CHAPTER 4.0 RESULTS

4.1 Soil sample pH

Seven soil samples obtained from Signy Island, Antarctica were undertaken for this study and the pH was determined. The pH of the soil samples ranged from slightly acidic to slightly alkaline (Table 4.1). Soils from Gourlay and Elephant Flats were slightly acidic soils and the acidic properties were probably caused by the presence of guano by penguins and seals. Backslope, which are with lichens and mosses and Spindrift Col, with Barren soils were also slightly acidic. The inland lake soils from Three Lakes Valley were slightly alkaline.

Table 4.1 pH of soil samples collected from Signy Island

Soil samples	Location	Soil pH reading			Average soil pH
		1	2	3	
BS-7	Backslope	5.41	5.4	5.42	5.41 ±0.01
EF-1	Elephant Flats	6.60	6.60	6.60	6.60
EF-2	Elephant Flats	6.81	6.8	6.78	6.80 ±0.02
G-1	Gourlay Peninsular	5.81	5.79	5.81	5.80 ±0.01
G-2	Gourlay Peninsular	5.10	5.10	5.10	5.10
LV-1	Three Lakes Valley	7.53	7.56	7.54	7.54 ±0.02
SD-1	Spindrift Col	5.58	5.62	5.63	5.61 ±0.03

4.2 Enumeration of bacteria and actinobacteria

Nine isolation media were used to isolate actinobacterial strains (Table 4.2). All the isolation media were incubated at 15℃ for one to four months. The highest actinobacteria count was 1.00 x 10^5 cfu/g wheareas the lowest actinobacteria count was (5.00 ± 0.71) x 10 cfu/g. Actinobacteria was not isolated from BS-7, G-1, G-2 and SD-1 using SM3 medium and from EF-1 using R2A with addition of 0.4% (w/v) sodium propionate.

Table 4.2 Bacteria and actinobacteria count (cfu/g) of soil samples isolated on various isolation media

Soil sample	**Media**	**Average bacteria count (cfu/g)**	**Average actinobacteria count (cfu/g)**
BS-7	SCN	(1.17 ± 0.55) x 10^5	(5.00 ± 0.71) x 10
	SCN with addition of 2% NaCl	(1.68 ± 0.25) x 10^4	(5.00 ± 0.41) x 10^3
EF-1	R2A	(1.06 ± 0.06) x 10^6	(5.00 ± 0.71) x 10^4
	R2A with addition of 50 µg/ml rose Bengal	(2.23 ± 0.18) x 10^4	(5.00 ± 0.71) x 10^2
	SM3	TNTC	(5.00 ± 0.71) x 10^2
EF-2	SM3	(1.91 ± 0.15) x 10^4	(5.00 ± 0.71) x 10
G-1	SCN	(2.38 ± 0.18) x 10^6	(5.00 ± 0.71) x 10^2
	SCN with addition of 2% NaCl	(5.70 ± 1.69) x 10^5	1.00 x 10^4
G-2	SCN	(2.44 ± 0.61) x 10^7	(5.00 ± 0.71) x 10^3
	SCN with addition of 2% NaCl	(1.16 ± 0.17)x 10^6	(5.00 ± 0.71) x 10^2
LV-1	R2A	(3.12 ± 0.59) x 10^4	(2.00 ± 1.41) x 10^2
	SM3	(6.00 ± 0.71)x 10^4	1 x 10^2
	R2A with addition of 50 µg/ml rose Bengal	(3.50 ± 0.71) x 10^4	(1.5 ± 0.71) x 10^2
	R2A with addition of 0.4% (w/v) sodium propionate	(6.70 ± 0.71) x 10^4	1 x 10^4
	TSA with addition of 0.1% (w/v) starch	TNTC	(6.00 ± 1.41) x 10^3
	TSA with addition of 0.1% (w/v) colloidal chitin	(2.24 ± 0.16) x 10^7	(5.00 ± 0.71) x 10
	TSA	(1.80 ± 0.28) x 10^7	(1.50 ± 0.71) x 10^2
SD-1	SCN	(9.70 ± 3.82) x 10^5	1.0 x 10^5
	SCN with addition of 2% NaCl	(1.18 ± 0.93) x 10^4	1 x 10^3

Note: R2A: Reasoner's 2 A agar, SM3: Gauze medium 2, TSA: Tryptic soy agar, SCN: Starch casein nitrate agar, TNTC: Too numerous to count.

4.3 Actinobacterial strains isolated from soil samples

The populations of actinobacteria strains isolated differed from each soil samples along with different culture media. A total of ninety five actinobacteria strains were isolated from seven soil samples. LV-1 soil sample yielded the highest number of actinobacteria, of which fifty four strains were isolated whereas only one strain was isolated from EF-2.

Table 4.3 Actinobacterial strains isolated from different soil samples and dilutions

Soil Sample (Total)	Isolation media	Dilution	Strain label
BS-7 (22)	SCN	10^{-1}	PSY021
		10^{-2}	PSY034, PSY073, PSY074, PSY075, PSY076
		10^{-3}	PSY031, PSY065, PSY078, PSY091
		10^{-4}	PSY087
	SCN with addition of 2% NaCl	10^{-2}	PSY025, PSY026, PSY027, PSY028, PSY029, PSY035, PSY036, PSY037, PSY052, PSY096
		10^{-3}	PSY044
EF-1 (5)	SM3	10^{-2}	PSY033
	R2A	10^{-4}	PSY012
	R2A with addition of 50 µg/ml rose Bengal	10^{-2}	PSY016
		10^{-4}	PSY040, PSY092
EF-2 (1)	SM3	10^{-1}	PSY019
G-1 (4)	SCN	10^{-2}	PSY066
		10^{-3}	PSY024
	SCN with addition of 2% NaCl	10^{-3}	PSY045, PSY095
G-2 (3)	SCN	10^{-3}	PSY086
	SCN with addition of 2% NaCl	10^{-2}	PSY079
		10^{-4}	PSY085

‘Table 4.3, continued’

LV-1 (54)	R2A	10^{-1}	PSY006, PSY014, PSY015, PSY057
		10^{-2}	PSY010
		10^{-3}	PSY023
	R2A with addition of 50 µg/ml rose Bengal	10^{-1}	PSY013, PSY020, PSY022
		10^{-2}	PSY002, PSY005, PSY008, PSY009, PSY011
	R2A with addition of 0.4% (w/v) sodium propionate	10^{-3}	PSY003, PSY004
	SM3	10^{-1}	PSY007, PSY041
		10^{-2}	PSY001, PSY017, PSY018
	TSA	10^{-1}	PSY071, PSY072, PSY090
		10^{-3}	PSY046, PSY050, PSY054, PSY061, PSY058, PSY080, PSY088
		10^{-4}	PSY056, PSY089
	TSA with addition of 0.1% (w/v) colloidal chitin	10^{-1}	PSY097
		10^{-4}	PSY043, PSY051
	TSA with addition of 0.1% (w/v) starch	10^{-2}	PSY042, PSY047, PSY048, PSY055, PSY059, PSY060, PSY062, PSY063, PSY069, PSY077, PSY081, PSY082
		10^{-3}	PSY049, PSY053, PSY064, PSY067, PSY068, PSY070
SD-1 (6)	SCN with addition of 2% NaCL	10^{-2}	PSY032, PSY039
		10^{-3}	PSY038, PSY094
	SCN	10^{-4}	PSY084, PSY093

4.4 Morphological observations of actinobacterial isolates

Morphological characteristics such as colour grouping, Gram stain and coverslip method were employed on all the actinobacterial strains.

4.4.1 Colony morphology of actinobacteria on isolation medium

Actinobacteria can be easily distinguished morphologically on isolation plates because they have distinct appearances which were dry and powdery. All ninety five actinobacteria exhibited colony morphology ranging from powdery, dry, undulate, and

irregular with white, yellow, orange, yellow and red aerial mycelia. The descriptions of actinobacteria, incubated at 15℃ on respective isolation medium were recorded as in Table 4.4. The colony appearance of actinobacterial isolates are shown in Figure 4.1-4.2 which exhibit white to whitish yellow, powdery colonies.

Table 4.4 Actinobacteria appearance on respective isolation medium

Isolation medium	**Strain label**	**Colony appearance on isolation media**
R2A with addition of 0.4% (w/v) sodium propionate	PSY003	Yellowish white, undulate, raised, irregular, powdery.
	PSY004	White, rough, irregular, powdery.
R2A with addition of 50µg/ml of rose Bengal	PSY002, PSY092	Whitish pink, undulate, raised, irregular, powdery.
	PSY008, PSY009, PSY016	Whitish pink, small, raised, dry, irregular, powdery.
	PSY005, PSY011, PSY013, PSY020, PSY022	Whitish pink, big, irregular, powdery, undulate, raised.
	PSY040	Orange red, round, entire, raised, circular, powdery.
R2A	PSY010	Yellow, raised, powdery, irregular, powdery.
	PSY012	Orange yellow, small, entire, circular, powdery.
	PSY023	Orange, irregular, raised, powdery.
	PSY006, PSY014, PSY015, PSY057	White, round, circular, convex, raised, powdery.
SCN	PSY086	Orange, small, round, flat, powdery.
	PSY024	Yellowish, irregular, raised, round, small, powdery.
	PSY021, PSY034, PSY066, PSY073, PSY074	Whitish yellow, dry, small, slight raised, powdery.
	PSY031, PSY065, PSY075, PSY076	Yellow, small, round, dry, flat, powdery.
	PSY084, PSY093	White, raised, dry, irregular, small, powdery.
	PSY087, PSY091	Yellow, small, raised, round, dry, powdery.
	PSY078	Orange yellow, small, flat, powdery.

‘Table 4.4, continued’

SCN supplemented with 2% NaCl	PSY025, PSY029	Whitish yellow, raised, slight raised, dry, powdery.
	PSY027, PSY028, PSY052	White, undulate, slight raised, small, round, powdery.
	PSY026, PSY035, PSY036, PSY037	White, undulate, small, slight raised, powdery.
	PSY032, PSY038, PSY039, PSY044, PSY045, PSY079	Orange, dry, big, raised, irregular, powdery.
	PSY085	Yellow, round, small, dry, powdery.
	PSY094	White, raised, dry, irregular, powdery.
	PSY095	Orange yellow, dry, raised, small, round, powdery.
	PSY096	Red, dry, small, round, powdery.
SM3	PSY007	Yellow, small, circular, powdery.
	PSY017, PSY018	White, raised, powdery, small, irregular, powdery.
	PSY019, PSY033	Orange, small, round, flat, powdery.
	PSY001, PSY041	Yellowish, irregular, raised, irregular, round, small, powdery.
TSA	PSY050, PSY088, PSY089	Yellowish, raised, dry, convex, powdery.
	PSY046, PSY054, PSY056, PSY058, PSY061, PSY071 PSY072, PSY080, PSY090	White, powdery, dry, undulate, irregular, powdery.
TSA with addition of 0.1% (w/v) starch	PSY042, PSY047, PSY048, PSY049, PSY053, PSY055, PSY077, PSY081	White, powdery, dry, undulate, irregular, slight raised, powdery.
	PSY059, PSY060, PSY082	Yellowish, raised, dry, convex, powdery.
	PSY062, PSY063, PSY064, PSY067, PSY068, PSY069	White, powdery, undulate, irregular, powdery.
	PSY070	Yellowish, white, powdery, raised, powdery.
TSA with addition of 0.1% (w/v) colloidal chitin	PSY043	Yellowish, raised, convex, powdery.
	PSY051, PSY097	White, powdery, undulate, irregular, big, raised, powdery.

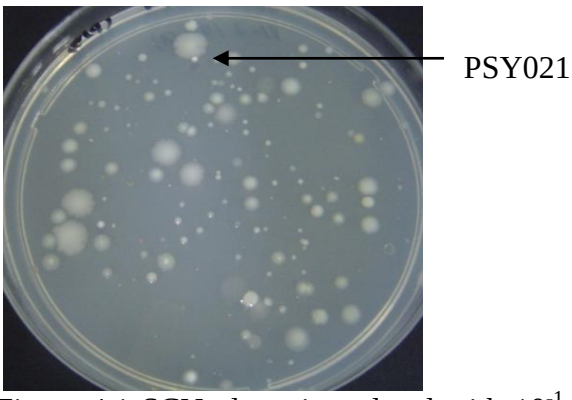


Figure 4.1 SCN plates inoculated with 10^{-1} dilution of soil sample BS-7 after 15℃, eight weeks incubation.

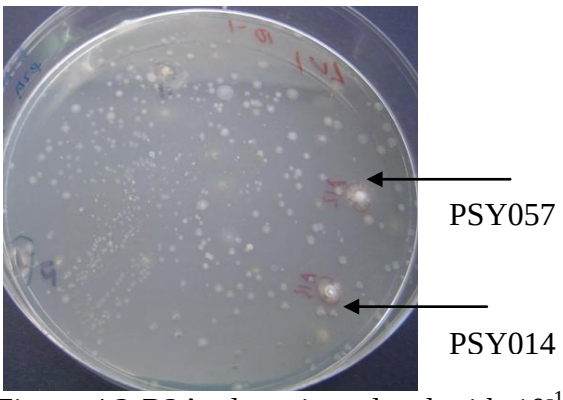
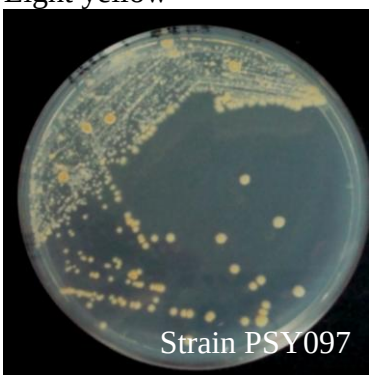
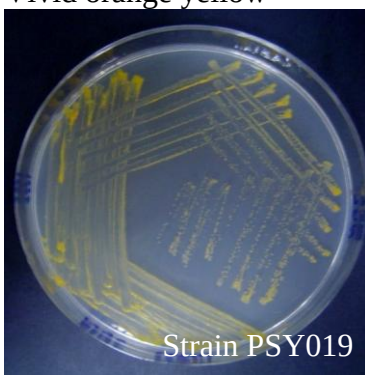
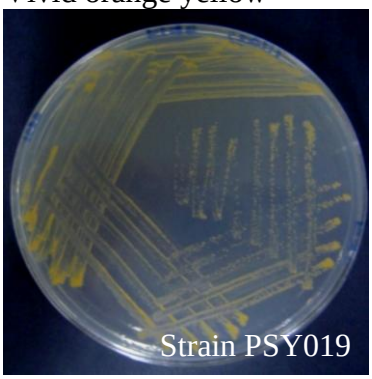


Figure 4.2 R2A plates inoculated with 10^{-1} dilution of soil sample LV-1 after 15℃, eight weeks incubation.

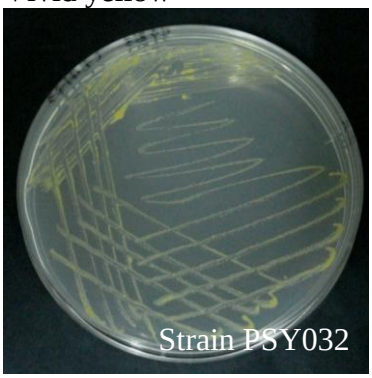
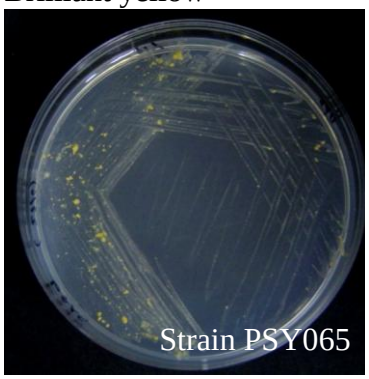
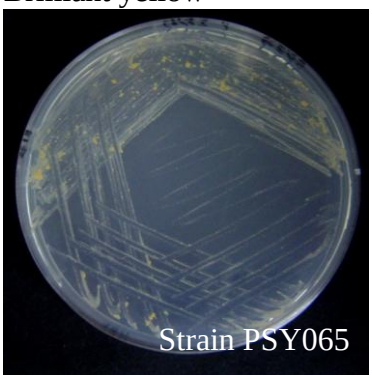
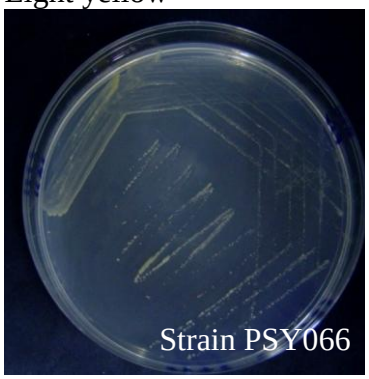
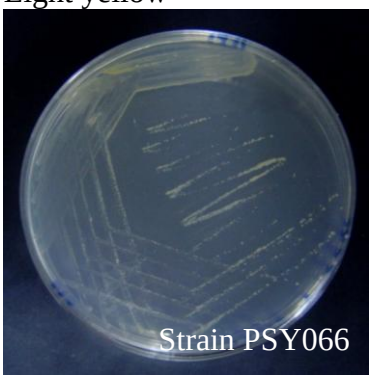
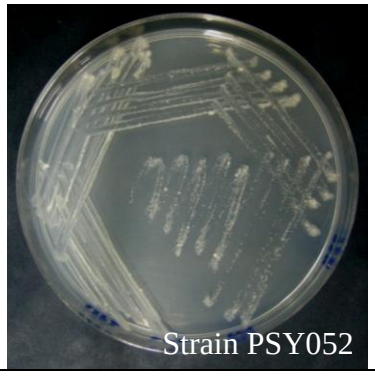
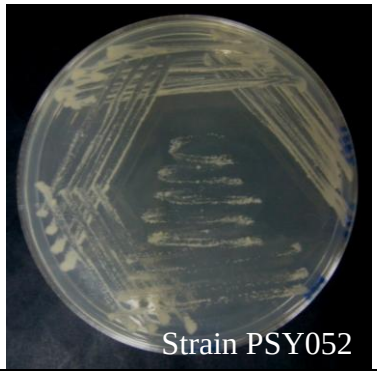
4.4.2 Colour grouping of actinobacterial isolates

The isolated actinobacteria strains were incubated at 15℃ for 20 days on ISP2 media and were assigned into fifteen colour groups respectively as shown in Table 4.5 and Table 4.6. All ninety five actinobacterial strains exhibited aerial mycelia colour ranging from deep orange yellow, deep yellowish pink, light brownish gray, light yellow, moderate orange yellow, pale yellow, strong reddish orange, vivid orange yellow, vivid yellow, white, and yellowish white. It was shown that colour group 1 had the largest collection of actinobacterial strains. Colour group 2, 4, 9, 10, 11, 12, 14 and 15 were clustered into single member group according to its aerial mycelia colour. Colour group 12 and 13 exhibited white aerial and substrate mycelia colour, but they exhibited different colony morphology. Strain PSY021 from colour group 12 exhibited dry, convex and powdery morphology whereas strain PSY084, PSY093 and PSY094 from colour group 13 exhibited dry, irregular and raised morphology. All ninety five actinobacterial strains did not exhibit diffusible pigments and thus indicated the strains did not produce melanoid pigments.

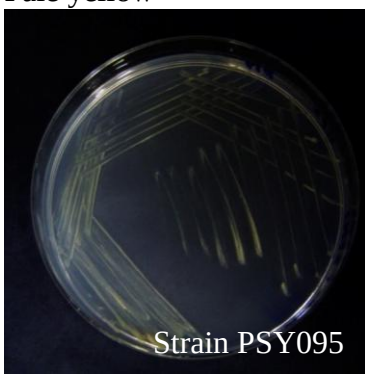
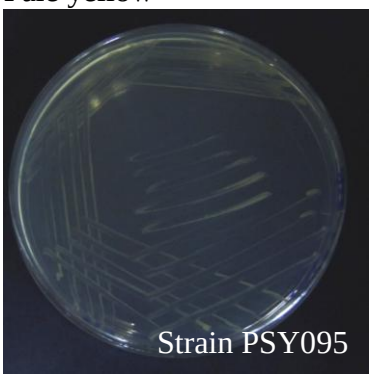
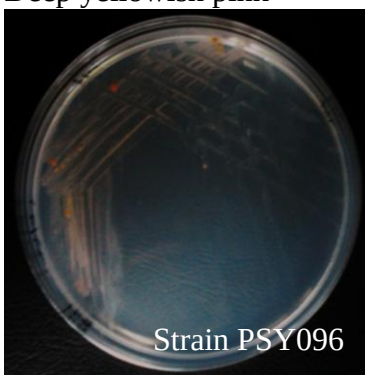
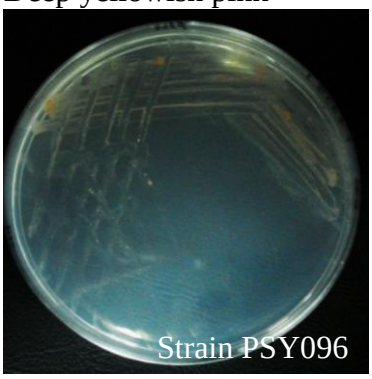
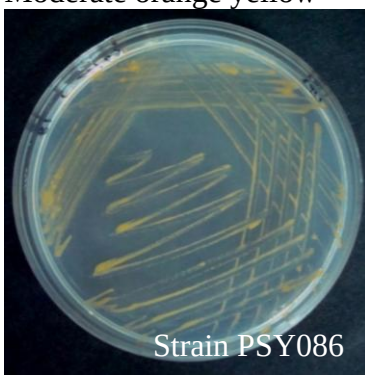
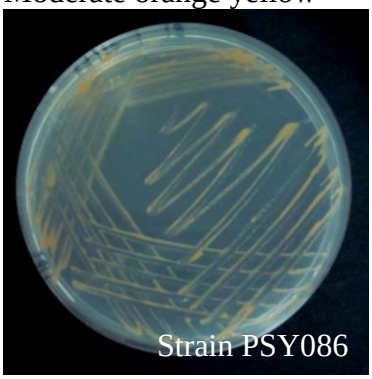
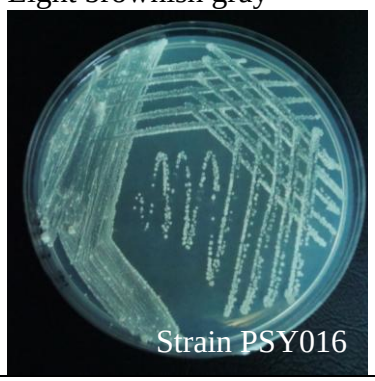
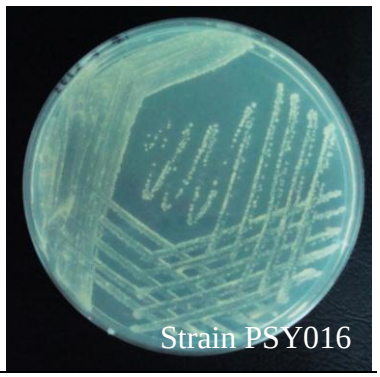
Table 4.5 Colour grouping of actinobacterial isolates on ISP2 media

Colour group	**Aerial mycelia Colour**	**Substrate mycelia colour**
1 (PSY001, PSY002, PSY003, PSY004, PSY005, PSY006, PSY007, PSY008, PSY009, PSY010, PSY011, PSY013, PSY014, PSY015, PSY017, PSY018, PSY022, PSY041, PSY042, PSY043, PSY046, PSY047, PSY048, PSY049, PSY050, PSY051, PSY053, PSY054, PSY055, PSY056, PSY057, PSY058, PSY059, PSY060, PSY061, PSY062, PSY063, PSY064, PSY067, PSY068, PSY069, PSY070, PSY071, PSY072, PSY077, PSY080, PSY081, PSY082, PSY088, PSY089, PSY090, PSY097)	Yellowish white 	Light yellow 
2 (PSY020)	Strong reddish orange 	Strong reddish orange 
3 (PSY012, PSY019, PSY023, PSY033, PSY040, PSY044, PSY045, PSY038, PSY039, PSY092)	Vivid orange yellow 	Vivid orange yellow 

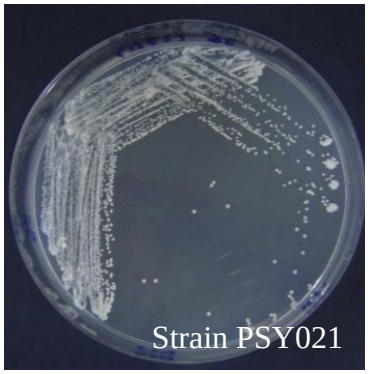
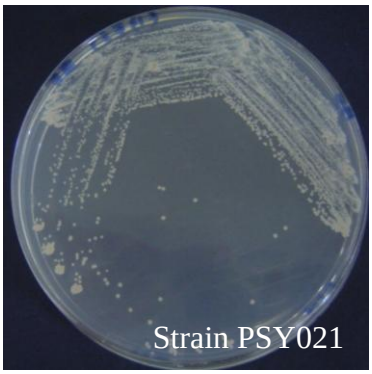
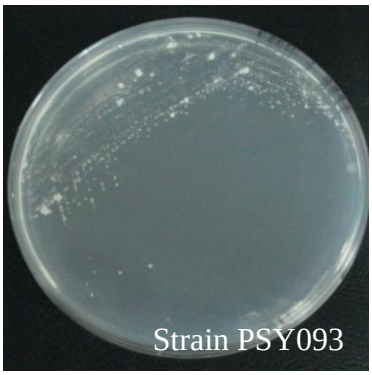
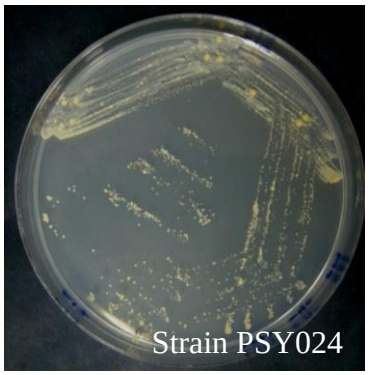
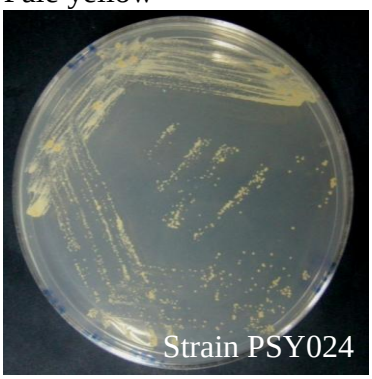
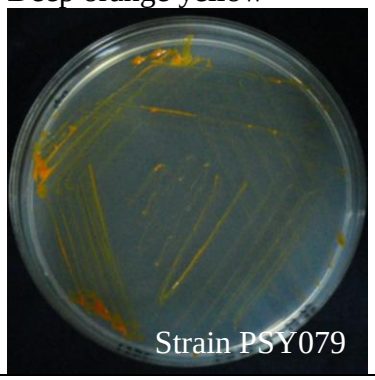
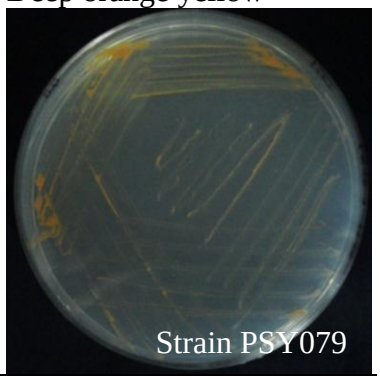
‘Table 4.5, continued’

4 (PSY032)	Vivid yellow	Vivid yellow
		
5 (PSY065, PSY073, PSY074, PSY075, PSY076, PSY078)	Brilliant yellow	Brilliant yellow
		
6 (PSY066, PSY085, PSY087, PSY091)	Light yellow	Light yellow
		
7 (PSY025, PSY026, PSY027, PSY028, PSY029, PSY034, PSY035, PSY036, PSY037, PSY052)	Yellowish white	Yellowish white
		

‘Table 4.5, continued’

8 (PSY031, PSY095)	Pale yellow 	Pale yellow 
9 (PSY096)	Deep yellowish pink 	Deep yellowish pink 
10 (PSY086)	Moderate orange yellow 	Moderate orange yellow 
11 (PSY016)	Light brownish gray 	Yellowish white 

‘Table 4.5, continued’

12 (PSY021)	White 	White 
13 (PSY084, PSY093, PSY094)	White 	White 
14 (PSY024)	Yellowish white 	Pale yellow 
15 (PSY079)	Deep orange yellow 	Deep orange yellow 

4.4.3 Growth characteristics at 25 ℃

All ninety five actinobacterial isolates were then incubated at 25 ℃. Results show that all the actinobacteria isolates showed good growth in 9 days of incubation (Figure 4.3 - 4.4). The strains exhibited the same colony morphology and colour texture as at 15 ℃ for 20 days.

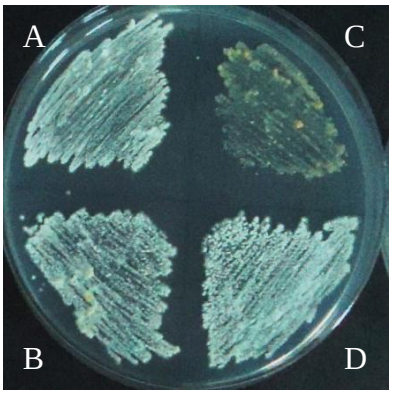


Figure 4.3 ISP2 plates inoculated with actinobacteria for 9 days, 25 ℃ incubation. A, Strain PSY042; B, Strain PSY053; C, Strain PSY024; D, Strain PSY051

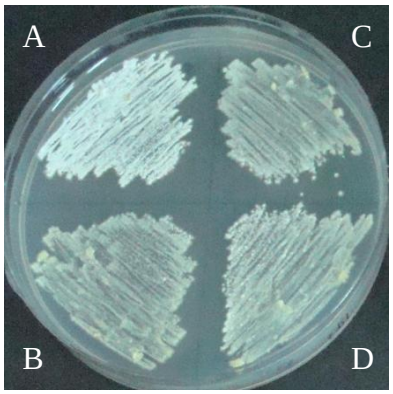
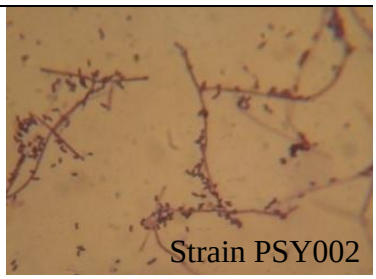
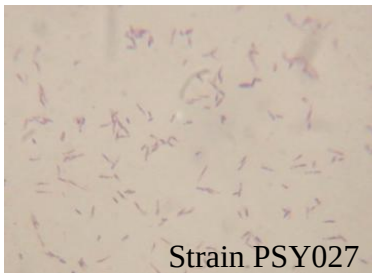
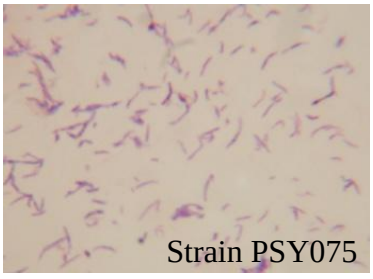


Figure 4.4 ISP2 plates inoculated with actinobacteria for 9 days, 25 ℃ incubation. A, Strain PSY067; B, Strain PSY060; C, Strain PSY006; D, Strain PSY080

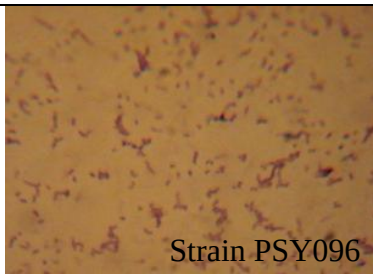
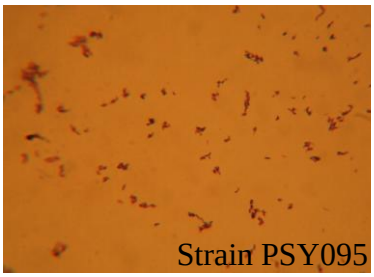
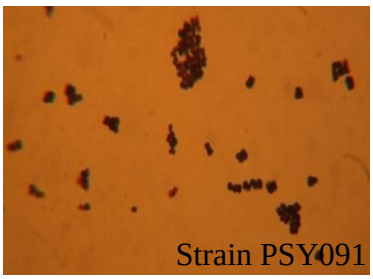
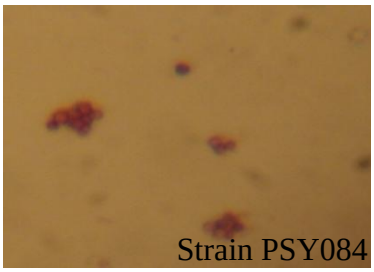
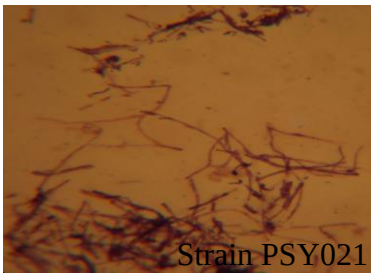
4.4.4 Gram stain examination

Microscopic features of all isolated actinobacterial strains through Gram staining technique were recorded in Table 4.6. All strains were assigned into eight different groups and showed Gram-positive, ranging from rods, long thin rods, branch filaments, irregular rod shape, cocci to branch like hyphae fragmenting into rods.

Table 4.6 Microscopic observation of actinobacteria through Gram staining technique

Microscopic observation	Strain	Representative strain
Gram-positive, rods	PSY001, PSY002, PSY003, PSY004, PSY005, PSY006, PSY007, PSY008, PSY009, PSY010, PSY011, PSY013, PSY014, PSY015, PSY016, PSY017, PSY018, PSY020, PSY022, PSY041, PSY042, PSY043, PSY046, PSY047, PSY048, PSY049, PSY050, PSY051, PSY053, PSY054, PSY055, PSY056, PSY057, PSY058, PSY059, PSY060, PSY061, PSY062, PSY063, PSY064, PSY067, PSY068, PSY069, PSY070, PSY071, PSY072, PSY077, PSY080, PSY081, PSY082, PSY086, PSY088, PSY089, PSY090, PSY097	 Strain PSY002
Gram-positive, long thin rods	PSY025, PSY026, PSY027, PSY028, PSY029, PSY034, PSY035, PSY036, PSY037, PSY052	 Strain PSY027
Gram-positive, rod or branched filaments	PSY024, PSY065, PSY073, PSY074, PSY075, PSY076, PSY078	 Strain PSY075

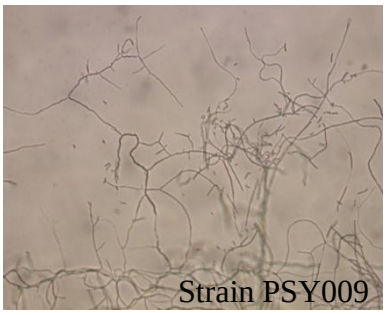
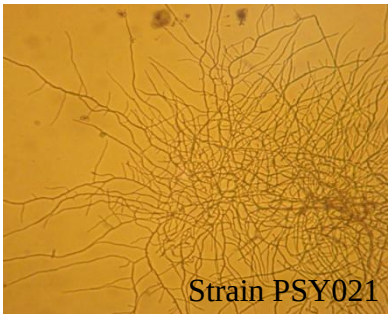
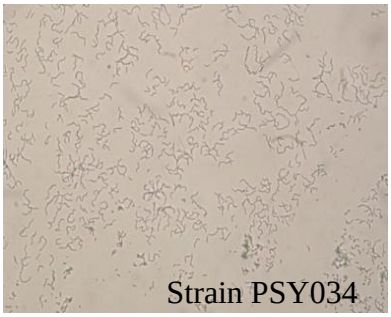
‘Table 4.6, continued’

Gram-positive, irregular rod shape.	PSY096	 Strain PSY096
Gram-positive, cocci to short rod shape	PSY079, PSY095	 Strain PSY095
Gram-positive, cocci	PSY012, PSY019, PSY023, PSY031, PSY032, PSY033, PSY038, PSY039, PSY040, PSY044, PSY045, PSY066, PSY085, PSY087, PSY091, PSY092	 Strain PSY091
Gram-positive, cocci in clusters.	PSY084, PSY093, PSY094	 Strain PSY084
Gram-positive, branched like hyphae fragmenting in rods	PSY021	 Strain PSY021

4.4.5 Coverslip examination

Coverslip examination enables the observation of mycelia and spore formation of actinobacteria. Microscopic features of actinobacterial through coverslip technique were divided into 4 different groups, recorded in table 4.7.

Table 4.7 Microscopic features of actinobacterial strains using the coverslip technique

Microscopic observation	Strain label	Representative
Long branching mycelium, spore production in long chains, straight to flexuous spore chains.	PSY001, PSY002, PSY003, PSY004, PSY005, PSY006, PSY007, PSY008, PSY009, PSY010, PSY011, PSY013, PSY014, PSY015, PSY016, PSY017, PSY018, PSY020, PSY022, PSY041, PSY042, PSY043, PSY046, PSY047, PSY048, PSY049, PSY050, PSY051, PSY053, PSY054, PSY055, PSY056, PSY057, PSY058, PSY059, PSY060, PSY061, PSY062, PSY063, PSY064, PSY067, PSY068, PSY069, PSY070, PSY071, PSY072, PSY077, PSY080, PSY081, PSY082, PSY088, PSY089, PSY090, PSY097	 Strain PSY009
Long branching mycelium.	PSY021	 Strain PSY021
Short branching mycelia, non-spore forming.	PSY024, PSY025, PSY026, PSY027, PSY028, PSY029, PSY034, PSY035, PSY036, PSY037, PSY052, PSY065, PSY073, PSY074, PSY075 PSY076, PSY078	 Strain PSY034
No mycelia present.	PSY012, PSY019, PSY023, PSY031, PSY032, PSY033, PSY038, PSY039, PSY040, PSY044, PSY045, PSY066, PSY079, PSY084, PSY085, PSY086, PSY087, PSY091, PSY092, PSY093, PSY094, PSY095, PSY096	

4.5 Analysis of Diaminopimelic acid isomers

Thin layer chromatography was carried out to analyse DAP isomers. The cell wall hydrolysates were distinguished by the separation of its cell wall types, either LL-DAP or *meso*-DAP. LL-DAP isomers are the major constituent of the cell wall of *Streptomyces* spp. (Becker *et al*., 1964). Representatives from each colour group were randomly selected to be analysed. Twenty nine isolated strains contained LL-DAP isomer while seventeen strains contained *meso*-DAP isomer (Table 4.8).

Table 4.8 Chemotaxonomic characterization of actinobacterial isolates

Cell wall Diamino acids	**Strain label**
LL-DAP	PSY001, PSY002, PSY003, PSY004, PSY005, PSY006, PSY007, PSY008, PSY011, PSY013, PSY014, PSY016, PSY017, PSY020, PSY032, PSY041, PSY042, PSY048, PSY050, PSY056, PSY057, PSY059, PSY060, PSY062, PSY068, PSY081, PSY093, PSY094, PSY097
Meso-DAP	PSY012, PSY019, PSY021, PSY024, PSY027, PSY028, PSY034, PSY036, PSY037, PSY039, PSY065, PSY074, PSY075, PSY078, PSY079, PSY092, PSY096
No DAP detected	PSY031, PSY045, PSY066, PSY085, PSY086, PSY087, PSY091, PSY095

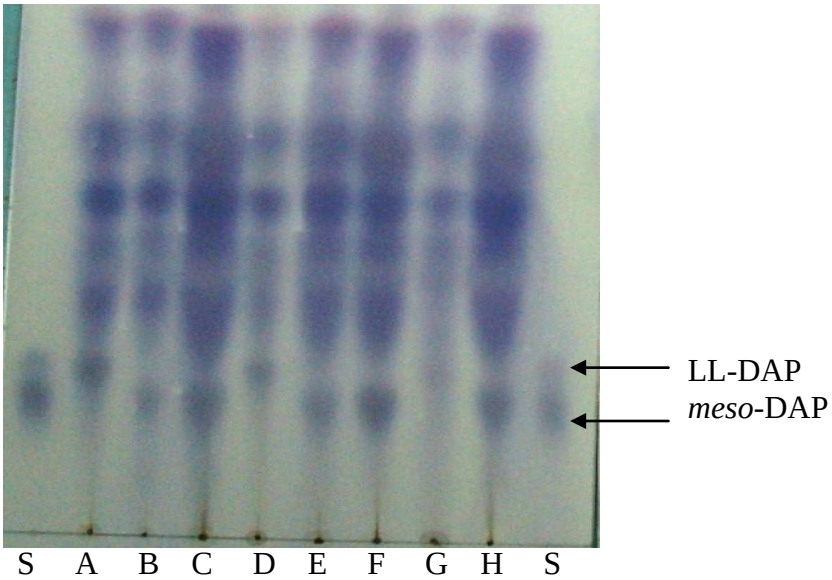


Figure 4.5 Separation of DAP isomers by TLC plates. S: Standard D, L-α, ε Diaminopimelic acid (Sigma, Germany), A: strain PSY013(LL-DAP), B: strain PSY075(*meso*-DAP), C: strain PSY078(*meso*-DAP), D: strain PSY003(LL-DAP), E: strain PSY037(*meso*-DAP), F: strain PSY019(*meso*-DAP), G: strain PSY020(LL-DAP), H: strain PSY074(*meso*-DAP), S: Standard D, L-α, ε Diaminopimelic acid (Sigma, Germany).

4.6 Molecular detection of actinobacteria in soil samples

DNA from the seven soil samples was successfully extracted. Figure 4.6 shows representatives of the DNA extracted from soil. Extracted DNA was used for 16S rRNA amplification followed by secondary PCR using actinobacteria specific primers.

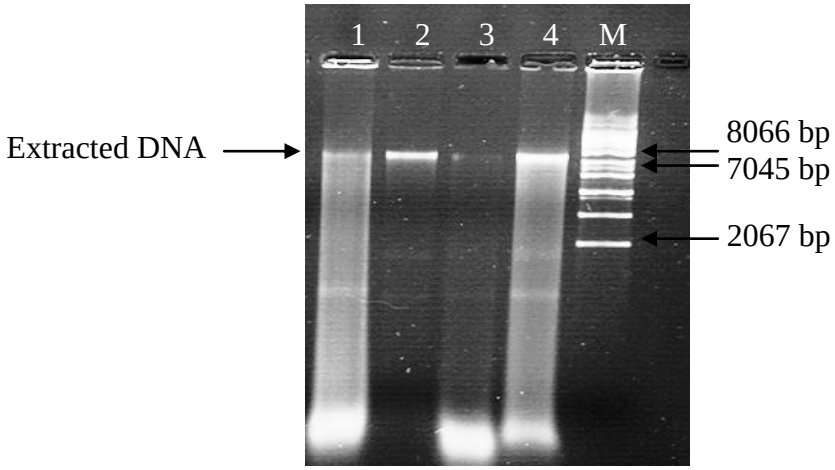


Figure 4.6 Total genomic DNA extraction using giving a 7045 bp to 8066 bp length on Antarctic soil samples. Lanes: 1, G-1; 2, EF-1; 3, BS-7; 4, SD-1; M, molecular marker (supercoiled DNA marker; Invitrogen, USA).

DNA templates from the seven soil samples were used for 16S rRNA amplification. All DNA soil templates were successfully amplified. Figure 4.7 show representatives of the soil 16S rRNA amplification products.

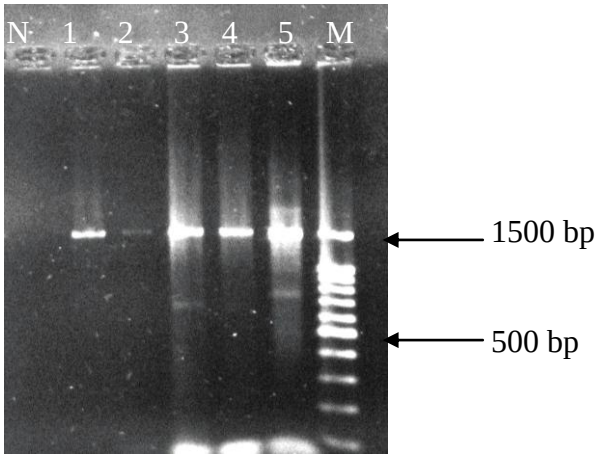


Figure 4.7 Agarose gel electrophoresis of PCR products using 27f and 1525r giving a 1500 bp length on Antarctic soil samples. Lanes: N, control reaction without DNA; 1, G-1; 2, LV-1; 3, SD-1; 4, EF-1; 5, EF-2; M, molecular marker (100 bp ladder; Promega, USA).

The amplified 16S rRNA PCR products were subjected to ten-fold dilution and used as template for secondary PCR using actinobacteria specific primers. All samples amplified a 640 bp length of the 16S rRNA gene. Results showed that the presence of actinobacteria in all the collected soil samples as shown in Figure 4.8.

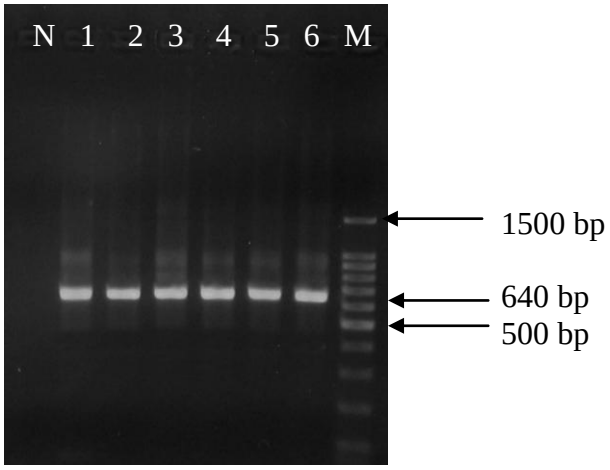


Figure 4.8 Agarose gel electrophoresis of PCR products derived from PCR using actinobacteria specific primers on Antarctic soil samples giving a 640 bp length. Lanes: N: control reaction without DNA; 1, EF-2; 2, BS-7; 3, SD-1; 4, G-1; 5, LV-1; 6, EF-1; M, molecular marker (100 bp ladder; Promega, USA).

4.7 Molecular characterization of pure cultures

4.7.1 DNA extraction from pure cultures

DNA from all ninety five isolated actinobacterial cultures was successfully extracted. Figure 4.9 shows representatives of the DNA extracted from pure cultures. Extracted DNA was used for 16S rRNA amplification followed by secondary PCR using actinobacteria specific primers.

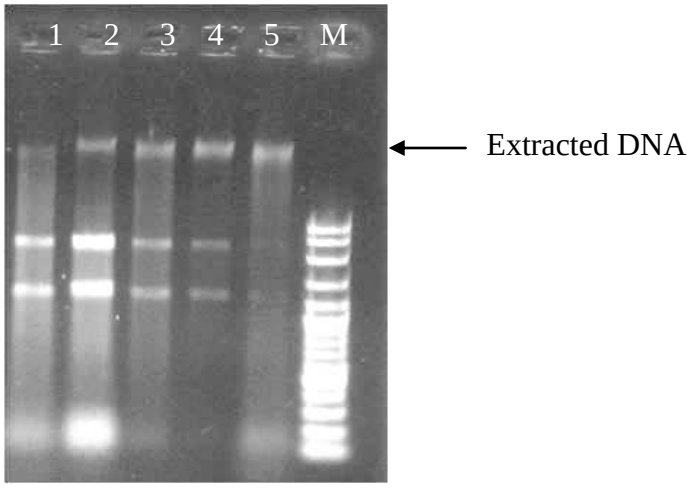


Figure 4.9 DNA extractions from pure cultures. Lanes: 1, strain PSY052; 2, strain PSY063; 3, strain PSY064; 4, strain PSY082, 5, strain PSY065; M, molecular marker (100 bp ladder, Fermentas, Lithuania).

4.7.2 16S rRNA gene amplification of pure cultures

DNA extracted from the actinobacterial strains were used as templates for 16S rRNA amplification. . All pure cultures were successfully amplified. Figure 4.10 show pure culture representatives of the of 16S rRNA amplification products.

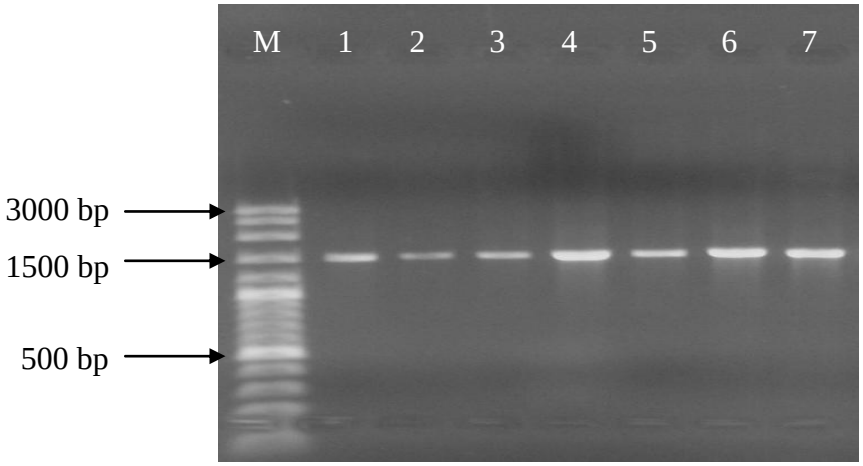


Figure 4.10 Agarose gel electrophoresis of PCR products using 27f and 1492r giving a 1500-bp length on actinobacterial pure cultures. Lanes: M, molecular marker (100 bp ladder, Vivantis, Malaysia), 1, PSY025; 2, PSY092; 3, PSY096; 4, PSY039; 5, PSY052; 6, PSY095; 7, PSY086.

4.7.3 Amplification using actinobacteria specific primers

The amplified 16S rRNA PCR products were subjected to ten-fold dilution and used as template for secondary PCR using actinobacteria specific primers. All samples amplified a 640 bp length of the 16S rRNA gene and thus confirmed all isolates were actinobacteria (Figure 4.11).

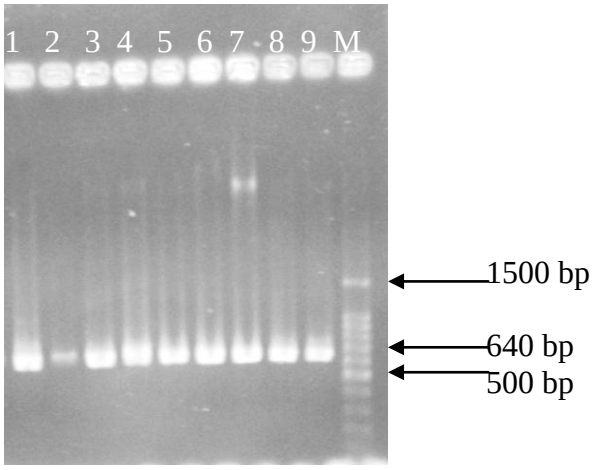
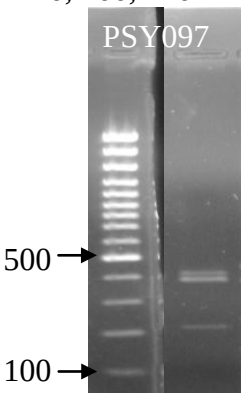
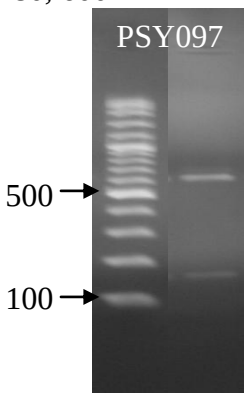
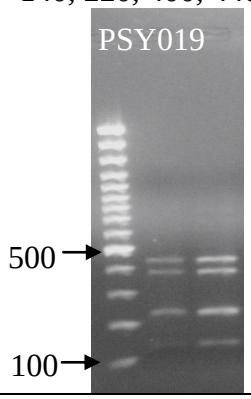
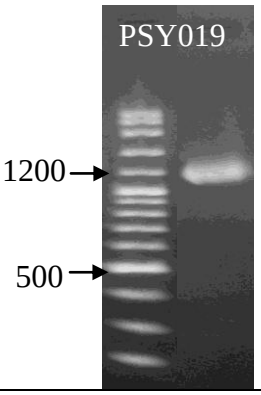


Figure 4.11 Agarose gel electrophoresis of PCR products derived from PCR using actinobacteria specific primers on actinobacterial cultures giving a 640-bp length. Lanes: 1, PSY085; 2, PSY072; 3, PSY071; 4, PSY081; 5, PSY042; 6, PSY049; 7, PSY032; 8, PSY053; 9, PSY093; M, molecular marker (100 bp ladder; Promega, USA).

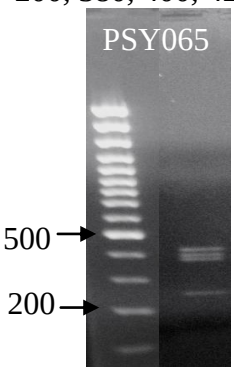
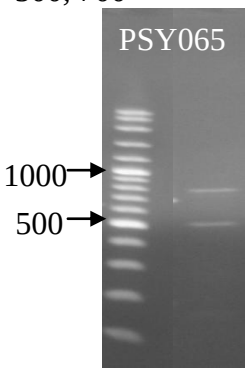
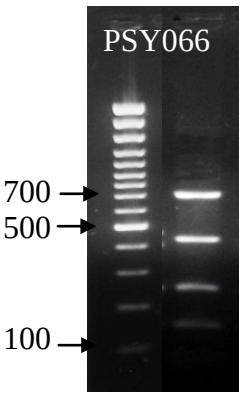
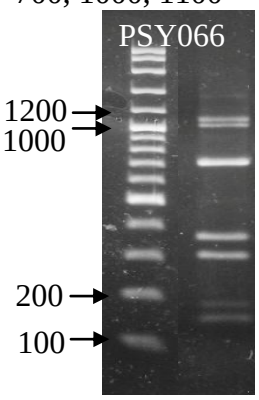
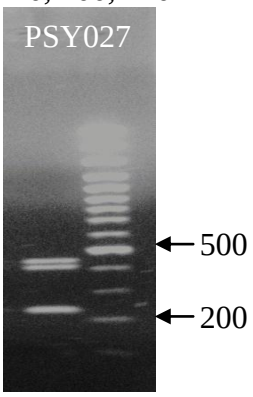
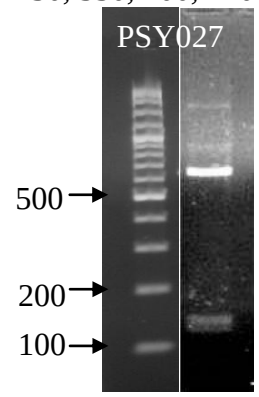
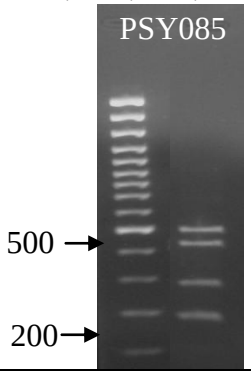
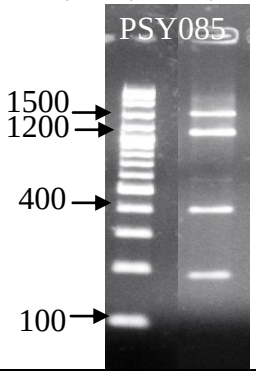
4.7.4 Dereplication of isolated actinobacterial strains using ARDRA

The 16S rRNA gene fragments from the primary PCR product were digested using two restriction enzymes, *BssM*I and *Hha*I. Fermentas 100 bp marker was used for *Hha*I digestions while Vivantis 100 bp marker was used for *BssM*I digestions. Based on the ARDRA pattern, the ninety five isolates were divided into sixteen groups. Each ARDRA banding pattern would indicate a genus (Table 4.9).

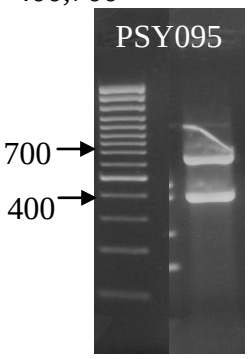
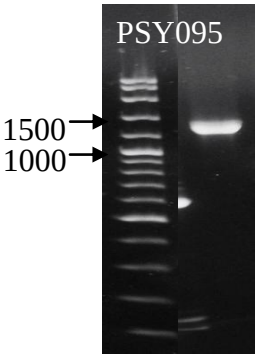
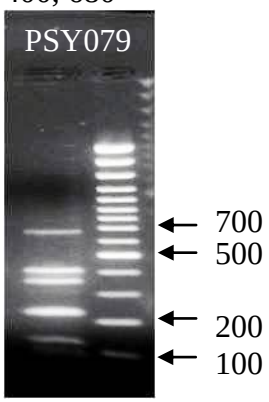
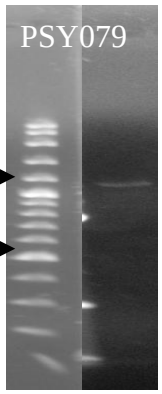
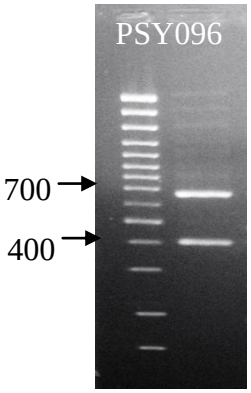
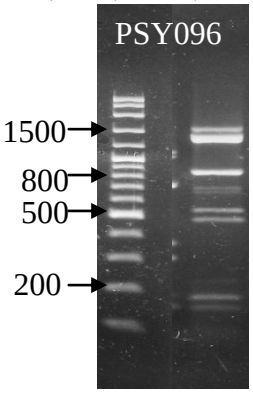
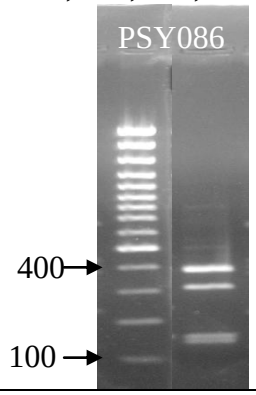
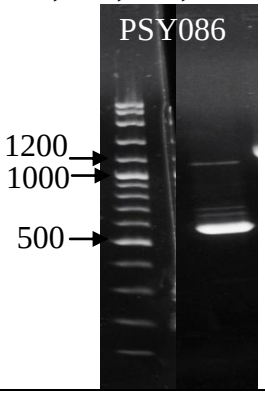
Table 4.9 ARDRA groups of isolated actinobacterial strains

Group	*Hha*I approx. restriction fragment lengths (bp)	*BssM*I approx. restriction fragment lengths (bp)	Strain label
1	220, 400, 440 	180, 600 	PSY001, PSY002, PSY003, PSY004, PSY005, PSY006, PSY007, PSY008, PSY009, PSY010, PSY011, PSY013, PSY014, PSY015, PSY017, PSY018, PSY020, PSY022, PSY041, PSY042, PSY043, PSY046, PSY047, PSY048, PSY049, PSY050, PSY051, PSY053, PSY054, PSY055, PSY056, PSY057, PSY058, PSY059, PSY060, PSY061, PSY062, PSY063, PSY064, PSY067, PSY068, PSY069, PSY070, PSY071, PSY072, PSY077, PSY080, PSY081, PSY082, PSY088, PSY089, PSY090, PSY097
2	140, 220, 400, 440 	1200 	PSY012, PSY023 PSY019, PSY033, PSY040, PSY038, PSY039, PSY092

‘Table 4.9, continued’

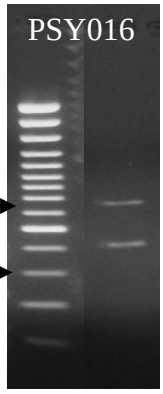
Group	*Hha*I	*BssM*I	Strain label
3	200, 380, 400, 420 	500, 700 	PSY065, PSY073, PSY074, PSY075, PSY076, PSY078
4	140, 220, 420, 700 	120, 150, 300, 350, 700, 1000, 1100 	PSY044, PSY045, PSY066
5	220, 400, 440 	150, 550, 700, 1100 	PSY025, PSY026, PSY027, PSY028, PSY029, PSY034, PSY035, PSY036, PSY037, PSY052
6	200, 280, 450, 500 	180, 400, 1200, 1500 	PSY085, PSY087, PSY091

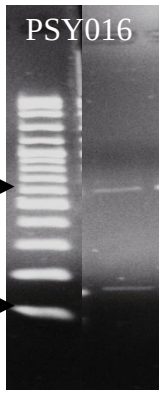
‘Table 4.9, continued’

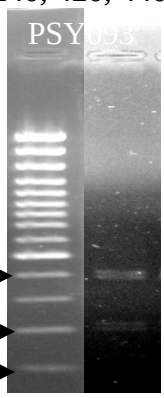
Group	*Hha*I	*BssM*I	Strain label
7	400,700 	1350 	PSY095
8	120, 220, 380, 400, 680 	1200 	PSY079
9	400, 650 	120, 150, 450, 500, 700, 800, 1200, 1400 	PSY096
10	150, 160, 320, 400 	600, 700, 800, 1200 	PSY086

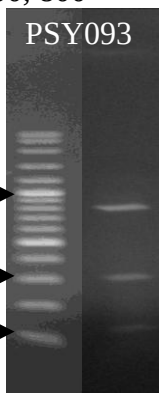
‘Table 4.9, continued’

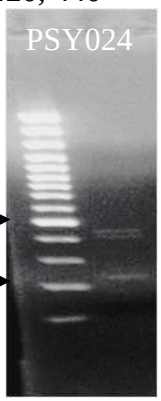
Group	*Hha*I	*BssM*I	Strain label
11	400,700	180, 600	PSY016
12	220, 240, 420, 440	150, 300, 800	PSY084, PSY093 , PSY094
13	220, 420, 440	200, 300, 800	PSY024
14	320, 420	300, 500, 700， 800	PSY031

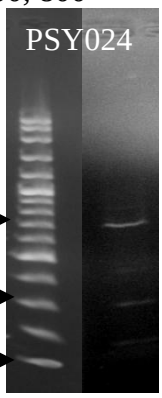


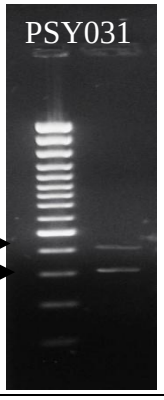


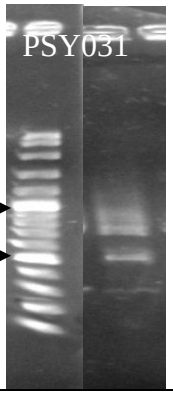






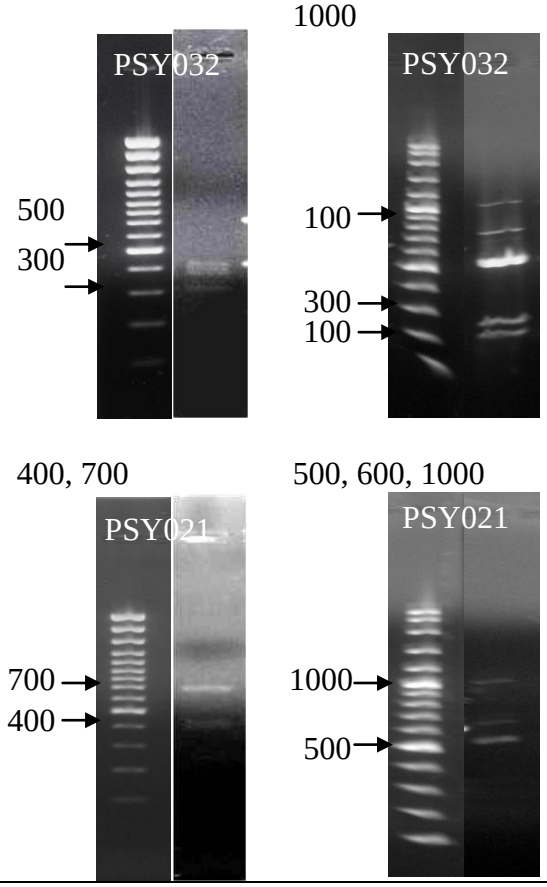






‘Table 4.9, continued’

Group	*Hha*I	*BssM*I	Strain label
15	300, 380, 400	200, 250, 500, 800, 1000	PSY032
16	400, 700	500, 600, 1000	PSY021



4.8 Sequence analysis of 16S rRNA actinobacterial isolates

Actinobacterial representatives chosen from each ARDRA group were partially sequenced and BLAST analysis was carried out through http://www.ncbi.nlm.nih.gov/. The sequences producing the most significant alignments were shown in Table 4.10.

Table 4.10 Identification of 16s rRNA isolated actinobacterial strains from Signy Island, Antartica

ARDRA group	Strains	Closest phylogenetic affiliation	Accession number	Length (bp)	Identity (%)	Source of sample
1	PSY013	*Streptomyces beijiangensis*	AB249973	810	98%	LV-1
	PSY020			659	98%	LV-1
	PSY056			770	98%	LV-1
	PSY059			780	98%	LV-1
	PSY081			810	98%	LV-1
	PSY097			650	99%	LV-1

‘Table 4.10, continued’

2	PSY019	*Rhodococcus* sp.	FJ195998	700	100%	EF-2
	PSY039			326	100%	SD-1
	PSY092			890	100%	EF-1
3	PSY065	*Mycobacterium* sp.	EU167989	838	98%	BS-7
	PSY074			850	98%	BS-7
	PSY075			830	98%	BS-7
	PSY078			830	98%	BS-7
4	PSY045	*Demetria terragena*	Y1452	440	91%	G-1
	PSY066			730	100%	G-1
5	PSY025	*Rhodococcus corynebacterioides*	X80615	860	96%	BS-7
	PSY027			830	96%	BS-7
	PSY028			830	96%	BS-7
	PSY037			850	97%	BS-7
	PSY052			879	97%	BS-7
6	PSY085	*Kocuria* sp.	FJ357623	700	100%	G-1
	PSY087			818	100%	BS-7
	PSY091			800	100%	BS-7
7	PSY095	*Glaciibacter superstes*	AB378302	810	97%	G-1
8	PSY079	*Humicoccus* sp.	EU939310	814	99%	G-2
9	PSY096	Actinobacterium P23	D1351736	860	96%	BS-7
10	PSY086	*Microbacterium* sp.	AB461113	809	100%	G-2
11	PSY016	*Streptomyces argenteolus*	EU570529	780	99%	EF-1
12	PSY093	*Marmoricola aequoreus*	AM295338	780	98%	SD-1
	PSY094			879	98%	SD-1
13	PSY024	*Tsukamurella* sp.	EF514880	840	100%	G-1
14	PSY031	*Micrococcus luteus*	FN984531	819	100%	BS-7
15	PSY032	Actinobacterium kmd_307	EU723162	760	97%	SD-1
16	PSY021	*Nocardia ninae*	DQ235678	370	97%	BS-7

4.9 Phylogenetic analysis of actinobacterial isolates

Representatives from the eighteen ARDRA groups i.e. strain PSY016, strain PSY019, strain PSY021, strain PSY024, strain PSY027, strain PSY031, strain PSY032, strain PSY065, strain PSY066, strain PSY079, strain PSY085, strain PSY086, strain PSY093, strain PSY095, strain PSY096 and strain PSY097 were chosen for phylogenetic analysis and good phylogenetic clustering were obtained (Figure 4.12).

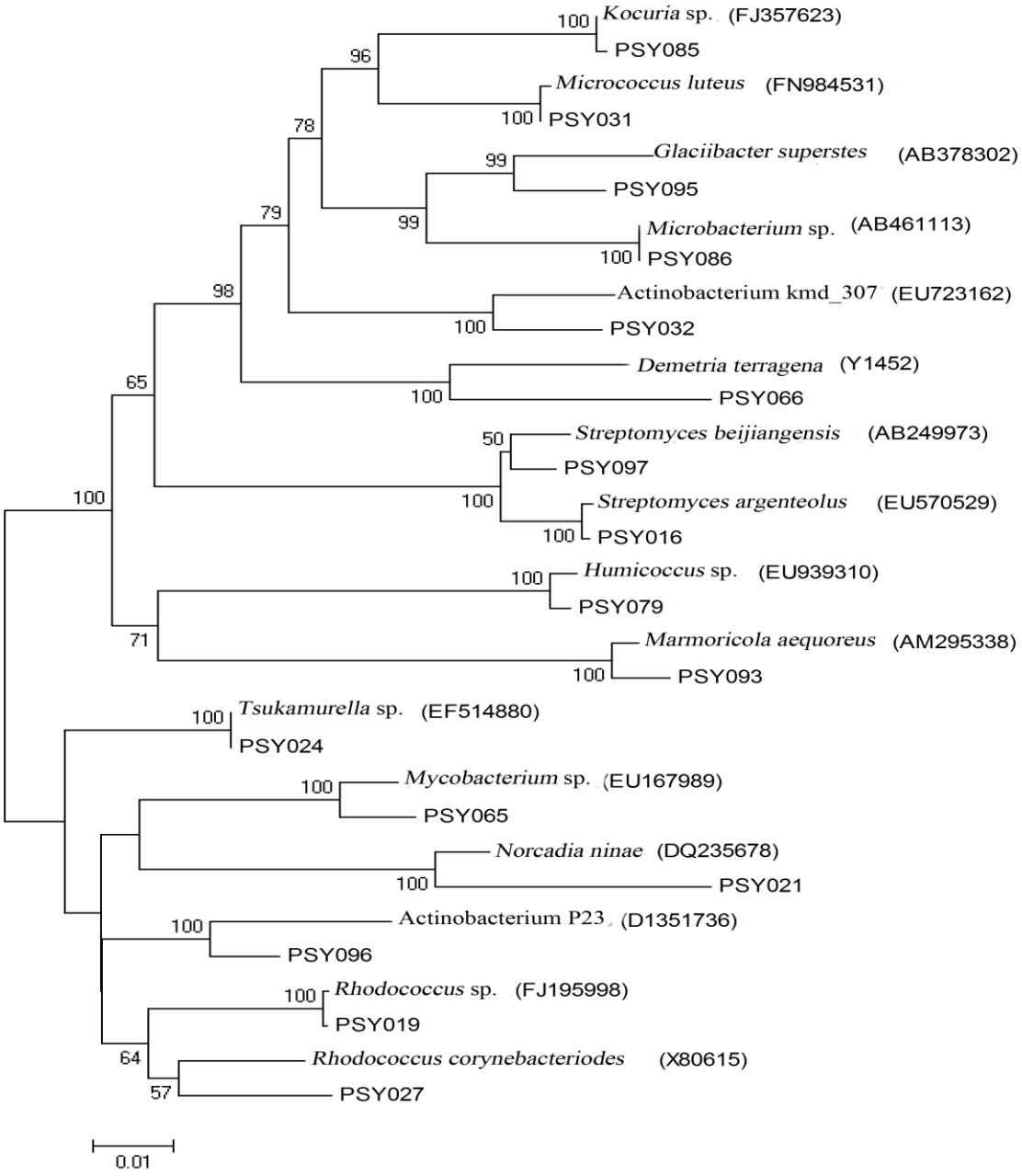


Figure 4.12 Phylogenetic analyses of actinobacterial isolates using Mega 4.1 (Tamura *et al*., 2007). Phylogenetic reconstruction was performed by using neighbor-joining. Bootstrap values indicated at branch points were 50% or more.

4.10 Primary screening of actinobacterial isolates for antimicrobial activity

The actinobacterial isolates were tested against six test bacteria. All actinobacterial isolates showed no antibacterial activity against two test bacteria, which were *E. coli* and *S. typhi* while *K. pneumoniae* was only susceptible to strain PSY013, with 17 mm inhibitory zones. Forty six of the isolated actinobacterial strains showed strong (>15mm), moderate (10-15mm) or weak (<10mm) antibacterial activity while fifty four actinobacterial isolates showed no activity against *S. aureus*, *S. epidermidis* and *P.vulgaris* (Figure 4.13, Figure 4.14 and Table 4.11). All the forty six isolates which showed activity were isolated from Three Lakes Valley. Generally, stronger inhibitions were observed against *S. aureus*, compared to those against *P. vulgaris* and *S. epidermidis* (Table 4.11).

Table 4.11 Antibacterial activity on tester organisms (The zones of inhibition were recorded as diameter in mm)

Strain label	*Staphylococcus aureus* ATCC 25923	*Staphylococcus epidermidis* ATCC 12228	*Proteus vulgaris* ATCC 13315
PSY001	9	30 ±1.41	14
PSY002	23 ±0.71	25	13
PSY003	-	-	7
PSY004	-	8	-
PSY005	19 ±1.41	25 ±1.41	8 ±1.41
PSY006	21	29	13 ±1.41
PSY007	24	30	14
PSY008	-	10	7
PSY010	-	6	-
PSY011	17 ±1.41	20	7 ±1.41
PSY013	25	29	19
PSY014	24 ±1.41	25 ±0.71	14 ±1.41
PSY041	20 ±1.41	8	9 ±0.71
PSY042	22	10 ±0.71	10
PSY043	20 ±1.41	10	-
PSY046	20	9	10 ±1.41
PSY047	21.5 ±2.12	12	11
PSY048	22	11 ±1.41	12 ±1.41
PSY049	23	14	9
PSY050	19 ±1.41	11 ±1.41	11 ±2.83
PSY051	21 ±1.41	30	14
PSY053	17	14	12
PSY054	17.5 ±2.12	14 ±0.71	10 ±1.41
PSY055	15	12	13

‘Table 4.11, continued’

Strain label	*Staphylococcus aureus* ATCC 25923	*Staphylococcus epidermidis* ATCC 12228	*Proteus vulgaris* ATCC 13315
PSY057	21 ± 1.41	11	10
PSY058	14.5 ± 2.12	14	12 ± 1.41
PSY059	17	15 ± 1.41	11 ± 4.24
PSY060	16.5 ± 2.1	14	12
PSY061	14 ± 5.66	13	11 ± 1.41
PSY062	25	11 ± 1.41	15
PSY063	21.5 ± 2.12	14 ± 1.41	16 ± 1.41
PSY064	21	14	15
PSY067	22 ± 2.12	20	11
PSY068	23 ± 1.41	13 ± 2.83	13 ± 1.41
PSY069	23	13 ± 1.41	12
PSY070	22	14	13 ± 1.41
PSY071	23	12 ± 1.41	14
PSY072	23 ± 1.41	14	14 ± 2.83
PSY077	18	14 ± 4.24	10
PSY080	21	11	10
PSY081	21 ± 1.41	11 ± 2.83	12
PSY082	17	15	13 ± 1.41
PSY088	20.5 ± 0.71	16 ± 2.83	16
PSY089	24	13 ± 1.41	16
PSY090	18	12	15 ± 1.41
PSY097	22	14	13 ± 1.41

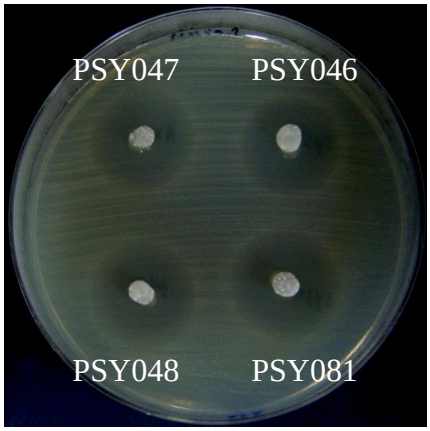


Figure 4.13 Strong inhibitions of *S. aureus* by actinobacterial representative strains.

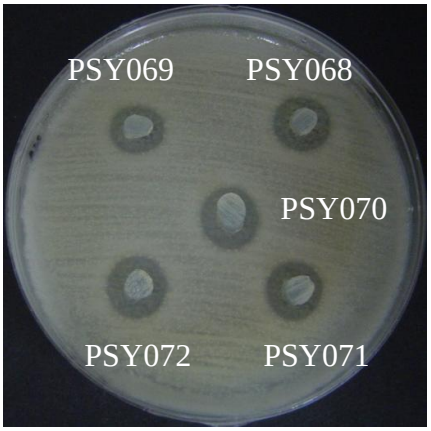


Figure 4.14 Moderate inhibitions of *S. epidermidis* by actinobacterial representative strains.

4.11 Screening of NRPS systems in actinobacterial isolates

All the isolated actinobacterial strains were furthered screened for NRPS systems. Results showed that out of the ninety five isolates, seventy nine isolates contain the NRPS genes. All the forty six strains which exhibited antibacterial activity against the three test organisms also contained the NRPS genes. NRPS genes were not detected in strain PSY025, strain PSY031, strain PSY032, strain PSY044, strain PSY045, strain PSY066, strain PSY074, strain PSY075, strain PSY076, strain PSY078, strain PSY079, strain PSY085, strain PSY086, strain PSY093, strain PSY095 and strain PSY096. Figure 4.15 shows representatives of isolates containing the NRPS gene.

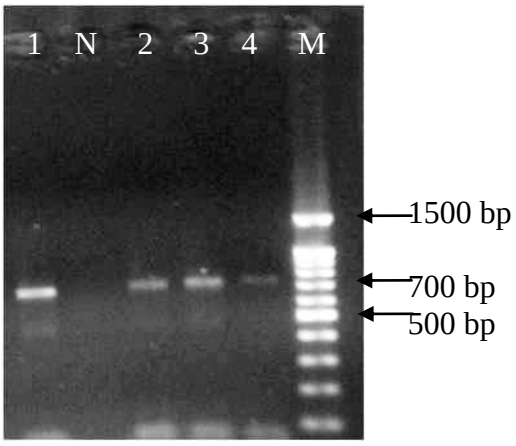


Figure 4.15 Agarose gel electrophoresis of PCR products using NRPS primers giving a 700-800 bp length. Lanes: N, negative control; 1, PSY062; 2, PSY058; 3, PSY077; 4, PSY004; M, molecular marker (100 bp marker iNtRON, Korea).