CHARACTERISATION AND PRELIMINARY BIOACTIVITIES OF ACTINOBACTERIA ISOLATED FROM SELECTED SOIL SAMPLES FROM SIGNY ISLAND, ANTARCTICA

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KUALA LUMPUR

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CHARACTERISATION AND PRELIMINARY BIOACTIVITIES OF ACTINOBACTERIA ISOLATED FROM SELECTED SOIL SAMPLES FROM SIGNY ISLAND, ANTARCTICA

ABSTRACT

Actinobacteria are one of the most prominent bacterial groups, even in harsh Antarctic environments, with important functional roles in soil. Signy Island, part of the South Orkney Islands archipelago in Maritime Antarctica, provides a unique environment due to its isolation and simple ecosystems which allow a deeper look into the effects of warming on actinobacterial communities particularly as this region is experiencing a trend of warming temperatures. This unique ecosystem is also a valuable source of potential new bioactive products and bacterial species. Aside from this, the harsh environment exerts some selection pressure favouring bacteria capable of adapting and thriving by utilizing diverse substrates at lower temperatures. This study uses actinobacteria isolated from the soils of Signy Island for assays of enzyme and biosurfactant production at low temperatures. Through this, it was found that certain strains were producing enzymes at low temperatures indicating a possible production of cold-active enzymes. This production is an advantageous adaptation of actinobacterial strains to the polar environment. This study also describes a novel actinobacterial species isolated from Signy Island. The new species, strain S63, shares a less than 97 % similarity with *Humibacillus xanthopallidus* KV-663^T; which is the only species in the genus. The whole genome of the strain has been sequenced and the draft genome shows the presence of genes coding for various stress tolerances. Phenotypic characterisation of the strain also evidences the potential of this strain being a novel species and genus of the family *Intrasporangiaceae*.

Keywords: Actinobacteria, Signy, Antarctic, Enzymes, Novel species

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PENCIRIAN DAN BIOAKTIVITI AWALAN AKTINOBAKTERIA YANG DIPENCILKAN DARI SAMPEL TANAH TERPILIH DARI PULAU SIGNY, ANTARTIKA

ABSTRAK

Aktinobacteria adalah salah satu kumpulan bakteria yang paling menonjol, walaupun dalam persekitaran Antartika yang sukar untuk kehidupan, dengan peranan dan fungsi penting dalam tanah. Signy Island, sebahagian dari Kepulauan Selatan Orkney di Maritime Antartika, mempunyai persekitaran yang unik kerana pemisahan secara geografi dan ekosistem yang tidak rumit yang membolehkan melihat secara lebih mendalam kesan pemanasan pada komuniti bakteria terutamanya apabila rantau ini mengalami trend suhu pemanasan. Ekosistem yang unik ini juga merupakan sumber berharga daripada produk bioaktif baru yang berpotensi dan spesies bakteria yang baru. Selain itu, persekitaran ini juga menimbulkan tekanan pemilihan yang memihak kepada bakteria yang mampu menyesuaikan dan berkembang dengan menggunakan substrat yang berbeza pada suhu yang lebih rendah. Kajian ini menggunakan aktinobakteria yang dipencilkan dari tanah Pulau Signy untuk pengujian pengeluaran enzim pada suhu rendah. Melalui ini, didapati bahawa terdapat isolat yang mengeluarkan enzim pada suhu sejuk yang menunjukkan penyesuaian isolat aktinobakteria kepada persekitaran polar. Kajian ini juga mengkaji spesies aktinobakteria baru yang dipencilkan dari Pulau Signy. Spesies baru ini, strain S63, mempunyai persamaan kurang daripada 97 % dengan *Humibacillus xanthopallidus* KV-663^T; yang merupakan satu-satunya spesies dalam genus tersebut. Genom penuh strain telah dijujukan dan draf jujukan menunjukkan kehadiran kod untuk protein antibeku. Pencirian fenotip dari strain ini juga membuktikan potensi strain ini menjadi spesies dan genus baru dari keluarga *Intrasporangiaceae*.

Kata kunci: Aktinobakteria, Signy, Antartika, Enzim, Species baru

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LIST OF SYMBOLS AND ABBREVIATIONS

Symbol / Abbreviation	Meaning
&	: *Ampersand* (and)
~	: Approximately
°C	: Degree Celsius
>	: More than
<	: Less than
µg/mL	: Microgram per millilitre
µL	: Microliter
µM	: Micromolar
%	: Percentage
±	: Plus minus
®	: Registered
x g	: Times gravity
AIA	: Actinomycetes Isolation Agar
a.s.l.	: Above sea level
BAS	: British Antarctic Survey
BEA	: Beef Extract Agar
bp	: Base pairs
C/N	: Carbon to Nitrogen
ca.	: Calculated
$CaCl_2.2H_2O$	: Calcium Chloride
$CaCO_3$	: Calcium Carbonate
CDS	: Coding sequence
CFU	: Colony forming unit
CFU/g	: Colony forming unit per gram
cm	: Centimetre
DGGE	: Denaturing gradient gel electrophoresis
DMSO	: Dimethyl sulfoxide
DNA	: Deoxyribonucleic acid
dNTPs	: Deoxynucleotide
e.g.	: *exempli gratia* (for example)
et al.	: *et alia* (and others)
etc.	: *et cetera* (and other similar things)

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g	: Gram
G+C	: Guanine-cytosine
HCl	: Hydrochloric
i.e.	: *id est* (that is)
ISP2	: International Streptomyces Project Medium 2
km	: Kilometres
km^2	: Kilometres
LL-DAP	: LL-Diaminopimelic acid
m	: Meter
mg	: Milligram
mg/mL	: Milligram per millilitre
$MgCl_2$	: Magnesium chloride
mL	: Millilitre
mm	: Millimetre
mM	: Millimolar
N	: Normality
N_{50}	: Shortest sequence length at 50 % of the genome
N/D	: Not determined
NaCl	: Sodium chloride
NaOH	: Sodium hydroxide
NCBI	: National Center for Biotechnology Information
ncRNA	: Non-coding RNA
ng	: Nanogram
nm	: Nanomole
OD	: Optical density
OTU	: Operational taxonomic unit
p.	: Page
PCR	: Polymerase Chain Reaction
pH	: Potential of hydrogen
psi	: Pounds per square inch
RAST	: Rapid Annotations using Subsystems Technology
RNA	: Ribonucleic acid
rpm	: Revolutions per minute
rRNA	: Ribosomal Ribonucleic Acid
SA	: *Streptomyces* Agar

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SB	: Sodium boric acid
sdH_2O	: Sterile distilled water
Sdn. Bhd.	: Sendirian Berhad (Private limited)
SIMCO	: Sea-ice microbial community
sp.	: Species
spp.	: Species
™	: Trade mark
T-RFLP	: Terminal restriction fragment length polymorphism
tRNA	: Transfer Ribonucleic Acid
TNTC	: Too numerous to count
U	: Unit
USA	: United States of America
UV	: Ultraviolet
V	: Voltage
vs.	: *Versus* (in contrast to)
w/v	: Weight per volume

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CHAPTER 1: INTRODUCTION

The Antarctic continent is a unique environment that has been of scientific interest since its discovery. The isolation and harsh environmental conditions pose a barrier to the establishment of biological life, yet it hosts thriving communities of microorganisms. With the lack of higher plant and animal life in the Antarctic, soil food webs are primarily comprised of microorganisms, which perform important roles in various niches and microhabitats. These generally simple microbial ecosystems in the Antarctic allow a deeper understanding of fundamental processes, diversity and ecosystem functions that would be difficult to study in more complex lower latitude ecosystems (Cary *et al.*, 2010).

Many microorganisms in Antarctic ecosystems remain understudied, evidenced by the large percentages of uncultured species discovered in molecular based community diversity studies (Makhalanyane *et al.*, 2013; Pearce *et al.*, 2012; Van Goethem *et al.*, 2016). As isolation techniques improve, more novel species are being described, which points towards the high potential of discovering novel microorganisms from Antarctic ecosystems (Yadav *et al.*, 2017).

Antarctica's typically harsh environmental conditions impose high selection pressures on the members of its microbial communities, which have led to various evolutionary adaptations to ensure survival (Shivaji *et al.*, 2017). These include the production of cold-active proteins and enzymes, cold shock and heat shock proteins, and enzymes for the degradation of complex substrates (de Pascale *et al.*, 2012; Deming, 2002). Production of these enzymes and other proteins allow the microbial community to utilise complex substrates and function effectively at low temperatures (Kügler *et al.*, 2015). This makes the Antarctic region a valuable and as yet largely untapped source of novel secondary metabolites and other compounds with potential to be applied in areas such as manufacturing industries and bioremediation.

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As a group that include some of the most prolific producers of secondary metabolites and products, the class Actinobacteria is of particular interest (Kügler *et al.*, 2015). A large percentage of antibiotics are derived from compounds originally isolated from actinobacteria (Berdy, 2005). This group of Gram-stain-positive bacteria are ubiquitous in many environments but are most commonly found in soil. Many actinobacteria form branching mycelia and sporulate (Stackebrandt *et al.*, 1997), allowing them to withstand periods of low water availability (Shivaji *et al.*, 2017). As this group is known for their combination of bioactive potential and stress tolerance, the current study focuses on the isolation and characterisation of actinobacteria from Antarctic soils.

Soil samples from Signy Island (South Orkney Islands, maritime Antarctic) were used to assess the biodiversity of the culturable terrestrial microbial community. Signy Island is relatively well characterised in terms of its terrestrial ecosystems and has experienced considerable warming over recent decades (Cannone *et al.*, 2017; Turner *et al.*, 2009). Coastal habitats on the island are heavily impacted by seals, penguins and other seabirds which come ashore to breed, rest, or moult (Favero-Longo *et al.*, 2011). Signy Island Research Station is operated by the British Antarctic Survey (BAS) and is a centre for terrestrial biological studies in the region.

In this study, actinobacterial strains isolated from Signy Island soils were further studied to assess their bioactive potential at low temperatures. The production of enzymes and biosurfactants at low temperature provide illustration of the adaptations of Antarctic actinobacteria to the chronically low temperatures of the polar environment. However, bacterial studies on Signy Island have previously centred on diversity or abundance studies with only limited studies of the functional properties of the bacteria from these communities. Thus, as part of this study, bacteria from Signy Island were studied for the production of extracellular amylase, protease, lipase and biosurfactants.

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Of the strains studied, one (strain S63) was found to be a potentially novel species and further studies were carried out describe the strain. These included characterisation tests to determine the phylogenetic, chemotaxonomic and phenotypic characteristics of the strain. The whole genome of the strain was sequenced, and the draft genome studied to identify genes of interest.

The objectives of this study were:

a) to characterise actinobacteria isolated from soil samples collected from Signy Island, Antarctica using phenotypic and genotypic methods

b) to screen selected actinobacterial strains for their ability to produce enzymes and biosurfactants

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CHAPTER 2: LITERATURE REVIEW

2.1 Introduction to the Antarctic

The Antarctic continent is one of the least explored continents in the world. One of the last recorded regions on Earth to be discovered and explored, this southernmost continent has since become one of the most interesting environments designated for scientific research. Since the signing of the Antarctic Treaty on the 1st of December 1959, military activities, mineral exploitation, nuclear explosions and nuclear waste disposal have been prohibited. Instead, the Treaty supports freedom of scientific research and protects the continent's environment and living resources (*The Antarctic Treaty*, Retrieved Dec. 29, 2016 from http://www.ats.aq/e/ats.htm). The Protocol on Environmental Protection to the Antarctic Treaty has designated Antarctica as a "natural reserve, devoted to peace and science" (Article 2) (*The Protocol on Environmental Protection to the Antarctic Treaty*, signed Oct. 4, 1991; Retrieved Dec. 29, 2016 from http://www.ats.aq/e/ep.htm).

The Arctic and Antarctic both include perennially ice-covered environments in the northern and southern polar regions, respectively (Figure 2.1). Although both share some characteristics (i.e. low temperatures throughout the year, long summer and winter seasons with periods of either 24-hour daylight or darkness, respectively, low vegetation cover largely dominated by lower plant forms), numerous differences set these regions apart from each other (Convey, 2013). The Arctic is an ocean surrounded by the northern continents. While much of the Arctic Ocean is covered by sea ice, especially in winter, the surrounding large landmasses, other than Greenland, are predominantly free of ice and snow in the summer. This, along with the continuous southwards land connections, has allowed the repeated colonization and movement of terrestrial biota into this region after ice retreat following successive glacial cycles, most recently after the Last Glacial Maximum (LGM) in the Pleistocene, when human colonists also followed the ice retreat

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northwards. The presence of large vertebrate grazers and predators also makes for more complex food webs in the Arctic.

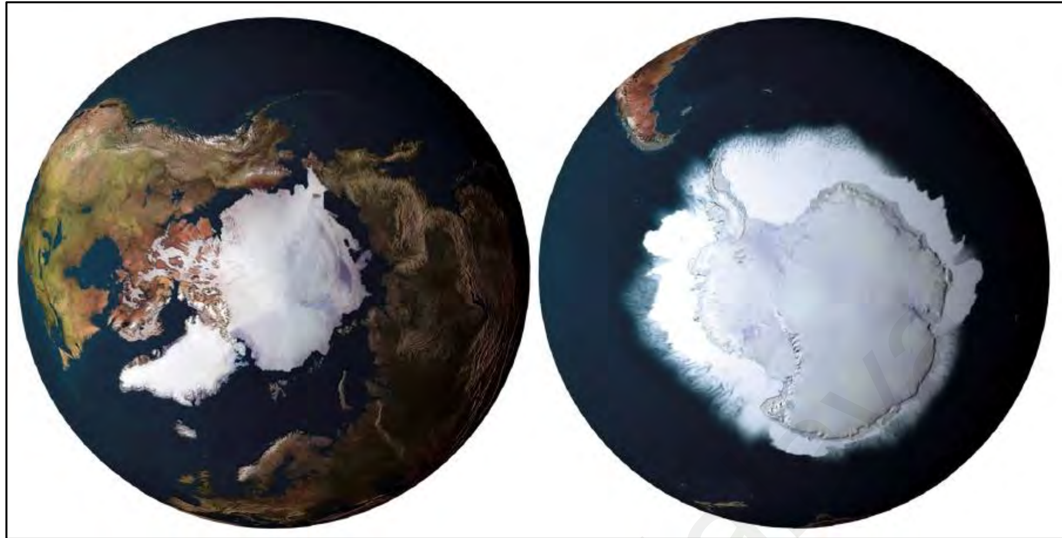

Figure 2.1: Satellite Earth imagery the Arctic (*left*) and the Antarctic (*right*). *Figure credits: ESA/AOES Medialab.*

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Unlike the Arctic, the Antarctic is an ancient continent currently located over the South Pole and surrounded and isolated from other continents by the vast Southern Ocean. It finally became separated from the last of the other continents derived from the supercontinent Gondwana around 30 - 35 million years ago (Livermore *et al.*, 2005). Its contemporary isolation due to the strong circumpolar winds and the Antarctic Circumpolar Current of the Southern Ocean has been a driving force in the evolution of its biota (Barnes *et al.*, 2006). It is only accessible to animals capable of flying or swimming, and to resistant biological propagules carried by air or ocean currents, or associated with other biota (Vincent, 2000). Viable propagules that manage to arrive on the continent may still be faced with unsuitable conditions for growth (Clarke *et al.*, 2007). These conditions pose significant barriers to colonization and establishment of organisms in the Antarctic. These key differences between the two poles affect the microbial ecosystem as well as larger organisms.

2.2 Signy Island

2.2.1 Background of Signy Island

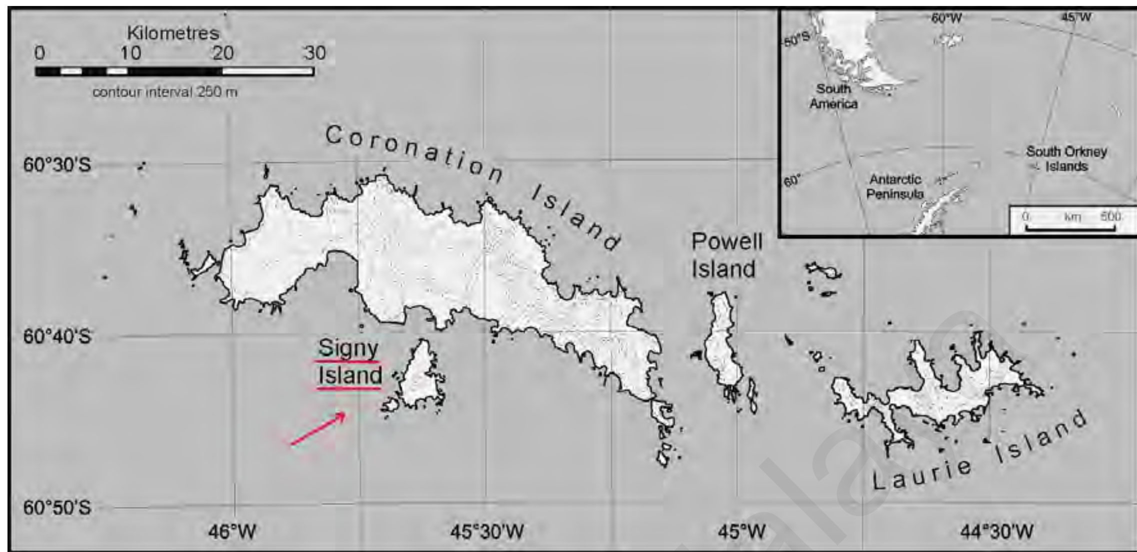

Figure 2.2: Signy Island is located in the South Orkney Island archipelago in Antarctica. *Figure credits: British Antarctic Survey*

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Signy Island lies in the South Orkney Islands archipelago, part of the Maritime Antarctic (Figure 2.2), and experiences a climate that is strongly influenced by the surrounding oceanic conditions. The island itself is relatively small, with a total surface area of around 20 km^2, a length of 8 km from north to south, a maximum width of 5 km and 280 m altitude at its highest. A whaling station was first set up on Signy Island in the 1920s (Convey & Lebouvier, 2009), and the Signy Research Station, now operated by the British Antarctic Survey (BAS), was opened on the same location in 1947 (Holdgate, 1967). A meteorological station was established first in Factory Cove and, in its early years, the station was used for mammalogical and ornithological studies. Later the station became the main centre for biological research carried out by BAS over several decades. After 1961, terrestrial, freshwater and inshore benthic marine ecosystems, which were research areas previously neglected in Antarctic biology, were studied from this station. A more modern laboratory with facilities was then constructed in 1963 – 1964 which allowed more in-depth studies to be carried out, especially in physiological and

autecological fields (Holdgate, 1967). Since then, newer facilities were built in the austral summer of 1995/96 with improved living accommodations, laboratories and offices as well as a fuel farm. It is now a summer-only station with 8 staff in summer including a maximum of five scientists at one time. Currently, research at Signy is primarily conducted to study penguin, seabird and seal biology, and terrestrial biology. Signy's research is vital for understanding biodiversity and biological trends in the region, and this is evidently seen in the long-term monitoring of several local species, such as Adélie, Chinstrap and Gentoo penguins as well as the southern giant petrel population, which has been monitored at Signy since the 1960s (*Signy Research Station*, Retrieved Jul. 27, 2017 from https://www.bas.ac.uk/polar-operations/sites-and-facilities/facility/signy/).

Coastal habitats on Signy Island are heavily impacted by seals, penguins and other seabirds which come ashore to breed, rest, or moult. Cape pigeons and snow petrels nest along cliffs in the coastal zone; the dove prion, Wilson's petrels and the black bellied storm petrels burrow in soil or breed in the interstices of coarse scree; and giant petrels, skuas, shags and penguins breed on the open ground (Holdgate, 1967). Non-breeding male Antarctic fur seals are found in large though variable numbers on Signy, with more than 20,000 individuals reported in 1994, 1995 and 2000 (Waluda *et al.*, 2010). These marine vertebrates are important vectors of nutrients from marine ecosystems into the terrestrial ecosystems of the island enriching the soil for microbiota and microfauna such as micro-arthropods and micro-invertebrates (Bokhorst *et al.*, 2007a; Chong *et al.*, 2009b; Holdgate, 1967; Teixeira *et al.*, 2013; Yew *et al.*, 2017), but they can also cause significant damage to moss and lichen communities (Favero-Longo *et al.*, 2011; Hodgson *et al.*, 1998a; Smith, 1988, 1997). The native flora of Signy includes only two vascular plants, *Deschampsia antarctica* and *Colobanthus quitensis*, cryptogamic communities of carpet- and turf-forming mosses, and fell-field communities consisting of mosses, liverworts and lichens (Convey, 2013; Guglielmin *et al.*, 2012; Smith, 1984, 1990).

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Terrestrial ecosystems on Signy Island, as is characteristic of the maritime Antarctic, lack many familiar faunal groups, including annelids, molluscs, winged insects and mammals (Convey, 2013; Davis, 1981).

Over recent decades, parts of the Antarctic continent have experienced a period of consistent strong warming, with Turner *et al.* (2009) stating "Surface temperature trends show significant warming across the Antarctic Peninsula and to a lesser extent West Antarctica since the early 1950s, with little change across the rest of the continent" (p. xvi). This includes Signy Island which is partially covered by an ice-cap that is shrinking, with a reduction of the snow cover by 40 % noted by Turner *et al.* (2009). The island has also air temperatures that are currently increasing at about 0.2 °C per decade (Guglielmin *et al.*, 2008; Guglielmin *et al.*, 2012; Turner *et al.*, 2005). Temperatures in this region tend to be highly variable, with mean annual air temperature (MAAT) around −3.5 °C (Cannone *et al.*, 2017; Guglielmin, 2012; Guglielmin *et al.*, 2012; Royles *et al.*, 2012) and with summer daily mean air temperatures ranging from 0 to 10 °C (Royles *et al.*, 2012). Warmer temperatures are recorded closer to the ground and soil temperatures range annually between -25 °C and > 25 °C (Wynn-Williams, 1996). Mean annual soil temperatures in 2003 and 2004 recorded at a 5 cm depth were around -1.68 °C and -2.22 °C (Yergeau *et al.*, 2007a).

The soils on Signy Island, as is typical of the Maritime Antarctic climatic zone, at any time of the year tend to be moist and unfrozen for short periods with ground temperatures rising above freezing and diurnal freeze-thaw cycles particularly in the spring and autumn (Campbell & Claridge, 1987; Wynn-Williams, 1996). A study of soils on Signy Island reported an average of 0.36 freeze-thaw cycles per day annually at a 5 cm depth (Yergeau *et al.*, 2007a) with a total of 130 freeze-thaw cycles annually (Convey *et al.*, in press). Ice-free ground on Signy Island tends to be dominated by fellfield habitats, including

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discontinuous moss and lichen cover as well as frost-sorted polygons (Block *et al.*, 2009; Smith, 1984, 1990; Vishniac, 1993).

Davey *et al.* (1992) reported that areas on the island covered by snow were insulated from low air temperatures and were more thermostable than areas without snow cover. Also, soil surfaces in summer recorded highs of around 17 ºC. Areas insulated by moss were found to be better protected against freeze-thaw events than those without any cover or with only lichen carpets. Yergeau *et al.* (2007a) also demonstrated that soils covered by moss-carpets, in comparison with bare soils, supported greater bacterial productivity and abundance by having greater nutrient availability and more favourable physical conditions.

2.2.2 Bacterial studies on Signy Island

With Signy Island's warming trend and ease of accessibility, it is a centre for biological studies in Maritime Antarctica. Many studies have been carried out to assess, for example, algae (Hodson *et al.*, 2008), fungi (Dennis *et al.*, 2012; Marshall, 1997; Newsham *et al.*, 2009; Upson *et al.*, 2009), lichens (Favero-Longo *et al.*, 2011; Singh *et al.*, 2015), mosses and grasses (Bokhorst *et al.*, 2007b; Cannone *et al.*, 2015; Convey & Smith, 1993; Convey, 1994b; Smith, 1990), nematodes (Newsham *et al.*, 2004; Yergeau *et al.*, 2007a), arthropods (Bokhorst *et al.*, 2008; Convey, 1994a; Worland, 2005), seals (Waluda *et al.*, 2010), and birds (Fijn *et al.*, 2012; Forcada *et al.*, 2006). Bacterial studies in Signy encompass a wide range of fields, and are not just limited to terrestrial bacterial studies, various ecological niches have been studied such as the freshwater lakes in Signy (Ellis-Evans & Wynn-Williams, 1985; Pearce, 2000; Pearce & Butler, 2002; Pearce, 2003; Pearce *et al.*, 2003; Pearce, 2005; Pearce *et al.*, 2005; Pearce *et al.*, 2007) as well as microbiomes associated with marine organisms (Banks *et al.*, 2014; Dewar *et al.*, 2013;

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Yew *et al.*, 2017). The focus of the current study is the terrestrial bacterial community of Signy Island.

Several studies have been carried out to assess the baseline microbial community of Signy Island. The use of molecular techniques to assess diversity has provided a better resolution of the baseline microbial community. The use of DGGE profiling studies in Signy Island diversity studies has suggested that as DGGE profiling only accurately describes the diversity of dominant bacterial phylotypes communities that are structurally significantly different at different sites around the island (Chong *et al.*, 2009b, 2010). However, these studies have found that Bacteroidetes was the most dominant phylotype retrieved with remaining sequences being assigned to Firmicutes, Cyanobacteria, Acidobacteria, Proteobacteria, Gemmatimonadetes and Actinobacteria, with many of the sequences obtained being affiliated to sequences originating from cold environments (Chong *et al.*, 2009b, 2010). In comparison, the use of microarray techniques to assess diversity in Signy soils indicated that they were dominated by Proteobacteria (~ 27 – 36 % of main phyla detected) compared to Bacteroidetes (only ~ 1.1 - 5.5 %) (Yergeau *et al.*, 2007c, 2009).

Signy Island terrestrial bacterial communities were also studied as part of an environmental gradient to understand the effects of key environmental factors on bacterial communities (Yergeau *et al.*, 2007a). Signy fell-field sites had lower abundance of 16S rRNA genes compared to the other sites examined along the gradient (Falkland Islands and Anchorage Island), and Signy vegetated sites had significantly different total bacterial phospholipid fatty acid (PLFA) abundances to those of fellfield sites, with Signy Island having relatively lower PLFA abundances in general. However, the bacterial colony forming unit (CFU) counts were consistently higher in Signy vegetated soils. This indicates the buffering effect vegetation cover has on bacterial communities in Signy

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soils. Fellfield soils on Signy were found to have higher number of unclassified bacteria compared to other sites along the same gradient (Yergeau *et al.*, 2007c)

A series of warming experiments has been carried out on Signy Island. Open-top chambers (OTCs) passively warm soil by ~1 °C (Bokhorst *et al.*, 2007b, 2011). This subtle passive warming causes shifts in soil bacterial communities. Bacteria are found to be more abundant in warmed vegetated soils. Although lower taxonomic levels are generally unaffected, the perturbation causes changes at phylum and class level (e.g. increases in the Alphaproteobacteria and decreases in the Acidobacteria) as well as affecting abundance and changes in microbial community functions (Dennis *et al.*, 2013; Yergeau *et al.*, 2012). These warming experiments increased microbial annual respiration rates by 10 % in Signy soils, suggesting some effects that climate changes will have on Antarctic microbial communities (Dennis *et al.*, 2013). *Ex-situ* studies have shown that increases in temperature increased microbial respiration rates as well as bacterial rRNA but did not affect associated functional genes (Laudicina *et al.*, 2015; Yergeau & Kowalchuk, 2008).

The actinobacterial community of Signy Island includes potentially rare and novel actinobacteria as well as species with antibacterial potential (Pan *et al.*, 2013). Methylotroph communities were widely detected in various soils, sediment and lake samples around Signy Island (Moosvi *et al.*, 2005a) as well as providing the first report of methanesulfonate-degrading bacteria from the Antarctic (Moosvi *et al.*, 2005b). These studies show the potential of Signy not only as a source of novel microorganisms but also a source of potential metabolites and enzymes. However, the island's terrestrial microbial ecosystems remain understudied particularly in comparison with its to the freshwater ecosystems.

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2.3 Bacterial diversity in Antarctic terrestrial ecosystems

The Antarctic's various terrestrial ecosystems are characterised by low temperatures, high temperature fluctuations, high desiccation stress, high periodic radiation fluctuations and low nutritional status (Convey *et al.*, 2014; Cowan & Ah Tow, 2004; Tindall, 2004). Of the total land area of Antarctica, only 0.18 ± 0.05 % is ice-free (Burton-Johnson *et al.*, 2016), meaning that the majority of terrestrial, particularly bacterial life is restricted to seasonally ice- and snow-free areas, such as the coastal regions of the lower latitude Antarctic Peninsula and associated archipelagos, coastal continental oases such as the Vestfold Hills and Schirmacher Oasis, the more extensive ice-free areas of the McMurdo Dry Valleys, the great mountain ranges such as the Ellsworth and Transantarctic Mountains, and isolated nunataks distributed throughout the region (Cary *et al.*, 2010; Convey, 2013). As these ice-free areas, and at smaller scales, the habitats within them, are typically discontinuous, the development of terrestrial and microbiological communities in the Antarctic is characterised by isolation (Chown & Convey, 2007; Terauds *et al.*, 2012). As the focus of the current study is bacterial, hereafter terms such as "microorganisms" and "microbial" refers to bacteria, excluding other microorganisms such as algae, fungi, *etc*.

The Antarctic continent is host to a wide variety of ecosystems each with its unique influences on the microbial communities whose members have adapted to survive in these habitats where they function to mineralise marine vertebrate excreta and release vital nutrients into the rhizosphere (Chong *et al.*, 2013). In most environments, the combination of physical factors and small-scale variations plays a large role in an organism's ability to survive (Fogg, 2008). Terrestrial microbiology has been of particular interest in ice-free areas across the continent (Table 2.1). The relative simplicity of Antarctic ecosystems potentially allows a deeper understanding of fundamental processes that would be difficult to study in complex ecosystems.

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Antarctic soils in general tend to be poorly developed and contain little organic material. The ice crystals that form in the winter heavily influence the formation, development and stability of the soil. The frequent formation and melting of ice moves and mixes soil particles and stones in the soil column (Convey, 2007). Soil microbiology is influenced by both climatic factors and edaphic factors. Maritime and Continental Antarctica generally possess a widespread permafrost layer, with organic (brown) soils only forming in proximity to limited fauna and ornithogenic depositions (guano depositions) and the limited stands of higher plant vegetation (Convey, 2007, 2013; Vishniac, 1993). Bacterial diversity tends to be highest in coastal and maritime Antarctica and reduces further inland and with increasingly higher latitudes (Wynn-Williams, 1996; Yergeau *et al.*, 2007a, 2009).

Microbial diversity studies in Antarctica inevitably initially relied on traditional culture methods. More recent technique developments such as microarrays, 16S rRNA gene clone libraries, and metagenomics have since been employed to study soil microbiota. Soil microbiology studies in Antarctica have tended to focus on establishing baseline diversity, community structure and functions in various environments, exploring environmental factors and patterns affecting soil microorganisms, and evaluating the significance of climate change on microbial populations (Brinkmann *et al.*, 2007; Chong *et al.*, 2015; Ma *et al.*, 2013; Rinnan *et al.*, 2009; Yergeau *et al.*, 2007a, 2007b, 2007c; Yergeau & Kowalchuk, 2008).

Soil ecosystems in the Antarctic were once considered to host extremely low numbers and diversity of bacteria (Heal, 1999; Vincent, 1989; Wynn-Williams, 1990). However, culture-dependent methods have proved that bacteria are able to thrive in the Antarctic despite the environmental pressures (Sjöling & Cowan, 2000; Tindall, 2004), whilst molecular microbial studies are increasingly supporting the presence of a considerably greater and largely unknown native bacterial diversity and biomass than previously

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suspected (Cary *et al.*, 2010; Cowan *et al.*, 2002; Cowan & Ah Tow, 2004; Hogg *et al.*, 2006; Wei *et al.*, 2016; Yergeau *et al.*, 2009) but still lower in diversity than temperate soils (Smith *et al.*, 2006).

More recent studies discussed in a review by Cowan *et al.* (2015) employ metagenomics and have revealed that, in the cold desert soils of Antarctica, "(i) standing microbial biomass is orders of magnitude higher than originally thought (Cowan *et al.*, 2002); (ii) microbial community complexity and diversity is much higher than predicted and soils have a high level of spatial heterogeneity (Aislabie *et al.*, 2008; Chong *et al.*, 2010; Lee *et al.*, 2012; Makhalanyane *et al.*, 2013) despite high levels of aeolian mixing and the absence of higher eukaryotes, and (iii) soil microbial communities exhibit surprisingly rapid structural changes in response to changing environmental conditions (Tiao *et al.*, 2012; Yergeau *et al.*, 2012)". A study carried out by Chong *et al.* (2012) analysing 3377 soil bacterial sequences from various molecular studies of Antarctic locations found that Bacteroidetes were the most widely distributed (detected in ~ 89 % of study sites) followed by Actinobacteria (~ 86 %) and Acidobacteria (~ 77 %).

Microorganisms in terrestrial ecosystems have been found inhabiting microhabitats in various niches, including on surfaces of plants and lichen; on, under or in rocks; and in between ice crystals (Convey, 2013). Despite the chronically cold climate, microhabitats, such as in or on the surface of rocks or small patches of exposed soil or vegetation, when exposed to sunlight can warm considerably, giving favourable conditions for microbial proliferation (Convey, 2013). These hardy microorganisms can withstand freeze-thaw cycles and are able to activate respiratory and growth metabolism even at near-freezing temperatures. Plant communities withstand freezing by producing soluble organic matter into the surrounding soil which can then be used by surrounding microbiota as both an energy source and as a cryoprotectant against freezing (Block *et al.*, 2009). Plant cells damaged by freezing also provide a source of nutrients for microorganisms. Freezing of

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water particles on wet rocks creates microhabitats and provides nutritional supply for opportunistic organisms as well as protection from harsh environmental features (Wynn-Williams, 1990). Terrestrial invertebrates are hosts to gut-associated microbial communities, some members of which are capable of ice-nucleation (Worland & Block, 1999; Worland & Lukešová, 2000).

In Antarctic terrestrial ecosystems, bacterial community diversity and composition are driven by the nutrient availability, with organic carbon and soluble salts in soils being major drivers as well as pH (Chong *et al.*, 2009a, 2010; Dennis *et al.*, 2013; Fierer & Jackson, 2006; Harris & Tibbles, 1997; Magalhães *et al.*, 2012; Pointing *et al.*, 2009; Sanyika *et al.*, 2012). Increasing water availability however, increases bacterial biomass (Convey *et al.*, 2014) but water availability is only a secondary factor in bacterial productivity (Harris & Tibbles, 1997) and has little direct influence on community composition (Newsham *et al.*, 2010; Stomeo *et al.*, 2012). A majority of soil communities are specialized and lower diversity communities, but with higher nutrient and water availability more diverse communities can develop (Chong *et al.*, 2012). Latitude, presumably as a proxy for various environmental drivers has also been found to play a role in structuring bacterial communities (Chong *et al.*, 2012; Yergeau *et al.*, 2007c). However, it is more likely that a combination of various factors is responsible for bacterial community structures instead of a single driving factor (Chong *et al.*, 2009b, 2015).

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Table 2.1: Selected studies of the diversity of bacterial phyla in Antarctic soils

Location / Region	Method	Dominant phyla	Reference
Darwin-Hatherton Glacier region, Transantarctic Mountains, Ross Sea Region	Cultivation and 16S rRNA gene clone libraries	Deinococcus-Thermus, Actinobacteria, Bacteroidetes	Aislabie *et al.* (2013)
Taylor Valley, McMurdo Dry Valleys, Victoria Land, East Antarctica	Metatranscriptomics using Illumina sequencing	Actinobacteria, Firmicutes, Proteobacteria, Bacteroidetes, Cyanobacteria, Tenericutes	Buelow *et al.* (2016)
Larsemann Hills, Northern Victoria Land, East Antarctica	Cultivation, 16S rRNA gene clone libraries, T-RFLP	Acidobacteria, Actinobacteria, Armatimonadetes, Bacteroidetes, Cyanobacteria, Chloroflexi, Deinococcus-Thermus, Firmicutes, Gemmatimonadetes, Nitrospira, Planctomycetes, Proteobacteria, Verrucomicrobia	Bajerski and Wagner (2013)
University Valley, McMurdo Dry Valleys, Victoria Land, East Antarctica	Metagenomics using Illumina sequencing	Actinobacteria, Firmicutes, Bacteroidetes, Proteobacteria	Goordial *et al.* (2017)
University Valley, McMurdo Dry Valleys, Victoria Land, East Antarctica	Culturable and small subunit rDNA pyrosequencing	Proteobacteria, Firmicutes, Actinobacteria, Bacteroidetes	Goordial *et al.* (2016)
Mitchell Peninsula, Windmill Islands, East Antarctica	Metagenomics using 454-tag pyrosequencing	Chloroflexi, Actinobacteria, Proteobacteria, Acidobacteria, WPS-2, AD3	Ji *et al.* (2015)

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Table 2.1, continued.

Location / Region	Method	Main phyla	Reference
Browning Peninsula, Windmill Islands, East Antarctica	Cultivation, soil substrate membrane system (SSMS), 454-tag pyrosequencing	Actinobacteria, Chloroflexi, Cyanobacteria, Proteobacteria, Acidobacteria, Gemmatimonadetes, Planctomycetes, Bacteroidetes, Firmicutes	Pudasaini *et al.* (2017)
Schirmacher Oasis, Dronning Maud Land, East Antarctica	PCR-DGGE	Acidobacteria, Proteobacteria, Bacteroidetes, Actinobacteria, Planctomycetes, Cyanobacteria, BRC1	Teo and Wong (2014)
Sør Rondane Mountains, Dronning Maud Land, East Antarctica	Metagenomics using Illumina sequencing	Acidobacteria, Actinobacteria, Armatimonadetes, Cyanobacteria, Chloroflexi, Bacteroidetes, FBP, Planctomycetes, Proteobacteria, Deinococcus-Thermus, TM7, Gemmatimonadetes, Verrucomicrobia	Tytgat *et al.* (2016)
Victoria Valley, McMurdo Dry Valleys, Victoria Land, East Antarctica	16S rRNA gene pyrosequencing	Actinobacteria, Acidobacteria, Cyanobacteria, Gemmatimonadetes, TM7, Verrucomicrobia	Van Goethem *et al.* (2016)
Miers Valley, McMurdo Dry Valleys, Victoria Land, East Antarctica	16S rRNA gene pyrosequencing	Actinobacteria, Proteobacteria, Cyanobacteria, Chloroflexi, Acidobacteria, Bacteroidetes, Gemmatimonadetes, Planctomycetes, Verrucomicrobia, Armatimonadetes, Chlorobi, TM7, Deinococcus-Thermus, Firmicutes, OD1	Wei *et al.* (2016)

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2.4 Actinobacteria in Antarctica

Actinobacteria are a well-known and diverse phylum of Gram-stain-positive bacteria generally containing a high G+C nucleic acid ratio (Gao & Gupta, 2012). They are commonly found in many terrestrial and marine environments. Actinobacteria are known to survive and adapt to a wide variety of environments, including polar environments (Cowan & Ah Tow, 2004; Makhalanyane *et al.*, 2016; Neufeld & Mohn, 2005; Smith *et al.*, 2006), and both culture-dependent and culture-independent studies show that Actinobacteria are one of the predominant taxa in the Antarctic (Aislabie *et al.*, 2006; Babalola *et al.*, 2009; Bottos *et al.*, 2014; Chong *et al.*, 2015; Goordial *et al.*, 2017; Lee *et al.*, 2012; Newsham *et al.*, 2010; Pointing *et al.*, 2009; Smith *et al.*, 2006; Steven *et al.*, 2007; Wei *et al.*, 2016). Actinobacteria in Antarctica were found in decreasing relative abundance with increasing latitude which is unsurprising due to their ability to withstand periods of desiccation and dormancy (Dennis *et al.*, 2013; Yergeau *et al.*, 2009). In most studies targeting the metagenome of terrestrial environments, actinobacterial communities have been detected (Table 2.1).

Actinobacteria are ubiquitous in the marine environment and are found in sea ice microbial communities (SIMCOs), the water column, sediment and components of the microbiomes of various marine organisms (Lee *et al.*, 2014; Mangano *et al.*, 2009; Murray *et al.*, 2016; Rodriguez-Marconi *et al.*, 2015; Webster & Bourne, 2007; Xin *et al.*, 2011; Yew *et al.*, 2017). In the Antarctic marine water column, Actinobacteria have been found in low frequencies (1.9 %) (Dickinson *et al.*, 2016) and diversity compared to surface waters in warmer climes (~ 5 %) (Biers *et al.*, 2009).

The actinobacterial communities in terrestrial Antarctica are found in various microhabitats where they perform many roles such as nitrogen cycling and decomposition (Chan *et al.*, 2013; Varin *et al.*, 2012; Wei *et al.*, 2014, 2016). In producing mycelia, they can initiate the process of degradation of rock surfaces, starting the formation of soil

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particles. In such microhabitats, bacterial community structure and diversity are strongly influenced by the pH of the soil as well as its salinity (Chong *et al.*, 2009b, 2010; Sanyika *et al.*, 2012). *Streptomyces*, the largest genus of the Actinobacteria class, have been reported to form the majority of cultured isolates in terrestrial Antarctica, however this is not always reflected in 16S rRNA clone libraries, suggesting there is a potential under-representation of this important genus in molecular-based studies (Babalola *et al.*, 2009; Sanyika *et al.*, 2012).

In response to Antarctic environmental stressors such as periods of nutrient and oxygen limitation, low osmotic potentials, low water availability and fluctuating temperatures, various stress response pathways have been discovered in actinobacteria. Many actinobacteria are capable of producing spores or mycelial forms, which enable their survival in permafrost and during periods of non-optimal growth conditions (Kochkina *et al.*, 2001). Actinobacteria possess pathways for:

i) Stress tolerance such as genes responsive to osmotic stress, heat shock, cold shock, radiation stress, nitrogen limitation, phosphate limitation;
ii) nitrogen cycling including nitrogen fixation, nitrification, denitrification, ammonification and nitrogen reduction and;
iii) carbon cycling including photoautotrophy, chemoautotrophy, acetogenesis, methane oxidation, carbohydrate catabolism, and aromatic compound catabolism (Wei *et al.*, 2014, 2016).

Further interest in these bacteria stems from their known abilities in the production of various bioactive compounds. *Streptomyces*, the largest genus of the class Actinobacteria, are known to be prolific producers of the clinically useful antibiotics of natural origin. A number of studies have been carried out to describe the antibacterial properties of actinobacteria, especially *Streptomyces* spp. and rare actinobacteria (Basilio *et al.*, 2003; Bull & Stach, 2007; Encheva-Malinova *et al.*, 2014; Lam, 2006; Lazzarini *et al.*, 2000;

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Pan *et al.*, 2013; Watve *et al.*, 2001). Besides antimicrobials, a diverse range of molecules and enzymes are also produced by actinobacteria such as anti-cancer compounds, anti-inflammatory compounds, anti-viral compounds, biosurfactants and various enzymes (El-Shatoury *et al.*, 2009; Gandhimathi *et al.*, 2009; Genilloud *et al.*, 2011; Kügler *et al.*, 2015; Lam, 2006; Reddy *et al.*, 2009; Subramani & Aalbersberg, 2012, 2013). Many of these have medical, biotechnological and industrial applications.

2.5 Antarctica as a source of novel organisms and secondary metabolites

The continent's isolation, along with the selective environmental pressures of the Antarctic and the warming temperatures make the Antarctic terrestrial environment unique and of interest as a potential source of novel microbial species and secondary metabolites. In many environments, culture methods are not able to fully capture the wide range of diversity of bacteria that exists, and molecular approaches can provide a better understanding of the diversity present (Cary *et al.*, 2010; Cowan *et al.*, 2002; Smith *et al.*, 2006). A study by Smith *et al.* (2006) of the Antarctic Dry Valleys found taxa such as Acidobacteria, Verrucomicrobia and Bacteroidetes were relatively common through the use of 16S rDNA clone libraries. These taxa were not discovered using culture dependant methods. In comparison, *Achromobacter*, *Bacillus*, *Corynebacterium*, *Micrococcus*, *Planococcus* and *Pseudomonas* were all cultured but were not detected in the clone libraries, indicating the shortfalls and biases introduced into diversity studies based on culture methods.

Many Antarctic molecular studies have shown the presence of a high percentage (between 2.9 – 36 %) of as yet unclassified bacteria (Bajerski & Wagner, 2013; Smith *et al.*, 2006; Yergeau *et al.*, 2012). A study by Chong *et al.* (2013) compared various studies that had identified Antarctic bacterial cultures based on the 16S rRNA gene and found

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that out of 2089 total sequences, 26 % were novel sequences indicating Antarctica's underdeveloped potential as a source of novelty. In another study, the microbial ecology of the Antarctic Dry Valleys was studied, concluding that a large ratio (48.5 – 72.2 %) of sequences were novel OTUs (Lee *et al.*, 2012).

The List of Prokaryotic with Standing in Nomenclature (LPSN) has recognised 29 phyla belonging to the domain bacteria at the time of writing, with Thermomicrobia reclassified as a class in the phylum Chloroflexi (Hugenholtz & Stackebrandt, 2004) (data accessed 20 Aug 2017 from http://www.bacterio.net/-classifphyla%20copy.html). However, as indicated in Figure 2.3, there are many more phyla that have yet to be cultivated and for which there are still no representative strains (Lasken & McLean, 2014). Novel species description papers have been regularly published for Antarctic species, with ~15 publications in 2017 at time of writing (data accessed 31 Aug 2017 from Google Scholar using search phrase "Antarctic AND bacteria AND novel"). Shivaji *et al.* (2017) reported and listed 209 novel bacterial species described from various Antarctic environments. Thirty-one of these are novel Actinobacteria from 16 genera, six of which were novel genera. Some of these include *Arthrobacter*, *Barrientosiimonas**, *Cryobacterium**, *Friedmanniella**, *Kocuria*, *Marisediminicola**, *Modestobacter**, *Mucilaginibacter*, *Nesterenkonia*, *Nocardioides*, *Planococcus*, *Rhodoglobus**, and *Streptomyces* (novel genera first isolated from Antarctica denoted with an asterisk) (Collins *et al.*, 2002; Ganzert *et al.*, 2011; Lawson *et al.*, 2000; Le Roes-Hill *et al.*, 2009; Lee *et al.*, 2013; Li *et al.*, 2010; Li *et al.*, 2011; Mevs *et al.*, 2000; Reddy *et al.*, 2002, 2003, 2010; Schumann *et al.*, 1997; Sheridan *et al.*, 2003; Suzuki *et al.*, 1997; Zheng *et al.*, 2016). Antarctica as a source of novel Actinobacteria is also as seen in a Marion Island study which isolated approximately 20 previously undescribed Actinobacteria (Sanyika *et al.*, 2012) and in a Signy Island study which isolated approximately six potential novel actinobacterial species (Pan *et al.*, 2013).

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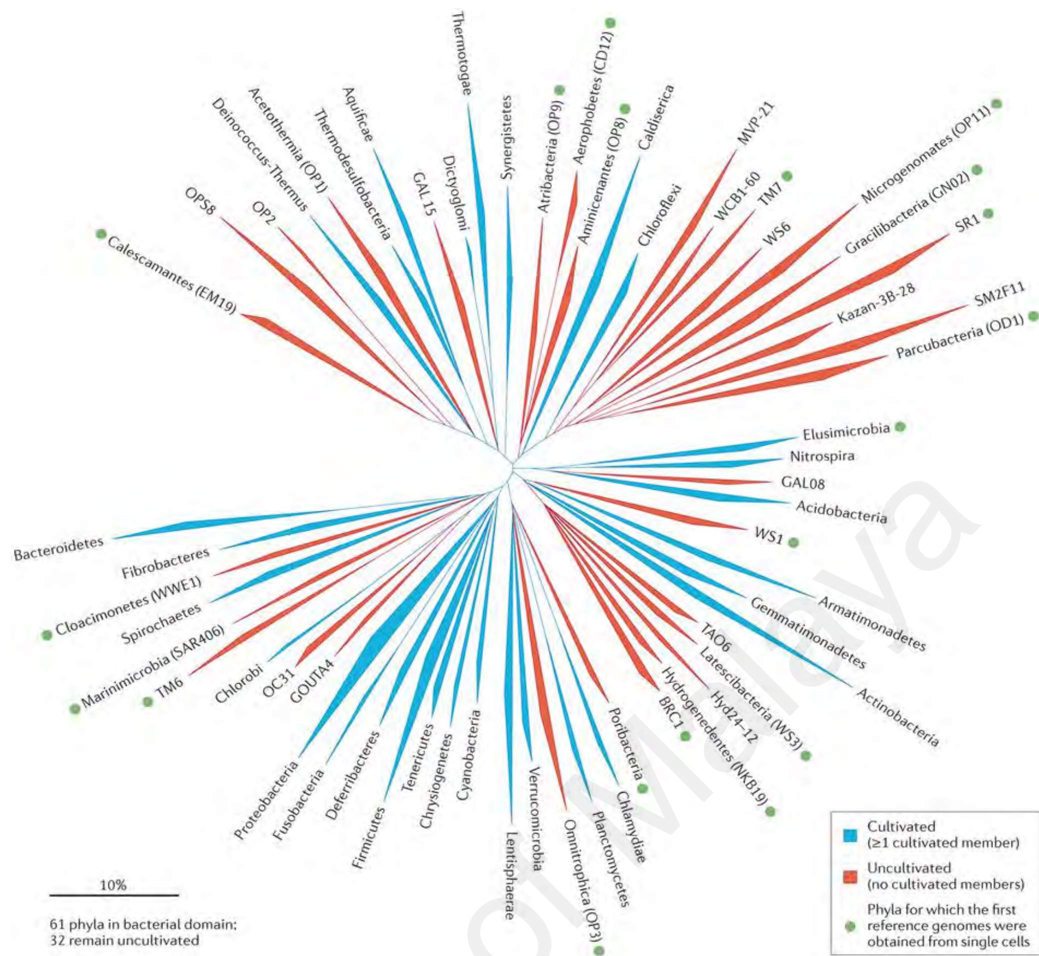

Figure 2.3: Bacterial phyla with cultivated vs. uncultivated members. Adapted from Lasken and McLean (2014).

Antarctic organisms are known to be a potential source of cold-active enzymes, molecules and compounds as seen in Table 2.2 below (de Pascale *et al.*, 2012). A large number of studies have been conducted to assess the potential of Antarctic bacteria in producing novel as well as cold-active molecules and proteins (Gesheva, 2010; Gesheva *et al.*, 2010; Gesheva & Negoita, 2011; Gesheva & Vasileva-Tonkova, 2012; Ma *et al.*, 2013; Tropeano *et al.*, 2012; 2013; Vasileva-Tonkova & Gesheva, 2004, 2005). Other studies have found extracellular enzyme producing bacteria from Signy lake sediments (Ellis-Evans, 1981). The bacteria were found to be proteolytic with smaller communities of amylolytic and lipolytic bacteria. In comparison, bacteria from a lake in Schirmacher Oasis (Queen Maud Land, Antarctica) was found to be mostly proteolytic and amylolytic

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with lipolytic and ureolytic bacteria in smaller components (Shivaji *et al.*, 2011). A novel antibiotic, frigocyclinone was isolated from an Antarctic *Streptomyces griseus* strain (Bruntner *et al.*, 2005).

2.6 Microbial enzymes, bioactive compounds and their uses

2.6.1 Microbial enzymes and biosurfactants

Enzymes are catalysts produced by biological organisms for metabolic and biochemical reactions (Adrio & Demain, 2014; Anbu *et al.*, 2013). Enzymes from microbial sources are favoured over enzymes of plant and animal origins as they are more active and stable, easily available and have fast growth rates (Singh *et al.*, 2016). Enzymes produced by microorganisms are useful in many fields, such as pharmaceuticals, food, animal feed, household care, fine chemicals, textiles, and technical industries (Adrio & Demain, 2014). Microbial enzymes are stable and can be processed in large quantities at low cost, making them highly sought after and profitable. They also work under mild reaction conditions, have a long half-life, highly specific, and can utilise a wide range of substrates (Adrio & Demain, 2014). Genetic and chemical modification can be used to enhance the use of the enzymes. The rising demands for industrial enzymes are being driven by industries looking for sustainable solutions. This is especially true for cold-adapted or cold-active enzymes which are able to catalyse the degradation of complex substrates at low temperatures.

Biosurfactants are derived compounds usually from microbial sources that share hydrophilic and hydrophobic moieties and are surface active. These surfactants are divided into low weight amphiphilic molecules that act as emulsifiers to efficiently lower surface tensions between different phases (e.g. in water-oil emulsions), and high molecular weight polymers that bind tightly to surfaces (Kügler *et al.*, 2015; Ron &

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Rosenberg, 2001, 2002). Biosurfactants are relatively easier to produce as well as being readily biodegradable and less toxic when compared to chemically derived surfactants (Marchant & Banat, 2012a). To that end, biosurfactants are utilised in various industries, as well in bioremediation (Beazley *et al.*, 2012; Gandhimathi *et al.*, 2009; Hasan *et al.*, 2006; King *et al.*, 2015; Kiran *et al.*, 2010; Kiran & Chandra, 2008; Kügler *et al.*, 2015; Marchant & Banat, 2012a, 2012b).

Actinobacteria are known to produce biosurfactants but their biological role in the surface-associated mode of growth, as well as their chemical nature and commercial applicability, are poorly investigated (Gesheva *et al.*, 2010; Kim *et al.*, 2000; Peng *et al.*, 2008; Richter *et al.*, 1998). It is suggested that biosurfactants are produced when growing on hydrophobic carbon sources or during poor growth conditions. Production of biosurfactants allows a wider utilization of available carbon sources and as a protection from harsh conditions (Kügler *et al.*, 2015). Various environmental sources have been studied to discover actinobacterial strains with enzymatic potential (Anderson *et al.*, 2002; Kurtböke, 2012; Meena *et al.*, 2013; Qin *et al.*, 2009; Ramesh & Mathivanan, 2009; Sigmund *et al.*, 2003). Aided by genome sequencing, five actinobacterial species were found to contain 20 or more natural product biosynthetic gene clusters for the production of (both known and predicted) secondary metabolites (Goodfellow & Fiedler, 2010).

2.6.2 Cold-active enzymes and biosurfactants

Cold-active enzymes and molecules are sought after particularly for industrial applications and bioremediation (Feller & Gerday, 2003; Morita *et al.*, 1997; Shivaji *et al.*, 2017). Cold active, but heat-labile, enzymes have a high catalytic activity at low temperatures, with some having an activity up to 10 times higher at low temperatures (Feller & Gerday, 2003). The production of cold-active enzymes allows psychrophiles to maintain enzyme-catalysed reactions despite the low temperatures. In industrial

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applications, the lower temperatures translate to lower costs in energy consumption. Cold-active enzymes prevent heat damage in reactions involving heat-sensitive substrates (de Pascale *et al.*, 2012; Jeon *et al.*, 2009). Table 2.2 highlights enzymes and molecules from polar bacterial sources which have been patented and used in biotechnological applications (de Pascale *et al.*, 2012). Most cold-active enzymes have originated from the polar regions (Gerday *et al.*, 2000), for example, cold-active amylases from an Antarctic actinobacterium, *Alteromonas haloplanktis*, are produced at temperatures as low as 4°C (Feller *et al.*, 1992).

Bacteria of polar origin have been shown to produce biosurfactants which are particularly active at low temperatures, and their applicability in hydrocarbon bioremediation has been investigated (Gesheva, 2010; Gesheva *et al.*, 2010; Gesheva & Negoita, 2011; Janek *et al.*, 2010; Nichols *et al.*, 1999; Ron & Rosenberg, 2001, 2002; Ryu *et al.*, 2006). The Antarctic environment is particularly vulnerable to human impacts as recovery can be slow (Aislabie *et al.*, 2004) and such bacteria can play an important role in removing hydrocarbons from impacted sites (Aislabie *et al.*, 1998, 2004; Bell *et al.*, 2011; Owsianiak *et al.*, 2009; Ruberto *et al.*, 2003; Stallwood *et al.*, 2005).

Biomining for cold-active enzymes and biosurfactants generally involve culturable bacteria isolated using selective media or pre-treatments and low incubation times (Tropeano *et al.*, 2012, 2013; Xiao *et al.*, 2005; Yu *et al.*, 2011). The search for cold-active enzymes is now aided by the use of metagenomics by screening community functions in order to target novel cold-adapted enzymes for particular applications (Berlemont *et al.*, 2011; Clark *et al.*, 2004; Heath *et al.*, 2009).

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Table 2.2: Molecules and enzymes from polar environments, recently patented and utilised in several biotechnological processes. Table adapted from de Pascale *et al.* (2012).

Enzyme	Source	Company
Glycoprotein	*Pseudoalteromonas antarctica*	Lipotec S.A., Spain
Beta-galactosidase	*Pseudoalteromonas haloplanktis*	University of Liege, Belgium
Alkaline phosphatase	Bacterium HK-47	Patent n. US4720458
Antifreeze Lipoprotein	*Moraxella* sp.	Kansai University, Japan
Lipase-catalyzed ester hydrolysis	*Pseudomonas* sp.	Nippon Paper Industries, Japan; Novozymes, Denmark
Anti-freeze proteins	*Marinomonas* sp., *Pseudomonas s*p.	Unilever, United Kingdom
Enzymes xylanolytic activity	*Pseudoalteromonas haloplanktis*	Puratos Naamloze Vennootschap, Belgium
Polyunsaturated fatty acid (PUFA) synthase systems	*Shewanella japonica* and *Shewanella olleyana*	Martek Biosciences Corporation, USA
Dehydrogenases	*Arthrobacter* sp., *Micrococcus* sp.	University of London, United Kingdom
Detergent compositions enzymes	Psychrophilic bacteria sp.	Procter & Gamble, USA
Chlamysin B antibacterial protein	*Chlamys islandica*	Biotec ASA, Norway
Thermostable isomerase	*Thermoanaerobacter mathranii*	Bioneer A/S, Denmark

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CHAPTER 3: MATERIALS AND METHODS

3.1 Introduction

This study sets out to investigate the diversity of the actinobacterial communities in soil samples from Antarctica as well as the bioactive potential of these communities. To achieve this, bacteria were isolated from Antarctic soil samples and screened at different temperatures to assess their bioactive potential in producing enzymes to degrade selected substrates. As the isolation did not yield many putative actinobacteria, actinobacterial strains from previous studies were also incorporated into this study and the bioactivity potential tested alongside the newly isolated strains. Further work was then carried out to sequence the genome of a putative novel actinobacteria and characterise it as a new species. The methods employed in this section are presented as a flowchart in Figure 3.1.

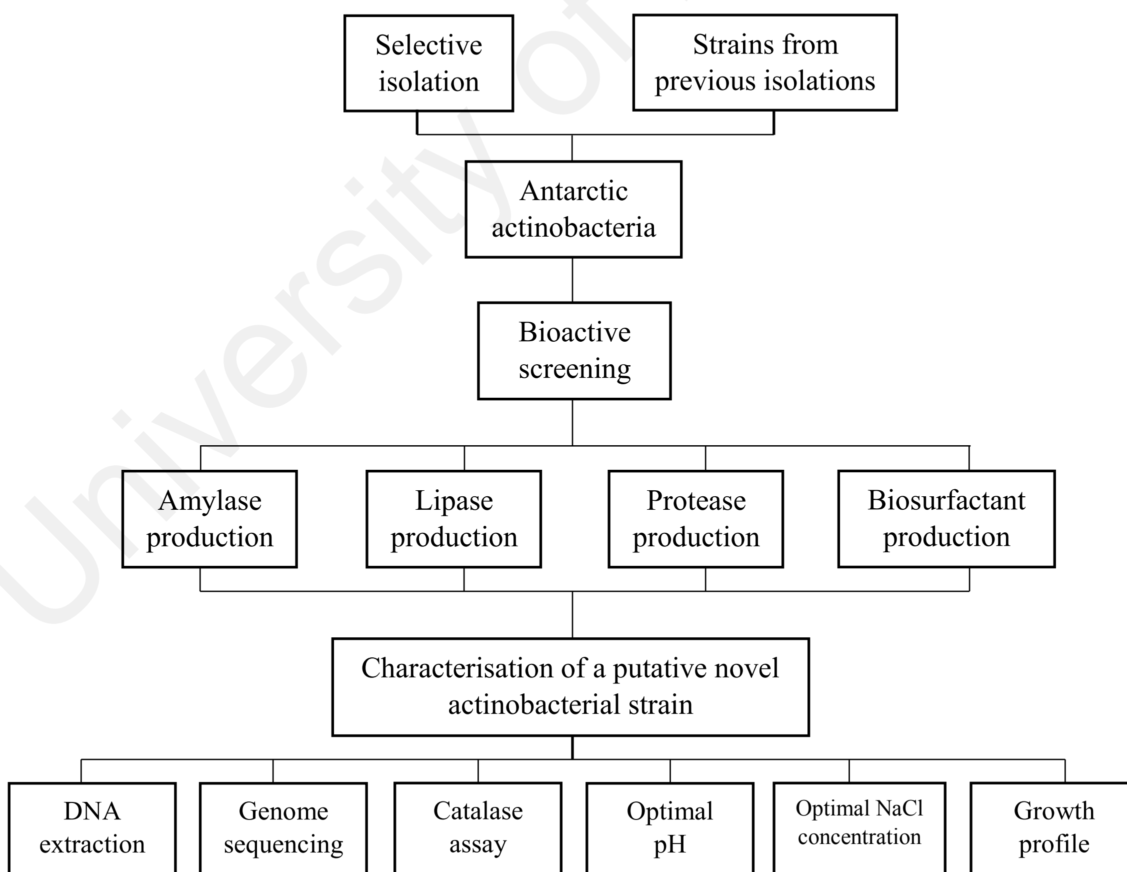

Figure 3.1: The flowchart above provides an overview of the work done in this study

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3.2 Study Area

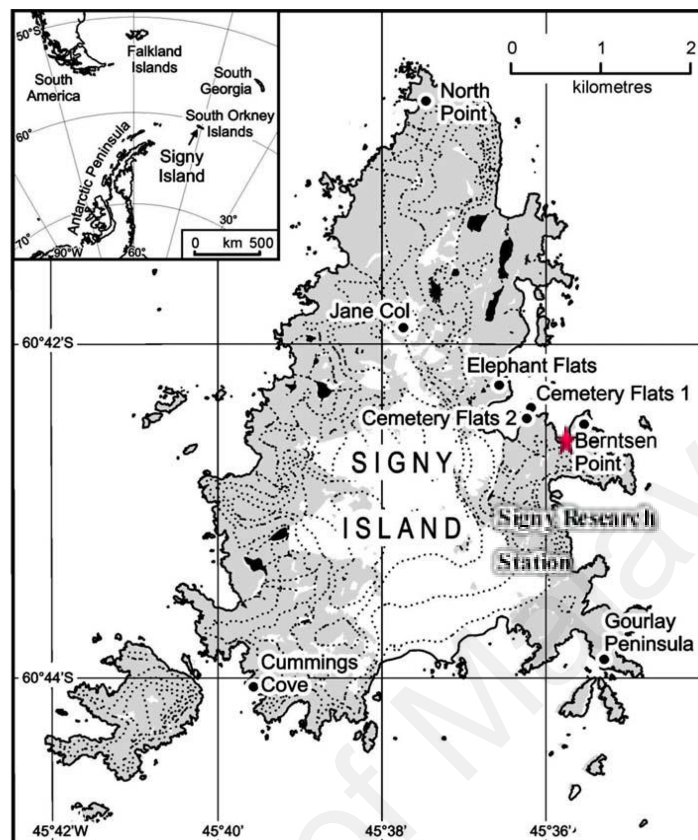

Figure 3.2: A map of Signy Island with the station marked by a star. The backslope sampling site is behind the station. Adapted from Chong *et al*. (2009).

The study area is Backslope, Signy Island (60°42.578' S; 45°35.594' W), South Orkney Islands, Antarctica. Backslope is near the Signy Research Station and covers an area that slopes upwards from the sea to around 150 m away and has and elevation ranging from ~ 5 – 100 m a.s.l., an average pH of 5.2, a percentage of soil nitrogen of 1.1 %, a percentage of soil carbon of 14.3 % and a C/N percentage ratio of 13.1 (Favero-Longo *et al.*, 2011). This area is vegetated by bryophytes and lichens as well as the two native flowering plants and receives nutrient input from local nesting birds (skuas, Wilson's petrels and prions in the area itself, and snow petrels and cape pigeons nesting on the adjacent Factory Bluffs). Although Signy Island terrestrial ecosystems are generally heavily and destructively impacted by the expansion of fur seal numbers in recent decades (Favero-Longo *et al.*, 2011; Hodgson & Johnston, 1997; Hodgson *et al.*, 1998b; Smith,

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1988) since the mid-1990s the Backslope has been protected from fur seal ingress by a fence constructed low on the slope and, as a result, now has very low seal impact and some of the best developed vegetation remaining on the island (Favero-Longo *et al.*, 2011, Convey, P., personal communication, 15 Sept. 2017). As part of Maritime Antarctica, Signy Island receives relatively high water input, with around 400 mm (water equivalent in annual precipitation) (Bockheim & Hall, 2002; Bokhorst *et al.*, 2008; Holdgate, 1967). Ecosystems close to the coast on Signy Island also receive water and nutrient input *via* sea-spray (Bokhorst *et al.*, 2007a).

3.3 Soil samples

The two soil samples used in this study were collected from Backslope, Signy Island during the austral summer of 2005/2006. Samples were stored at -20 ^{o}C until use. Two soil samples were used in this study, samples BS-5 and BS-7 (Table 3.1). These samples were clayish, muddy soils surrounded by lichens and mosses. The soil samples from this location were selected due to a previous study that isolated high numbers of actinobacteria as well as multiple potential new species (Pan, 2010).

Table 3.1: Sampling history for the soil samples from Backslope, Signy Island used in this study

Sample	BS-5	BS-7
Date of collection	19 December 2005	06 January 2006
Temperature at time of collection	1.0 ^{o}C	9.1 ^{o}C

Prior to use, the pH of each sample was determined by suspending two grams of soil in sterile distilled water with a 1:1 ratio and allowing it to settle before measurement. Readings were taken three times and the average value recorded. Due to the low

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availability of each of the soil samples, the two samples from the same location were treated separately with Sample BS-5 pre-treated by air-drying for a month whereas sample BS-7 was left untreated. Drying soil samples is a fairly well-established method to selectively isolate more rare genera of actinobacteria as well as reduce the number of non-actinobacterial bacteria (Subramani & Aalbersberg, 2013).

3.4 Washing and Sterilization

Clean glassware, pipette tips, tubes and bottles used in these methods were sterilized by autoclaving at 121 ºC, 15 psi for 20 minutes. Bench-tops and table surface were chemically disinfected using 70 % ethanol.

3.5 Culture media

3.5.1 Isolation media

All ingredients were added to 1 L of distilled water and mixed thoroughly. The pH was then adjusted to pH 7.0. All media were then autoclaved at 121 °C and 15 psi for 20 minutes. Sterilized media were then cooled prior to the addition of 50 µg/mL cycloheximide and 50 µg/mL nystatin before being poured into sterile Petri dishes. Cycloheximide and nystatin were both dissolved in dimethyl sulfoxide (DMSO) prior to filter sterilization.

Beef Extract Agar (BEA)

BD Bacto™ Beef Extract	10.0 g
BD Bacto™ Peptone	10.0 g
NaCl (Systerm®)	5.0 g
BD Bacto™ Agar	15.0 g

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1/10 Strength Beef Extract Agar (1/10 BEA)

BD Bacto™ Beef Extract	1.0 g
BD Bacto™ Peptone	1.0 g
NaCl (Systerm®)	0.5 g
BD Bacto™ Agar	15.0 g

Actinomycetes Isolation Agar (AIA) (Difco™)

Glycerol (Riendemann Schmidt Chemical) (w/v)	5.0 g
Powdered medium	22.0 g

1/10 Strength Actinomycetes Isolation Agar (1/10 AIA) (Difco™)

Glycerol (Riendemann Schmidt Chemical) (w/v)	0.5 g
Powdered medium	2.2 g
BD Bacto™ Agar	13.5 g

***Streptomyces* Agar (SA) (Atlas, 2004)**

Glucose (Duchefa Bichemie)	10.0 g
BD Bacto™ Beef Extract	4.0 g
BD Bacto™ Peptone	4.0 g
NaCl (Systerm®)	2.5 g
BD Bacto™ Yeast Extract	1.0 g
$CaCO_3$ (Riedel-de Haën)	2.0 g
BD Bacto™ Agar	15.0 g

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1/10 Strength *Streptomyces* Agar (1/10 SA) (Modified from Atlas (2004))

Glucose (Duchefa Bichemie)	1.0 g
BD Bacto™ Beef Extract	0.4 g
BD Bacto™ Peptone	0.4 g
NaCl (Systerm®)	0.25 g
BD Bacto™ Yeast Extract	0.1 g
$CaCO_3$ (Riedel-de Haën)	0.2 g
BD Bacto™ Agar	15.0 g

3.5.2 Purification medium

1/2 strength ISP2 agar (Modified from Shirling and Gottlieb (1966))

BD Bacto™ Yeast Extract	2.0 g
BD Bacto™ Malt Extract	5.0 g
Dextrose (Duchefa Bichemie)	2.0 g
BD Bacto™ Agar	15.0 g

All ingredients were added to 1 L distilled water and mixed thoroughly. The pH was then adjusted to pH 7.0. The medium was then autoclaved at 121 °C and 15 psi for 20 minutes. Sterilized medium was then cooled prior to being poured into sterile petri dishes.

3.5.3 Assay media

Starch Agar (Atlas, 2004)

Soluble starch (Sigma-Aldrich)	2.0 g
BD Bacto™ Nutrient Agar	23.0 g

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All ingredients were added to 1 L distilled water and mixed thoroughly. The pH was then adjusted to pH 7.0. The medium was then autoclaved at 121 °C and 15 psi for 20 minutes. Sterilized medium was then cooled prior to being poured into sterile petri dishes.

Lipase agar (Ramteke *et al.*, 2005)

$CaCl_2.2H_2O$ (Merck)	0.1 g
Tween 80 (Merck)	10 mL
BD Bacto™ Nutrient Agar	23.0 g

The $CaCl_2.2H_2O$ and nutrient agar were dissolved in 1 L of distilled water. The pH was then adjusted to pH 7.0. The medium was then autoclaved at 121 °C and 15 psi for 20 minutes. Tween 80 was autoclaved separately. Sterilized Tween 80 was added to the molten medium at 45 °C. The medium was shaken to dissolve the Tween 80 completely prior to being poured into sterile petri dishes.

Skim-Milk Agar (Staneck & Roberts, 1974)

Skim-milk powder	50.0 g
BD Bacto™ Agar	18.0 g

Skim-milk powder was dissolved in 500 mL of distilled water. In a separate bottle, agar was added then dissolved in 500 mL of distilled water. The pH was adjusted to pH 7.0. The skim-milk powder and agar was then autoclaved separately at 121 °C and 15 psi for 15 minutes. Sterilized agar was then added to the sterilized skim-milk before being cooled and poured into sterile petri dishes.

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1/2 strength ISP2 Broth (Modified from Shirling and Gottlieb (1966))

BD Bacto™ Yeast Extract	2.0 g
BD Bacto™ Malt Extract	5.0 g
Dextrose	2.0 g

All ingredients were added to 1 L distilled water and mixed thoroughly. The pH was then adjusted to pH 7.0. The media was then autoclaved at 121 °C and 15 psi for 20 minutes. Sterilized media was then cooled prior to inoculation.

ISP2 agar (Modified from Shirling and Gottlieb (1966))

BD Bacto™ Yeast Extract	4.0 g
BD Bacto™ Malt Extract	10.0 g
Dextrose	4.0 g
BD Bacto™ Agar	15.0 g

All ingredients were added to 1 L distilled water and mixed thoroughly. The pH was then adjusted to pH 7.0. The medium was then autoclaved at 121 °C and 15 psi for 20 minutes. Sterilized medium was then cooled prior to being poured into sterile petri dishes.

3.6 Serial dilution and plating

One gram of soil was first suspended in 10 mL sterile 1/4 strength Ringer's solution (Oxoid™). The suspension was vortexed thoroughly then allowed to settle for 30 minutes. Upon settling, 1 mL of the suspension was serially diluted with 9 mL of sterile 1/4 strength Ringer's solution to create a first tenfold dilution (10^{-1}). One mL of this was then pipetted into 9 mL of sterile 1/4 strength Ringer's solution for a 10^{-2} dilution. This was repeated to obtain a 10^{-3} dilution. For both soil samples, 0.1 mL of the dilutions 10^{-1} until

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10^{-3} were spread-plated by pipetting the suspensions on to isolation media (described in section 3.5) then a sterilised glass spreader was used to evenly spread the suspension on the agar surface. All plates were prepared in triplicates to ensure accuracy. To prevent an over-growth of fungi, all isolation media were supplemented with antifungals which were 50 µg/mL each of cycloheximide and nystatin. Inoculated plates were incubated at one of three temperatures, 5 ºC, 15 ºC or 25 ºC for 1 month. True psychrophiles are known to grow at temperatures as low as 5 ºC. The optimum temperature for isolation of psychrophiles tend to be around 15 ºC with an upper limit of around 25 ºC (Pan *et al.*, 2013; Pulschen *et al.*, 2017; Russell & Cowan, 2006).

3.7 Isolation and purification

Numbers of colonies were enumerated and CFU/g of soil was calculated. As the objective was to isolate actinobacteria, colonies with dry, powdery, compact and raised appearance were selected from the isolation plates. Morphologically distinct colonies were picked and streaked onto 1/2 strength ISP2 agar using an inoculation needle. Pure colonies that were cultured from isolation plates were observed for colony appearance. Shape, colour, shine, texture, elevation and transparency were noted. Cultures were Gram-stained, and the slides were observed microscopically. Gram-stain-negative isolates were not included in the following steps.

3.8 Strain preservation

Putative actinobacterial strains were then cultured and grown at 15 ℃ for 14 days on 1/2 strength ISP2 agar. Pure colonies of the strains used were preserved for short periods on 1/2 strength ISP2 agar slants. For long term storage, isolates were stored in 1.5 mL centrifuge tubes containing 20 % (w/v) glycerol stocks and kept frozen at -20 ºC (Russell & Cowan, 2006).

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3.9 Strains used in this study

To supplement the strains isolated from this study, actinobacterial strains isolated in previous studies from other locations on Signy Island were included in the enzyme production assays. These strains had been maintained in 20% glycerol stocks at -20 ºC prior to use in this study. Strains were revived by plating on ISP2 at 15 ºC. The list of strains used in this study including isolates from this study are shown in Table 3.2 below. These strains were selected based on the soil sample they originated from as well as strains of varied morphology.

Table 3.2: Antarctic actinobacterial strains used in this study (ND = Not determined).

Strain	Putative Identification	Soil sample	Isolation media	Reference
5	ND	Three Lakes Valley	Reasoner's 2A + Sodium propionate	Pan, 2010
9	*Rhodococcus* sp.	Three Lakes Valley	Reasoner's 2A	Pan, 2010
19	*Rhodococcus* sp.	Elephant flats	Reasoner's 2A + rose Bengal	Pan, 2010
21	*Streptomyces beijiangensis*	Three Lakes Valley	Reasoner's 2A + rose Bengal	Pan, 2010
A17	*Tsukamurella* sp.	Gourlay Peninsula	Starch Casein Nitrate Agar	Pan, 2010
A18	*Kocuria* sp.	Gourlay Peninsula	Starch Casein Nitrate Agar + 2 % NaCl	Pan, 2010
B25	*Rhodococcus corynebacterioides*	Backslope	Starch Casein Nitrate Agar + 2 % NaCl	Pan, 2010
C3	*Streptomyces beijiangensis*	Three Lakes Valley	Tryptic Soy Agar + 0.1 % starch	Pan, 2010
HK8	ND	Elephant Flats	SM3 + Sodium propionate + trehalose	Tan, 2008
J4	*Nocardia* sp.	Backslope	Starch Casein Nitrate Agar	Lim, 2005
J5	ND	Backslope	Starch Casein Nitrate Agar	Lim, 2005

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Table 3.2, continued.

Strain	Putative Identification	Soil sample	Isolation media	Reference
J32	ND	Underneath *Deschampsia antarctica*	Starch Casein Nitrate Agar	Lim, 2005
L17	ND	Underneath *Deschampsia antarctica*	Soil Medium 1	Lim, 2005
L6	ND	Spindrift Col	Glycerol Asparagine agar	Lim, 2005
S63	This study	Spindrift-Col	Starch Casein Nitrate Agar + 2 % NaCl	Pan, 2010
SMT 1	This study	Backslope	This study	This study
SMT 4A	This study	Backslope	This study	This study
SMT 51	This study	Backslope	This study	This study
SP38	ND	Spindrift-Col	SM3 + Sodium propionate + trehalose	Toh, 2008
SP39	ND	Spindrift-Col	SM3 + Sodium propionate + trehalose	Toh, 2008
SY30	*Streptomyces drozdowiczii*	Three Lakes Valley	Tryptic Soy Agar + 0.1 % starch	Pan, 2010
SY62	*Marmoricola aequoreus*	Spindrift-Col	Starch Casein Nitrate Agar + 2 % NaCl	Pan, 2010
SY73	*Demetria terragena*	Gourlay Peninsula	Starch Casein Nitrate Agar	Pan, 2010
SY105	*Rhodococcus* sp.	Spindrift-Col	Starch Casein Nitrate Agar + 2 % NaCl	Pan, 2010
THL38	ND	ND	ND	Tan Hui Ling

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3.10 Amylase production assay

To assess the production of amylase by Antarctic actinobacteria, two methods were employed. The first method, using starch agar, is a qualitative method and is based on absence or presence of amylase. In comparison, a second method employed (the micro-plate based starch-iodine assay) provides a more quantitative look into the production of amylase. Incubation times were selected based on a few factors including the time required by strains to achieve maturation, pilot studies conducted (data not shown) and previously published studies (Dang *et al*., 2008; Sánchez-Porro *et al*., 2003; Yu *et al*., 2009).

3.10.1 Starch Agar

Actinobacterial strains were cultured and grown at 15 °C for 14 days on 1/2 strength ISP2 agar. Pure colonies were inoculated on starch agar (Atlas, 2004) containing 0.2 % soluble starch. Plates were incubated for 7 days at 4 ºC, 15 ºC, 25 ºC or 37 ºC. After incubation, plates were flooded with Lugol's iodine. Lugol's iodine was prepared by dissolving 10 g of potassium iodide in 100 mL of distilled water, after which 5 g of iodine crystals were added while shaking. The formation of clear halo zones around the colonies indicating a positive test was observed and recorded after 14 days.

3.10.2 Micro-plate based starch-iodine assay

The micro-plate based starch utilization assay is modified from methods described in Xiao *et al.* (2006). Actinobacterial strains were cultured and grown at 15 °C for 14 days on 1/2 strength ISP2 agar. A single loopful of fresh cultures were inoculated into tubes containing 1/2 strength ISP2 broth supplemented with increasing concentrations of soluble starch (0.2 %, 0.5 % or 1.0 % soluble starch) to assay for amylase production at different concentrations of starch. Inoculated tubes were incubated at 15 ºC, 25 ºC or 37 ºC for 3 days. Absorbance was measured after 3, 5 and 7 days of incubation. This was

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carried out by first removing one mL of broth and placing it into sterile 1.5 mL centrifuge tubes. This was then centrifuged at 8000 *x g* for 5 minutes. One hundred microliters of supernatant were added to individual 96-well microtiter plates containing 100 µL of Lugol's iodine solution. Absorbance was measured at 600 nm using a microplate reader (Infinity 200 Pro, Tecan Group Ltd, Austria).

3.11 Lipase production assay

Actinobacterial strains were cultured and grown at 15 °C for 14 days on 1/2 strength ISP2 agar. Pure colonies were inoculated on lipase plates (Ramteke *et al.*, 2005). Inoculated plates were then incubated at 4 ºC, 15 ºC or 25 ºC for 14 days after which the formation of opaque halo zones around the colonies, indicating a positive test, was observed.

3.12 Protease production assay

Actinobacterial strains were cultured and grown at 15 °C for 14 days on 1/2 strength ISP2 agar. Pure colonies were streaked on skim milk agar (Staneck & Roberts, 1974) to observe for the production of protease. Inoculated plates were then incubated at 4 ºC, 15 ºC, 25 ºC or 37 ºC for 14 days. The incubation temperatures were modified from the original protocol by Staneck and Roberts (1974) to cover a wider temperature range, allowing studies of production by psychrophiles. The formation of clear halo zones around the colonies indicating a positive test was observed and recorded after 14 days.

3.13 Biosurfactant production assay

The oil-drop collapsing test to screen for the production of biosurfactants is modified from methods described in Gandhimathi *et al.* (2009) and Youssef *et al.* (2004).

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Actinobacterial strains were cultured and grown at 15 °C for 14 days on 1/2 strength ISP2 agar. A single loopful of pure colonies was inoculated into 1/2 strength ISP2 broth. This was then incubated for 7 days at 4 ºC, 15 ºC or 25 ºC. At the end of the incubation period, the cultures were centrifuged at 8000 *x g* for 5 minutes. The resulting pellet was discarded, and the supernatant was used for the assay.

Two microliters of mineral oil (bioMérieux, France) were placed in each well of 96-well microtiter plates. The plates were allowed to equilibrate at 37 ºC for 1 hour. After this, 5 µL of culture supernatant was added to the wells containing mineral oil. The shape of the drop was observed after 1 minute. The resulting drop shape was compared to positive and negative controls, with soap as a positive control and sterile distilled water as a negative control.

3.14 Characterisation of a putative novel actinobacteria

The methods in this section were carried out to identify the characteristics of a putative novel actinobacteria strain. This includes DNA extraction, identification of the 16S rRNA gene sequence, whole genome sequencing, catalase test, NaCl and pH tolerance, and the growth profile.

3.14.1 DNA extraction

The strain was cultured and grown at 15 °C for 14 days on 1/2 strength ISP2 agar. Genomic DNA extraction was performed following the manufacturer's protocol for the NucleoSpin® Tissue kit (Macherey-Nagel, Germany). A loopful of pure single colonies was placed in a microcentrifuge tube containing 180 µL of Buffer T1. Twenty microlitres of lysozyme (50 mg/mL) were added, then the tube was gently vortexed before being incubated at 37 °C for 60 minutes. Twenty-five microliters of Proteinase K (0.6 mg/mL) were then added to the same tube and the sample was incubated again for at least 1 to 3

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hours (overnight where necessary) at 56 °C. After incubation, sample lysis was carried out by adding Buffer B3, followed by vortexing and incubating at 70 °C for about 10 minutes. Following that, the suspension was centrifuged at 11000 *x g* for 5 minutes and 200 µL of molecular-grade ethanol were added to the supernatant to adjust the DNA binding conditions. Upon DNA binding to the silica membrane of the NucleoSpin® Tissue Column, washing steps were carried out using Buffer BW and Buffer B5. The genomic DNA was then eluted using pre-warmed Buffer BE and its concentration was determined using a NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Fisher Scientific, USA). Gel electrophoresis was also performed to check the integrity of extracted DNA using a 0.8 % (w/v) agarose gel and 1× SB buffer, at 100V for 30 minutes. The gel was then stained using GelRed™ Nucleic Acid Gel Stain (Biotium, USA) before being viewed in UV Transilluminator MUV21 (Major Science, USA). The genomic DNA was stored at -20 °C until further usage.

3.14.2 Amplification and sequencing of the 16S rRNA gene

Amplification of the 16S rRNA gene was carried out by Polymerase Chain Reaction (PCR) using the Swift™ Maxi thermal cycler (ESCO, Singapore). The concentrations and final volumes of the PCR reagents used are listed in Table 3.3. The PCR reagents used were the GoTaq® Flexi Buffer and GoTaq® Flexi DNA Polymerase (Promega, USA) with the PCR conditions as listed Table 3.4. The universal primers used in this study were 27f (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492r (5'-TACGGYTACCTTGTTACGACTT-3') (Lane *et al.*, 1985).

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Table 3.3: Concentration and final volume of reagents used for the amplification of 16S rRNA gene

Components	Final concentration	Final volume (µL)
5× Green GoTaq® Flexi Buffer (Promega, USA)	1×	10.0
GoTaq® Flexi DNA Polymerase (Promega, USA)	1.5 U	0.15
dNTPs	0.2 mM	1.0
Magnesium chloride ($MgCl_2$)	1.5 mM	3.0
Forward primer (27f)	0.2 µM	1.0
Reverse primer (1492r)	0.2 µM	1.0
Sterile distilled water (sdH_2O)	-	31.85
DNA template	~ 50 ng	2.0
Total	**-**	**50**

Table 3.4: PCR conditions used for 16S rRNA gene amplification

Step	Temperature (°C)	Duration (m)	Number of cycles
Initial denaturation	95	2	1
Denaturation	95	0.5	35
Annealing	55	0.5	
Extension	72	1.5	
Final Extension	72	10	1

The quality of the amplified product was determined using gel electrophoresis using 1 % (w/v) agarose gel and 1× SB buffer. A working concentration of 1× SB buffer was prepared by diluting a 20× SB buffer. The initial stock of 20× SB buffer was prepared according to Brody and Kern (2004) by adding 450 mL distilled water to 10 mL of 10N NaOH and the pH was adjusted to 8.5 by gradually adding boric acid. This is then topped up with 500 mL distilled water, before being filter sterilised and diluted to 1× working

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concentration. The gel electrophoresis was run at 100V for 20 minutes, followed by gel staining using GelRed™ Nucleic Acid Gel Stain (Biotium, USA) before being viewed in UV Transilluminator MUV21 (Major Science, USA). The PCR product obtained was then purified using the NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel, Germany) before being sent to First BASE Laboratories Sdn. Bhd. (Selangor, Malaysia) for DNA sequencing. The sequencing services were performed using the BigDye® Terminator v3.1 Cycle Sequencing kit (Thermo Fisher Scientific, USA) and results were analysed using the Applied Biosystems Sequence Scanner v2.0 (Thermo Fisher Scientific, USA). The sequences were compared to type strain 16S rRNA gene sequences using the EzBioCloud database available at http://www.ezbiocloud.net/taxonomy (Yoon *et al.*, 2017). Phylogenetic analysis of the strains was carried out *via* alignment using the Molecular Evolutionary Genetics Analysis (MEGA) software version 6 (Tamura *et al.*, 2013).

3.14.3 Whole genome sequencing of a putative novel actinobacterial strain

Genome sequencing was carried out by First BASE Laboratories Sdn. Bhd. (Selangor, Malaysia). Total genomic DNA extraction from pure colonies was carried out following the manufacturer's instructions using the MG™ DNA Purification kit (Macrogen Inc., Korea). The obtained genomic DNA was then fragmented using adaptive focused acoustic technology (AFA; Covaris) to a targeted size of 250 base pairs. These DNA fragments were then end-repaired, before ligation to Truseq adapters, and PCR-enriched using Truseq PCR master mix and Primer cocktail according the manufacturer's protocol. The quantification of the final sequencing library was carried out using qPCR as per the qPCR Quantification Protocol Guide (KAPA Library Quantification kits for Illumina Sequencing platforms). The library size was confirmed using the Agilent 1000 DNA Kit on an Agilent 2100 Bioanalyzer (Agilent Technologies, Böblingen, Germany). The resulting library was sequenced on the Illumina HiSeq2000 platform using a standard

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flow cell and 200 cycles (100 paired end). Genome assembly was performed using Spades version 3.8.1 with the "careful" option enabled (Nurk *et al.*, 2013). The genome sequences were annotated using the RAST (Rapid Annotation using Subsystem Technology) server (Aziz *et al.*, 2008).

3.14.4 Optimal growth pH and NaCl percentage assay

ISP2 medium was prepared containing a range of increasing pH (from pH 4 to pH 12 with a single pH interval). The pH was adjusted using 1M NaOH and 1M HCl to achieve the desired pH prior to autoclaving. Sterilized medium was poured into 24-well plates. Once the medium had solidified, pure cultures of the strain were swabbed using sterile cotton swabs on to the medium then incubated at 15 ^{o}C. The assay was carried out in triplicate to ensure accuracy.

Similarly, ISP2 medium supplemented with increasing percentages of NaCl were prepared (from 0 % to 10 % w/v at 1 % intervals). Autoclaved medium was poured into 24-well plates. Once the medium had solidified, pure cultures were swabbed using sterile cotton swabs on to the medium then incubated at 15 ^{o}C. The assay was carried out in triplicate to ensure accuracy.

3.14.5 Catalase test

A drop of 3 % (w/v) hydrogen peroxide solution was placed on a glass slide. A small amount of bacterial isolate was added to the drop, mixed and observed for the presence of bubbles.

3.14.6 Growth profile of a putative novel actinobacterial strain

The growth profile of the putative novel strain of was generated to examine the effects of incubation temperatures on the strain. Three temperatures were included in the profile; 4 ^{o}C, 15 ^{o}C and 25 ^{o}C. The profile was obtained by first preparing lawns of the strain on

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1/2 strength ISP2 agar. Upon maturation, agar plugs of the strain were aseptically placed into 250 mL conical flasks containing 50 mL 1/2 strength ISP2 broth media. The broth containing the inoculum was incubated in an Innova® 44 shaking incubator (New Brunswick Scientific, United States) at 100 rpm for 14 days. The absorbance was measured at 580 nm at frequent intervals by removing 1 mL of the media and placing it in a clean 3 mL bijoux bottle containing 1 mL of sterile 1/4 strength Ringer's solution. The broth was diluted to give a more accurate reading. Readings were taken in triplicates using a Spectronic 20D+ spectrophotometer (Milton Roy, Europe) and the average absorbance was recorded.

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CHAPTER 4: RESULTS

4.1 Isolation and purification

The bacterial communities in soil samples from Backslope, Signy Island were isolated on a range of media and incubated at a range of temperatures. The number of isolates were enumerated as CFU/g and the counts are presented in Table 4.1.

Table 4.1: Isolation of bacteria from Backslope soil samples (AIA: Actinomycetes Isolation Agar, 1/10 AIA: 1/10 strength of Actinomycetes Isolation Agar, BEA: Beef Extract Agar, 1/10 BEA: 1/10 strength of Beef Extract Agar, SA: *Streptomyces* Agar, 1/10 SA: 1/10 strength of *Streptomyces* Agar, TNTC: Too numerous to count).

Soil sample	Media	Mean bacteria count (CFU/g)			Putative actinobacteria count		
		4°C	15°C	25°C	4°C	15°C	25°C
BS-7	AIA	5.3×10^3	6.3×10^4	4.6×10^4	1	6	2
	1/10 AIA	0	1.8×10^4	5.6×10^3	0	3	1
	BEA	9.4×10^4	TNTC	TNTC	2	0	0
	1/10 BEA	5.8×10^3	8.2×10^5	2.8×10^5	1	7	2
	SA	2.4×10^4	TNTC	1.6×10^5	3	0	3
	1/10 SA	4.7×10^3	8.3×10^4	7.6×10^4	1	3	2
BS-5 (air-dried)	AIA	0	9.7×10^3	0	1	10	0
	1/10 AIA	0	5.8×10^3	0	2	8	0
	BEA	2.5×10^4	2.8×10^4	2.5×10^4	0	0	0
	1/10 BEA	6.0×10^3	1.6×10^4	6.8×10^3	4	6	2
	SA	4.5×10^3	1.5×10^4	1.4×10^4	2	5	2
	1/10 SA	0	7.0×10^3	0	0	0	0
TOTAL					17	48	14

The bacterial count (CFU/g) was enumerated for all plates with the numbers of colonies obtained being between 30 and 300. Examples of isolation plates are seen in Figure 4.1. All dilutions were plated in triplicate and the numbers in Table 4.1 have been averaged. Due to the low availability of each of the soil samples, the two samples from

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the same location were treated separately with Sample BS-5 pre-treated by air-drying for a month whereas sample BS-7 was left untreated. The pH of the soil samples used was slightly acidic. Sample BS-7 had an average pH of 5.5 whereas sample BS-5 had an average pH of 5.0. In the isolation, one of the samples (BS-5) was pre-treated by air-drying the soil. This was found to reduce the overall number of bacteria isolated, particularly the more mucoidal bacteria. This is a fairly well-established method to selectively isolate more rare genera and Actinobacteria (Subramani & Aalbersberg, 2013). This sample also had higher putative actinobacterial counts.

The total number of putative actinobacteria isolated from both isolations was 79. However, many of the isolated strains did not withstand subsequent purifications. Upon purification of the isolates and Gram-staining, the number of Gram-stain-positive bacteria were found to be much lower, with 23 being Gram-stain-positive. However, none of the isolates presented the typical dry, sporulating appearance of actinomycetes. Most strains were waxy or mucoidal in appearance with bright colouration (e.g. strains SMT 1 and SMT 51 in Figure 4.2). Actinobacteria, particularly the members of the *Streptomyces* genera have a typically dry, sporulating appearance, while others have a soft, waxy appearance, as such bacteria without the characteristic appearance (such as mucoidal bacteria) were not selected for subsequent purification.

From this isolation, after dereplication based on morphology, three isolates (SMT 1, SMT 4A and SMT 51) were included in the following assays for selected enzyme and biosurfactant production. Due to the low numbers of typical actinomycetes in this isolation, Antarctic actinobacteria from previous studies were also included in the subsequent tests and screenings. Strains were included in this study from stock cultures maintained in the laboratory. Strains were selected to represent other sampling sites on Signy Island as well as cultures with varying morphology from different genera. The observed colony morphology is shown in Table 4.2 below.

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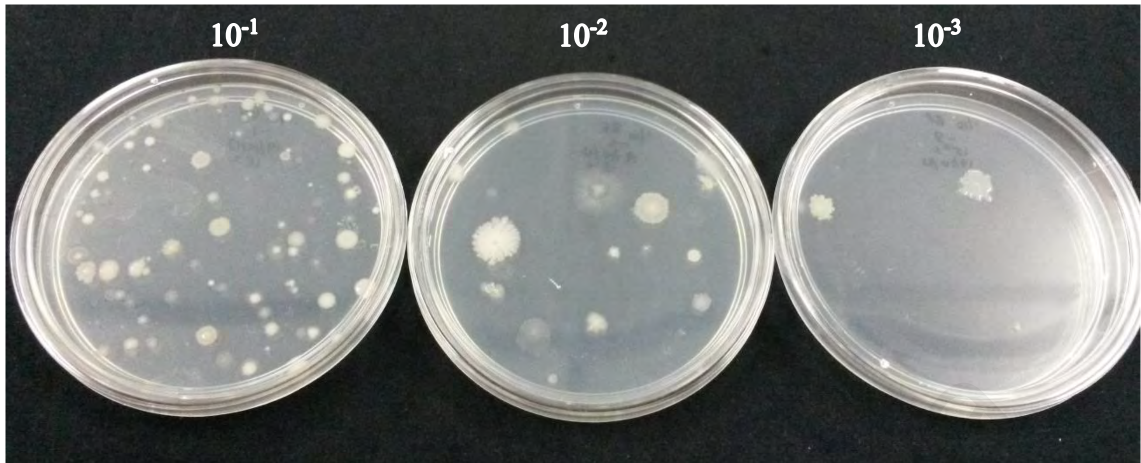

Figure 4.1: Isolation plates showing colonies isolated at the three dilutions. Media used was 1/10 strength AIA incubated at 15 ºC.

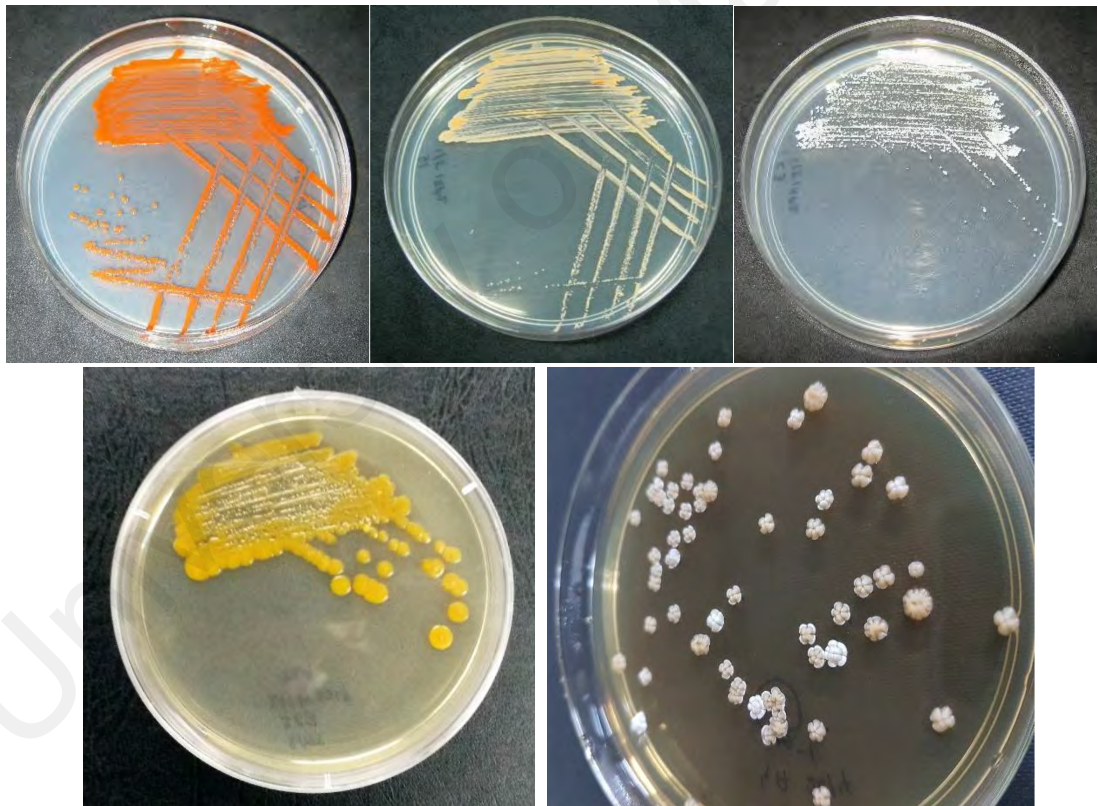

Figure 4.2: Actinobacterial strains used in this study. Top row: left to right are strains SMT 1, SMT 51, C3. Bottom row shows strains S63 (left) and 21 (right).

Table 4.2: Colony morphology Antarctic actinobacterial strains used in this study. Putative identification provided where available, otherwise indicated by ND.

Strain	Putative Identification	Appearance on ISP2
5	ND	Yellowish white, dry colonies with no observable diffusible pigment
9	*Rhodococcus* sp.	Vivid reddish orange waxy colonies
19	*Rhodococcus* sp.	Vivid yellowish orange mucoidal colonies
21	*Streptomyces beijiangensis*	Yellowish white, dry colonies with no observable diffusible pigment
A17	*Tsukamurella* sp.	Yellowish white waxy colonies
A18	*Kocuria* sp.	Light yellow waxy colonies
B25	*Rhodococcus corynebacterioides*	White waxy colonies
C3	*Streptomyces beijiangensis*	Yellowish white, dry colonies with no observable diffusible pigment
HK8	ND	Pale yellow, mucoidal colonies with no diffusible pigment
J4	*Nocardia* sp.	White, dry colonies with no observable diffusible pigment
J5	ND	Violet brownish, dry colonies with light brown diffusible pigment
J32	ND	White brownish, dry colonies with light brown diffusible pigment
L17	ND	White, dry colonies with light brown diffusible pigment
L6	ND	White brownish, dry colonies with brown diffusible pigment
S63	This study	Orange, mucoidal colonies with no diffusible pigment
SMT 1	This study	Vivid orange red waxy colonies
SMT 4A	This study	Yellow, waxy colonies with no diffusible pigment
SMT 51	This study	Vivid yellowish orange mucoidal colonies

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Table 4.2, continued.

Strain	Putative Identification	Appearance on ISP2
SP38	ND	Vivid reddish orange waxy colonies
SP39	ND	Vivid reddish orange waxy colonies
SY30	*Streptomyces drozdowiczii*	Yellowish white, dry colonies with no observable diffusible pigment
SY62	*Marmoricola aequoreus*	White dry colonies
SY73	*Demetria terragena*	Light yellow waxy colonies
SY105	*Rhodococcus* sp.	Vivid reddish orange waxy colonies
THL38	ND	White dry sporulating colonies

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4.2 Amylase production assay

4.2.1 Starch Agar

Twenty-five actinobacterial strains were assayed for amylase production on Starch Agar. Pure colonies were streaked onto Starch Agar and incubated at four different temperatures; 4 ºC, 15 ºC, 25 ºC and 37 ºC. After 2 weeks of incubation, the plates were flooded with Lugol's Iodine. The results of the assay are shown in Table 4.3. Plates showing positive tests are shown below in Figure 4.3. A total of 15 strains produced amylases with four strains producing amylase at all four temperatures tested. Four other strains only produced amylase at lower temperatures. Three strains (strains SMT1, SY73 and SP39) were found to be producing amylase at 25 ºC and/or 37 ºC. Four strains (strains C3, SMT 51, 21 and J32) were found to produce amylase at all experimental temperatures. Four strains tested produced amylase at 4 ºC and 15 ºC but not at higher temperatures (strains 19, SY30, L6, and SP38).

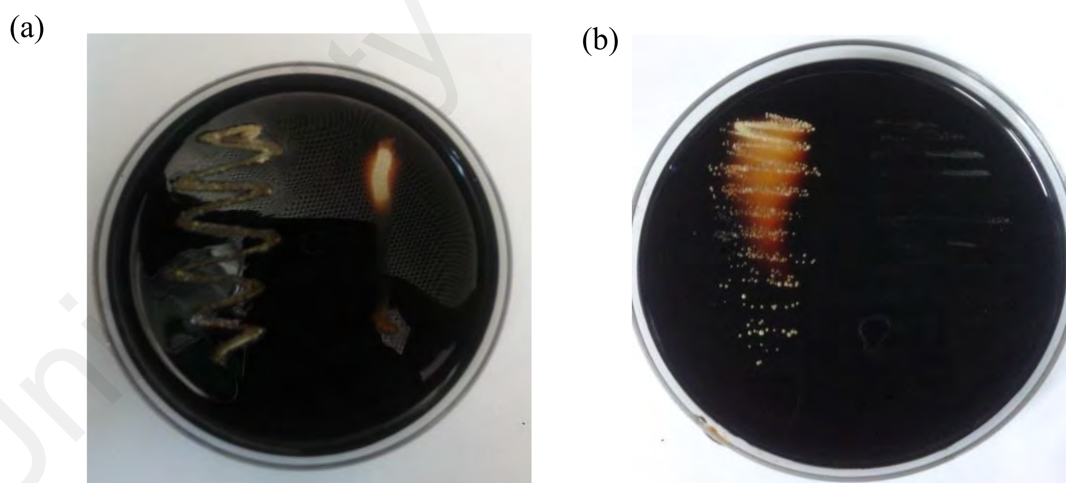

Figure 4.3: Starch assay plates with positive results indicated by the presence of halo zones around colonies. Figure 4.3 (a) shows a negative result for strain THL 38 and a positive result for strain C3, and (b) shows a clear positive result for strain 21 and a negative for strain 9.

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Table 4.3: Results of amylase production assay (+ : presence of halo zone; +/- : presence of a faint halo zone; - : absence of halo zone)

Strain	Temperature			
	4 ºC	15 ºC	25 ºC	37 ºC
19	+	+	+/-	-
21	+	+	+	+
C3	+	+	+	+
HK8	-	+	+	+
J4	-	+	+	+
J5	-	+	+	+
J32	+	+	+	+
L6	-	+	-	-
S63	-	+	+	+
SMT 1	-	-	-	+
SMT 51	+	+	+	+
SP38	-	+	-	-
SP39	-	-	+	-
SY30	+	+	-	-
SY73	-	-	+	+

4.2.2 Micro-plate based starch-iodine assay

Eighteen strains were selected for screening for production of amylase at 15 ºC, 25 ºC and 37 ºC. Strains that did not grow well in 1/2 strength ISP2 broth at one or more temperatures were excluded from this study to prevent false negatives. A reduction of absorbance readings indicated a reduction of starch contained in the supernatant. The plates used are shown in Figure 4.4 and a summary table of results is shown in Table 4.4.

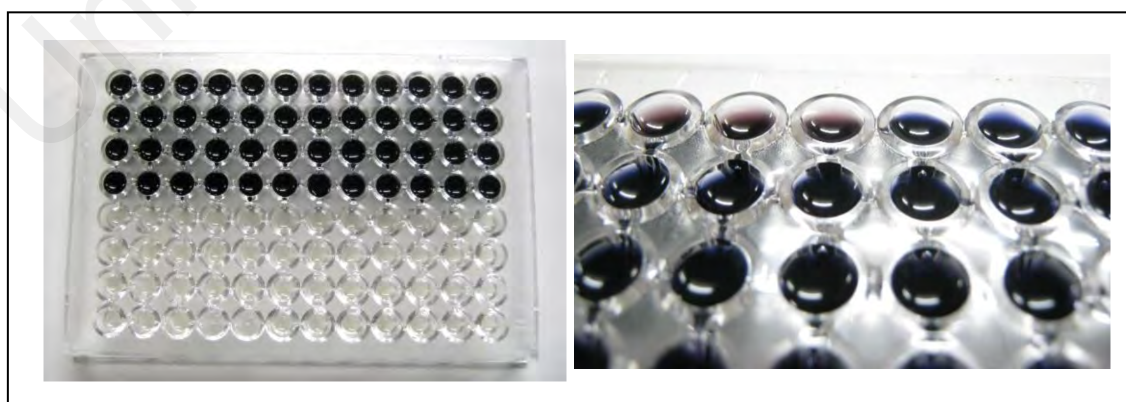

Figure 4.4: 96-well plates used for the micro-plate based starch-iodine assay. A faint discolouration is seen in the positive wells as seen in the second image.

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Table 4.4: Micro-plate based starch assay summary table. The table summarises the results for amylase production at different starch concentrations at three temperatures.

Strain	Starch Concentration (%)								
	0.2			0.5			1		
	15 °C	25 °C	37 °C	15 °C	25 °C	37 °C	15 °C	25 °C	37 °C
5	+	-	-	+	+	+	+	+	+
9	+	+	+	-	+	+	+	+	+
19	+	+	+	+	-	-	+	-	+
21	+	+	+	+	+	+	+	+	+
A17	+	+	+	-	+	+	+	+	+
A18	+	+	+	+	+	-	-	+	+
C3	+	+	+	+	+	+	+	+	+
HK8	+	+	-	-	-	+	+	-	+
J32	+	+	+	+	+	+	+	+	+
L17	+	+	+	+	+	+	+	+	+
S63	+	+	+	+	+	+	-	+	+
SMT 1	-	+	+	-	-	+	-	+	+
SMT 4A	-	-	+	-	-	+	+	+	+
SMT 51	+	+	+	+	+	+	+	+	+
SY105	-	+	+	+	-	+	+	+	+
SY30	+	+	+	+	+	-	+	+	+
SY62	+	+	+	+	+	-	+	+	+
THL38	-	-	-	+	-	-	-	+	+

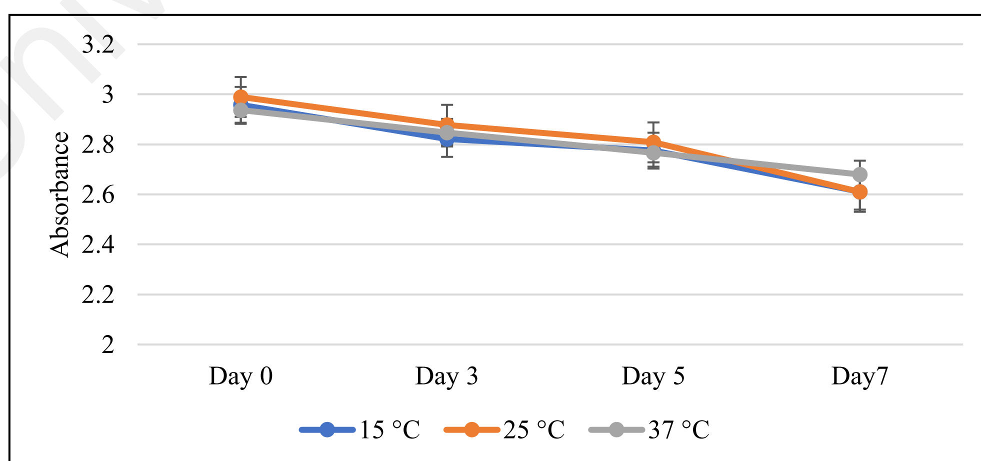

Figure 4.5: Starch utilization by strain 21. Strain 21 utilised starch at all three temperatures tested (the starch concentration shown here is 0.2 %).

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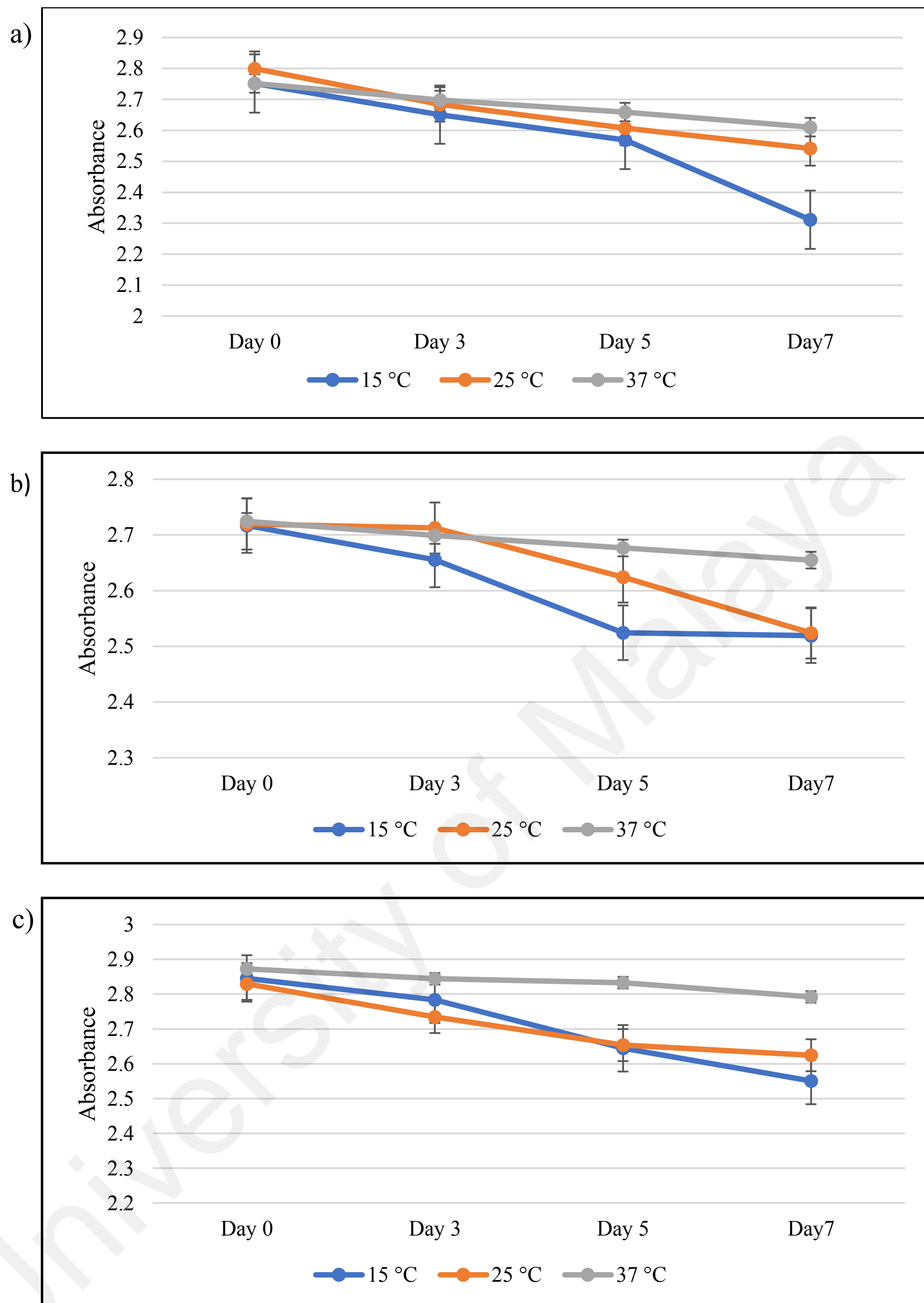

Figure 4.6: Starch utilization by strain SY30. The three graphs show utilization at three concentrations of starch (a: 0.2 %, b: 0.5 % and c: 1%)

The figures 4.5 and 4.6 above show results of amylase production for selected strains. Most of the strains were found to be positive for amylase production. Five strains (strains L17, C3, SMT 51, 21 and J32) were positive for production of amylase at all three

temperatures and at all three tested starch concentrations (Figure 4.5). A few strains were only able to reduce starch at low temperatures (e.g. strain 21 seen in Figure 4.6). Strain SMT1 only utilized starch at 25 ºC and 37 ºC at all three concentrations of starch. Overall, production was best at higher concentrations of starch. Complete results are given in Appendix A.

4.3 Lipase production assay

A total of 25 actinobacterial strains were tested for production of lipase (Table 4.5) below. Positive results are indicated by presence of opaque halo zones around colonies as seen in Figure 4.7. A total of 12 strains were positive for lipase production. Two strains, SMT 1 and SMT 4A, were positive for lipase production at all three temperatures tested. One strain, HK 8, was only positive for lipase production at low temperatures. Four strains were positive for production of lipase at only 15 ºC.

Table 4.5: Results for lipase production assay (++ : presence of intense halo zone; + : presence of halo zone; +/- : presence of a faint halo zone; - : absence of halo zone)

Strain	Temperature		
	4 ºC	15 ºC	25 ºC
21	-	+	+
A17	-	-	+
C3	-	+	-
HK8	+	+	-
J32	-	-	+
L6	-	+	+
S63	-	+	-
SMT 1	+	+	+
SMT 4A	+	++	+
SP39	-	+/-	-
SY105	-	+	-
SY30	-	++	+

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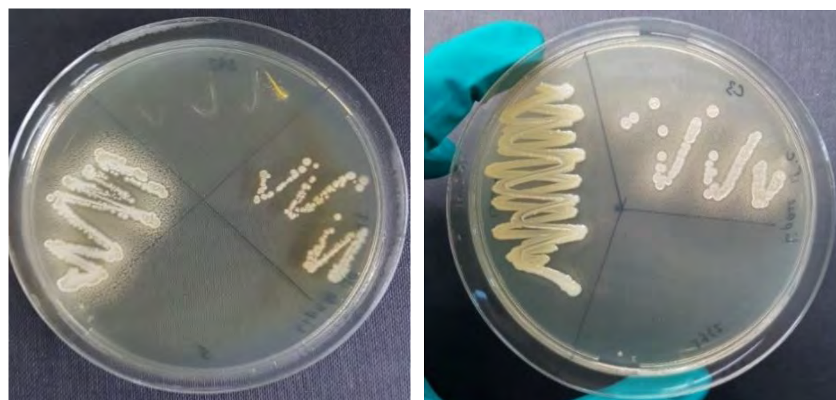

Figure 4.7: Opaque halo zones produced indicating a positive lipase production assay

4.4 Protease production assay

A total of 23 actinobacterial strains were tested for protease production (Table 4.6). Two strains were excluded to prevent false negatives as no growth was observed at all temperatures tested. The production of protease was observed at four temperatures; 4 ^{o}C, 15 ^{o}C, 25 ^{o}C and 37 ^{o}C. Positive results are indicated by presence of halo zones around colonies (Figure 4.8). Eight strains were positive for production at all temperatures tested with 11 strains positive in total. One strain was positive for production at only 15 ^{o}C and 25 ^{o}C. More strains were found to be producing protease at 37 ^{o}C than 4 ^{o}C. Strong production was seen in a number of strains, particularly at 25 ^{o}C. Strains L6 and SY73 were found to be producing protease best at 15 ^{o}C and 25 ^{o}C with slightly poorer production at 4 ^{o}C and 37 ^{o}C.

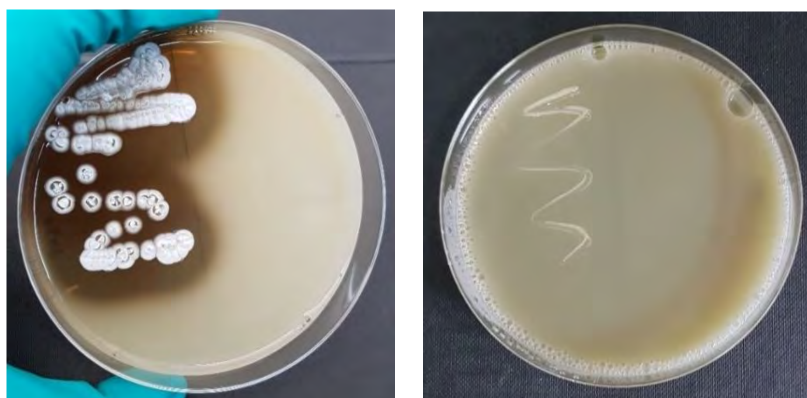

Figure 4.8: Protease production assay plates. The translucent halo zone (left) indicates a positive test and the absence of the halo zone (right) indicates a negative test.

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Table 4.6: Results for protease production assay (++ : presence of intense halo zone; + : presence of halo zone; - : absence of halo zone)

Strain	Temperature			
	4 °C	15 °C	25 °C	37 °C
21	+	+	+	+
C3	-	-	+	+
J32	+	+	++	+
J4	-	+	+	-
J5	+	++	++	++
L6	+	++	++	+
S63	+	+	+	+
SMT 51	-	-	+	+
SP39	-	-	+	+
SY30	+	+	++	+
SY73	+	++	++	+

4.5 Oil-drop collapsing test

Twenty-five strains were tested for production of biosurfactants that reduce surface tension of the oil drop. At all three temperatures tested, no positive results were observed (Figure 4.9).

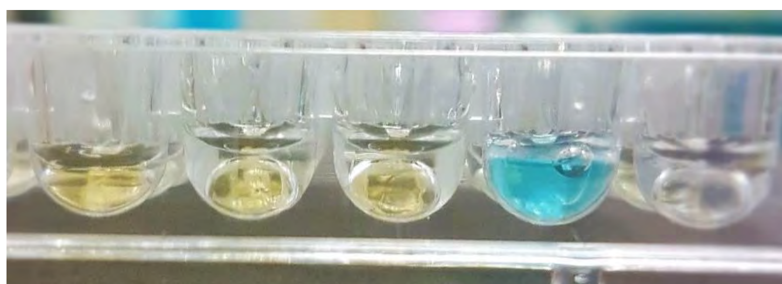

Figure 4.9: Oil drop collapsing test. The blue well contains soap as a positive control. In the wells next to the control, the supernatant forms a bead, indicating a negative test.

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4.6 Summary of enzymatic production by tested strains

As seen in Table 4.7, only a few strains were capable of producing multiple enzymes at any of the temperatures tested. Most of the strains the could produce amylase, lipase and protease had previously been identified as belonging to the *Streptomyces* genus with the exception of strain S63.

Table 4.7: Summary table of production assays carried out on Antarctic actinobacteria. (+ : presence of activity; - : absence of activity)

Strain name	Amylase Production Assay		Lipase Production Assay	Protease Production Assay	Oil-drop collapsing test
	Qualitative	Quantitative			
5	-	+	-	N/D	-
***Rhodococcus* sp. strain 9**	-	+	-	-	-
***Rhodococcus* sp. strain 19**	+	+	-	-	-
***Streptomyces beijiangensis* strain 21**	+	+	+	+	-
***Tsukamurella* sp. strain A17**	-	+	+	-	-
***Kocuria* sp. strain A18**	-	+	-	-	-
***Rhodococcus corynebacteroides* strain B25**	-	N/D	-	-	-
***Streptomyces beijiangensis* C3**	+	+	+	+	-
HK8	+	+	+	-	-
***Nocardia* sp. strain J4**	+	N/D	-	+	-
J5	+	N/D	-	+	-
J32	+	+	+	+	-
L17	-	+	-	-	-
L6	+	N/D	+	+	-
S63	+	+	+	+	-
SMT 1	+	+	+	-	-
SMT 4A	-	+	+	-	-
SMT 51	+	+	-	+	-
SP38	+	N/D	-	-	-

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Table 4.7, continued.

Strain name	Amylase Production Assay		Lipase Production Assay	Protease Production Assay	Oil-drop collapsing test
	Qualitative	Quantitative			
SP39	+	N/D	+	+	-
***Streptomyces drozdowiczii* strain SY30**	+	+	+	+	-
***Marmoricola aequoreus* strain SY62**	-	+	-	N/D	-
***Demetria terragena* strain SY73**	+	N/D	-	+	-
***Rhodococcus* sp. strain SY105**	-	+	+	-	-
THL38	-	+	-	-	-

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4.7 Phylogenetic and phenotypic characteristics of strain S63

4.7.1 Identification of 16S rRNA gene sequence

As one of the strains with enzyme production for amylase, lipase and protease, strain S63 was identified as a strain of interest. To further study this strain, the genomic DNA was extracted and the full 16S rRNA gene was sequenced. The initial 16S rRNA gene sequencing produced a unit of 1366 bp after trimming the tail ends of the sequence using the Applied Biosystems Sequence Scanner Software v2.0 (Thermo Fisher Scientific, USA). However, upon sequencing the genome, the full length of the 16S rRNA gene sequence was obtained and used instead. The full length of the gene was 1519 bp. The complete 16S rRNA gene sequence can be found in Appendix B.

Using the EzBioCloud database, strain S63 was most closely related to *Humibacillus xanthopallidus* KV-663^T with a 96.97 % similarity. Strain S63 was also closely related (< 97 % similarity) to multiple members of the genus *Terrabacter*. The closest relatives of the strain and the similarity percentages are shown in Table 4.8. Strain S63 lies in the family *Intrasporangiaceae*, sub-order *Micrococcineae*, Micrococcales order and Class Actinobacteria. The phylogenetic tree (Figure 4.10) was inferred using the Neighbour-Joining method (Saitou & Nei, 1987) and is based on 16S rRNA gene sequences of strain S63, closest relatives and representatives of the family. Strain S63 formed a separate branch from the closest relatives of the strain. The *Terrabacter*, *Humibacillus* and *Monashia* genera were clustered together, while the *Oryzobacter* genus also branched away. The closest relatives were all isolated from non-polar environments with most of them being from agricultural related soils (Table 4.8). As such, this strain is most likely a new species and phenotypic and chemotaxonomic criteria delineated in Schumann *et al.* (2009) are required to characterise and differentiate strain S63 as a new species within the *Intrasporangiaceae* family.

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Table 4.8: This table lists the strains most closely related to strain S63. The data was exported on the 5th of July 2017 from the EzBioCloud 16S database. This list only includes species that have been validly described and are the type strains for the species (as indicated by the T symbol in uppercase).

Rank	Name	Strain	Authors	Isolation Source	Accession	Pairwise Similarity (%)	Completeness (%)
1	*Humibacillus xanthopallidus*	KV-663^{T}	Kageyama *et al.* (2008)	Paddy field soil, Japan	AB282888	96.97	98.07
2	*Terrabacter terrae*	PPLB ^{T}	Montero-Barrientos *et al.* (2005)	Soil mixed with Iberian pig hair	AY944176	96.88	100
3	*Terrabacter tumescens*	DSM 20308 ^{T}	Collins *et al.* (1989)	Soil	X83812	96.86	99.38
4	*Terrabacter ginsenosidimutans*	Gsoil 3082 ^{T}	An *et al.* (2010)	Ginseng farm soil, Korea	EU332827	96.80	100
5	*Terrabacter lapilli*	LR-26 ^{T}	Lee *et al.* (2008)	Stone from agricultural field, Korea	AM690744	96.67	100
6	*Terrabacter aerolatus*	5516J-36 ^{T}	Weon *et al.* (2007)	Air sample, Korea	EF212039	96.64	97.02
7	*Monashia flava*	MUSC 78 ^{T}	Azman *et al.* (2016)	Mangrove soil, Malaysia	KF682157	96.60	100
8	*Oryzobacter terrae*	PSGM2-16 ^{T}	Kim *et al.* (2015)	Pot of paddy soil, Korea	KP100643	96.55	100
9	*Terrabacter terrigena*	ON10 ^{T}	Yoon *et al.* (2009)	Soil around wastewater treatment plant, Korea	FJ423552	96.53	100
10	*Terrabacter carboxydivorans*	PY2 ^{T}	Kim *et al.* (2011)	Roadside soil, Korea	FJ717334	96.53	100

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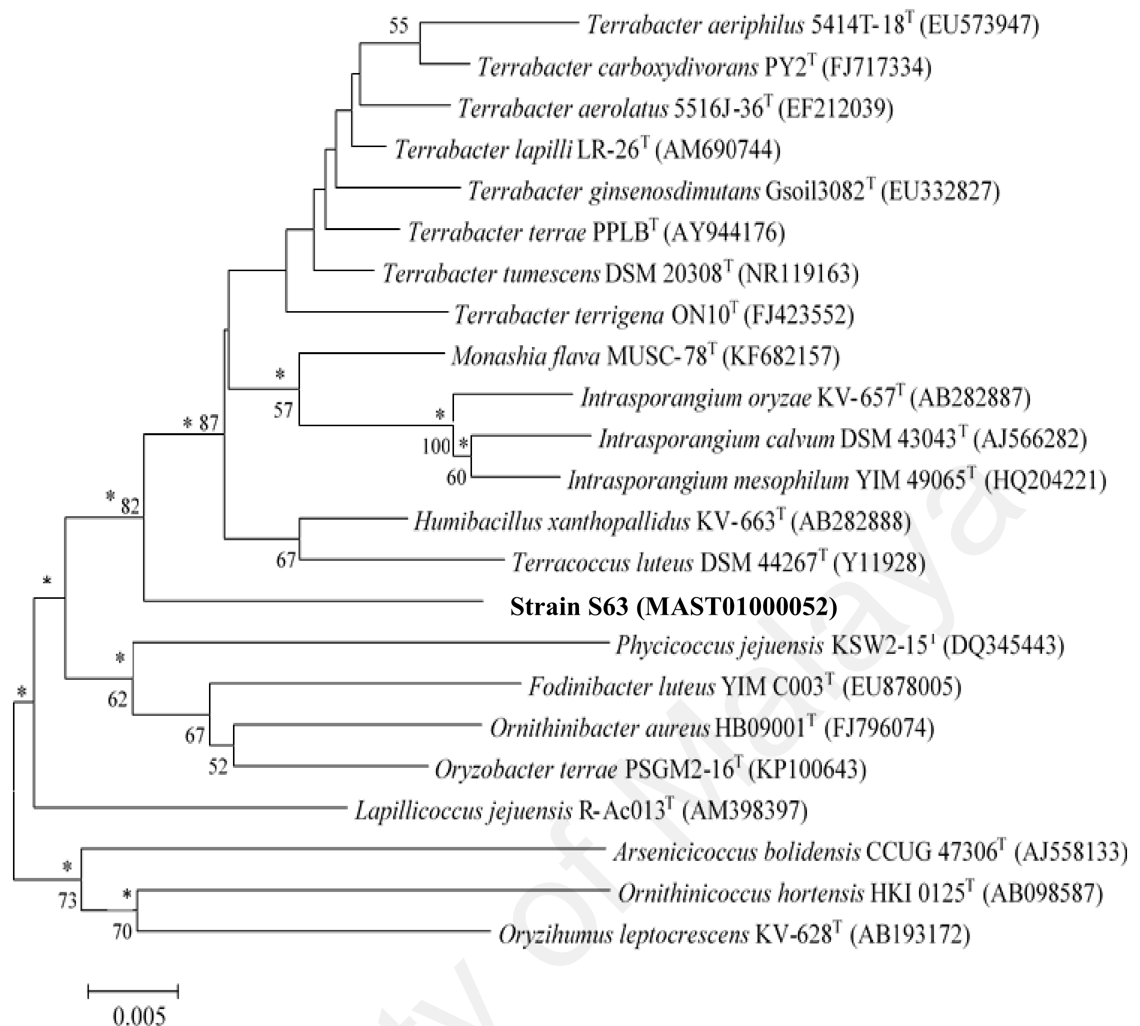

Figure 4.10: Phylogenetic tree of strain S63 and other type strain members of *Intrasporangiaceae* inferred using the Neighbour-Joining method. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Jukes-Cantor model. The bootstrap values (based on 1000 replicates) are shown as percentages at each node for values above 50 %. The scale bar length indicates 0.005 substitutions per nucleotide position. Asterisks next to nodes indicate corresponding nodes recovered using the maximum-likelihood tree-making algorithm. The tree was built using the MEGA 6 software.

The partial 16S rRNA gene sequence of strain S63 has been deposited at DNA Data Bank of Japan (DDBJ), the European Nucleotide Archive (ENA) and GenBank under the accession number KJ547654. Pure cultures have been deposited in The Leibniz Institute DSMZ - German Collection of Microorganisms and Cell Cultures GmbH, Germany

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(DSMZ) under the accession number DSM 29435 and in Thailand Bioresource Research Center, Thailand (TBRC) under the accession number TBRC 5146.

4.7.2 Whole genome sequencing of strain S63

The Illumina HiSeq2000 platform generated a total of 47,882,286 paired-end reads (average read length of 100 bp) of which 6,000,000 reads were subsequently subsampled for de novo assembly. The genome assembly was performed using Spades version 3.8.1 with the "careful" option enabled (Nurk *et al.*, 2013). The draft assembly was subsequently improved using in-silico scaffolding and gap-closing (Boetzer *et al.*, 2011; Boetzer & Pirovano, 2012). The final draft genome had an accumulated genome length of 5,023,713 bp of which 69.33 % were GC. This was contained in 54 contigs with an N_{50} of 194,740 bp. The genome features are listed in Table 4.9. The NCBI Prokaryotic Genome Annotation Pipeline was used for annotation leading to the prediction of 4,529 coding sequences of which 2,671 had annotated functions and 1,858 were hypothetical proteins. One of copy of each ribosomal RNA (5S, 16S and 23S) was identified in the draft genome, along with 47 tRNAs and 3 ncRNAs. This Whole Genome Shotgun project has been deposited at DNA Data Bank of Japan (DDBJ), the European Nucleotide Archive (ENA) and GenBank under the accession MAST00000000. The version described in this section is version MAST01000000. The tree above was drawn using the 16S rRNA gene sequence obtained from the whole genome shotgun sequencing with the accession number: MAST01000052.

Annotation and analysis was also carried out on the RAST server. The server identified 4621 coding sequences and 2672 subsystems. The SEED viewer version 2.0 (Overbeek *et al.*, 2005) was used to browse the annotated genome. The feature counts displayed in Figure 4.11 were obtained from the SEED annotation environment (http://rast.nmpdr.org/seedviewer.cgi). The analysis and annotation revealed the presence

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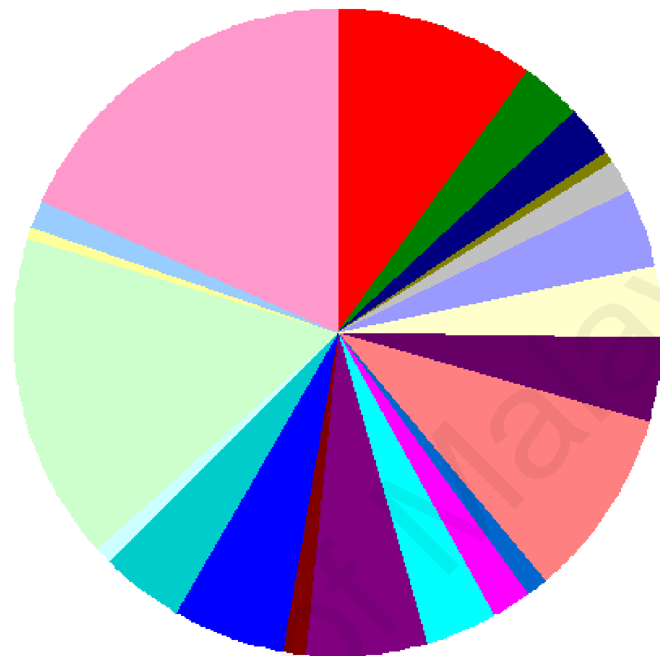
of multiple genes for the degradation of various substrates including amylases, lipases and/or esterases, proteases, chitinases and cellulase precursors. Also found were genes encoding proteins involved in stress responses such as heat shock proteins, cold-shock proteins, antifreeze proteins and trehalose biosynthesis related proteins.



Subsystem Feature Counts

- Cofactors, Vitamins, Prosthetic Groups, Pigments (268)
- Cell Wall and Capsule (84)
- Virulence, Disease and Defense (65)
- Potassium metabolism (18)
- Photosynthesis (0)
- Miscellaneous (42)
- Phages, Prophages, Transposable elements, Plasmids (0)
- Membrane Transport (103)
- Iron acquisition and metabolism (3)
- RNA Metabolism (102)
- Nucleosides and Nucleotides (110)
- Protein Metabolism (248)
- Cell Division and Cell Cycle (32)
- Motility and Chemotaxis (4)
- Regulation and Cell signaling (50)
- Secondary Metabolism (4)
- DNA Metabolism (98)
- Fatty Acids, Lipids, and Isoprenoids (161)
- Nitrogen Metabolism (33)
- Dormancy and Sporulation (3)
- Respiration (151)
- Stress Response (110)
- Metabolism of Aromatic Compounds (21)
- Amino Acids and Derivatives (436)
- Sulfur Metabolism (14)
- Phosphorus Metabolism (42)
- Carbohydrates (470)

Figure 4.11: Subsystem and features of strain S63 were annotated using the RAST server.

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Table 4.9: Genome features of strain S63

Features	
N_{50}	194,740
Genome Size (bp)	5,023,713
GC content (%)	69.33
Number of contigs	54
Number of CDS	4,529
Number of RNAs	53
Number of tRNAs	47
Number of rRNAs	3
Number of ncRNAs	3

4.7.3 Catalase test

Strain S63 produced oxygen bubbles in the catalase test, indicating a positive test for the production of the catalase enzyme. This assay was only carried out for this strain as part of the requirements to describe this strain as a novel species/genus.

4.7.4 Optimal growth pH and NaCl percentage assay

As seen in the Figure 4.12 below, pure colonies of strain S63 were swabbed onto media in 24-well plates. Strain S63 had an optimum NaCl concentration percentage of 0 % and 1 %, poor growth at percentages of 2 and 3 and did not grow at higher concentrations. The optimum pH for growth was a pH of 8, followed by pH 7. Strain S63 grew poorly at pH 6 and 9 and did not grow at a pH lower than 6 or higher than 9.

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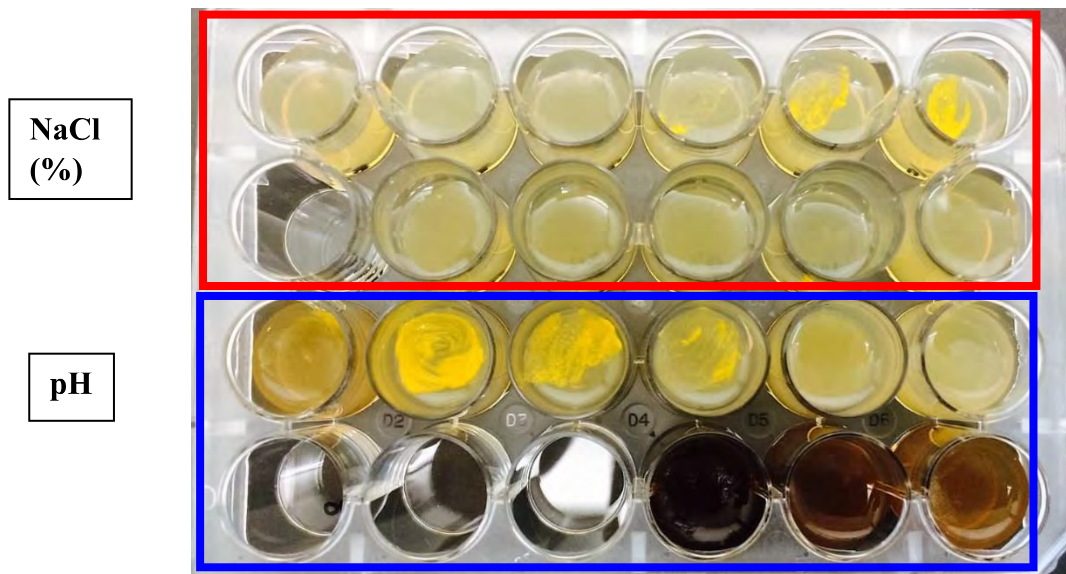

Figure 4.12: Strain S63 on 24-well plates. The top row of wells contains ISP2 media supplemented with increasing concentrations of NaCl (%, w/v) reading from right to left: 0, 1, 2, 3, 4, 5. The second row continues the increasing concentrations reading from right to left: 6, 7, 8, 9, 10. The third row contains ISP2 media with increasing pH, reading from right to left: pH 4, 5, 6, 7, 8, 9. The fourth row continues the increasing pH, reading from right to left: pH 10, 11 and 12.

4.7.5 Growth profile of strain S63

Strain S63 was cultured in broths incubated at three different temperatures to obtain a growth profile. The absorbance was measured at 550 nm and results are given in Table 4.6 below. The growth profile is presented in Figure 4.13. Strain S63 grew optimally at 15 ^{o}C, with good growth at 25 ^{o}C but growth was poor at 4 ^{o}C.

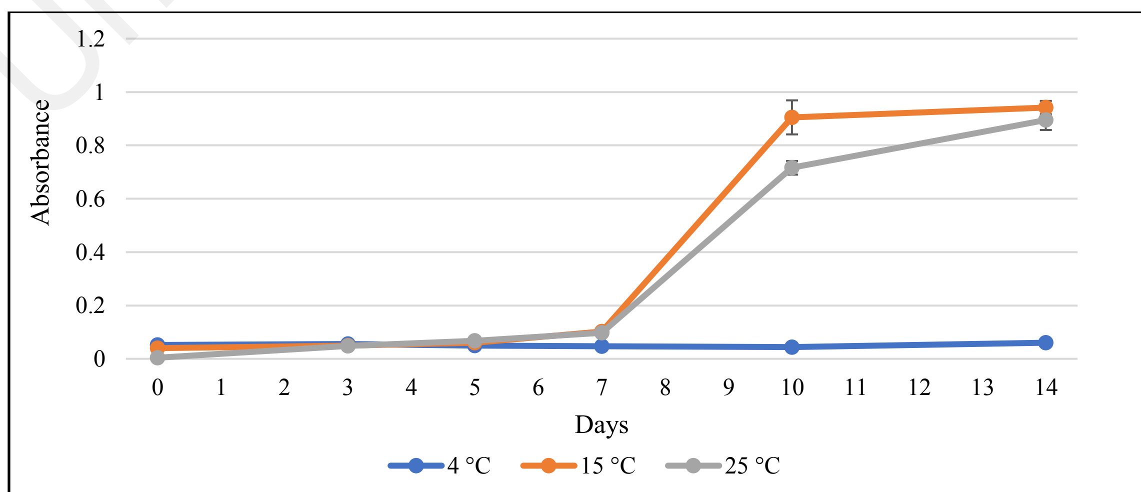

Figure 4.13: Growth profile of strain S63 at three different temperatures

4.7.6 Phenotypic and chemotaxonomic studies carried out on strain S63

As part of the formal description of novel species, the morphological, physiological and biochemical features of strain S63 were studied. Some of these characteristics were determined separately from this study and as such, the methods were not shown here. The results are summarised in Table 4.10 and Figure 4.14 along with the characteristics observed in this study.

Table 4.10: Characteristics morphological, physiological and biochemical features of strain S63 (* = denotes tests done in this study)

Feature	Result
Gram-stain	Positive
Shape	Cocci
Oxygen requirement	Aerobic
Spore production	Non-spore forming
Motility	Non-motile
Whole cell sugar	Glucose
Cell wall peptidoglycan	LL-diaminopimelic acid
Dominant cellular fatty acids	iso-$C_{15:0}$, $C_{17:1\omega8c}$ and iso-$C_{16:0}$
Salinity tolerance*	Up to 3 %
pH tolerance*	Growth at 6 – 9 (optimum at pH 8)
Optimum temperature*	15 – 25 °C
Catalase*	Positive

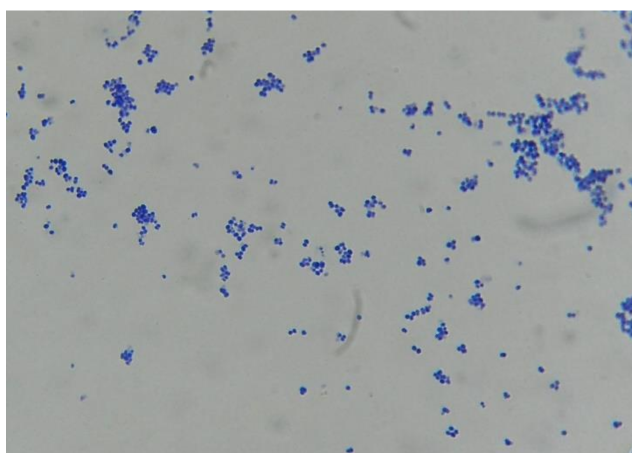

Figure 4.14: Strain S63 under 1000x magnification showing Gram-stain-positive cocci

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CHAPTER 5: DISCUSSION

5.1 Isolation of Actinobacteria from Signy soil samples

This study set out to investigate the diversity of the actinobacterial communities in soil samples from Antarctica as well as the bioactive potential of these communities. Actinobacteria are ubiquitous in the Antarctic and are found in diverse ecosystems. As prolific producers of various secondary metabolites and bioactive molecules, the isolation of this group of bacteria could yield novel species as well as secondary metabolites and bioactive molecules of interest.

In the isolation, one of the samples was pre-treated by air-drying the soil. This was carried out to reduce the number of mucoidal bacteria as well as selectively isolate more rare genera and Actinobacteria (Subramani & Aalbersberg, 2013). The air-dried samples did have a higher ratio of putative actinobacteria compared to the untreated soil, showing that the pre-treatment was effective in reducing the number of mucoidal bacteria. However, despite the pre-treatment, the expected dry colonies were not observed which could be due to a number of reasons, such as storage and isolation conditions, comparatively high water availability on Signy Island and soil pH.

The largest number of putative actinobacteria were found on the Actinomycetes Isolation Agar (AIA) and the *Streptomyces* Agar (SA). Full strength AIA and SA were better isolation media compared with Beef Extract Agar (BEA). Some of the 1/10 strength isolation media, such as 1/10 strength BEA, proved to be better isolation media for actinobacteria. To improve actinobacterial yield in future studies two strategies might be employed, (i) using low temperature incubations for longer periods of time, possibly in combination with (ii) using media with lower nutritional status than those used in this study (Pulschen *et al.*, 2017).

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The most effective temperature for isolation was found to be 15 ^{o}C, which is not unusual and also suggests that the isolates were generally psychrotolerant rather than being obligate psychrophiles (Franzmann, 1996; Morita, 1975; Peeters *et al.*, 2011). Growth at 4 ^{o}C was extremely slow and smaller numbers of isolates, and actinobacteria specifically, were obtained. Antarctic isolates have been found in a number of studies to have higher optimum temperatures than obligate psychrophiles (whose optimum growth temperatures are 4 – 8 ^{o}C) (Chong *et al.*, 2013; Cowan *et al.*, 2011). Eurythermal bacteria or psychrotrophic bacteria are terms referring to bacteria capable, but not requiring, cold temperatures for growth (Chong *et al.*, 2013; Morita, 1975). The lower isolation success noted here at 25 ^{o}C may indicate that the bacterial communities studied had a larger representation of psychrotolerant bacteria rather than mesophilic bacteria. Nevertheless, due to the long storage time of the samples at -20 ^{o}C, the viable community isolated here may not be a true representation of the culturable bacterial community *in situ*.

The comparatively high water availability on Signy Island compared to many other Antarctic terrestrial systems may be another factor leading to the low numbers of actinobacterial isolates obtained. The availability of water may alleviate the environmental selection pressure on some bacteria, thus the communities would potentially have fewer members able to tolerate desiccation (such as members of the *Streptomyces* genus) (Aislabie *et al.*, 2008, 2009; Cary *et al.*, 2010). Actinobacteria are found in significantly higher proportions at more southerly sites, which generally receive less precipitation and experience higher desiccation stress, compared to northerly sites on an Antarctic Peninsula/Scotia Arc latitudinal gradient (Yergeau *et al.*, 2009). This trend is also seen in other studies which included Ellsworth mountains at the southern limit of the Antarctic Peninsula (Chong *et al.*, 2015; Yergeau *et al.*, 2007c) as well as studies comparing Antarctic Peninsula communities with Dry Valley communities (Bottos *et al.*, 2014; Cary *et al.*, 2010). The effects of water availability are seen in studies by Newsham

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et al. (2010) on soils from Mars Oasis, Maritime Antarctica, and Niederberger *et al.* (2015) on soils from the Dry Valley. Although soils from the Mars Oasis site showed minimal influence of water availability on microbial community composition, the actinobacterial community was larger in the drier soils (~ 33.2 %) compared to wetter soils (~ 24 %). Similarly, wetted soils in the hyper arid Dry Valley had a lower percentage of actinobacterial OTUs (~ 7.5 %) compared to dry soils (~ 4.7 – 37.0 %). Combined, these studies suggest that actinobacterial presence could be higher in higher latitudes where the desiccation stress is more pronounced compared to Signy Island soils.

Previous studies have shown that soil Actinobacteria are more commonly found in soils with neutral and slightly alkaline pH (Basilio *et al.*, 2003; Labeda & Shearer, 1990; Waksman, 1967). The soils of Signy Island in general tend to be acidic to neutral (pH 4.79 – 7.54) (Chong *et al.*, 2010; Pan *et al.*, 2013). As soil pH is one of the factors that influence bacterial community structures, this could also contribute to the low numbers of actinobacteria isolated (Chong *et al.*, 2009b; Chong *et al.*, 2011; Fierer & Jackson, 2006). Consistent with this, in a study of culturable Actinobacteria from Signy Island, the largest number of cultured Actinobacteria were obtained from a site with a more neutral pH (Pan *et al.*, 2013). Similarly, this was also noted in other studies of Antarctic terrestrial communities. Soils with neutral to alkaline pH were found to have more Actinobacteria than soils with lower pH (Aislabie *et al.*, 2013; Sanyika *et al.*, 2012).

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5.2 Screening for production of enzymes and biosurfactants

Antarctic Actinobacteria are known to be sources of enzymes and biosurfactants with many of these having activity at low temperatures (Feller & Gerday, 2003; Gerday *et al.*, 2000; Kügler *et al.*, 2015; Nichols *et al.*, 1999; Shivaji *et al.*, 2017). The genera with known producers are *Arthrobacter*, *Micrococcus*, *Rhodococcus*, *Nocardia*, *Gordonia*, *Streptomyces* and *Corynebacterium*. Cold-active enzymes and biosurfactants are of use in many biotechnological, manufacturing, medical and pharmaceutical industries as well as having an application in bioremediation (Adrio & Demain, 2014; Cavicchioli *et al.*, 2002; de Pascale *et al.*, 2012). As these industries grow, the demands for economical alternatives to chemically-manufactured, plant- or animal-sourced enzymes and biosurfactants are increasing (Gerday *et al.*, 2000; Shivaji *et al.*, 2017). To meet these demands, various Antarctic ecosystems are currently being explored for more effective microbial enzymes and biosurfactants (Coker, 2016; Podar & Reysenbach, 2006; Shivaji *et al.*, 2017; Taylor *et al.*, 2012). A range of Antarctic bacteria have been found to produce the enzymes targeted in the current study, namely amylases, proteases and lipases as well as a host of other enzymes such as pectinases, cellulases and xylanses (Ramteke *et al.*, 2005; Shivaji *et al.*, 2004, 2011; Tropeano *et al.*, 2012, 2013). Bacteria isolated from Signy Island lake systems were also found to produce amylases, proteases and lipases (Ellis-Evans, 1981).

In this study, various Signy Island isolates were confirmed to be able to produce amylase. Based on the starch agar assay, four strains (strains C3, SMT 51, 21 and J32) were found to produce amylase at all experimental temperatures, suggesting that temperature did not influence production for these strains. Other strains tested amylase at 4 ^{o}C and 15 ^{o}C but not at higher temperatures (strains 19, SY30, L6, and SP38). This could be either due to the production of a cold-active amylase which allows these bacteria to utilize starch despite the low temperature or the production of amylase as a response

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to cold temperatures. However, the mode of action and mechanisms involved are beyond the scope of this study. Three strains (strains SMT1, SY73 and SP39) were found to be producing amylase at 25 ºC and/or 37 ºC. These were more likely to be mesophiles which do not possess adaptations for amylase production in the cold.

The microplate assay was conducted to provide a more quantitative assay of the digestion of starch at different temperatures. The assay also was more sensitive in detecting changes in starch concentrations than the plate assay as weaker producers are still detected. From the assay results, all strains tested had some production depending on the temperature and starch concentrations provided. Similar to the starch agar assay, certain strains were unaffected by the changes in temperature and concentration and four strains (strains C3, SMT 51, 21 and J32) had similar results in both assays, showing the reproducibility and usefulness of the quantitative method in lieu of the qualitative starch plate assay. Amylase production in Antarctic bacteria is highly beneficial for survival. The ability to produce amylase in low nutritional status environments allows for the breakdown and utilization of the complex macromolecule, starch. Aside from that, amylase production is also linked to trehalose, a disaccharide widely involved in stress tolerance as well as a source of carbon (Block *et al.*, 2009; D'Amico *et al.*, 2006; Iturriaga *et al.*, 2009).

Cold-active lipases are generally fairly understudied compared to other lipases (Joseph *et al.*, 2008). Industrial applications of cold-active lipases are held back by high enzyme cost, low activity levels, low reaction yields and poor stability under environmental conditions (Yu & Margesin, 2014). As such, psychrophilic bacteria are investigated to overcome this *via* the use of recombinant lipases and metagenomics (Koo *et al.*, 2016; Seo *et al.*, 2014; Yu & Margesin, 2014; Yuan *et al.*, 2014). Of the strains tested in this study, strains producing lipase at all three temperatures tested are potential sources of thermostable lipases as well as potentially having wide applications in bioremediation of

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polluted soils and waste water and in biotechnological applications (Cavicchioli *et al.*, 2002; Feller & Gerday, 2003). Of the three temperatures tested, lipase production was most commonly seen at 15 ^{o}C and the strains showing strong production would be the best candidates for further studies into cold-active expression as well as enhancing production. A deeper study into the quantitative aspects of lipase production would be needed to assess if these are indeed cold-active lipases as well as identifying potential uses for these strains (Joseph *et al.*, 2008).

Proteases are the most widely used class of enzyme and unlike cold-active lipases, cold-active proteases have been more extensively studied and are commercially available (Adrio & Demain, 2014; Coker, 2016; Taylor *et al.*, 2012). Widely found in Antarctic, these hydrolytic enzymes breakdown organic matter to allow uptake by bacteria (Shivaji *et al.*, 2011; Tropeano *et al.*, 2013). Antarctic actinobacteria have been known to be involved in the production of this enzyme (Ma *et al.*, 2013; Yu *et al.*, 2011; Zhou *et al.*, 2013). In line with this, the production of protease was tested at four temperatures to identify potential sources of cold-active proteases. Most of the strains tested were found to be producing protease at wide temperature ranges with notably strong production at 15 ^{o}C and 25 ^{o}C. The strains with strong production at these temperatures would perhaps be the best sources of cold-active proteases among the strains tested. Similar to the other two assays, this may indicate the presence and production of thermostable cold-active enzymes which could have potentials in a wide range of applications with further studies. The production at wide temperature ranges may indicate that some of these are indeed psychrotrophic bacteria that could rely on protease production for survival regardless of the surrounding temperature. This is would also explain why more strains were found to produce protease at 37 ^{o}C than 4 ^{o}C.

Out of 25 strains tested, 14 produced two or all three of the tested enzymes at various temperatures. Notable among these were strains S63, 21 and C3. Strains 21 and C3 were

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previously identified using 16S rRNA gene sequencing as having a 98.5 % sequence similarity to *Streptomyces beijiangensis* (Pan *et al.*, 2013). The same study also initially identified strain S63 as a potentially novel species and with the ability to produce all three enzymes tested, this strain was selected for further studies using full genome sequencing. Enzyme production at 4 ^{o}C was low in general with most strains showing positive results at 15 ^{o}C and 25 ^{o}C. Strains 21, J32 and SY30, however, were producing both lipase and protease at 4 °C. Strain SY30 was putatively identified as *Streptomyces drozdowiczii* (Pan, 2010) while strain J32 was putatively identified as a *Streptomyces* sp. based on the morphology.

The oil-drop collapsing test was carried out to detect the presence of surfactants produced by actinobacteria. However, the results for the tested strains were negative. This could have been due to a lack of an isolation media to selectively isolate for biosurfactant producers. Alternatively, the strains could have first been grown with a hydrocarbon as the main carbon source to induce the production of biosurfactants, as an Antarctic *Rhodococcus* sp. has been known to have biosurfactant production induced under these conditions (Gesheva *et al.*, 2010).

5.3 Strain S63: Species characterisation and whole genome sequencing

Strain S63 was initially isolated from Signy soils as part of an earlier study (Pan *et al.*, 2013). The strain did not produce anti-microbial compounds when tested, and preliminary sequencing found it to have a 97 % similarity with Actinobacterium kmd_307 (EU723162) (Pan *et al.*, 2013). As part of this study, the enzymatic potential of strain S63 was studied alongside other strains. The 16S rRNA gene was fully sequenced and had a closest (96.7 %) similarity to *Humibacillus xanthopallidus* KV-663^{T}. Strain S63 lies in the family *Intrasporangiaceae*, sub-order *Micrococcineae*, order Micrococcales and

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Class Actinobacteria. At present, although it sits within the *Intrasporangiaceae* family, the strain does not fall under any currently described genera. When compared to the closest relatives (Table 4.8), strain S63 was not grouped with the closest relatives as seen in the phylogenetic tree (Figure 4.10). As such it may represent not just a new species but a new genus.

To ascertain this, the minimum standards for describing new genera and species of the suborder *Micrococcineae* (Schumann *et al.*, 2009) have been used to compare phenotypic and chemotaxonomic criteria with those of other members of the suborder (Table 5.1). Although the phylogenetic data supports the description of strain S63 as a new species, this is not adequate for formal descriptions of species and the genotypic, chemotaxonomic, cultural and biochemical criteria are still required.

The *Intrasporangiaceae* is a family with a wide range of phenotypic and chemotaxonomic properties and are found in a wide range of habitats (Stackebrandt *et al.*, 2014). Members of the family have been detected in various Antarctic environments (Pulschen *et al.*, 2017; Selbmann *et al.*, 2010; Smith *et al.*, 2006; Tytgat *et al.*, 2016; Yuan *et al.*, 2014). The family consists of 21 recognised genera at time of writing with 16 genera summarised in Schumann *et al.* (2009) (note: the genus *Humihabitans* has since been reclassified (Yang *et al.*, 2012)), as well as five newly described genera; *Aquipuribacter* (Toth *et al.*, 2012), *Fodinibacter* (Wang *et al.*, 2009), *Monashia* (Azman *et al.*, 2016), *Ornithinibacter* (Xiao *et al.*, 2011), and *Oryzobacter* (Kim *et al.*, 2015). Some of the main differentiating features include cell morphology, cell wall diamino acid, DNA G+C content (%), major menaquinones, major fatty acids and polar lipids.

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Table 5.1: Criteria for the description of a new species or genera of the suborder *Micrococcineae* (adapted from Schumann *et al.* (2009))

Criteria
Cultural and biochemical criteria
o General morphology
o Gram reaction
o Relation to oxygen and oxidative or fermentative metabolism
o Catalase activity, oxidase activity
o Utilization of different carbon sources, acid production from carbohydrates and other carbon sources and enzymic activities and decomposition of selected substrates which must be tested concurrently with the most closely related species on the basis of the same method
Chemotaxonomic criteria
o Peptidoglycan structure (qualitative amino acid composition of peptidoglycan mandatory for genus descriptions)
o Cellular fatty acids
o Polar lipids
o Respiratory quinone pattern
Genotypic criteria
o Phylogenetic position based on 16S rRNA gene sequence comparisons (different treeing methods should be applied). For species allocation within an established genus, the 16S rRNA gene sequence similarity values to the most closely related species (and the exact methods of calculation) must be given
o DNA base composition (G+C content % of the DNA) (mandatory for genus descriptions)
o DNA–DNA hybridizations with the most closely related species (showing a 16S rRNA gene sequence similarity > 97 %) within an established genus (mandatory for species descriptions)

Selected differentiating features of strain S63 and the type strains of the two most similar species *Terrabacter terrae* PPLB^T (Montero-Barrientos *et al.*, 2005) and *Humibacillus xanthopallidus* KV-663^T (Kageyama *et al.*, 2008) are shown in Table 5.2. Based on the studies describing them, strain S63 shares a number of similarities with *T. tumescens and H. xanthopallidus*. All three are Gram-stain-positive, catalase positive,

aerobic, non-spore forming, and contain LL-DAP. The differences in the fatty acid profiles strongly indicate that strain S63 is a new species as the profile is one of the main differentiating factors between genera. Other features seen in Table 5.1, including major respiratory quinones and polar lipid components, remain to be examined as time and budget constraints prevented the determination of these properties. These characteristics will be able to conclusively place strain S63 as either a novel species or genus within the family.

Table 5.2: Selected differentiating features of strain S63, *Terrabacter terrae* PPLB^T and *Humibacillus xanthopallidus* KV-663^T

Feature	Strain S63	*T. terrae*	*H. xanthopallidus*
Cell morphology	Cocci	Long rods	Irregular rods
Salinity tolerance	Up to 3 %	Up to 7 %	Up to 3 %
pH tolerance (optimum)	6 – 9 (8)	N/D	5 – 11 (7)
Motility	Non-motile	Non-motile	N/D
Whole Cell Sugar	Glucose	Fucose and galactose	Galactose, glucose, rhamnose
DNA G+C content (%)	69.3	71	69 - 70
Major fatty acids	iso-$C_{15:0}$, $C_{17:1\omega8c}$, iso-$C_{16:0}$	iso-$C_{15:0}$, anteiso-$C_{15:0}$, iso-$C_{16:0}$, anteiso-$C_{17:0}$	iso-$C_{15:0}$, iso-$C_{14:0}$, iso-$C_{16:0}$, $C_{17:1\omega8c}$

Although not outlined in the minimal standards for describing a novel species of the suborder, newer publications describing new species now include whole genome sequences. Sequencing the whole genome allows further understanding of the genetic makeup of the strain as well as of its possible functional adaptations and potential biotechnological applications. Through the analysis of the annotated genes of strain S63, presence of protein domains commonly found in cold-shock proteins and anti-freeze proteins were detected, indicating adaptations that allow continued function in cold

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environments. Strain S63 also possesses genes coding for enzymes involved in the conversion of maltose to trehalose. Trehalose is a source of energy as well as being a cryoprotectant. It is also involved in many groups of organisms in anhydrobiosis and would in this case assist this bacterium to withstand prolonged desiccation (Convey, 1996; Everatt *et al.*, 2015; Wynn-Williams, 1983, 1996). The genome also contained alpha-amylase and glucoamylase genes, which are part of the gene complex for the biosynthesis of trehalose. This may indicate that the utilization of starch could be linked to low temperatures, as the production of trehalose increases survival of cold stress, for example an *Arthrobacter* sp. isolated from an alpine permafrost sample was found to accumulate trehalose upon cold shock (Chen *et al.*, 2011). Aside from amylase, strain S63 also possessed the genes for the production of lipase and/or esterase, including putative genes for secreted lipase, and genes for the production of proteases as well as putative genes for secreted proteases. This genotypic potential was supported in the phenotypic tests in this study, which confirmed production of amylase, lipase and protease in the assays conducted. Also detected were the presence of genes for chitinase production and cellulase precursors. The ability to utilize multiple substrates is advantageous in environments with poor nutritional sources.

A second tree was built (Figure 5.1) including strain S63 and the next 5 most similar strains in the National Center for Biotechnology Information (NCBI) database as well as other similar strains (*H. xanthopallidus*, *T. terrae*, *T. tumescens*, and *T. ginsenosdimutans*). The NCBI database includes the 16S rRNA gene sequences of strains that have not yet been described and uncultured bacterial clones. This tree was built to highlight the relationship between the unclassified *Intrasporangiaceae* strains and S63 as all four originated from Antarctic soil samples.

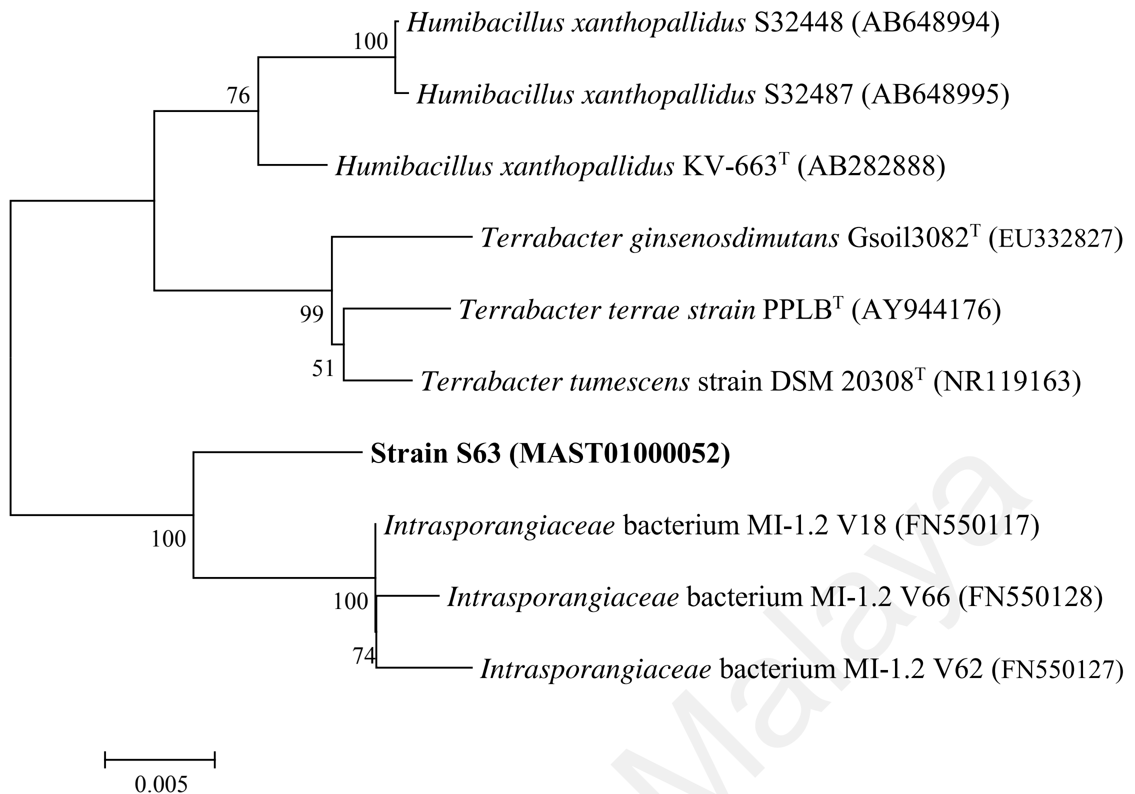

Figure 5.1: Phylogenetic tree of strain S63 and selected members of *Terrabacter*, *Humibacillus* and unclassified *Intrasporangiaceae* inferred using the Neighbour-Joining method. Tree is drawn to scale (branch lengths in same units as evolutionary distances used to infer the phylogenetic tree). Evolutionary distances were computed using the Jukes-Cantor model. Bootstrap values (based on 1000 replicates) are shown as percentages at each node for values above 50 %. Scale bar length indicates 0.005 substitutions per nucleotide position. The tree was built using the MEGA 6 software.

The three closest sequences from the NCBI database were cultured *Intrasporangiaceae* bacteria which shared a 98 – 99 % similarity with strain S63. These all originated from Marion Island, a sub-Antarctic island in the Indian Ocean sector of the Southern Ocean. In a separate study by Zdanowski *et al.* (2012), a bacterial isolate from the top of an old glacial river bank in proximity to Ecology Glacier on King George Island had a 98 % similarity to *Intrasporangiaceae* bacterium MI-1.2 V18 (Accession number: FN550117) and a 98 % similarity to strain S63. This strain was excluded from the tree in Figure 5.1 as the gene sequence was only 860 bp long.

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In contrast, all three of the *Humibacillus xanthopallidus* strains were isolated from soil originating from a paddy field in Japan (Kageyama *et al.*, 2008) and the *Terrabacter* strains were all also from non-polar soils. The similarity and clustering of strain S63 to the Marion Island and King George Island isolates could potentially be a novel polar genus. The similarity between these strains could also point to the presence of shared genes for cold adaptations including common cold environment stress tolerance mechanisms. The growth profile further supports this as the strain grows well at 15 ^{o}C. However, the lack of growth at 4 ^{o}C and the abundant growth at 25 ^{o}C indicates that this strain is more likely a psychrotolerant bacterium instead of an obligate psychrophile.

5.4 Future considerations and future work

As actinobacteria are well known producers of enzymes, wider ranges of substrates could be selected for sourcing further strains and screening for cold-active enzymes. For example, actinobacteria may be a source of cold-active cellulase enzymes (Yergeau *et al.*, 2009). Alternatively, screening could be conducted at low temperatures for a wider range of actinobacteria to find bacteria with strong enzyme production characteristics at low temperature.

Enrichments and pre-treatments could also be employed to selectively isolate strains possessing biosurfactant or specific enzyme production potential, such as using media with specific carbon sources or hydrocarbons. The use of newer (more recently collected) soil samples would also be preferable in future studies to assess diversity. This would be a better measure of the soil microbial communities. As actinobacterial communities are still of interest in various fields and have been demonstrated to express compounds and enzymes with potential biotech applications, the actinobacterial communities of Continental Antarctica could be studied with greater depth. The continent may host a larger actinobacterial community than Signy, though this remains to be clearly proven.

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In looking for a specific group or specific functions such as enzyme production, the use of metagenomics and metatranscriptomics can also be employed to guide isolation of culturable bacteria for biotechnological applications (Berlemont *et al.*, 2011; Vester *et al.*, 2015). The genome sequences of bacteria can also be used to identify strains with this potential. Aside from the presence of genes for the production of amylase, lipase and protease, strain S63 also possesses various other genes such as chitinase production genes and cellulase precursor genes. The availability of this genomic information can direct future screening for enzyme production. However, when dealing with large numbers of isolates, obtaining whole genome sequences can be costly and screening based on phenotypic production is still favoured. This may change as sequencing costs become less prohibitive (Vincent *et al.*, 2016). Transcriptomic approaches could also be used to study gene expression linked to enzyme production at different temperatures.

Downstream enzymatic studies could be carried out on strains capable of producing enzymes at 4°C. This would include quantification and identification of the enzymes being produced, the biochemical properties of the enzyme (e.g. half-life, temperature optima, activation energies), and elucidating the enzyme structure with focus on structural modifications for cold adaptations.

This study has shown evidence that strain S63 is highly likely to be a new species and possibly a new genus. However, the current body of work is inadequate to conclusively make this claim. To further confirm the placement of strain S63 as a new species or genus, further work to characterise strain S63 as a novel species will be carried out in comparison with closely related strains. This will include determinative features such as major respiratory quinone patterns and polar lipid components as well as other physiological and biochemical features outlined in Table 5.1, such as carbon utilization and acid production.

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CHAPTER 6: CONCLUSIONS

The Antarctic continent is a region of scientific interest as it remains understudied, physically isolated from other land masses and has a largely untapped potential in the search for novel bacteria and compounds. Much work remains to be done in describing the microbial communities of Antarctica. The continent's isolation and harsh environmental features have resulted in the evolution of microbial species and communities that are highly specialised, potentially endemic, and well adapted to survive under the stresses of this region. These adaptations include the production of cold-active enzymes as seen in this study. Novel bacteria are also isolated from these environments presenting yet another source of novel compounds and enzymes.

Despite the low number of actinobacterial isolates obtained in this study, Signy Island bacterial strains were active producers of extracellular amylases, lipases and proteases. Out of 25 strains tested, 15 were positive for amylase, 12 for lipase and 11 for protease production. Fourteen strains produced more than one of the enzymes tested, with many of these doing so at cold temperatures, indicating that some of the strains are capable of producing cold-active enzymes. However, none of the strains examined produced biosurfactants under the culture and experimental conditions used.

One of the strains producing all three enzymes was strain S63, a potential novel species from the *Intrasporangiaceae* family which has a < 97 % 16S rRNA gene sequence similarity with its closest relative recorded in GenBank, *Humibacillus xanthopallidus*. The draft whole genome sequence and phenotypic characteristics of strain S63 were obtained in order to formally describe this strain as a new species. The genome sequence revealed the presence of genes associated with cold-shock proteins, anti-freeze proteins, trehalose biosynthesis, chitinase, and the three enzymes tested in this study. Further analysis of these enzymes could provide deeper insight into enzyme adaptations to

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environments with extreme conditions. Further work to supplement the characterisation in this study will also be carried out formally describe and publish strain S63 as a novel species. Analysis of the draft genome to mine for genes of interest will also be carried out.

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LIST OF PAPERS PRESENTED

Oral presentation

Thomas, S. M., Convey, P., Pearce, D. A. and Tan, G. Y. A. (2013, Oct). *Temperature effects on amylase activity in Antarctic Actinobacteria.* Paper presented at Malaysian International Seminar on Antarctica 6, Pulau Pinang, Malaysia.

Poster presentations

Thomas, S. M., Convey, P., Pearce, D. A. and Tan, G. Y. A. (2013, Oct). *Biosurfactant Production in Antarctic Actinobacteria*. Paper presented at Malaysian International Seminar on Antarctica 6, Pulau Pinang, Malaysia.

Tan, G. Y. A. and Thomas, S. M. (2015, Aug). *Polar Actinobacteria*. Paper presented at Regional Conference on Culture Collections, Serdang, Malaysia.

Thomas S. M. and Tan, G. Y. A. (2015, Dec). *Draft genome sequence of a Humibacillus Strain S63 isolated from Signy Island, Antarctica.* Paper presented at International Conference for Microbiology, Penang, Malaysia.

Tan, G. Y. A., Thomas, S. M. and Convey, P. (2016, Aug). Novel actinobacterium strain S63 isolated from Signy, Antarctica. Paper presented at Scientific Committee on Antarctic Research: Biennial Meetings and Open Science Conference 2016, Kuala Lumpur Malaysia

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