fungal growth. A higher dilution until 10^{-4} of soil suspension, were employed and plated for the isolation of actinobacteria (Table 4.3). Higher dilutions are able to avoid overgrowth of other bacteria, thus allowing the growth of actinobacteria.

All the isolates were isolated and grown at 15℃ because studies done by members of our research group had shown that Antarctic actinobacteria could be cultured at 4℃ but the time taken for good growth was at least one month. All the isolates had good growth with 20 days at 15℃. The predominance of psychotolerant isolates can be found in permanently cold environments (O'Brien *et al.*, 2004). Although the temperature of most Antarctic soils seldom rises above 10˚C, exposed soils may reach to 20-25˚C during summer (O'Brien *et al.*, 2004). A 20℃ moderate temperature shortens the incubation time and thus allows a higher growth rate of the psychrotolerant microorganisms (Ruberto *et al.*, 2005). Actinobacterial isolates were then cultured on ISP2 and incubated at 25℃ for temperature tolerance tests. Results showed that all the actinobacterial strains showed good growth within 9 days of incubation. All the actinobacterial strains exhibits similar colony morphology and colour texture compared to the actinobacterial strains grown on 15℃ for 20 days. Our results suggest that for the rapid growth of actinobacteria, the strain can be grown at 25℃ for 9 days, as compared to 20 days at 15℃.

5.2 Microscopic and chemotaxonomic characterization of actinobacteria

Gram stain is the most basic and widely employed method to distinguish Gram-positive bacteria from Gram-negative bacteria. Fifty four isolated actinobacterial strains were Gram-positive, rods (Table 4.6) and they showed high homology to *Microbacterium* spp., *Streptomyces beijiangensis* and *Streptomyces argenteolus* (Table 4.10). Other strains which exhibited Gram-positive rods, rods or branched filaments, long thin rods, irregular rod shaped showed high homology to Actinobacterium P23, *Mycobacterium* sp., *Nocardia ninae*, *Rhodococcus corynebacterioides* and *Tsukamurella* sp. (Table 4.6). Nineteen actinobacterial strains which were Gram-positive cocci showed high homology to Actinobacterium kmd_307, *Demetria terragena*, *Kocuria* sp., *Marmoricola aequoreus*, *Rhodococcus* sp. (Table 4.10). Previous reports showed that members from the genera *Kocuria* and *Micrococcus* were Gram-positive cocci, genera *Microbacterium*, *Mycobacterium* and *Tsukamurella* were Gram-positive, rods, genera *Nocardia* showed hyphae with aerial mycelia while rod-cocci morphology was observed in *Rhodococcus* spp. (Midayoh *et al*., 1997). Groth *et al.* (1997) described *Demetria terragena* as irregular-coccoid to short rod shaped and Lee (2007) described *Marmoricola aequoreus* as Gram-positive cocci which occurred either as singly, pairs, cluster or short chains.

Coverslip method was also employed to observe the mycelia and the spores of actinobacteria. Actinobacterial strains were divided into four different groups based on their spore or mycelia formation. Fifty four actinobacterial strains possessed long branching mycelia, spore production in long chains with straight to flexuous spore chains and were showed high homology to *Streptomyces beijiangensis* and *Streptomyces argenteolus* (Table 4.7, page 47 and Table 4.10, page 57) whereas strain PSY021 possess long branching mycelia without spore formation and were closely related to *Nocardia ninae* (Table 4.10). Majority of *Streptomyces* spp. produce long

chains frequently having more than 50 spores (Midayoh *et al*., 1997) and *Nocardia* species was described to form branching mycelia (Williams *et al*., 1989). Short branching mycelia and non-spore forming were recorded in seventeen of our isolated actinobacterial strains and showed high homology to *Mycobacterium* sp., *Tsukamurella* sp. and *Rhodococcus corynebacterioides* (Table 4.7, Table 4.10). *Mycobacterium* sp., *Rhodococcus* sp., and *Tsukamurella* sp. are non-spore formers and their cells are rods or branched filaments without the formation of aerial mycelia (Midayoh *et al*., 1997; Yassin *et al*., 1997). Hence, the short branching mycelia observed should be of the branching filaments of the cells. Twenty three strains which did not possess any mycelia showed high homology to *Actinobacterium* P23, Actinobacterium kmd_307, *Demetria terragena*, *Glaciibacter superstes*, *Humicoccus* sp., *Kocuria* sp., *Marmoricola aequoreus*, *Micrococcus luteus Microbacterium* sp. and *Rhodococcus* sp. (Table 4.7, Table 4.10). According to previous reports, *Rhodococcus* spp., *Marmoricola aequoreus*, *Microbacterium* spp. and *Kocuria* spp. are non-spore formers and do not form aerial mycelia (Lee, 2007; Matsuyama *et al*., 2003; Speck, 1943; Tvrzová, 2005). Coverslip examination serves as one of the morphological characterization of actinobacteria.

In our study, a simplified method of thin layer chromatography was employed to identify the diaminopimelic acid (DAP) in the cell walls of actinobacteria (Staneck and Roberts, 1974). The cell wall hydrolysates were distinguished by the separation of its cell wall types, either LL-DAP or *meso*-DAP. LL-DAP was detected in four ARDRA groups whereas *meso*-DAP were detected in seven groups (Table 5.1, page 64). The actinobacteria which contained LL-DAP showed high homology to *Streptomyces beijiangensis*, *Streptomyces argenteolus* and *Marmoricola aequoreus* (Table 5.1). Our data is consistent with previous reports which indicated *Streptomyces* spp. and *Marmoricola* spp. which contained LL-DAP (Staneck and Roberts, 1974; Lee, 2007) while *Mycobacterium* spp., *Nocardia* spp., *Rhodococcus* spp., *Tsukamurella* spp.

(Midayoh *et al*., 1997) and *Humicoccus* spp. (Yoon *et al*., 2007) contained *meso*-DAP. However, neither LL-DAP nor *meso*-DAP was detected in some of our actinobacterial strains. Our strains which did contain LL or *meso*-DAP showed high homology to *Demetria terragena*, *Kocuria* sp., *Glaciibacter superstes*, *Microbacterium* sp. and *Micrococcus luteus*. Previous researches showed that some actinobacteria only contain L-lysine diamino acids. Examples of actinobacteria containing L-lysine diamino acids were *Micrococcus* spp., *Kocuria* spp., *Demetria* spp. and *Microbacterium* spp. (Midayoh *et al*., 1997; Stackebrandt and Schumann, 2006).

5.3 Characterization of actinobacterial isolates based on colour and ARDRA grouping

Descriptions of colour of both aerial and vegetative mycelia are important in characterizing actinobacteria since the beginning of streptomycete taxonomy (Lyons and Pridham, 1965). All actinobacterial isolates were assigned into fifteen colour groups and no diffusible pigment was observed in all the actinobacterial isolates (Table 4.5). No diffusible pigments were observed in all ninety five actinobacterial isolates. Colour grouping and ARDRA were used to dereplicate the strains. Fifteen different colour groups were identified (Table 4.5). ARDRA method using two restriction enzymes, *BssM*I and *Hha*I identified sixteen groups based on restriction patterns (Table 4.9). This shows that colour grouping method is not as discriminative as the ARDRA grouping method.

Representatives from each ARDRA group were partially sequenced (370-890 nucleotides) and showed high homology to Actinobacterium P23, Actinobacterium kmd_307, *Demetria terragena*, *Glaciibacter superstes*, *Humicoccus* sp., *Kocuria* sp., *Marmoricola aequoreus*, *Microbacterium* sp., *Micrococcus luteus*, *Mycobacterium* sp., *Nocardia ninae*, *Rhodococcus* sp., *Rhodococcus corynebacterioides*, *Streptomyces*

argenteolus, *Streptomyces beijiangensis* and *Tsukamurella* sp. (see Table 4.10, page 57). ARDRA technique was employed previously and shown to be reliable and valuable for phylogenetic and taxonomic studies of large sets of strains (Heyndrickx *et al*., 1996). Previous reports showed that ARDRA is a rapid and convenient method which can be very useful in grouping actinobacterial isolates efficiently and it can also effectively reduce the number of isolates required for sequencing whilst screening for their diversity (Jiang *et al*., 2008). All our actinobacterial isolates were well grouped to genus level by using *BssM*I and *Hha*I restriction endonucleases. Cook and Meyers (2003) indicated that actinobacteria could be identified at genus level by using four restriction enzymes without sequencing.

5.3.1 Colour group 1- ARDRA group 1

Fifty two actinobacterial strains isolated from the Three Lakes Valley site were grouped into colour group 1, which exhibited yellowish white aerial mycelia and light yellow substrate mycelia with dry, convex, irregular and powdery colony morphology (Table 4.5). All these strains showed Gram-positive rods with long branching mycelia, spore production in long chains, straight to flexuous spore chains and contained LL-DAP (Table 4.6, Table 4.7 and Table 5.1). All the strains generated the same ARDRA restriction patterns. 16S rRNA genes of representatives from ARDRA group 1 (strains PSY013, PSY020, PSY056, PSY059, PSY081, PSY097) were partially sequenced and closely matched to *Streptomyces beijiangensis* (AB249973), with 98-99% homology (Table 4.10 and Table 5.1). Strain PSY097 clusters together with *Streptomyces bejiangensis* with a bootstrap value of 50% (Figure 4.12). Our data is consistent to those of Li *et al*. (2002) who isolated *Streptomyces beijiangensis* from soils in low-temperature habitats collected from Beijiang, western China. It was described with long spore chains were straight to flexuous and occasionally retinaculiaperti which bear on

aerial mycelia and contained LL-DAP and traces of meso-A2pm. The aerial mycelia was pale white with moderate yellow substrate mycelia on ISP2 media.

5.3.2 Colour group 2 – ARDRA group 1

Strain PSY020 was clustered into single member group in colour group 2, which exhibited strong reddish orange aerial and substrate mycelia with dry, convex, irregular and powdery colony morphology (Table 4.5). The strain showed Gram-positive rods with long branching mycelia, spore production in long chains, straight to flexuous spore chains and contained LL-DAP (Table 4.6, Table 4.7 and Table 5.1). However, the strain generated a same ARDRA restriction pattern as all actinobacterial strains in colour group 1. 16S rRNA genes of strain PSY020 showed 98% homology to *Streptomyces beijiangensis* (AB249973) (Table 4.10 and Table 5.1). Based on our results, *Streptomyces beijiangensis* may possess two different phenotypes, which are yellowish white and strong reddish orange aerial mycelia.

5.3.3 Colour group 3 – ARDRA group 2 and ARDRA group 4

All isolated actinobacterial strains of colour group 3 exhibit vivid orange yellow aerial and substrate mycelia colour with circular, flat and powdery colony morphology (Table 4.5). However, two different restriction patterns were generated by ARDRA and were grouped to ADRDA group 2 and 4. Actinobacterial strains from ARDRA group 2 showed Gram-positive cocci without mycelia and contained *meso*-DAP (Table 4.6, Table 4.7 and Table 5.1). 16S rRNA genes of representatives from ARDRA group 2 (strains PSY019, PSY039 and PSY092) were partially sequenced and closely matched to *Rhodococcus* sp. (FJ195998), with 100% homology (Table 4.10 and Table 5.1). Strain PSY019 clusters together with *Rhodococcus* sp. with a bootstrap value of 100% (Figure 4.12). These strains were isolated from Elephant Flats (EF-1 and EF-2), Three

Lakes Valley (LV-1) and Spindrfit Col (SD-1) site. This showed that genus *Rhodococcus* is present in various locations in Signy Island. Genus *Rhodococcus* possess rods and form filaments or show elementary branching in the early growth phase and are mostly cocci in the stationary phase (Matsuyama *et al*., 2003) and they contain *meso*-DAP (Midayoh *et al*., 1997). Strain PSY045 and PSY066 from ARDRA group 4 are Gram-positive cocci without mycelia and do not contain LL or *meso*-DAP (Table 4.6, Table 4.7 and Table 5.1). Strain PSY045 was partially sequenced and showed 91% homology to *Demetria terragena* (Figure 4.12). Our data is consistent with those of Groth *et al*. (1997) who isolated *Demetria terragena* from compost soils collected from Germany. It was described as Gram-positive with irregular coccoid to short rod shaped and contained lysine cell wall peptidoglycan.

5.3.4 Colour group 4 – ARDRA group 15

Strain PSY032 isolated from Spindrift Col site was clustered into single member group in colour group 4 and ARDRA group 15, which exhibited vivid yellow aerial and substrate mycelia with flat, small and smooth colony morphology (Table 4.5). Strain PSY032 showed Gram-positive with long thin rods, short branching mycelia, non-spore forming and contained *meso*-DAP (Table 4.6, Table 4.7 and Table 5.1). 16S rRNA genes of strain PSY032 was partially sequenced and closely matched to Actinobacterium kmd_307 (EU723162) (Table 4.10 and Table 5.1). Strain PSY032 clusters together with Actinobacterium kmd_307 with a bootstrap value of 100% (Figure 4.12) and this indicates that strain PSY032 could be a novel actinobacteria and further research could be conducted to identify the strain.

5.3.5 Colour group 5 – ARDRA group 3

Six actinobacterial strains (strain PSY065, PSY073, PSY074, PSY075, PSY076 and PSY078) isolated from soil samples of Backslope site were grouped into colour group 5, which exhibited vivid yellow aerial and substrate mycelia with dry, irregular and slight raised colony morphology (Table 4.5). All these strains showed Gram-positive rods or branch filaments with short branching mycelia, non-spore forming and contained *meso*-DAP (Table 4.6, Table 4.7 and Table 5.1). All the strains generated the same ARDRA restriction patterns and were grouped into ARDRA group 3. 16S rRNA genes of representatives (strains PSY065, PSY074, PSY075 and PSY078) from ARDRA group 3 were partially sequenced and were closely matched to *Mycobacterium* sp. (EU167989), with 98% homology (Table 4.10 and Table 5.1). Strain PSY065 clusters together with *Mycobacterium* sp. with a bootstrap value of 100% (Figure 4.12). Previous reports indicated that genus *Mycobacterium* belongs to the family *Mycobacteriaceae* and the cells of *Mycobacterium* are rods or branch filaments without formation of aerial mycelia. They are aerobic and acid fast (Midayoh *et al*., 1997; Stackebrandt and Schumann, 2006).

5.3.6 Colour group 6 – ARDRA group 4 and ARDRA group 6

Four isolated actinobacterial strains were grouped into colour group 6, which exhibited light yellow aerial and substrate mycelia with circular, small, flat and dry morphology (Table 4.5). Two different restriction patterns were generated by ARDRA and were grouped into ARDRA group 4 and group 6. Strain PSY066 from ARDRA group 4 strain showed Gram-positive cocci without mycelia and do not exhibit LL or *meso*-DAP (Table 4.6, Table 4.7 and Table 5.1). 16S rRNA genes of strain PSY066 were partially sequenced and were closely matched to *Demetria terragena* (Y1452) (Table 4.10, Table 5.1). However, strain PSY044 and PSY045 which also showed

similarity to *Demetria terragena* were grouped into colour group 3 (Table 5.1). This might indicate that *Demetria terragena* might exhibit two different phenotypes, which are vivid orange yellow and light yellow aerial mycelia (Table 4.5). Actinobacterial strains from ARDRA group 6 (strain PSY085, PSY087 and PSY091) isolated from soils samples of Gourlay and Backslope site were partially sequenced and were closely matched to *Kocuria* sp, with 100% homology (Table 4.10 and Table 5.1). All these strains are Gram-positive rods, no mycelia, non-spore forming and do not exhibit LL or *meso*-DAP (Table 4.6, Table 4.7 and Table 5.1). Genus *Kocuria* belongs to the family *Micrococcaceae* (Stackebrandt and Schumann, 2006). It was first classified under the genus *Micrococcus* and *M. Kristinae*, *M. roseus* and *M. Varians* was then proposed under the genus *Kocuria* (Stackebrandt *et al*., 1995). Our data is consistent with Tvrzová *et al*. (2005) who reclassified *Kocuria carniphila* sp. nov.. It was described as Gram-positive cocci, either in pairs and tetrads, non-motile, non-acid-fast and non-spore forming and exhibited yellow, circular, convex and opaque colony morphology. *Kocuria polaris* sp. nov. was also isolated from Antarctic cyanobacterial mat sample. It was described as Gram-positive cocci, occurring in pairs, tetrads or clusters, L-Lys-Ala_3 cell wall peptidoglycan with smooth, round, uniformly edge, translucent, mucoid and orange in colour (Reddy *et al*., 2003).

5.3.7 Colour group 7 – ARDRA group 5

Nine actinobacterial strains isolated from soils of Backslope site were grouped into colour group 7, which exhibited yellowish white aerial and substrate mycelia with dry, irregular and powdery colony morphology (Table 4.5). All these strains showed Gram-positive long thin rods, short branching mycelia, non-spore forming and contained *meso*-DAP (Table 4.6, Table 4.7 and Table 5.1). These phenotypic characteristic is similar to *Rhodococcus* sp. which exhibit rods or abundantly branched

filaments, non-spore forming with *meso*-DAP (Midayoh *et al*., 1997). All these strains generated a same ARDRA pattern and were grouped into ARDRA group 5. 16S rRNA genes of representatives from ARDRA group 5 (strains PSY025, PSY027, PSY028, PSY037 and PSY052) were partially sequenced and were closely matched to *Rhodococcus corynebacteriodes* (X80615), with 96-97% homology (Table 4.10 and Table 5.1). Strain PSY027 clusters together with *Rhodococcus corynebacteriodes* with a bootstrap value of 57% (Figure 4.12). Our strain is consistent to *Rhodococcus corynebacterioides* which was formally known as *Nocardia corynebacterioides*, described as Gram-positive, with rod and cocooid like shape and contained *meso*-DAP cell wall (Yassin and Shaal, 2005).

5.3.8 Colour group 8 – ARDRA group 7 and ARDRA group 14

Two actinobacterial strains were clustered into colour group 8, which exhibited pale yellow aerial and substrate mycelia with circular, small, flat and smooth colony morphology (Table 4.5). Two different restriction patterns were generated by ARDRA and were grouped into ARDRA group 7 and group 14. Strain PSY095 from ARDRA group 7 isolated from soil samples of Gourlay (G-1) site showed Gram-positive cocci to short rod shaped without mycelia and do not exhibit LL or *meso*-DAP (Table 4.6, Table 4.7 and Table 5.1). 16S rRNA genes of strain PSY095 were partially sequenced and were closely matched to *Glaciibacter superstes* (AB378302), with 97% homology (Table 4.10 and Table 5.1). Strain PSY095 clusters together with *Glaciibacter superstes* with a bootstrap value of 99% (Figure 4.12). This work is consistent to those of Katayama *et al*. (2009) who isolated *Glaciibacter superstes* from a permafrost ice wedge in Alaska. It was described as Gram-positive irregular rod shaped, aerobic and non spore forming. Strain PSY031 from ARDRA group 14 isolated from Backslope was partially sequenced and were closely matched to *Micrococcus luteus*, with 100%

homology (Table 4.10 and Table 5.1). Strain PSY031 from ARDRA group 14, isolated from Backslope soils, showed Gram-positive cocci, no mycelia and do not contain LL or *meso*-DAP (Table 4.6, Table 4.7 and Table 5.1). 16S rRNA gene of strain PSY031 was partially sequenced and was closely matched to *Micrococcus luteus* (FN984531) (Table 4.10 and Table 5.1). Strain PSY031 clusters together with *Micrococcus luteus* with a bootstrap value of 100% (Figure 4.12). *Micrococcus* spp. belongs to the family *Micrococcaceae* (Stackebrandt and Schumann, 2006) and can be found in mammalian skin, meat, dairy products and water (Midayoh *et al*., 1997). Our data is significant to Midayoh *et al*. (1997) who described *Micrococcus luteus* as Gram-positive cocci, non-motile, non-spore forming and contain lysine cell wall with yellow colony morphology.

5.3.9 Colour group 9 – ARDRA group 9

Strain PSY096 was clustered into single member group in colour group 9 and ARDRA group 9, which exhibited deep yellowish pink aerial and substrate mycelia with dry, small, entire and flat morphology (Table 4.5). Strain PSY096 isolated from soil samples of Backslope (BS-7) site showed Gram-positive irregular rod shape, no mycelia and contained *meso*-DAP (Table 4.6, Table 4.7 and Table 5.1). Strain PSY096 was partially sequenced and was closely matched to Actinobacterium P23 (D1351736), with a 96% homology (Table 4.10 and Table 5.1). Strain PSY096 clusters together with Actinobacterium P23. Strain PSY096 clusters together with Actinobacterium P23 with a bootstrap value of 100%. This indicates that strain PSY096 could be a novel actinobacteria and further research could be conducted to identify the strain.

5.3.10 Colour group 10 – ARDRA group 10

Strain PSY086, isolated from soil samples of Gourlay (G-2) site was clustered into single member group in colour group 10 and ARDRA group 10, which exhibited

moderate orange yellow aerial and substrate mycelia with dry, flat and smooth colony morphology (Table 4.5). The strain showed Gram-positive rods, no mycelia and do not contain LL or *meso*-DAP (Table 4.6, Table 4.7 and Table 5.1). 16S rRNA genes of strain PSY086 was partially sequenced and was closely matched to *Microbacterium* sp. (AB461113), with 100% homology (Table 4.10 and Table 5.1). Strain PSY086 clusters together with *Microbacterium* sp. with a bootstrap value of 100% (Figure 4.12). Midayoh *et al* (1997) described genus *Microbacterium* as Gram-positive rods and contained L-lys and L-Hsr cell wall DAP.

5.3.11 Colour group 11 – ARDRA group 11

Strain PSY016, isolated from soil samples from Elephant Flats (EF-1) site was clustered into single member group in colour group 11 and ARDRA group 11, with light brownish gray aerial and yellowish white substrate mycelia (Table 4.5). Strain PSY016 showed Gram-positive, rods, long branching mycelia, spore production in long chains, straight to flexuous spore chains and contained LL-DAP cell wall (Table 4.6, Table 4.7 and Table 5.1). The strain was partially sequenced and showed high homology to *Streptomyces argenteolus*, with 99% similarity (Table 4.10 and Table 5.1). Strain PSY016 showed a bootstrap value of 100% to *Streptomyces argenteolus* (Figure 4.12). Genus *Streptomyces* belongs to the family *Streptomycetaceae* (Waksman and Henrici, 1943). Till now, more than 500 species have been described. During their life cycle, Streptomycete spore germinates into well-developed, branched and rarely fragmented substrate mycelia followed by fragmenting into chains of spores (Midayoh *et al*., 1997; Tarkka and Hampp, 2008). *Streptomyces* possesses variety of spore chains, ranging from short to long chains, straight to flexuous, hooked to looped or spiral (Midayoh *et al*., 1997; Waksman and Henrici, 1943). They are the main and genus producing antibiotics commercially (Watve *et al*., 2001; and produce proteolytic

enzymes as well (Antonova-Nikolova *et al*., 2005). According to Liu *et al*. (2005), *Streptomyces argenteolus* was described with spore production in long chains, straight to flexuous spore chains. The aerial mycelia were yellowish grey with yellow-brown substrate mycelia with no diffusible pigment on oatmeal agar.

5.3.12 Colour group 12 – ARDRA group 16

Strain PSY021, isolated from Backslope (BS-7) site was clustered into single member group in colour group 12 and ARDRA group 16, which exhibited white aerial and substrate mycelia with dry, convex and powdery colony morphology (Table 4.5). Strain PSY021 showed Gram-positive branched like hyphae fragmenting into rods with long branching mycelia, non-spore forming and contain *meso*-DAP (Table 4.6, Table 4.7 and Table 5.1). Strain PSY021 was partially sequenced and was closely matched to *Norcardia ninae* (PSY021), with 97% homology (Table 4.10, Table 5.1). Strain PSY021 clusters together with *Norcardia ninae*, with a bootstrap value of 100% (Figure 4.12). Genus *Nocardia* belong to Nocardiaceae family (Stackebrandt and Schumann, 2006) and are strictly aerobic, non acid fast or partially acid fast. The cells are filamentous, fragmenting into rod or coccoid elements (Midayoh *et al*., 1997). *Nocardia ninae* was described to be isolated from a human respiratory sample, which was Gram-positive, aerobic, branched like hyphae fragments into rod or cocci and formed orange substrate mycelia with white aerial hyphae (Laurent *et al*., 2007).

5.3.13 Colour group 13 – ARDRA group 12

Strains PSY084, PSY093 and PSY94 isolated from soil samples of Spindrift Col (SD-1) were grouped into colour group 13, which exhibited white aerial and substrate mycelia with dry, irregular and raised colony morphology (Table 4.5). All these strains were Gram-positive cocci in clusters, no mycelia and contained LL-DAP (Table 4.6,

Table 4.7 and Table 5.1). All the strains generated the same ARDRA pattern and were grouped into ARDRA group 12. 16S rRNA genes of representative strains (strains PSY093 and PSY094) were partially sequenced and were closely matched to *Marmoricola aequoreus* (AM295338) (Table 4.10 and Table 5.1). Strain PSY093 clusters together with *Marmoricola aequoreus* with a bootstrap value of 100% (Figure 4.12). Two *Marmoricola* species were proposed, namely *Marmoricola aurantiacus* was first proposed into the family *Nocardioidaceae* due to its characteristics and phylogenetic position (Urz ì *et al.*, 2000) followed by *Marmoricola aequoreus* (Lee, 2007). Our data showed similarity to Lee (2007) who isolated *Marmoricola aequoreus* sp. nov. from marine sediments, Samyang beach in Jeju, Republic of Korea. It was described as Gram-positive spherical, occurred singly in pairs, clusters or short chains, aerobic, non-motile, non-spore forming and contained LL-DAP.

5.3.14 Colour group 14 – ARDRA group 13

Strain PSY024 isolated from soil samples of Gourlay (G-1) site was clustered into single member group in colour group 14 and ARDRA group 13, which exhibited yellowish white aerial and pale yellow substrate mycelia with dry, irregular, slightly raised colony morphology (Table 4.5). The strain showed Gram-positive rod or branched filaments, short branching mycelia, non-spore forming and contained *meso*-DAP (Table 4.6, Table 4.7 and Table 5.1). Strain PSY024 was partially sequenced and closely matched to *Tsukamurella* sp. (EF514880), with 100% homology (Table 4.10 and Table 5.1). Genus *Tsukamurella* belongs to the family *Tsukamurellaceae* (Stackebrandt and Schumann, 2006) and they exhibit Gram-positive rod shape, non-spore forming and do not form true branching mycelia (Yassin *et al*., 1996 and 1997).

5.3.15 Colour group 15 – ARDRA group 8

Strain PSY079 isolated from soil samples of Gourlay (G-2) site was clustered into single member group in the colour group 15 and ARDRA group 8, which exhibited deep orange yellow aerial and substrate mycelia with circular, dry and flat colony morphology (Table 4.5). Strain PSY079 showed Gram-positive cocci to short rod shaped, no mycelia and contained *meso*-DAP cell wall (Table 4.6, Table 4.7 and Table 5.1). 16S rRNA gene of PSY079 was partially sequenced and closely matched to *Humicoccus* sp. (EU939310), with 99% homology (Table 4.10, Table 5.1). Strain PSY079 clusters together with *Humicoccus* sp. with a bootstrap value of 100% (Figure 4.12). Yoon *et al*. (2007) isolated and classified the new genera *Humicoccus flavidus* gen. nov., sp. nov. from soils in Korea. Genera *Humicoccus* was described as coccus isolated from soils It was described as Gram-positive non-spore forming cocci and exhibited *meso*-DAP.

5.4 Molecular characterization of soil DNA and pure cultures

DNA was extracted from the seven soil samples collected on Signy Island, Antarctica (Figure 4.6). The amount of DNA in extracted soil samples varied among soil sample sites. A good DNA band was obtained from soil Elephant Flats (EF-1) and Spindrift Col (SD-1) soil samples. Less amount of DNA from Gourlay (G-1) and Backslope (BS-7) was extracted and some of the DNA was degraded and little amount of DNA were extracted. This might be due to excessive lysing of soil with glass beads on a beat beater. The usage of CTAB for soil samples can successfully extract soil DNA. Six of the soil samples were successfully amplified by 16S rRNA gene amplification. Only soil samples of Backslope site could not be amplified by this method. The amplified 16S rRNA products were then diluted to 10^{-1} and were then successfully amplified by 16S rRNA gene amplification (Figure 4.7). Actinobacteria

were known to be widely distributed in soil (Labeda, 1990). All soil samples amplified a 640-bp length and this indicates the presence of actinobacteria in all soil samples (Figure 4.8).

DNA was successfully extracted from all ninety five isolated actinobacterial strains and this indicates that lysozyme and proteinase K can successfully extract DNA (Figure 4.9). All the strains were successfully amplified by 16S rRNA gene amplification (Figure 4.10) followed by 10^{-1} dilution of 16S rRNA products for actinobacteria amplification. All isolated actinobacterial strains from gave a positive amplification on genus specific actinobacteria primers (Stach *et al.*, 2003), thus confirming that they are actinobacteria (Figure 4.11). These actinobacteria specific primers (Stach *et al*., 2003) were also used in other research in identifying actinobacterial strains (Gavrish *et al*., 2008; Pathom-aree *et al*., 2006). Usage of these specific primers rapidly confirms the presence of actinobacteria in environmental samples or the identity of actinobacteria tentatively assigned to specific taxa (Stach *et al*., 2003).

5.5 Primary screening of actinobacterial isolates for antimicrobial activity

All ninety five isolated actinobacterial strains were tested for antibacterial activity. All the actinobacterial isolates were cultured at 15˚C for 20 days and tested on the test organisms by the agar plug method. Forty six of the isolates showed antibacterial activity and it shows that actinobacteria are potential producers of secondary metabolites (Table 4.11). All actinobacterial isolates showed no antibacterial activity against *E. coli* and *S.typhi* while *K. pneumoniae* was only susceptible to strain PSY013, with 17 mm inhibitory zones. Our observation was that actinobacteria were more active against Gram-positive bacteria compared to Gram-negative bacteria. Different inhibitory patterns were shown against all the tester organisms especially

strain PSY013, which was the most potent strain and was active against four out of six tester organisms. All the isolates which showed antibacterial activity showed high homology to *Streptomyces beijiangensis*, with 98-99% homology (Table 5.1) and this is the first report indicating that *Streptomyces beijiangensis* possess antibacterial activity.

Actinobacteria which exhibit antimicrobial properties have been isolated from various places in Antarctica (Brutner *et al*., 2005; Moncheva *et al*., 2002; Nedialkova and Naidenova, 2005). Antarctica could represent an unexploited environment for the discovery of biotechnologically exploitable microorganisms (Giudice *et al.*, 2007). The results of present investigation showed that Antarctica is a valuable region of exploiting valuable sources of bioactive compounds.

5.6 Screening of NRPS systems on actinobacterial isolates

All the isolated actinobacterial strains were furthered screened for NRPS systems. NRPS genes are largely involved in the synthesis of secondary metabolites which can contribute to the medical field. Previous research also indicated a wide distribution of NRPS genes in *Streptomyces* spp. (Ayuso-Sacido and Genilloud, 2005). NRPS systems were detected in seventy nine actinobacterial strains. In our study, forty six out of fifty three strains from ARDRA group 1 showed antibacterial activity through primary screening. All fifty three strains from ARDRA group 1, which were closely matched to *Streptomyces beijiangensis* contained NRPS genes. NRPS genes were also detected in *Kocuria* sp., *Marmoricola aequoreus*, *Mycobacterium* sp., *Nocardia ninae*, *Rhodococcus* sp., *Rhodococcus corynebacterioides*, *Streptomyces argenteolus* and *Tsukamurella* sp. (Table 5.1).

Antibiotic resistance is not a new problem, but in recent past there is growing concern of increase of antibiotic resistance (van den Bogaard and Sobberingh, 2000) Hence, different approaches such as usage of NRPS systems to detect new secondary

metabolites can be employed. Bacteria use NRPS to produce peptides of broad structural and biological activity (Finking and Marahiel, 2004). Screening of NRPS genes is of medical importance hence, actinobacteria were used to screen for NRPS genes. However, most of the actinobacteria which contain NRPS are slow growers. Therefore expression of genes in the fast growers such as *E.coli* would be recommended, and a large amount of genes may be expressed in a short period of time which would benefit the medical or agricultural field.