Potential of Cave Bacteria in Drug Discovery: Investigation of *Sphingopyxis terrae* and other Bacteria from a BC Cave

By

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Abstract

The discovery of new and more effective antibiotics continues to be a priority given the frequency of the emerging multi-drug resistant pathogenic microorganisms. Thus, scientists are searching for new antibiotics from microorganisms selected from extreme habitats such as very old caves. Various cave bacteria species were isolated and could be sources of new antibiotics. The objective of our work is to isolate cave bacteria from a volcanic cave in Wells Gray Provincial Park in BC and to test if they produce metabolites with antimicrobial activity against some microorganisms including multi-drug resistant pathogens. This study used 16 cave strains previously isolated and screened against a panel of microorganisms including drug resistant pathogens. Upon retesting, 4 out of 16 cave bacterial isolates, RA001, RA003, RA004, and PM58B-RA, demonstrated antimicrobial activity against *Mycobacterium smegmatis*, *Micrococcus luteus*, *Acinetobacter baumannii* and MDR-*Staphylococcus aur*eus. To study the conditions for best growth and antimicrobial production, these four bacteria were cultured in different fermentation media (namely Hickey-Tresner, R_2A, V-8 juice and ISP-2) and incubated at 12 and 25°*C* for 14 days. During the course of fermentation, the percentage of packed cell volume (%PCV), antimicrobial activity and pH were observed and recorded daily. It was found that each of the bacteria demonstrated antimicrobial activity against different microorganisms at various times of fermentation and temperature. Overall, R_2A broth

medium and the lower temperature of 12°*C* appear to be best for antimicrobial production by the cave bacteria used in this study. Isolation and purification of the antimicrobial compounds produced by these isolates is under investigation using the best growth conditions determined in this study. We identified these bacteria using chemotaxonomic studies; 16S rRNA sequencing and Matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF), all isolates were identified to the species level. PM58B was found to be *Bacillus licheniformis*, RA001, and RA004 were identified as *Arthrobacter agilis* that may be of different variants. While RA003 was identified as *Sphingopyxis terrae*. Active compounds from RA003 fermentation broth were further studied by extraction and purification.

In conclusion, cave bacteria are promising sources of potential novel antimicrobial compounds. Isolation, optimization of screening, growth media and conditions of cave bacteria may be useful in the discovery of new antimicrobial drugs. Additionally, the knowledge obtained from this study with respect to cave bacteria and their roles in cave formation and degradation will add to existing information on cave conservation.

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1 Introduction

1.1 Summary

The objective of this study was to isolate bacteria from a volcanic cave in Wells Gray Provincial Park in BC, and investigate whether these bacteria can produce metabolites with antimicrobial activity against various tested microorganisms including pathogens and non-pathogens. Previous studies demonstrated that a diverse community of microorganisms inhabiting the Helmcken Falls Cave could inhibit various tested microorganisms and indicated that some of these microorganisms have the ability to produce antimicrobial agents; Rule and Cheeptham, 2013; Cheeptham et al., 2013). The present study confirms that a wide variety of microorganisms inhabiting the Helmcken Falls Cave possess antimicrobial activities that can inhibit the growth of some tested bacteria.

Sixteen cave bacterial isolates were examined using the disc-diffusion (Kirby-Bauer) method against the tested microorganisms such as *Mycobacterium smegmatis*, *Micrococcus luteus*, multi-drug resistant *Staphylococcus aureus* (MDR-MRSA), *Acinetobacter baumannii*, ESBL *E. coli* and *Candida albicans*. In total, four isolates demonstrated effective antimicrobial activity, as they had the ability to kill and inhibit the growth of tested microorganisms such as Gram-positive and/or negative-bacteria. Interestingly, one of the isolates (RA003) displayed antimicrobial activity against MDR-MRSA.

This study also demonstrated that the media selection was critical in the antimicrobial activity of the isolates. R_2A broth was the optimal medium for the antimicrobial activity of all four isolates selected for further study. Production of antimicrobial activity was not observed for some isolates in some of the media tested, and some of the isolates displayed varying levels and/or loss of antimicrobial activity during the fermentation course when cultured in various media. Notably, the RA001, RA004 and PM58B-RA isolates lost their antimicrobial activity on specific days of culture and then antimicrobial activity reappeared on subsequent days of culture. These observations were

consistent with repeating experiments. The reasons are not clear as to why they lost their activity, these need to be further studied. Additionally, the characteristics of secondary metabolites are not completely understood at this time, their chemical structures need to be further studied. One plausible explanation may be due to their functions, the antimicrobial activity of secondary metabolites in the laboratory may not reflect their role in nature (Bibb, 2005).

Temperature, pH, fermentation period, seed inoculum volume and the type of media all had effects on the antimicrobial activity, growth and production of pigments by the four isolates. In general, when inoculated using a 2% seed culture, cultured at 12 or 25°C and pH 7.8-8.5 with shaking at 250 rpm over 14 days, the optimum fermentation period for antimicrobial activity was between days 3-11 for all four isolates. Production of pigments by the isolates was also affected by various physical factors: culture media, fermentation period and temperature; previous studies have shown that a variety of factors can affect bacterial pigmentation(Reasoner and Geldreich, 1985) (Reasoner and Geldreich, 1984; Goswami, 2010).

All in all, this study has confirmed using a combination of techniques; we were able to reliably identify with 16S rRNA sequencing, three isolates identified to the species level and one isolate to the genus level matching with 99% similarity were identified on the basis of 16S rRNA gene sequencing, enabling identification of the PM58B-RA, RA001 and RA004 isolates to the species level. MALDI-TOF MS confirmed the results of the 16S rRNA gene sequencing; however, MALDI-TOF MS also identified the RA003 isolate to the species level.

In conclusion, four isolates with antimicrobial activity isolated from the Helmcken Falls Cave were studied and identified, and the effect of different fermentation conditions on the antimicrobial activity of the isolates was investigated. In the future, a variety of temperatures such as 7°C, 12°C, 15°C, 25°C and 30°C should be tested in order to optimize the antimicrobial activity of each isolate, as 12°C and 25°C may not be close to the optimal temperatures for each isolate. Additionally, further research is required to purify and identify chemical structures of the antimicrobial secondary metabolites, and investigate the mechanisms underlying the effects of different

fermentation conditions on the production of antimicrobial secondary metabolites by these isolates. It will also be important to determine the mode of action study of these antimicrobial compounds and investigate their antimicrobial activities against different tested microorganisms using in vitro assays.

1.2 The Objectives of This Research

1- To isolate and screen cave bacteria to determine if they produce metabolites with antimicrobial activity against the pathogenic microorganisms *Micrococcus luteus*, MDR-MRSA, *Acinetobacter baumanni*, *Mycobacterium smegmatis*, *Candida albicans*, *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*.

2- To partially purify the secondary metabolites produced by the cave isolates to study their structure.

1.3 Literature Review

1.3.1 Antibiotics

Antimicrobial agents are "chemical substances that kill or inhibit the growth of microorganisms" (Cushnie and Lamb, 2005), and the term includes antibacterial, antifungal, antiparasitic and antiviral compounds (Kujumgiev et al., 1999). Professor Selman A. Waksman first defined antibiotics in the 1940s as "chemical substances of microbial origin that possess antibiotic capacity". In general, antibiotics are substances derived from plants, animals or microbes that can inhibit bacterial growth (Kannel et al., 1971; Strohl, 1997; Davies, 2006).

There are three classifications of antibacterial agents: natural, semi-synthetic and synthetic. Natural antibiotics are antibiotics originally derived from microorganisms, fungi, plants or animals (Topliss et al., 2002); microorganisms may develop resistance to natural antimicrobials more rapidly as they have been pre-exposed to these compounds in nature. Semi-synthetic drugs are natural compounds that have been chemically altered to reduce their toxicity and increase their effectiveness. Synthetic drugs may be advantageous as bacteria have not previously been exposed to these compounds (Topliss et al., 2002). However, on the other hand, natural antibiotics and semi-synthetic drugs are less effective than synthetic antibiotics (Topliss et al., 2002).

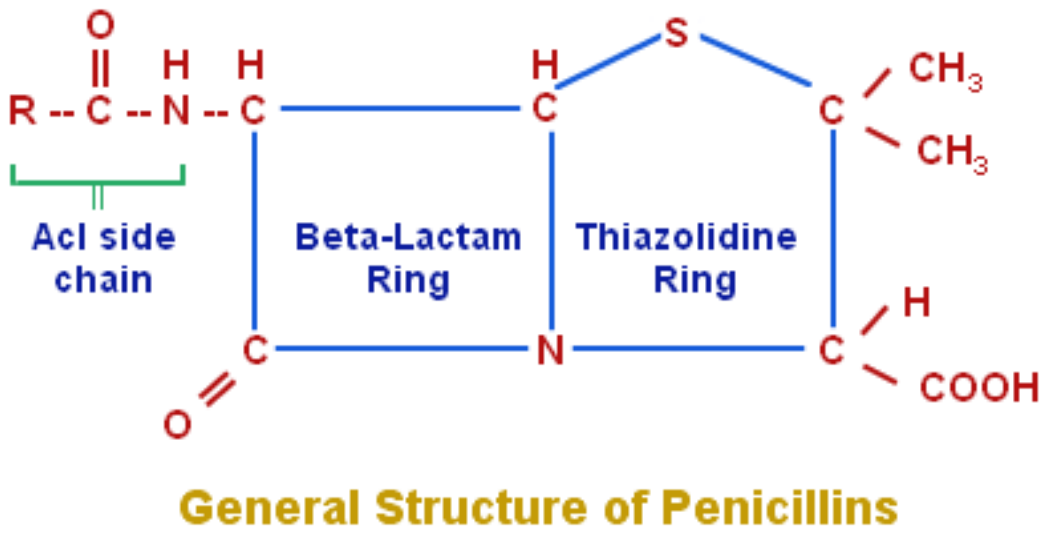


Figure 1: General structure of penicillin (Baldo, 1999)

Penicillin and tyrothricin were the first antibiotics used to kill gram-positive bacteria (Figure 1; Spector et al., 2012; Brozak, 2013). During the 1940s, Selman Waksman first isolated the antibiotic streptomycin, which is an inhibitor of several gram-negative and -positive bacteria and provided the first effective treatment for tuberculosis (Davies, 2010). Most modern antibiotics were discovered between 1945 and 1960, a period regarded as "The Golden Era of Antibiotic Discovery"; a wide range of antibiotics effective against a variety of pathogens including gram-positive and gram-negative bacteria were identified during this time (Spector et al., 2012).

1.3.2 Mechanism of Action of Antibiotics

Different antibiotics have varying mechanisms of action and target sites within bacterial cells, based on their structure. The major targets of common antibiotics are proteins related to DNA synthesis, protein synthesis, cell wall synthesis, nucleic acid synthesis and other metabolic processes (Bauer and Dicks, 2005). For example,

penicllins, cephalosporins and vancomycin inhibit bacterial cell wall synthesis, and these antibiotics do not affect human and animals cells as they do not have cell walls.

1.3.3 Antibiotic Resistance

1.3.3.1 The Appearance of Antibiotic Resistance

Antibiotic resistance is a significant threat to human health, as it counteracts the beneficial effects of the antibiotic compound targeting the pathogen (Cushnie and Lamb, 2005), often resulting in treatment failure, which can cause severe health consequences and may lead to death (Tenover, 2006). Resistance both pathogenic and non-pathogenic microorganisms can develop rapidly following the deployment of a new antibiotic (Figure 2).

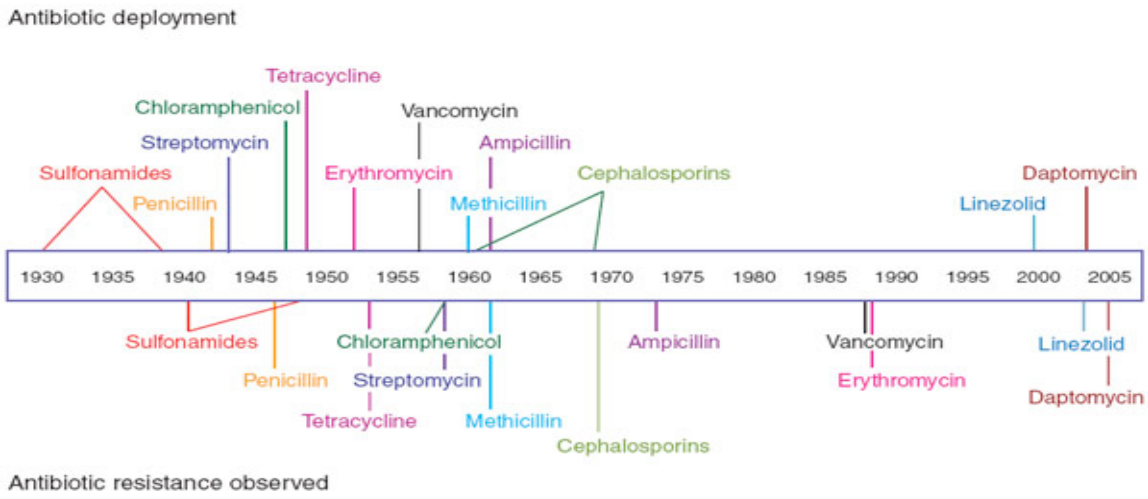


Figure 2: Timeline showing the discovery of antibiotics and the first appearance of resistance (Clatworthy et al., 2007).

More than 70 years ago, the antibiotic era started with sulfonamide (Figure 2),

and scientists identified bacterial resistance to sulfonamide in the 1940s (Fleming, 1945). Resistance to almost every available antibiotic has been observed in both Gram-negative and Gram-positive bacteria (Ash 2002; Rice, 2006; Baquero et al., 2008;). Today, the growing emergence of antibiotic-resistant bacterial strains makes antibiotics ineffective for the treatment of infectious diseases. The Infectious Disease Society of America reported that approximately 70% of hospital-acquired infections in the United States are resistant to one or more antibiotic (Chadwick et al., 1996; Davies, 2010).

The continual discovery of resistance against novel antimicrobial agents is not unexpected, as antibiotics and other related organic molecules are similar to other natural products. Nevertheless, bacterial resistance is a particular problem in healthcare settings, and the appearance of novel resistance genes in the clinic is alarming as it necessitates the controlled use of antibiotics (Levy, 2002).

1.3.3.2 Major Species of Antibiotic-Resistant Bacteria

The increasing prevalence of multidrug-resistant (MDR) strains, such as methicillin-resistant *Staphylococcus aureus* (MRSA), MDR *Acinetobacter baumannii*, and extended-spectrum beta-lactamase (ESBL)-producing *Escherichia coli*, *Mycobacterium tuberculosis*, *Klebsiella pneumoniae*, *Salmonella* spp. and *P. aeruginosa*, is a serious risk to human health. Such pathogens are resistant to most available antibiotics, reducing the number of curative options and making treatment of these infections more expensive (Conly, 2002; Dapkevicius, 2013).

Acinetobacter species are common nosocomial Gram-negative pathogens, which cause blood stream infections, secondary meningitis and urinary tract infections (Camp et al., 2010). Three *Acinetobacter* species cause disease; however, 80% of these infections are associated with *A. baumannii* (Camp et al., 2010). Approximately 12,000 healthcare-associated *Acinetobacter* infections occur in the United States every year (Camp et al., 2010). MDR *Acinetobacter* strains are frequently resistant to penicillin, ampicillin,

aminoglycosides, erythromycin, cephalosporins, chloramphenicol, norfloxacin, streptomycin and tetracycline (Camp et al., 2010).

MRSA is a Gram-positive bacterium and a major cause of nosocomial and society-acquired infections (Matsui et al., 2011). MRSA causes bloodstream infections, pneumonia and surgical-site infections, and is resistant to methicillin and other penicillin-class agents (Pray et al., 2008; Russo and Johnson, 2003). In 2004, the Centers for Disease Control reported that 63% of Staphylococcus infections were due to MRSA (Camp et al., 2010).

Even though the Gram-negative bacterium *E. coli* is a component of the normal human and animal intestinal flora (Tadesse et al., 2012), it is the second-most common cause of human infections after S. aureus (Russo & Johnson, 2003). *E. coli* causes intestinal, extra-intestinal and urinary tract infections; such infections kill around 7200 people in the United States each year (Russo & Johnson, 2003). Furthermore, *E. coli* causes diarrhea, which kills about 1 million people each year worldwide; most of these cases are children and immuno-compromised individuals (Russo & Johnson, 2003). Tadesse et al., (2012) studied the evolution of drug resistance in *E. coli* strains from the 1950s through to 2002 and found resistance to ampicillin, sulfonamide and tetracycline. Another recent study by Olson et al. (2009) reported that *E. coli* is resistant to a sulfonamide used to treat urinary tract infections.

1.3.3.3 Genetics of Antibiotic Resistance

Antibiotic resistance genes acquired via horizontal gene transfer between bacteria are responsible for the phenomenon of resistance (Levy, 2002; Davies, 2010). Antibiotic exposure provides a pressure that selects for bacteria possessing resistance genes. Resistance develops as a result of two factors: the antibiotic itself and the resistance gene. The antibiotic acts as a selective agent that helps to select for organisms possessing the resistance gene; both of these factors are required for resistance to develop.

Scientists have conducted functional studies of bacteria isolated from different environments to identify the sources of antibiotic-resistance genes (White, 2013; Bhullar, 2011). Bacterial DNA was collected from different environments such as soil, processed sewage and the human intestine, and antibiotic-resistance genes were found in each environment tested (White, 2013). In 2012, Forsberg and colleagues described natural environments as reservoirs of antibiotic-resistance genes that enable the genes to be transferred between soil bacteria and clinical pathogens. Additionally, seven resistance genes found in soil microbes were highly similar to the resistance genes found in clinical isolates of human pathogens.

Bhullar (2011) investigated microbes isolated from the Lechuguilla cave, New Mexico. The microorganisms inhabiting this 'isolated cave' are unlikely to have had contact with surface water or influences for 4-6 million years, and even today, human access to this cave is extremely limited. The authors reported that genetic diversity was a critical measure of resistance in the cave isolates. For example, some of the bacteria were highly resistant to different commercially available antibiotics, which the cave bacteria are extremely unlikely to have encountered in their natural environment. Together, these studies support the suggestion that antibiotic resistance is a naturally occurring process which is encoded for by ancient microbial genes (Hughes and Datta, 1983; Barlow and Hall, 2002; Bhullar, 2011).

Numerous recent studies have indicated that resistance genes arise due to the frequent use of antibiotics (Levy, 2002; Davies, 2010; Bhullar, 2011). However, other studies suggest that some resistance genes existed before humans used antibiotics to treat diseases (White 2013). Several studies of antibiotic-resistance genes have reported that decreasing the use of antibiotics will help alleviate the problem of resistance (White 2013). Furthermore, using an alternative approaches such as antimicrobial vaccines or bacteriophages may help to decrease the spread of drug resistance and provide a tool for combating highly resistant infections (Dantas et al., 2008).

1.3.3.4 Mechanisms Contributing to the Evolution of Antibiotic Resistance

Microorganisms use various mechanisms to protect themselves from other organisms. Bacteria can transfer the genes responsible for antibiotic resistance into other bacteria via plasmids. A single plasmid can carry genes conferring resistance to multiple antibiotics (Bennett, 2008). *A. baumannii* expresses a single enzyme, β-lactamase, that confers resistance to penicillins, cephalosporins and carbapenems (Levy, 2002). *A. baumannii* can also acquire other genes, such as acetyltransferases, phosphotransferases and nucleotidyltransferases, which confer resistance to fluoroquinolones and aminoglycosides (Levy, 2002). Extra-chromosomal elements such as plasmids can transfer genes between bacteria, and resistance genes can be transferred between Gram-positive and Gram-negative bacteria (Levy, 2002). Other mechanisms, such as bacteriophages and naked-DNA mechanisms, also contribute to the evolution of resistance by moving resistance genes between different bacterial species and populations; however, these mechanisms do not function in all types of bacteria (Levy, 2002).

1.3.3.5 The Impact of Human Activities on the Evolution of Drug Resistance

The increasing prevalence of antimicrobial resistance is the result of bacteria evolving over time in response to natural and societal pressures that constrain their ability to grow and disperse (Spellberg, 2012). The role played by human activities is a central issue in antibiotic resistance. Antibiotic resistance began to spread soon after humans started using antibiotics widely in medicine and agriculture. The misuse of drugs to promote human and animal health and increase food production has accelerated the evolution of antimicrobial resistance (Bhullar, 2011). In addition, the same antibiotics have been used to treat humans as well as animals and agricultural crops, which may have contributed to the high levels of resistance observed today (Bhullar, 2011).

The frequent release of antibiotics into bodies of water and wastewater is also believed to contribute to the spread of antibiotic-resistance genes (Baquero et al., 2008). For example, an increase in the use of avoparcin, a glycopeptide used as a growth promoter in poultry farms, was found to lead to the spread of vancomycin-resistant *Enterococci* in the intestinal flora of poultry animals. When the use avoparcinuse was reduced, glycopeptide resistance diminished in the animal flora (Bhullar, 2011). There are similar examples of human activity contributing to resistance to tylosin, virginiamycin and other antibiotics (Bhullar, 2011). It is important to educate people by arranging workshops and training, especially farmers and workers, about community safety to reduce antibiotic resistance in human and agricultural populations. Education may be the most effective means to reduce the transmission of antibiotic-resistant organisms within communities (Bhullar, 2011).

Additionally, antibiotic-resistant organisms can spread within healthcare communities. For example, MRSA has become a widespread problem in healthcare facilities and hospitals around the world. Antibiotic-resistant bacteria can spread via the transfer of patients between hospitals or more widely via medical tourism. In addition, 40, 25, and 10% of patients receiving antibiotic treatments carried two or more, three or more, and four or more drug resistant-bacteria, respectively, in 10% of their *E. coli* (Bhullar, 2011). Additionally, the discovery of resistance in commensal strains may indicate future problems with resistance to an agent in clinical pathogens in that hospital or community (Levy, 2002).

1.3.4 The Need for New Antibiotic Agents

Drug discovery must focus on finding novel structures, mechanism and targets to inhibit bacterial growth (Strohl, 1997; Brozak, 2013). There are a number of reasons driving scientists to discover and develop new antibiotics with novel structures and activities (Strohl, 1997). New infectious diseases that were previously unknown to

scientists have emerged. Additionally, some crucial antibiotics such as gentamicin and other aminoglycosides have high toxicities that constrain their effectiveness (Strohl, 1997). However, the increase in the spread of antibiotic-resistant bacteria, particularly MDR pathogens, has become one of the most serious problems in healthcare (Wright, 2007; Davies, 2010; Cheeptham, 2013; Strohl, 1997) and is the major reason driving the search for new antibiotics.

Between 1983 to 1987, 16 new antibiotics were approved for use in humans. In contrast, only two new antibiotics were approved between 2008 and 2012 (Spellberg, 2012). **F**luoroquinolones, a class of drugs discovered 40 years ago and used to treat Gram-negative bacilli infections, were the last new class of antibiotics to be discovered **(**Spellberg, 2012). Hence, due to the lack of novel antibiotic agents, the prevalence of antibiotic resistance among Gram-negative bacteria is increasing (Pallett and Hand, 2010; Spellberg, 2012).

Strohl (1997) stated that the continued development of new antibiotics over the next decade is required to enter a "new era of antibiotics"; otherwise, antibiotics may become increasingly ineffective against drug-resistant pathogens. Dr. Margaret Chan, the head of the World Health Organization, declared that the world faces a post-antibiotic era in which infectious diseases will be increasingly difficult to treat using antibiotics (Brozak, 2013). Dr. Keiji Fukuda (2013), the Assistant Director General of the World Health Organization, declared that the rapid emergence of antibiotic resistance is a global risk to human health. Therefore, researchers, companies and governments must help to develop new methods for drug discovery in order to provide novel, more effective antibiotics. Otherwise, we may become unable to treat a number of pathogenic microorganism infections. For example, novel antibiotics with new mode of action need to be developed to enable effective combination therapy against MRSA.

New sources of microorganisms must be found to increase the chances of discovering and developing new antibiotics. Natural resources are a major source of drugs. Over half of the drugs used today were discovered as natural products in plants,

animals, the oceans, caves or soil (Ji et al., 2009). It is important to search for new sources of drugs in extreme environments to broaden our chances of finding novel antimicrobial agents (Strohl, 1997).

For example, Mars could be a potential source of pharmacologically active agents (Gabriel and Northup, 2013). Microbiologists have hypothesized about the existence of cave-dwelling microorganisms on Mars and the potential of such organisms to be a new source of secondary metabolites with useful properties (Boston et al., 2001; Leveille and Datta, 2010). Mars is an extreme environment with a strong surface radiation, making life on the surface of Mars impossible. Some of the conditions on Mars are similar to those on Earth, such as warm temperatures and the presence of water (Baker et al., 1991; Beaty et al., 2005). Cushing et al. (2007) and Deak (2010) presented high-quality pictures of lava-tube caves beneath the surface of Mars, and Boston et al. (2006) reported that the Martian lava tubes might be much larger than those on Earth because of the lower gravity on Mars. One day, looking for microorganisms in the Martian lava-tube caves may lead to the discovery of new antibiotics.

1.3.5 The Cave Habitat as a Source of Novel Antibiotic-Producing Bacteria

Caves are unique in nature, unexploited, and poorly studied (Cheeptham, 2013; Gabriel and Northup, 2013). Caves are extreme environments in which highly specialized microorganisms grow, making them attractive places to look for new microorganisms that could produce novel bioactive compounds. Several novel organisms have been found in caves (Gabriel & Northup, 2013; see Table 1). Lee et al. (2000) identified two new species belonging to the genus *Saccharothrix*, *Saccharothrixviolacea* sp. nov. and *Saccharothrixalbidocapillata* comb. nov., in soil samples collected from a gold mine in Korea**.** Lee (2006) also studied the biodiversity of cave bacteria in a natural cave in Korea and discovered a novel *actinomycete*, *Nocardiajejuensis* sp. Cheng et al. (2013) isolated the novel species *Microlunatuscavernae* sp. nov. from an ancient cave in China. Margesin et al. (2004) discovered a new species belonging to the genus *Arthrobacter*,

Arthrobacter psychrophenolicus sp. nov., in an alpine ice cave. These microorganisms were distributed among a wide variety of caves (Table 1).

Table 1: Examples of microorganisms found in various caves (adapted from Cheeptham, 2013)

Cave location	**Microorganisms found**
Altamira and Tito Bustillo, northern Spain	*Streptomyces* sp. (predominant), *Nocardia* sp., *Rhodococcus, Nocardioides* and *Amycolatopsis*.
Cesspool caves, Virginia, USA	Genus *Thiothrix*, the flxibacter- Cytophaga-Bacteriodes phylum and possibly *Helicobacter* or the *Thiovulum* group for some remaining strains
Basalticsea caves, Kauai Hawaii, USA	Bacteria and cyanobacteria
Various Japanese caves (20 limestone and volcanic caves)	Members of *Trichosporon* species, *Candida palmioleophila*, *Clusitaniae*, *Debaryomyceshansenii* and *Hanseniaspora* spp.
Basaltic Lava caves in Azores (Portugal), Hawaii and NewMexico (USA)	*Bacteroidetes*, *Chloroflexi*, *Nitrospirae* and *Verrucomicrobia*
Buracos cave (Lava tube), Terceira island (Azores), Portugal	Mainly *Proteobacteria* and *Actinobacteria; Gallionella* sp. and *Leptothrix* sp. were observed by SEM but not using other techniques

Actinomycetes are one of the most common bacteria that produce secondary metabolites. For example, the genus *Streptomyces*, which was isolated from a volcanic cave, has a high yield of secondary metabolites (Kay et al., 2013; Cheeptham, 2013). Therefore, is therefore necessary to discover more about the microorganisms and

environmental conditions inside caves.

1.3.5.1 Effect of the Cave Habitat on Microorganisms and Antibiotic Production

The environmental conditions inside caves include various factors that could influence microbial communities (Cheeptham, 2013). The factors influencing cave conditions include light, temperature, energy and nutrient availability. Caves are harsh environments, deficient in light and nutrients (Dapkevicius, 2013). Previous studies of volcanic environments revealed that the lack of sunlight in caves forced some common bacteria to adapt (Northup et al., 2011; Cheeptham, 2013).

The ability of different microbes to grow in caves suggests that caves may be ideal environments that provide diverse habitats for microbes. However, it is not known how organisms obtain energy in caves, where energy sources are often rare. Heterotrophic bacteria have a variety of mechanisms to obtain organic carbon (Barton and Jurado, 2007). For example, running streams and rivers may bring organic carbon in the form of plant material into caves from the surface. In addition, airflow can carry various carbon sources into caves. Animals and humans can also bring organic carbon into caves (Gabriel and Northup, 2013); such visitors may induce the production of antimicrobial agents in caves (Montano and Henderson, 2013).

Chemolithoautotrophs are bacteria that obtain energy by oxidizing inorganic compounds such as ammonia, iron, nitrogen, hydrogen and hydrogen sulfide (Gabriel and Northup, 2013). Chemolithoautotrophs inhabit caves, such as the arid-land caves in New Mexico, where microbes obtain energy from reduced gases that pass through springs (Gabriel and Northup, 2013). While energy is essential for the production of bacterial metabolites, inorganic compounds such as nitrogen, iron, sulfur and carbon dioxide also play an important role in the production of bacterial metabolites (Northup at el., 2011).

Temperature, humidity, and light are also important factors determining bacterial diversity in cave systems. Temperature and humidity vary more inside lava caves than

outside the cave (Cropley, 1965). Northup et al. (2011) studied the microbial communities in three lava-tube caves that differed significantly in temperature (14-19°C in Hawaii, 15-16°C in the Azores and -2 to 9°C in New Mexico) and humidity. Despite the diversity of conditions between these caves, there were similarities in the bacterial species compositions at the phylum level, as indicated by a combination of SEM and molecular methods (DNA extraction, PCR, 16S rRNA sequencing and phylogenetic analysis; Northup et al., 2011).

Gabriel and Northup (2013) reported that the low temperatures in some lava caves could cause microorganisms to produce secondary metabolites related to cold resistance, although they did not specifically examine the influence of temperature on the production of secondary metabolites. Cheeptham et al. (2013) investigated antimicrobial activity in actinomycetes isolated from a cold volcanic cave (7.5-12.2°C) in British Columbia, Canada, and found that the cave bacteria had the ability to produce secondary metabolites that possessed anti-microbial activity against pathogens. Their results also suggested that the same species of cave bacteria can be found in caves with different conditions and in different locations (Cheeptham et al., 2013).

Surface microbes have developed mechanisms to defend themselves against the damaging effects of UV radiation (Walter, 1983; Yasui and McCready, 1998). Cave bacteria, which exist mostly in the dark, have adapted to low-light conditions. Gabriel and Northup (2013) hypothesized that there is correlation between such adaption and the depth of the cave: bacteria in deeper caves are more adapted to low-light conditions (Gabriel and Northup, 2013). In contrast, Snider et al. (2009) hypothesized that the lack of light does not affect the adaptation of microbes living in caves (Snider et al., 2009). In addition, Cheeptham and Rule (2013) studied the effects of UV light on the antimicrobial activities of cave-dwelling actinomycetes, and found that UV light influenced the antimicrobial activity of the cave actinomycetes, suggesting that UV light exposure could potentially be used as a new screening strategy for antibiotic discovery (Cheeptham and Rule, 2013).

1.3.6 Techniques Used to Analyze and Identify Cave Bacteria

Various techniques have been used to identify cave isolates. It is important to determine if the isolates have been previously identified and to evaluate their production of new or previously identified antibiotic compounds. In previous studies, various soil isolates were phenotypically and genotypically characterized (Fakruddin and Mannan, 2013).

A number of studies have suggested that microbial diversity and species composition differ significantly between limestone caves and lava tubes (Cheeptham, 2013). An enormous number of techniques have been used to analyze the microorganisms which inhabit these two types of caves, including molecular methods and cultivation-independent methods. Limited data exists regarding mineral-microorganism interactions in caves, and community composition analyses have primarily been based on culture techniques (Aman et al., 1995; Hugenholtz et al., 1998).

Groth et al. (1999) and Laiz et al. (2003) found that a wide diversity of microorganisms inhabit Karstic caves using culture methods. However, these studies used different media and aerobic Petri films for isolation, while the first group also applied morphological, and physiological, techniques for identification.

Molecular methods are now considered standard analyses for identifying organisms (Fakruddin and Mannan, 2013; Sacchi, 2002). For example, 16S rRNA gene sequences have been widely used to determine bacterial phylogenies and identify unknown isolates at the genus and species levels (Sacchi, 2002). Recently, Northup et al. (2011) identified various phyla of bacteria (Chloroflexi, Nitrospirae, Verrucomicrobia, etc.; see Table 1) in three diverse lava-tube caves using a combination of scanning electron microscopy (SEM) and molecular techniques (DNA extraction, PCR, 16S rRNA sequencing and phylogenetic analysis). Despite some problems and drawbacks such as high cost and technical considerations, 16S rRNA gene sequencing has several

advantages for microbial identification, as it is accurate, objective and comprehensive, and the 16S rDNA gene is present in all bacteria and does not contain extensive mutations. Analysis of the nucleotide sequences of bacterial 16S rRNA genes allows the determination of phylogenetic relationships that could not be obtained by phenotypic methods (Clarridge, 2004; Song et al., 2005). However, none of these approaches are 100% accurate for the identification of bacteria (Clarridge, 2004).

In general, methods for studying bacterial metabolism vary. Bacterial metabolism can be studied through different processes, including isolation, purification, optimization of conditions, screening for antimicrobial activity, and identification of unknown organisms (Ripa et al., 2009; Bhullar, 2011; Rule, 2013; Ababutain et al., 2013; Lueschow et al., 2013). We studied cave bacteria using traditional methods and a combination of other techniques including culture-based techniques for isolation, liquid enrichment for optimization, and morphological, chemotaxonomic, molecular (16S rRNA sequencing) and matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) techniques for identification (see Chapter 2 for further details of these techniques) (Kay et al., 2013; Cheeptham, 2013).

Recently, MALDI-TOF has used to identify unknown bacteria at the species level by characterizing the components of cellular proteins (Sacchi, 2002). Peptide spectra produced by MALDI-TOF were used to identify Arcanobacterium and Trueperella isolates collected from pigs and cows (Hijazin et al., 2012). MALDI-TOF has several clear advantages: it is fast, accurate and a large database of bacterial reference spectra is available (Sacchi, 2002). However, MALDI-TOF has some disadvantages. One important requirement for MALDI-TOF is that the bacterial cultures have to be pure. The resolving power of MALDI-TOF is lower than 16s r RNA analysis, and the technique is based on a chemical testing method which is not always 100% accurate for the identification of bacterial species.

It is important to use a combination of complementary techniques to study microbial communities in different environments (Fakruddin and Mannan, 2013). The

advantage of a combined strategy is that it increases our knowledge and ability to study bacteria that are difficult to culture (Fakruddin and Mannan, 2013), and such an approach may help to avoid rediscovering antibiotics and increase the chances of discovering novel organisms and/or antibiotics. By using a combination of techniques, Gurtner et al. (2000) reduced the instances of finding common organisms, in confirmation of the importance of using a combination of methods to study bacterial communities (Cheeptham, 2013). Using molecular techniques in combination with MALDI-TOF may help confirm bacterial identities. It is very important to confirm bacterial taxonomies using different techniques and strategies to determine if the target bacteria have been previously identified.

1.3.7 Approaches for Screening Bacteria for the Production of Novel Antimicrobial Agents

With the widespread increase in antibiotic resistance, there is an urgent need for rapid methods to determine antibiotic resistance and metabolite production in novel microorganisms. Scientists and laboratories in the United States, Japan, Britain and Russia have developed screening procedures to identify new compounds based on the ability of microorganisms to produce secondary metabolites that can inhibit the growth of microbes (Porter, 1997). Such screening methods are considered a traditional approach for antibiotic discovery and are still in widespread use today (Guo et al., 2013).

Agar dilution and disc diffusion are a primary method of screening for selected antimicrobial agents (Holasva et al., 2004). An inhibition zone around a disc on an agar plate indicates a positive result (see Chapter 2 for further details of these techniques). Valgaerts et al., (2007) reported the advantages of the disc-diffusion method, including its simplicity, low cost, absence of the need for specialist equipment and high flexibility, including the ability to test newly-available drugs. The effectiveness of the disc-diffusion method for identifying antimicrobial activities was supported by the results of Holasva et al. (2004), who used the disc-diffusion method as the primary method of screening the

antimicrobial activity of 16 cave isolates, and found that the method was an easy way of observing the inhibition zone produced by cave bacteria against microorganisms and against each other. The disc-diffusion method can also be used to qualitatively assess drug susceptibility and resistance (Valgaerts et al., 2007). These characteristics make the disc-diffusion method a good technique for preliminary screening of the antimicrobial activity of environmental isolates (Reller et al., 2009). However, Valgaerts et al. (2007) reported that the disc-diffusion method has several disadvantages, including long assay times and high cost, although the method does produce clear inhibition zones.

1.3.8 Study of Culture Conditions for Cave Bacteria

Many studies have attempted to optimize antimicrobial production by microorganisms (Bajpai and Reuss, 1981), and the effects of media components on different traits such as cell growth, cellulose production, and the production of metabolites and antimicrobial compounds by cave isolates have been studied (Judaibi, 2011; Dayal et al., 2013). Optimizing the media and fermentation conditions will increase the production of antimicrobial compounds by bacteria. The production of antimicrobial compounds can be influenced by the nature and concentration of the carbon, nitrogen and phosphorus sources (Yegneswaran et al., 1988). In general, the nutrient sources, which include carbon, nitrogen, ammonia, phosphate can inhibit or enhance antibiotic production by microorganism in culture media (Omura et al., 1986).

In general, optimizing the culture media is a challenging process by which researchers try to create an ideal environment for microbial communities. In particular, bacteria isolated from cave habitats are adapted to environments that contain limited nutrients and organic compounds, and therefore optimization can be challenging. Development of an optimized medium is important for growing cave-adapted microorganisms, which generally grow very slowly (Pankratov et al., 2008).

1.3.8.1 Carbon sources

Gram-negative and Gram-positive bacteria need carbon for energy and structural components (Sánchez et al., 2010); however, the carbon source used in bacterial fermentation is very important for the production of antimicrobial compounds. Different carbon sources such as glucose have different effects on the production of antimicrobial compounds by bacteria (Sánchez et al., 2010). For example, using glucose as a carbon source had a positive effect on phenazine production by *Pseudomonas chloraphis*, whereas fructose, sucrose and ribose had a negative effect (Rij et al., 2004), and *E. coli* exhibited strong antimicrobial activity when grown on medium containing fructose and yeast extract (Vijavakumar et al., 2012).

Gram-positive bacteria have the ability to produce drugs through ribosomal and non-ribosomal mechanisms (Sánchez et al., 2010). Gram-positive cell walls have multiple layers of peptidoglycan, protein and teichoic acid. Nevertheless, Gram-positive bacteria respond differently to growth on glucose (Sánchez et al., 2010). In particular, the antimicrobial activity of *Bacillus* sp. significantly decreased in the absence of a carbon source in the fermentation media (Vijavakumaret al., 2012). In another example, anthracycline production by *Streptomyces peucetius* was inhibited by high concentrations of glucose (Escalante et al., 1999).

1.3.8.2 Nitrogen sources

The nitrogen source is also an important factor in antimicrobial production. Vijavakumar et al. (2012) studied the effects of nitrogen sources on antibacterial production by *Bacillus* sp., and concluded that growth and the production of antibacterial compounds were maximized when nitrogen sources such as yeast extract, calcium nitrate, ammonium sulfate and potassium nitrate were included in the fermentation media separately. Ripa et al. (2009) concluded that yeast extract was the optimal nitrogen source for the production of bioactive metabolites in *Streptomyces* sp. RUPA-08PR, which was isolated from soil in Bangladesh.

1.3.8.3 Temperature, pH and incubation period

There have been many studies of the effects of temperature and pH on the production of antimicrobial compounds. In particular, Laiz et al. (2003) studied microorganisms isolated from different caves with varying temperatures in Spain (5, 13, 20, 28, and 40°C), and reported that most bacteria could grown in the range 13-40°C; however, lower temperatures (13°C) were optimal for the growth of a higher diversity of species. Khizhnyak et al. (2011) isolated bacteria from water in a cave where the air temperature varied between 0 and 5°C, and found that the bacteria could grow at a low temperature (7°C), but the bacteria altered their morphology and consequently died at the higher temperature (35°C), indicating that the cave bacteria were adapted to the cold, which is also referred to as psychrophilic.

Ripa et al. (2009) incubated a strain of actinomycetes isolated from the soil in northern Bangladesh at different temperatures (25-49°C), and found that the optimum temperature and pH for a new *Streptomyces* species, RUPA-08PR, were 39°C and 8, respectively, indicating that the new organism was mesophilic. In addition, the bacteria began producing bioactive metabolites after 7 days of incubation in fermentation broth, and optimal production was observed after 10 days and slowly decreased thereafter. Usha et al. (2011) found an initial pH of 7 and 30°C to be the optimal conditions for bioactive metabolite production by the rare actinomycete strain *Pseudonocardia* VUK-10.

Rule and Cheeptham (2013) reported that 100 actinomycete strains, which were collected from a volcanic cave, showed the greatest metabolite production when their fermentation broth was incubated at 25°C for 10 days. Kay et al. (2013) incubated actinomycetes collected from a volcanic cave for 10 days, and found that strain E9 in the genus *Streptomyces* produced a high yield of secondary metabolites at pH 7 on day 4 of incubation at 28°C.

Cladera et al., (2004) studied bacteriocin production by *Bacillus licheniformis* P40 when cultured under aerobic conditions. During 50 h of cultivation, the organism showed the highest level of bacteriocin production after 15 h. Pattnaik et al. (2001) studied bacteriocin production by *B. licheniformis* 26 L-10/3RA cultured under anaerobic and aerobic conditions, and observed aerobic growth within 18 h and gradually increasing antimicrobial production after 1 day. However, it is not necessary that bacteria achieve optimal growth in order to maximize the production of antibacterial compounds (Leal et al., 2002) as, for example *B. licheniformis* 26 L-10/3RA lost its antimicrobial activity when exposed to an aerobic environment.

1.3.8.4 Salts and minerals

The salt concentration also affects cell growth and the production of bioactive metabolites. Ripa et al. (2009) studied the optimum culture conditions for a new species of *Streptomyces*, RUPA-08PR, and showed that 1% NaCl maximized the production of bioactive metabolites, but the growth of the strain decreased when the NaCl concentration was greater than 1%. Vijayakumar et al. (2012) reported that the marine *Streptomyces* sp. VPTS3-1 produced maximum levels of secondary metabolites at a NaCl concentration of 4%, which is a suitable concentration for the growth of the organism. However, higher NaCl concentrations did not improve secondary metabolite production. Ripa et al. (2009) reported that K_2HPO_4 enhanced the production of bioactive metabolites by the actinomycete *Pseudonocardia* VUK-10 and the *Streptomyces* species RUPA-08PR; however, 5% K_2HPO_4 was optimal.

In conclusion, optimization of the sources and concentration of carbon, nitrogen, salts and minerals, as well as the temperature, pH and incubation period are very important during the discovery and isolation of novel antibiotics (Himabindu and Jetty, 2006).

2.0 Materials and Methods

2.1 Isolation of microorganism from cave

2.1.1 Collecting samples

A total of 15 samples were previously collected from different sites of a volcanic cave at Wells Gray Provincial Park in British Columbia (BC), Canada (Figure 3). The samples collected included rocks, sterile swabs of percolating material, sediment, cave popcorn. Aseptic techniques were used to collect the samples and the samples were placed in sterile falcon tubes and zip-lock bags. All samples were kept cold in a refrigerator at 5^{o}C until the isolation process was complete. (This work was done by Paul Moote and Devon Rule)

2.1.2 Isolation of cave microorganisms

One gram of each of the cave samples was diluted in sterile saline solution and plated onto different selective media using the glass spreading technique (Moote, 2010). These media consist of Actinomycetes Agar, Bennett's Agar, Hickey Tresner (HT), Starch Casein Nitrate Agar, and Modified Soil Agar (Appendix A-1). After incubating all of the sample plates at 25^{o}C for four weeks, microorganism colonies appeared. The colonies were sub-cultured and then purified on R_2A media using the streak plate method (Katz 2010).

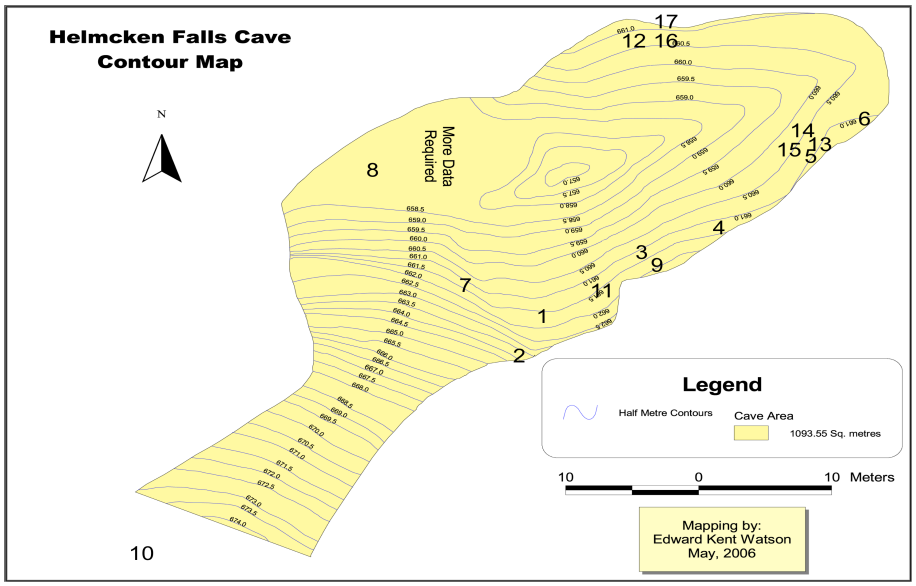


Figure 3: Cave contour map of the Helmcken Falls Cave in Wells Gray Provincial Park in British Columbia, Canada. Seventeen cave samples were collected from different sites in the cave. 1, 2 and 10 were collected near the cave's entrance; 3, 4 and 9 were collected from its south wall; 5 and 6 were collected from its east corner; 7 was collected from its centre; 8 was collected from its northwestern side; 12 and 17 were from close to its northern wall; and 13, 14 and 15 were collected from its east wall (Moote 2009 and Rule 2010).

2.2 Optimizing the fermentation conditions of the cave strains

2.2.1 Primary optimization of the cave strains

The fermentation conditions of the cave strains were optimized to maximize their metabolite production. To do this, 16 cave isolates (from initial studies of Dr. Cheeptham's former students, Paul Moote and Devon Rule) were inoculated into small test tubes (150 x 25 mm) containing 15 ml each of four different broths: Hickey-Tresner, ISP2 (International Streptomycetes Project), V-8 juice, and R_2A (Appendix B). Also, extra tubes (150 x 25 mm) containing these broths were diluted tenfold. The aim of performing the dilutions was to create media similar to that of the parental environment, which contains a very low nutrient level, so as to increase results. All inoculated broths were incubated at 25^{o}C with 250 rpm reciprocal shaking for 14 days. The isolates were then tested for antimicrobial production using the bioassay method described below.

2.2.2 Growing tested microorganisms for the antimicrobial assay

The tested microorganisms used in this project include: *Mycobacterium smegmatis* JVC 1213 (MC^2155), Extended spectrum beta-lactamase (ESBL) *E.coli* JVC1195 strain 1841, *Micrococcus luteus*, multi-drug resistant methicillin-resistant *Staphylococcus aureus* (MDR-MRSA), *Acinetobacter baumanni* strain 14394, *Candida albicans*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*. Each tested microorganisms was inoculated onto the appropriate medium (Table 2) and was incubated in a 35°C shaker until growth appeared. The resulting tested microorganisms suspensions were then diluted to reach an absorbance of 0.132 at 600 nm (OD_{600}) (Bollela *et al*.,1999). Afterwards, 1% (2.50 mL) of each tested microorganisms at the appropriate log phase OD_{600} was transferred to three beakers, 250 mL beakers containing nutrient, LB and HT molten agar (Appendix A). The microorganisms agar mixtures were then poured onto bioassay plates to test the antimicrobial activity of the cave bacteria against these microorganisms.

Table 2: The list of tested microorganisms, the media they were grown in, their Gram reactions and the positive controls used in this project.

Tested Microorganisms	Gram Reaction	Media	Antibiotic (Positive Control)
Mycobacterium smegmatis 1213 (MC^2155)	+	NB	SXT (Sulfamethoxozole-23.75 μg JVC trimethoprim- 1.25 μg)
ESBL *E. coli* 1841	_	LB	C (Chloramphenicol 30 μg)
Micrococcus luteus	+	NB	E (Erythromycin 15 μg)
MDR-MRSA ATCC 43300	+	NB	VA-30 (Vancomycin VA-30 μg)

Acinetobacter baumannii ATCC19606 (JVC 119)	_	LB	C (Chloramphenicol 30 μg)
Candida albicans	Mold	H.T	N/A – antifungal (Nystatin- 20 μg)
Klebsiella pneumoniae ATCC BAA 1705	_	LB	E (Erythromycin 15 μg)
Pseudomonas aeruginosa	_	MH	E (Erythromycin 15 μg)

Hickey Tresner (HT), Nutrient Broth (NB), Mueller Hinton (MH), Luria Bertani (LB)

2.2.3 Primary screening of the cave bacteria isolates for antimicrobial production

To determine whether any of the cave isolates produced antimicrobial compounds in pure culture, we searched for the presence of any diffusible materials that inhibited the growth of the tested microorganisms in the bioassay plates. The procedure for testing 16 microbial isolates for antimicrobial activity is the disc diffusion method (Pieboji 2009). This method uses sterile, 8 mm discs that are impregnated with bacterial fermentation broth and are placed on a glass plate in a fume hood to air dry (Figure 2). The positive control used for each bacterium is listed in Table 2, and the negative controls were discs soaked in the sterile broth medium appropriate for each bacterium.

Figure 4: The discs used to test the antimicrobial activity of the cave bacteria with the disc diffusion assay. The sterile discs were dipped in bacteria fermentation broth and were left to dry on a glass plate in the fume hood.

Every 3 to 5 days, the bacteria isolates were screened against the tested microorganisms. The isolates that showed antimicrobial activities against the tested microorganisms were then further optimized to encourage these isolates to maximize their production.

2.3 Further of the secondary metabolite production of four cave bacteria isolates

Four cave bacteria isolates, PM58B-RA, RA001, RA003, and RA004, which showed the highest antimicrobial activity against the tested microorganisms listed above, were studied further to determine whether the antibiotics they produced against the tested microorganisms in this study were new. To do this, at least two to four well-isolated colonies of the four isolates were selected from R_2A agar plate culture. Then, the top of each colony was touched with a loop, and the growth was transferred into R_2A broth. In the end, the culture broth was incubated at different temperatures to see if these bacteria can grow at low temperature (12˚C) that is similar to actual environment or at normal temperature (25˚C), with reciprocal shaking at 250 rpm, for 14 days. To determine the best growth temperature, pH, media and fermentation period of the four isolates in producing secondary metabolites, the high and low temperatures of 25 $^{\circ}$C and 12 $^{\circ}$C were used to grow them.

2.3.1 Study of the fermentation periods of the cave bacteria

The fermentation periods of the cave bacteria strains were optimized using a method described by Nanjwade and Shamarez (2010). The purpose of this procedure was to find the harvesting time at which the bacteria produced the highest yield of the antimicrobial agent (Fermentation time course). To do this, the primary seed cultures of the four isolates were incubated at 25˚C until growth occurred. Then, the seed cultures were used to inoculate 166 ml into 500 ml flasks and 333 ml into 1000 ml Erlenmeyer flasks containing R_2A broth (Tabaraie et al., 2012). After that, the sterilized R_2A broth was inoculated with 1% (1.66 ml) and 2% (6.66 ml) of the PM58B, RA001, RA003, and RA004 isolates, separately. Different inoculum volume (1% and 2%) was used to decide the most suitable volume for producing antimicrobial compound(s). Then, the flasks were incubated at 12˚C and 25˚C for 14 days. The production was determined daily by testing the packed cell volume (%PCV), the pH value, and the antimicrobial activities of the inoculated broths.

Note that the antimicrobial production by cave microorganisms is normally an aerobic procedure. In addition, the broth cultures were centrifuged at 3000 rpm for 5 minutes and then the supernatant was filtered by syringe (Kumari et al., 2013). The resulting filtrates were used for further antimicrobial activity.

2.3.2 Testing the percentage of packed cell volume (%PCV) and the pH value of the inoculated broths

The expected growth times of the bacteria strains were determined daily during the fermentation process by using a %PVC cell counting kit. Three milliliters of broth of each of the four isolates was transferred into 15 ml centrifuge tubes, which were centrifuged at 250 rpm for 1 minute to measure the cell pellet volume after centrifugation. After that, the antimicrobial activities of the isolates were tested against those of the tested microorganisms mentioned below. In addition, the pH value of the fermentation broth of each isolate was recorded daily.

Another method, employed by Stettler *et al*., 2006 and Jordan, 2005, was used to determine the PCV measurements when the first method gave negative results first of all, 1 ml of each isolate broth was transferred into a PCV tube (13 mm x 43 mm) (Techno Plastics Products AG, Trasadingen, Switzerland) whose maximum capillary volume is 5 µl. Then, the tubes were centrifuged using a microcentrifuge for 1 minute at 5000 rpm. After centrifugation, the isolate cells were pelleted within the capillary. The height of the cell pellet defines the volume of the cells in the capillary. This volume can be converted into the PCV (%) using the formula described by Jordan (2005): PCV (%) = (volume of cell pellet/volume of sample) x 100 (1000 µl was the sample volume for each tube).

2.4 The antimicrobial activity detection assay

2.4.1 The cross-streak assay

In this study, the agar diffusion method (or the cross-streak assay) was used to determine how cave isolates communicate through their metabolites. This method was used by Northup *et al*. (2013) when they tested the antimicrobial activity of cave isolates against that of such tested microorganisms as *Staphylococcus aureus* (ATCC 6538), *Kelbsiella pneumoniae* (ATCC 13883), *Shigella flexneri* (ATCC 9199) and *Streptococcus pneumoniae* (ATCC 6303). In this study, they inoculated the cave isolates onto a nitrate broth and incubated them at 15-20˚C for 48 hours until visible growth appeared. After that, the cave cultures were inoculated using the cross-streak assay as described below.

In our study, to carry out the agar diffusion assay, the cave isolates RA001, RA003, RA004 and PM58B were inoculated onto a R_2A broth and were incubated at 25˚C until growth was observed. Then, the cave isolates were streaked in one vertical line onto HT agar and R_2A agar plates and incubated at 25˚C for 48 hours in order to obtain a thin line of growth. Subsequently, another layer of R_2A and HT medium was added over the first layer of the medium on the original R_2A and HT plates, and the plates were solidified. After that, each cave isolate was horizontally inoculated onto its vertical streak

and the plates were incubated at 25˚C for 48 hours. Then, any zones of inhibition were evaluated.

2.4.2 Paper disc diffusion method

The first use of the paper disc diffusion method was in the 1940s (Vincent 1944). This method can be easily used in the laboratory to detect the antimicrobial activity of bacteria culture and its low cost. In addition, it is suitable for use if there is no requirement to determine the concentration or type of the present antibiotic (Driscoll 2012). For these reasons, this method was used instead of the cross-streaking assay method that was previously described to detect the communication of the four isolates through their metabolites. Sterile, 8 mm discs were impregnated with 80 µl of broth from the four bacterial isolates that exhibited the most antimicrobial activity against the tested microorganisms. The discs were placed on a glass plate in a fume hood to air dry. Then, 1% (2.50 mL) of the isolates was transferred into 250 ml flasks containing the R_2A agar. The isolate-agar mixture was poured onto bioassay plates to test the four isolates' antimicrobial activities against one another. When the plates were solidified, the paper discs of the strains that were not combined with the R_2A Agar were placed onto the bioassay plates. For example, if the R_2A agar was inoculated with the RA003 isolate, the papers discs were soaked with RA001, RA004, PM58B-RA and Streptomyces griseus, which was used as a positive control. After that, the plates were incubated at 25˚C and were checked daily for one week for results.

2.5 Identification of antimicrobial production by the cave isolates

Cave isolate identification is essential to determine if these isolates are novel species, have new type of antimicrobial compounds and new microbial interaction. Different traditional methods were used to identify the four isolates, including phenotypic identification using Gram staining, culturing and the biochemical method. However,

these methods were insufficient to yield accurate results. Thus, a 16S rRNA sequencing method and software for the four isolates identifications was used to attain the definitive isolates identification.

2.5.1 Morphological observations

Both the microscopic and macroscopic characteristics of isolates RA001, RA003, RA004, and PM58B-RA were observed and used to identify the isolates. Macroscopic morphologies such as the shapes and colours of the colonies and the diffusible pigmentation of the isolates were recorded. In addition to the observation of morphological traits, chemotaxonomy was also used to enhance the identification of the strains.

2.5.2 Chemotaxonomy

In addition to the observation of morphological traits, chemotaxonomy was also used to enhance the identification of the cave bacteria strains used in this project. This method is simple in principle, and separates the amino acids in the cell walls of the four isolates; it was employed to investigate whether these substances, which belong to the four isolates, are similar to those of Streptomyces griseus, which was used as a positive control (see 3.0 Results Figures 17, 18, 19 and 20).

2.5.2.1 Thin Layer chromatography of whole cell hydrolysates

To better identify the bacteria isolates used in this project, the hydrolysates in the cell walls of the four antimicrobial-producing actinobacterial strains were analyzed for amino acid content using thin layer chromatography and cell wall hydrolysis (Staneck, 1974).

First, the four isolates were grown on R_2A media at 25˚C with reciprocal shaking at 250 rpm. Then, the cells were collected at the maximal growth stage by centrifuging them at 2000 rpm for 10 minutes, washing them with 10 mL of saline water, and centrifuging them again at 2000 rpm. One ml of distilled water was then added to the collected cells and the cells were hydrolyzed and sonicated. Hydrolysis occurred when 1 ml of 6M of HCl was added to 5 mL of the cells' pellets and then placed in a 161x300 glass 15 mL test tube, which was placed in an autoclave at 121˚C for 15 minutes. After cooling, the tube was opened and its contents were centrifuged. Then, the liquid hydrolysate was evaporated in a 50˚C water bath to remove most of the HCl.

Subsequently, ascending chromatography was performed on the pellets of the cells for approximately 18 to 22 hours using the solvent system of methanol: distilled water: 6M HCL: pyridine (80:26:4:10, v/v). Then, the chromatogram was air-dried and its amino acid spots were visualized by spraying the chromatogram with 0.2% ninhydrin in acetone, leaving it to dry for 3 hours, and then placing it in the chromatography oven at 100˚C for three minutes to visualize the spots. The cells were also sonicated, after washing them with 10 mL of saline water for about one minute to remove cell components, and were subsequently centrifuged at about 10,000 rpm for 10 to 20 minutes. Streptomyces griseus was used as a positive control in the TLC. Its cells, except the amino acid standard, were also hydrolyzed and sonicated. The migration of the standard DAP spot and the other amino acids was observed in the TLC after three minutes of heating the TLC.

2.5.3 Molecular identification of the antimicrobial-producing cave strains using 16S rRNA gene sequencing

To prepare the four antimicrobial-producing strains, RA001, RA003, RA004 and PM58B-RA, for 16S rRNA sequencing, the strains were cultured in R_2A agar medium for three days at 25 ˚C. When the cave bacteria appeared on the plates, the plates were sent to Seoul, Korea for 16S rRNA gene sequencing. There, polymerase chain reaction (PCR)

was used to clarify the taxonomic status of these isolates, as described herein. At Macrogen, Korea, template DNA of each of the isolates was prepared and colonies of each isolate were collected with a sterilized toothpick and suspended in 0.5 mL of sterile saline in a 1.5 mL centrifuge tube. The cultures were then centrifuged at 10,000 rpm for 10 minutes. After removal of the supernatant, the pellet was suspended in 0.5 mL of Insta Gene Matrix (BIO-Rad, USA), incubated at 56 ˚C for 30 minutes, and then heated to 100˚C for a further 10 minutes.

After heating, the supernatant was used for PCR. For the PCR process, 1 µl of each template DNA sample was added to 20 µl of the PCR reaction solution. 518F/ 800R primers were used to amplify the 1400 bp bacterial 16S rRNA gene (Table 3) and the amplification cycle was performed 35 times at 94 ˚C, for 2 minutes. at 94 ˚C, for 45 s, at 55 ˚C, for 60s 72 ˚C for 60 s, and at 72 ˚C for 5 minutes. Then, the PCR products of each isolate were removed from the unincorporated PCR primers and dNTPs by using the Montage PCR Clean up Kit (Millipore).

For sequencing, 518F/ 800R primers were used as seen in Table 5. Sequencing was performed by using Big Dye terminator cycle sequencing products, which were resolved on an Applied Biosystem model 3730XL automated DNA sequencing system (Applied Biosystem, USA) at Macrogen, Inc., in Seoul, Korea. The resulting 16S rRNA sequences were compared with the GenBank databases by using BLAST.

Table 3: Primers used in amplification and 16S rRNA sequencing of the four cave strains

Primers	Sequences	Amplification	Sequencing
518F	CCA gCAgCCgCggTAATA Cg		Used
800R	TAC CAgggT ATC TAA TCC		Used

2.4.3.1 Sequence and phylogenetic analysis

The NCBI BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to identify the strains. The DNA sequences generated in this study (1,000 to 1,400 bp long) were submitted to BLAST and RDB to identify their closest relatives based on their 16S rDNA sequences. The nucleotide sequences of the four isolates obtained from this study have been deposited in the GenBank database under the Accession Numbers Table 4.

Table 4: The Accession Numbers of PM58B-RA, RA001, RA003, and RA004

Sequence ID	Organism ID	Isolate	GeneBank accession number
Seq1	*Bacillus licheniformis*	PM58B-RA	KF991616
Seq2	*Sphingomonadaceae*	RA003	KF991617
Seq3	*Arthrobacter agilis*	RA001	KF991618
Seq4	*Arthrobacter agilis*	RA004	KF991619

2.5.4 Using Matrix-assisted laser desorption/ionization (MALDI-TOF) to identify the four antimicrobial-producing isolates

The aim of using the Matrix-assisted laser desorption/ionization (MALDI-TOF) was to get an accurate isolates identification and confirm it with the other identification results.

2.4.4.1 Growing bacterial isolates

To confirm the identity of the isolates using MALDI-TOF mass spectrometry, the isolates were first freshly grown. RA001, RA003, RA004, and PM58B-RA and *Streptomces griseus* were grown in R_2A broth and were incubated at 25˚C with 250 rpm

shaking until growth appeared, as described previously. The isolates were also cultured on R_2A agar plates at 25˚C for 4 days. *Streptomces griseus* was used as a positive control in both cases. The purpose of using different growth conditions is to find out which growth conditions show accurate results.

2.5.4.2 Sample preparation for formic acid extraction

Sample preparation of two different growth conditions of the four strains for formic acid extraction was performed for direct identification of bacteria as described previously by Bruker and Daltonik (2013). First, strains colonies present in the R_2A broth were collected by centrifugation. To do this, 1 mL of each bacteria isolate was transferred into separate Eppendorf tubes using a sterile pipette tip. Then, the tubes were centrifuged for 3 minutes at 13,000 rpm. Next, the supernatant was removed, and 300 µL of deionized water and 900µL of EtOH were added to the remaining liquid. After centrifugation at 14,000 rpm for 2 minutes, the supernatant was decanted and centrifuged again. Then, the residual EtOH was removed by carefully pipetting it off without disturbing the pellet. Following this, the pellet was allowed to dry at room temperature for three minutes. Twenty-five µL of 70% formic acid was added to the pellet and was mixed well by vortexing. After that, 25 µL of ACN was added, and the tube was mixed carefully and centrifuged for 2 minutes at 14,000 rpm. Then, 1 µL of the supernatant was transferred onto a MALDI target plate (Bruker Daltonics, Breman Germany), the plate was air-dried and was overlaid with 1 µL of matrix solution. After air-drying, the target plate was placed in the MALDI-TOF mass spectrometer for analysis.

The samples of the four isolate colonies grown on solid R_2A media were then prepared, one single colony (5-10 mg) of each bacteria isolate was transferred into separate Eppendorf tubes using a sterile pipette tip. Then, 300 microliters (µL) of deionized water was added into each tube and then was mixed well by vortexing. After that the tubes were centrifuged for 3 minutes at 13,000 rpm. Next, 900 µL €uof EtOH were added to the remaining liquid. After centrifugation at 14,000 rpm for 2 minutes the supernatant was decanted and centrifuged again. Then, the residual EtOH was removed by carefully pipetting it off without disturbing the pellet.

Following this, the pellet was allowed to dry at room temperature for three minutes. Twenty-five μL of 70% formic acid was added to the pellet and was mixed well by vortexing. After that, 25 μL of ACN was added, and the tube was mixed carefully and centrifuged for 2 minutes at 14,000 rpm. Then, 1 μL of the supernatant was transferred onto a MALDI target plate (Bruker Daltonics, Breman Germany), the plate was air-dried and was overlaid with 1 μL of matrix solution. After air- drying, the target plate was placed inside the MALDI-TOF mass spectrometer for analysis

2.5.4.3 MALDI spotting protocol

The protocols described below for the preparation of bacterial samples prior to MALDI analysis were provided by Bruker Daltonics (Bremen, Germany).

2.5.4.4 Direct transfer method

The matrix, alpha-cyano-4-hydroxycinnamic acid (HCCA), was prepared by dissolving 2.5 mg in 250 μL standard solvent that was composed of 50% acetonitrile (ACN), 47.5% H_2O, and 2.5% trifluoroacetic acid (TFA). The dissolved matrix was vortexed at room temperature for 1 minute and sonicated for 5 minutes to ensure complete dissolution. A single colony (5–10 mg) of bacteria was directly spotted as a thin film onto a stainless steel MALDI target plate (Bruker Daltonics). The smeared bacterial sample was then overlaid with 1 μL of 70% (v/v) formic acid and allowed to dry at room temperature before pipetting 1 μL of HCCA solution on top of it. The sample was dried at room temperature and analyzed immediately on the MALDI instrument.

Formic acid extraction method

A single colony (5-10 mg) was placed into an Eppendorf tube containing 300 μL of deionized water. The sample was vortexed for 2 minutes before adding 900 μL of ethanol. The sample was mixed again and centrifuged at 13,000 rpm for 2 minutes. The resulting pellet was recovered by decanting most of the ethanol, and then dried at room temperature for 3 minutes. Depending on the size of the pellet, 50-80 μL of 70% formic acid was added to dissolve the pellet. The same volume of acetonitrile was added and the

sample was centrifuged at maximum speed until all insoluble material was collected in a single pellet on the bottom of the vial. The resulting supernatant (1 μL) was spotted on the target plate and allowed to dry at room temperature before spotting 1 μL of the HCCA solution.

2.5.4.5 Data acquisition and analysis

Mass spectra were obtained using a bench-top Microflex MALDI-TOF MS from Bruker Daltonics® (Bremen, Germany) equipped with a pulsed nitrogen laser at 355 nm wavelength. Spectra were recorded from 2 kDa to 20 kDa (positive mode) using FlexControl 3.3 software (ion source 1: 20 kV; ion source 2: 18.25 kV; lens voltage: 7 kV; laser frequency: 60 Hz; pulsed ion extraction (PIE) delay: 10 ns). A mass gate of 500 Da was set for all experiments. A bacteria test standard was used to calibrate the instrument. Individual mass spectrum from each spot was acquired by averaging 240 laser shots. The data acquisition was set to automate and the "random walk" movement was activated at 10 shots per raster during the sequence. Peak lists, intensities, and peak areas were calculated using the peak-picking centroid algorithm in FlexAnalysis 3.3 software. Mass spectra were exported to Biotyper 3.1 for evaluation by comparing the bacterial protein fingerprints to an existing database. The same Bruker bacterial test standard which is composed of Escherichia coli DH5 alpha peptide was used as a reference to maintain the validity of the results.

2.6 Extraction and Purification of the Secondary Metabolite

The aim of this study is to partially identify the secondary metabolites present in the isolates that showed activity against tested microorganisms. In collaboration, Dr. Arjun Banskota of NRC in Halifax has greatly contributed to this section.

The isolates, RA001, RA003, RA004, and PM58B-RA were grown in 2 L of R2A broth, separately, at both temperature 12 °C and 25 °C as previously mentioned. Next, the time course of fermentation in each isolate was determined and the day that showed the highest result of the antimicrobial activity of the isolates was chosen for harvesting time. For instance, on day 10th for RA001, on day 5th for RA003 and PM58B-RA, and on day 9th for RA004, and then these culture broths were centrifuged at 4000 rpm for 10 minutes. After discarding the supernatant, the pellets in each of the centrifuge tube were resuspended in the rest of the isolates culture (20 mL). All pellets were sent to National Research Council at Halifax, NS for further purification of secondary metabolites.

2.6.1 Extraction and Purification of Secondary Metabolites

RA004: The culture broth (2.0 L) was evaporated under reduced pressure to 250 mL and extracted with EtOAC (250 mL x 2). The combined EtOAc fraction further dried under reduced pressure yielding 31.0 mg EtOAc extract. The EtOAc extract was dissolved in MeOH filtered through a 13 mm nylon membrane filter with 0.20 μm pore size (VWR, USA) and subjected for HPLC purification. Semi-preparative HPLC was performed on an Agilent 1200 series HPLC using an Nova Pack C18 column (5μm, 7.8 × 300 mm) with a H_2O/Acetonitrile linear gradient (1:9 to 0:1 between 0 to 20 minutes). Nine fractions were collected (Fraction 1 to Fraction 9) eluted at 2.0, 2.3, 3.0, 3.8, 4.9, 5.8, 7.4, 7.8 and 9.7 minutes, respectively. The flow chart 1 is shown in Appendix B. The proton NMR spectra of all the above fractions were recorded on Bruker 700 MHz spectrometer, indicating none of the fraction has pure secondary metabolites. Thus, LC/MS and NMR used for purification of all isolates RA001, RA003, RA004, and PM58B-RA instead of HPLC purification.

PM58B: The mycelia received from TRU were lyophilized yielding 460 mg dry mycelia, which were extracted with MeOH (25.0 mL x 2). The combined MeOH extract were dried under reduced pressure yielding MeOH extract (50.0 mg). The MeOH extract was further fractionated by solid phase extraction using Discovery DIC-18 cartridge eluting with H_2O/MeOH/EtOAc gradient into four fractions [fraction 1 (27.9 mg, H_2O 10 mL), fraction 2 (13.0 mg, H_2O/MeOH, 1:1, 10 mL), fraction 3 (2.3 mg, MeOH, 10 mL) and fraction 4 (2.0 mg, EtOAC, 10 mL)]. Further LC/MS and NMR study revealed that

these fractions contain multiple components and subjected for further anti-bacterial activity test. The flow chart 2 is shown in Appendix B.

RA003: The mycelia received from TRU were lyophilized yielding 470 mg dry mycelia, which were extracted with MeOH (25.0 mL x 2). The combined MeOH extract were dried under reduced pressure yielding MeOH extract (20.0 mg). The MeOH extract was further fractionated by solid phase extraction using Discovery DIC-18 cartridge eluting with H_2O/MeOH/EtOAc gradient into three fractions [fraction 1 (10.4 mg, H_2O 10 mL), fraction 2 (5.3 mg, H_2O/MeOH, 1:1, 10 mL), fraction 3 and (3.9 mg, MeOH, 10 mL). Further LC/MS and NMR study revealed that these fractions contain multiple components and subjected for further anti-bacterial activity test. The flow chart 3 is shown in Appendix B.

RA004: The mycelia received from TRU were lyophilized yielding 450 mg dry mycelia, which were extracted with MeOH (25.0 mL x 2). The combined MeOH extract were dried under reduced pressure yielding MeOH extract (90.0 mg). The MeOH extract was further fractionated by solid phase extraction using Discovery DIC-18 cartridge eluting with H_2O/MeOH/EtOAc gradient into four fractions [fraction 1 (71.3 mg, H_2O 10 mL), fraction 2 (4.3 mg, H_2O/MeOH, 1:1, 10 mL), fraction 3 (8.8 mg, MeOH, 10 mL) and fraction 4 (3.2 mg, EtOAc 10 ml). Further LC/MS and NMR study revealed that these fractions contain multiple components and subjected for further anti-bacterial activity test. The flow chart 4 is shown in Appendix B.

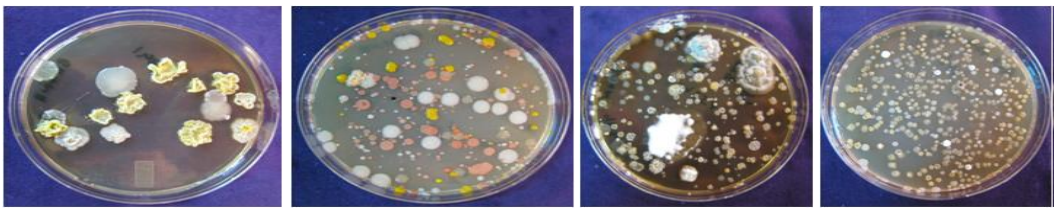
RA001: The mycelia received from TRU were lyophilized yielding 260 mg dry mycelia, which were extracted with MeOH (25.0 mL x 2). The combined MeOH extract were dried under reduced pressure yielding MeOH extract (120 mg). The MeOH extract was further fractionated by solid phase extraction using Discovery DIC-18 cartridge eluting with H_2O/MeOH/EtOAc gradient into three fractions [fraction 1 (90.4 mg, H_2O 10 mL), fraction 2 (4.1 mg, H_2O/MeOH, 1:1, 10 mL), and fraction 3 (16.5 mg, MeOH, 10 mL). Further LC/MS and NMR study revealed that these fractions contain multiple components and subjected to further anti-bacterial activity test. The flow chart 5 is shown in Appendix B.

Next, RA003 isolate grew again in the TRU lab for isolation. Pellets sent to National Research Council at Halifax, NS for further isolation of secondary metabolites. The mycelia of RA003 received from TRU were lyophilized yielding 580 mg dry mycelia, which were extracted with MeOH (25.0 mL x 2). The combined MeOH extract were dried under reduced pressure yielding MeOH extract (160 mg).

3.0 Results

3.1 Sample collection and isolation of cave microorganisms

In total, 16 cave samples were obtained from different sites within the Helmcken Falls Cave. The samples collected included rocks, and sterile swabs of percolating material, soil and cave popcorn. After incubation of the plates at 25^{o}C for 4 weeks, a variety of colonies were observed (Figure 5). Of the four culture media tested, Actinomycete agar, SCNA agar and Bennett's agar were found to be optimal in terms of supporting the growth of the all different kind of bacteria. Out of a total of 317 cave microorganisms, 16 isolates selected based on their morphological features under the light microscope by using a Gram stain, the viability, consistent activity and some unique features in their growth. Thus, these isolates were selected for further antimicrobial activity assays.



Hickey Tresner Bennett's agar SCNA agar Actinomycete agar

Figure 5: Isolation of cave microorganisms by culture on different selective media (Moote 2010).

3.2 Primary screening of the antimicrobial activity of the sixteen bacterial isolates

The antimicrobial activity of the sixteen bacterial isolates was examined using the disc-diffusion (Kirby-Bauer) method against *Mycobacterium smegmatis, Micrococcus luteus*, MDR-MRSA, *Acinetobacter baumannii*, ESBL *E. coli* and *Candida albicans*. The inhibition zones were compared with the inhibition zones of the positive control (see Table 2 in Chapter 2).

The effect of different fermentation broths on the antimicrobial activity of the 16 cave isolates were examined. Seven of the 16 isolates (NC18, PM184, PM58B-RA, RA003, RA001 and RA004; Table 5) demonstrated various antimicrobial activities against the tested microorganisms when cultured in Hickey Tresner (HT), R_2A and ISP#2 broth.

In particular, PM58B-RA, RA003, RA004, NC18 and 245 demonstrated high antimicrobial activities when cultured in HT broth, while PM58B-RA, RA001, RA003, A1A3 and 46A demonstrated high antimicrobial activities in R_2A broth, and PM184 and RA003 demonstrated high antimicrobial activities with ISP#2; none of the strains demonstrated any antimicrobial activity with V8-juice. These results indicate that the media can affect the production of antimicrobial secondary metabolites by the isolates.

In contrast, when cultured in broth that had been diluted ten times with the aim of increasing the production of antimicrobial secondary metabolites, the 16 cave isolates failed to demonstrate any antimicrobial activity.

The isolates PM58B-RA, RA001, RA003 and RA004 were selected for further study as they exhibited high antimicrobial activities against various tested microorganisms in the primary screening test and consistency in antimicrobial agent production (Table 5).

Table 5: The zones of inhibition (mm) observed in the 16 bacterial isolates against the various tested microorganisms: *Mycobacterium smegmatis, Micrococcus luteus*, MDR-MRSA, *Acinetobacter baumannii*, ESBL *E. coli* and *Candida albicans* when cultured in Hickey Tresner, R_2A, ISP#2 and V-8 juice broth.

Isolate	**Gram Positive**			**Gram Negative**		**Yeast**
	M. luteus	*M. smegmatis*	MDR-*MRSA*	*A. baumannii*	*ESBL E. coli*	*C. albicans*
NC18	0	13.00	0	0	0	0
PM184	0	10.00	0	0	0	0
245	8.99	0	0	0	0	0
PM58B-RA	**18.00**	**16.00**	0	0	0	**15.00**
PMA1A3	0	0	0	0	0	0
RA003	15.62-11.00	0	**13.45**	0	0	0
A1A3 (3)	8.50	0	0	0	0	0
RA001	9.00	0	0	0	0	0
NC18(p-2)	0	0	0	0	0	0
RA002	0	0	0	0	0	0
RA004	0	0	0	**11.40**	0	0
126	0	0	0	0	0	0
46A	0	9.14	0	0	0	0
A1A2(2)	0	0	0	0	0	0
E9	0	0	0	0	0	0
SK119	0	0	0	0	0	0
Positive control	20.44	17.9	19.87	25.3	27.35	24

Notes: Sterile 8 mm paper discs were used to test the antimicrobial activity of each strain. The four highlighted isolates exhibited the highest degree of antimicrobial activity; the highlighted values indicate the best media for that isolate, as follows: HT, R_2A and ISP#2.

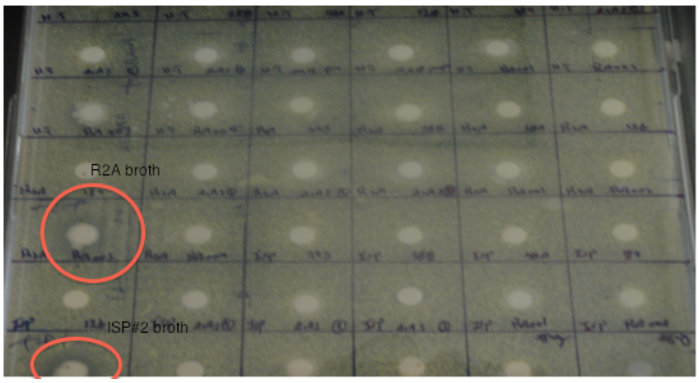


Figure 6: Primary screening of the 16 cave strains against *M. luteus*. The RA003 isolate showed antimicrobial activity against *M. luteus* when cultured in R_2A and ISP#2 broth. The diameters of the zones were measured using sliding calipers.

3.3 Further screening of the antimicrobial activity of the four selected isolates

3.3.1 Effect of fermentation broth on antimicrobial activity

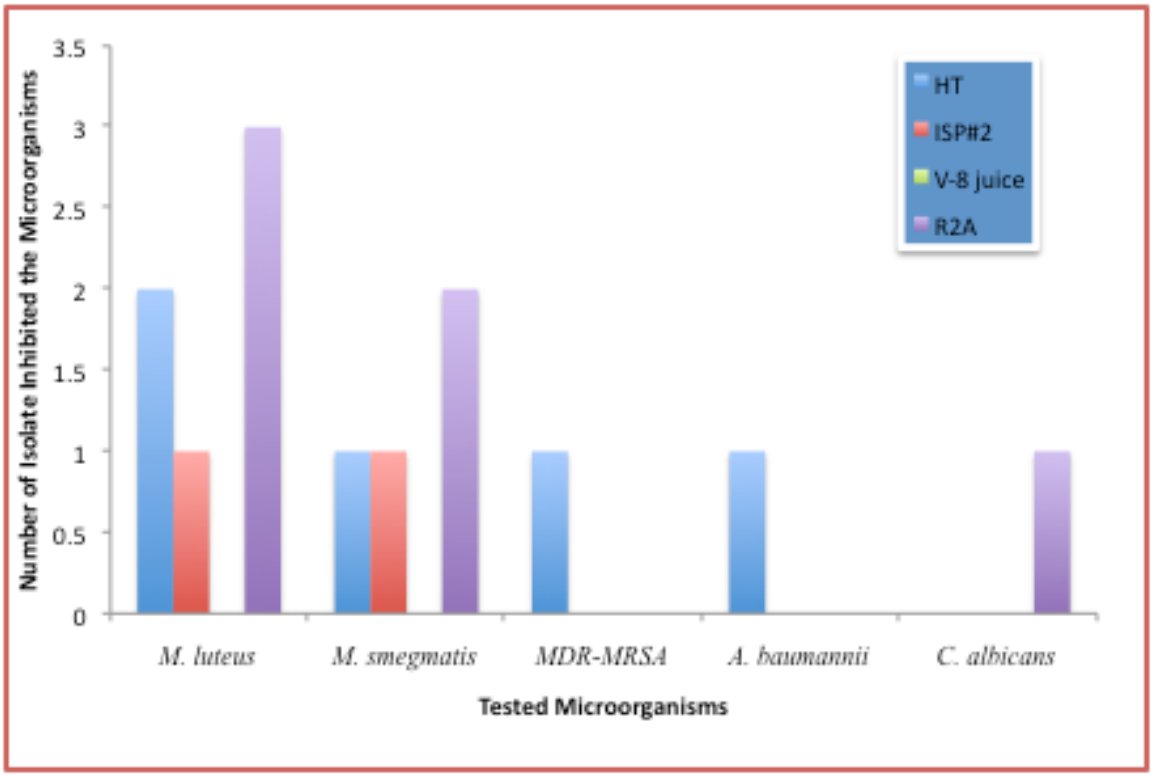
The effect of different fermentation broths on the antimicrobial activity of the isolates is presented in Figure 7. The four selected cave isolates (PM-58BRA, RA001, RA004, RA003) displayed various degrees of antimicrobial activity when cultured in each of the four fermentation broths, as shown in Table 6 and Figure 7. In particular, Hickey Tresner (HT) broth and R_2A broth were best fermentation media, as these broths led to the highest antimicrobial activity for most of the cave bacterial isolates; 46% of the cave isolates showed antimicrobial activity when grown in R_2A broth, 39% showed antimicrobial activity when grown in HT broth, 15% showed antimicrobial activity when grown in ISP#2 broth, while none of the cave bacterial isolates demonstrated

antimicrobial activity against the various tested microorganisms when grown in V-8 juice (Table 6 and Figure 7). R_2A broth was identified as the optimal fermentation media for the cave isolates rather than HT broth, as the zones of inhibition for the PM58B-RA, RA001, RA004 and RA003 isolates were larger when grown in R_2A broth than HT broth. In addition, these strains produced pigmentation in R_2A medium but did not produce any pigmentation when cultured in the other media. These results indicated that of the four fermentation media examined, R_2A broth may enhance the production of secondary metabolites with antimicrobial activity by the isolates.

Table 6: Evaluation of the impact of growth medium on the antimicrobial activity of the sixteen isolates towards Gram-positive and Gram-negative bacteria and yeast.

Media	***M. luteus***	***M. smegmatis***	**MDR-MRSA**	***A. baumannii***	***C. albicans***
HT	18.00-8.99	13.00	13.45	11.04	0
ISP#2	10.00-11.00	0	0	0	0
R2A	15.62-9.00-8.50	16.00-9.14	0	0	15.00
V8-juice	0	0	0	0	0

HT = Hickey Tresner, ISP#2 = International *Streptomyces* Project #2 (Yeast-Malt Extract). The highlighted values indicate the various degree of inhibition zones produced by strains. HT, R_2A and ISP#2 are the best media that enhance the production of secondary metabolites with antimicrobial activity by the isolates.



3.3.2 Effect of inoculation volume on antimicrobial activity

As shown in Figures 8, 9, 10 and 11, the use of different seed inoculum volumes (1% and 2%) had apparent effects on the growth and inhibition zones of the PM-58B-RA, RA001, RA004 and RA003 isolates. Different amounts (1% and 2%) of seed inoculate were tested to determine the appropriate harvesting day for maximal antimicrobial activity.

The antimicrobial activity of the PM58B-RA, RA001, RA003 and RA004 isolates was higher when inoculated at 2% than 1%. For example, when PM58B-RA was inoculated at 2%, this isolate started to display antimicrobial activity against *M. luteus* on day 4 of culture; however, the same isolate did not exert any antimicrobial activity against *M. luteus* when inoculated at 1% (Figure 8). When inoculated at 2%, the RA003 isolate started to show antimicrobial activity against MDR-MRSA on day 5 of culture; however,

this isolate also displayed antimicrobial activity against MDR-MRSA on day 6 when inoculated at 1% (Figure 11). In general, and as shown in Figures 8, 9, 10 and 11, all four of the isolates showed faster production of antimicrobial activity and more rapid growth when inoculated at 2% than 1%. However, the PM58B-RA isolate started to display antimicrobial activity on day 8 when inoculated at both 1% and 2% (Figure 8).

When the seed inoculum volume was 2%, the maximum inhibition zones were 15.00 mm for PM-58BRA against *M. luteus*, 9.92 mm for RA001 against *M. luteus*, 12.35 mm for RA004 against *A. baumannii* and 19.17 mm for RA003 against *M. luteus*. When the seed inoculum volume was 1%, the maximum inhibition zones were 8.87 mm for PM-58BRA against *M. smegmatis* and 13.83 mm for RA003 against MDR-MRSA; RA001 and RA004 did not show any antimicrobial activity at this temperature.

3.3.3 Effect of the length of fermentation on antimicrobial activity

Determination of the optimal fermentation day is also significant for the maximal production of antimicrobial compounds. Antibiotic production can take place on specific days during the stationary phase. Therefore, the production of antimicrobial secondary metabolites may not increase as the number of days of culture increases. In fact, increasing the number of days of culture may lead to the production of toxins which inhibit the production of antimicrobial secondary metabolites.

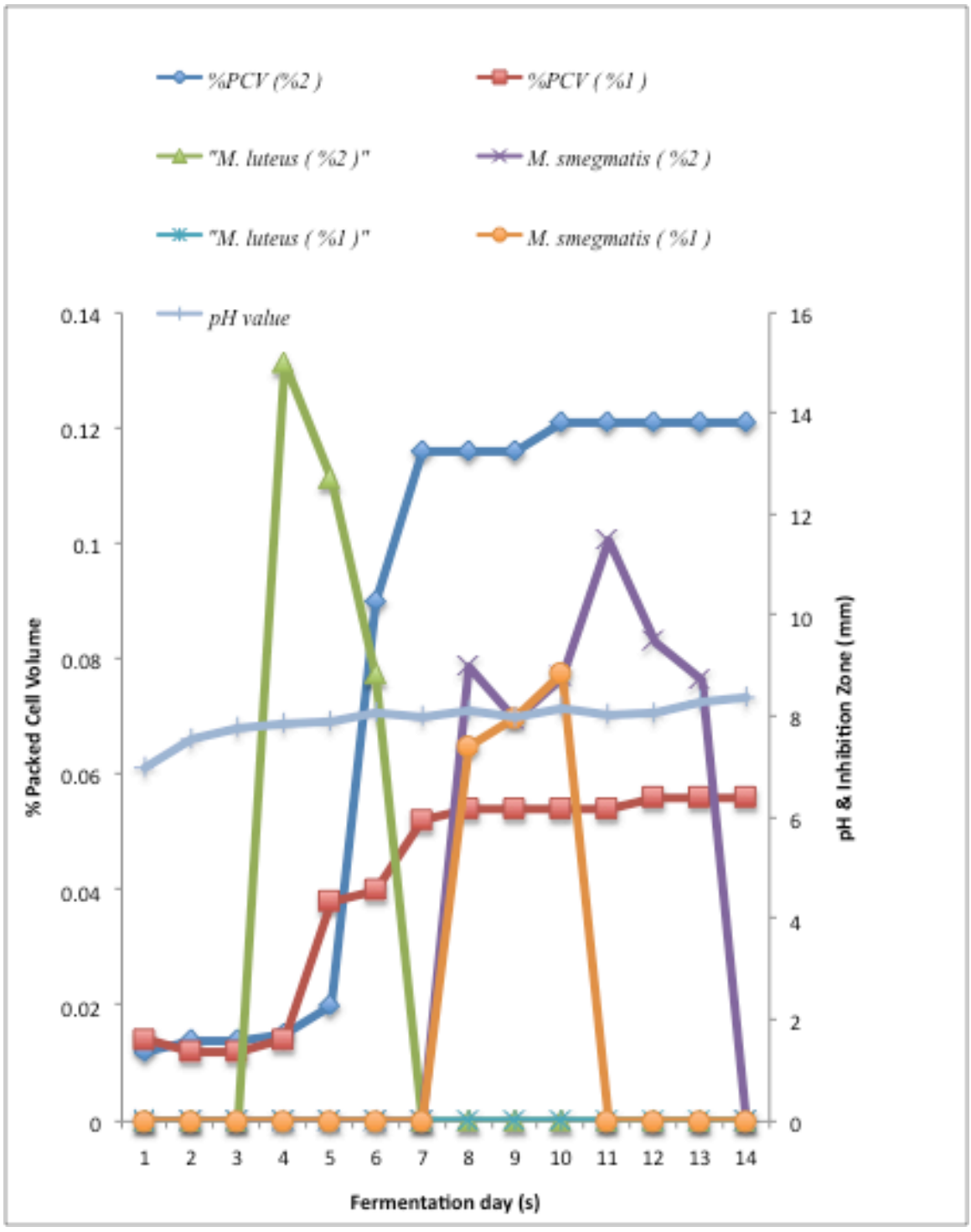
Figures 8, 9, 10 and 11 illustrate the effects of the number of days of fermentation on the antimicrobial activity of the isolates. The optimal days were day 4 for PM-58BRA, RA001 and RA004 and day 7 for RA003. The maximum inhibition zone of the PM-58BRA isolate against *M. luteus* and *M. smegmatis* (15.00 mm and 11.5 mm) occurred on days 4 and 11, respectively (Figure 8). The maximum inhibition zones of the RA001 isolate (9.92 mm) against *M. luteus* occurred on day 4 (Figure 9). The maximum inhibition zones of the RA004 isolate (12.85 mm) against *A. baumannii* occurred on day 9 (Figure 10). The maximum inhibition zones of the RA003 isolate (19.17 mm and 17.19) against *M. luteus* and MDR-MRSA occurred on day 7 (Figure 11). After these

times, the production of antimicrobial secondary metabolites by the PM58B-RA, RA001, RA004 and RA003 isolates decreased. However, there were obvious differences in antimicrobial production by the PM58B-RA, RA001, RA004 and RA003 isolates between 4 and 11 days of culture, as the inhibition zones of these isolates changed over time. For example, the inhibition zone of the PM58B-RA isolate reached 9.00 mm on day 8, decreased to 7.93 mm on day 9 and then increased to 8.87 mm on day 10; similar patterns were also observed for the RA001, RA004 and RA003 isolates.

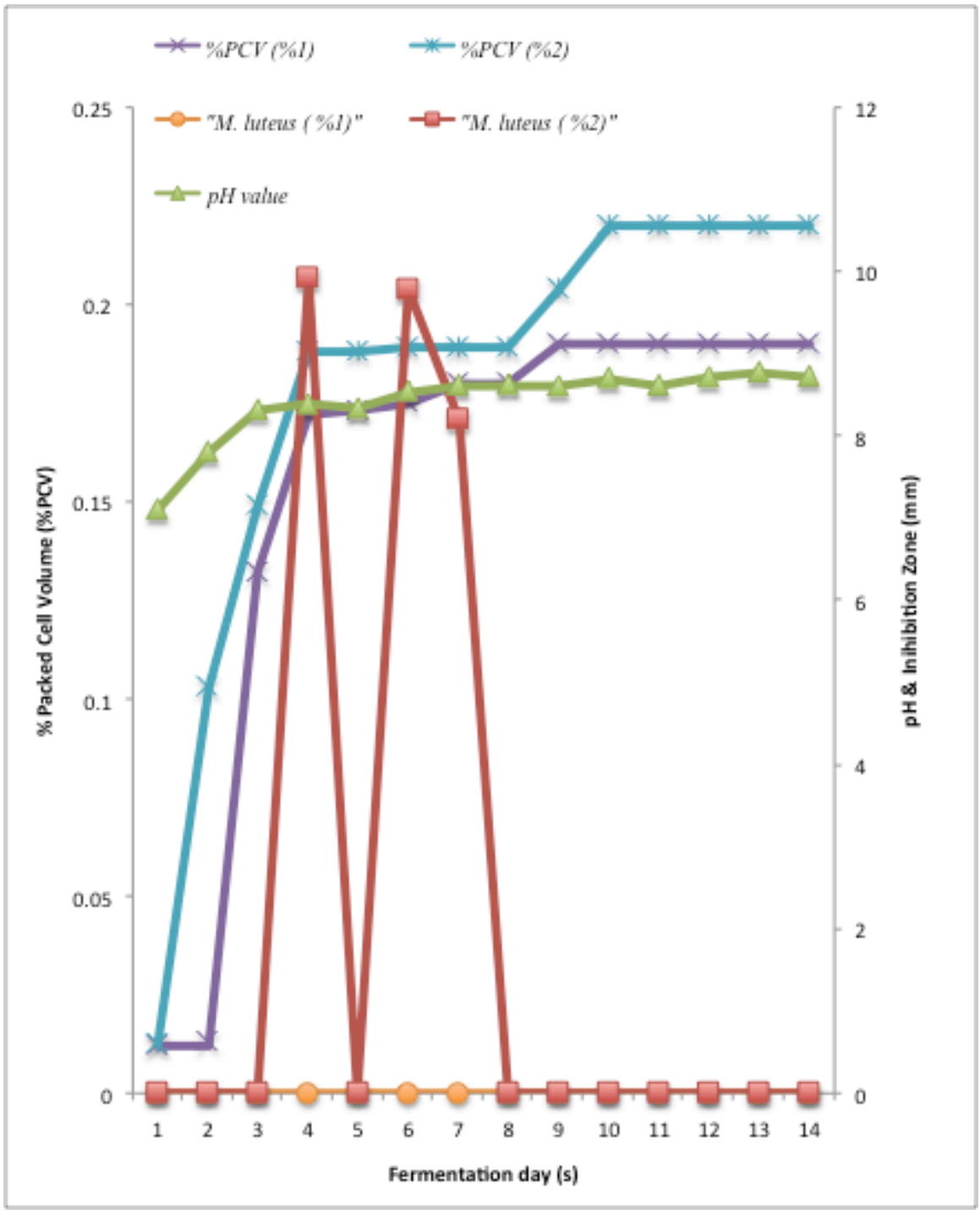
3.3.4 Effect of pH and packed cell volume (PCV%) on antimicrobial activity

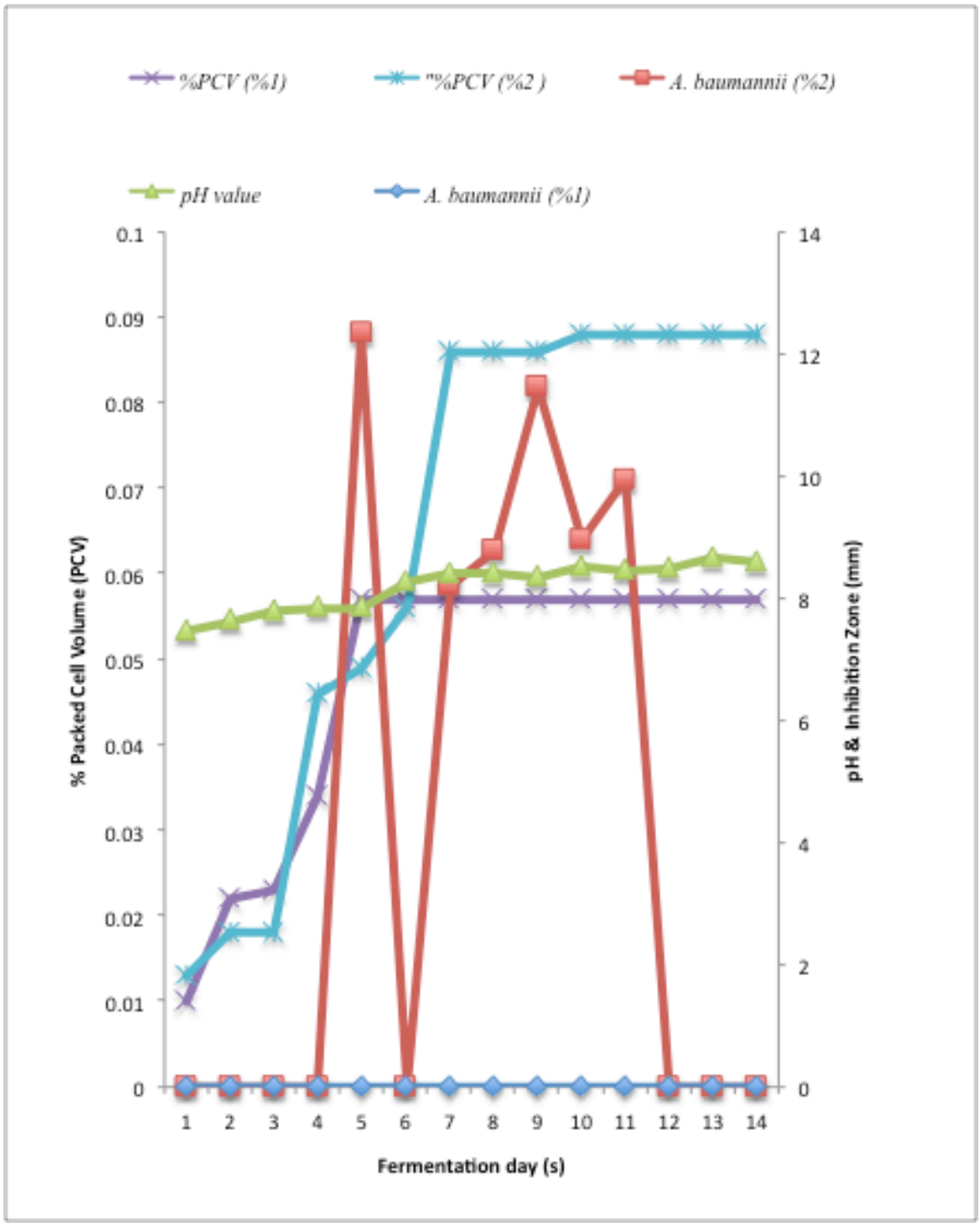
The effect of pH on the antimicrobial activity of the PM58B-RA, RA001, RA004 and RA003 isolates is shown in Figures 8, 9, 10 and 11. In general, growth and antimicrobial activity are optimal at around pH 7 for actinomycetes (Usha et al., 2011; Ababutain et al., 2013). In present study antimicrobial activity was observed for most of the isolates between pH 7.8 and 8.5. In terms of antimicrobial activity, the optimal pH values for the PM58B-RA, RA001, RA004 and RA003 isolates were 7.8, 8.3, 8.3 and 8.5, respectively.

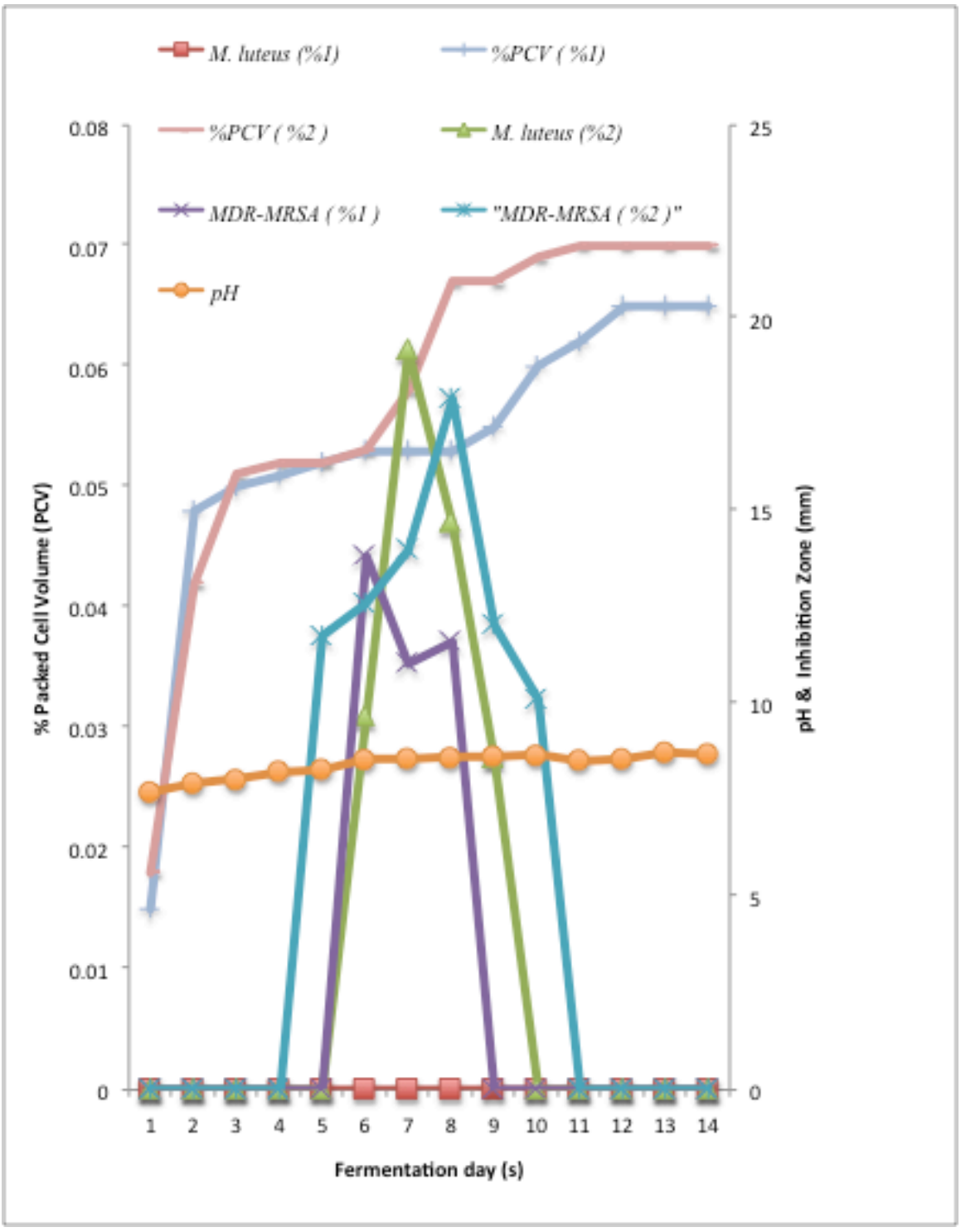
The microtube system manufacture was used to measure the total of cell volumes of the isolate cultures in uL. Glass PCV tubes were found to be better than 15 mL centrifuge tubes as the isolates in this study grew very slowly and resulted in low PCV% values. In general, inoculum volumes of 2% led to higher PCV (%) than inoculum volumes of 1%. The best temperature for the growth of the isolates was 12°C; however, the maximal growth of each isolate occurred on different days of culture. The maximum growth of PM58B-RA , RA001, RA003 and RA004 occurred after day 9. It is worth noting that these PCV (%) values are very low compared with the results of other studies; this is addressed in the discussion (Chapter 4).


%PCV (%2)
%PCV (%1)
"M. luteus (%2)"
M. smegmatis (%2)
"M. luteus (%1)"
M. smegmatis (%1)
pH value
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
% Packed Cell Volume
16
14
12
10
8
6
4
2
0
pH & Inhibition Zone (mm)
1 2 3 4 5 6 7 8 9 10 11 12 13 14
Fermentation day (s)

.


%PCV (%1)
%PCV (%2)
"M. luteus (%1)"
"M. luteus (%2)"
pH value
0.25
0.2
0.15
0.1
0.05
0
12
10
8
6
4
2
0
% Packed Cell Volume (%PCV)
pH & Inhibition Zone (mm)
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Fermentation day (s)


%PCV (%1)
"%PCV (%2)
A. baumannii (%2)
pH value
A. baumannii (%1)
% Packed Cell Volume (PCV)
pH & Inhibition Zone (mm)
Fermentation day (s)
0.1
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
14
12
10
8
6
4
2
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14

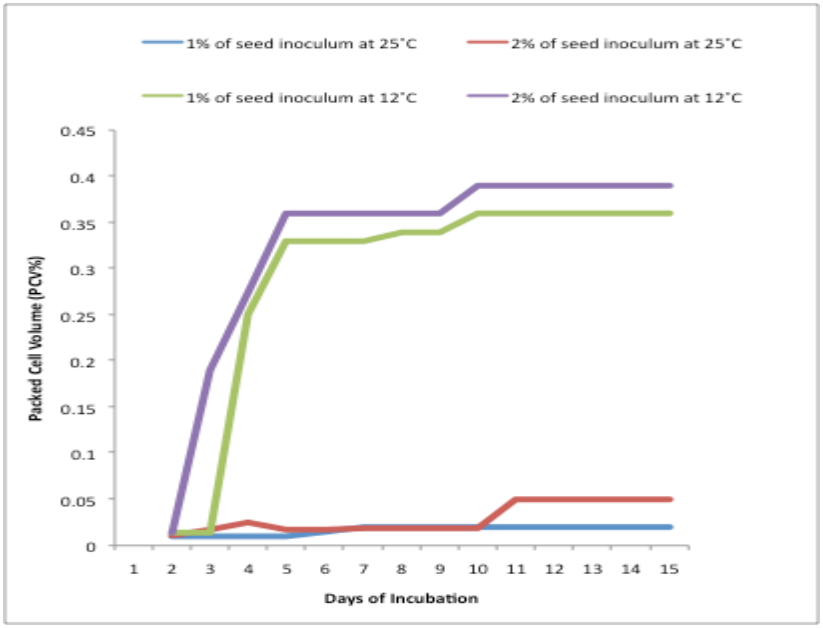

M. luteus (%1)
%PCV (%1)
%PCV (%2)
M. luteus (%2)
MDR-MRSA (%1)
"MDR-MRSA (%2)"
pH
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
25
20
15
10
5
0
% Packed Cell Volume (PCV)
pH & Inhibition Zone (mm)
1 2 3 4 5 6 7 8 9 10 11 12 13 14
Fermentation day (s)

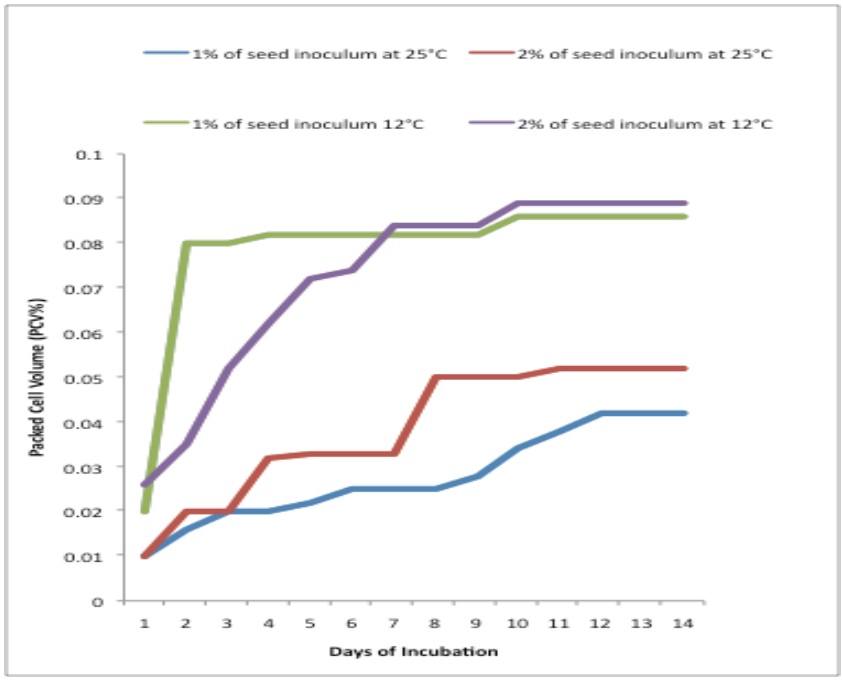
3.3.5 Effect of fermentation temperature on antimicrobial activity

To investigate the effect of fermentation temperature on the antimicrobial activity of the four isolates, RA001, RA003, RA004 and PM58B were cultivated at 12˚C and 25˚C. As shown in Table 6, RA001, RA003, RA004 and PM58B demonstrated antimicrobial activity against some microorganisms including *Mycobacterium smegmatis*, *Micrococcus luteus*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, MDR-MRSA and *K. pneumoniae.* The largest zones of inhibition against the Gram-positive and Gram-negative bacteria were observed when the isolates were cultured in R_2A broth at 12˚C or 25˚C. The inhibition zones for RA003 (19.17 mm), RA001 (9.92 mm) and PM58B-RA (15 mm) were larger when cultured at 12˚C than 25˚C; however, the RA003, RA004 and RAPB58B-RA isolates also showed some antimicrobial activity when cultured at 25˚C. The highest degree of inhibition was observed for RA003 (13.83 mm) and RA004 (12.85 mm) when cultured at 12˚C. These results suggest that 12˚C is a better temperature for fermentation of the RA003 and PM58B-RA and RA001 isolates than 25˚C (Table 7 and Figures 12,13,14 and 15).

Table 7: The inhibition zone diameter of the paper discs used (8 mm) for the four bacterial isolates against a variety of various tested microorganisms when cultured at 12˚ and 25˚C

Isolate	*M. luteus*		*M. smegmatis*		MDR-*S.aureus*		*A. baumannii*		*P. aeruginosa*		*K. pneumoniae*	
	12˚C	25˚C	12˚C	25˚C	12˚C	25˚C	12˚C	25˚C	12˚C	25˚C	12˚C	25˚C
RA001	9.92	0	0	0	0	0	0	0	0	0	0	0
RA003	19.17	0	0	0	17.91	13.83	0	0	0	0	0	0
RA004	0	0	0	0	0	0	10.85	12.85	0	0	0	0
PM58B-RA	15	0	11.5	8.87	0	0	0	0	0	0	0	0





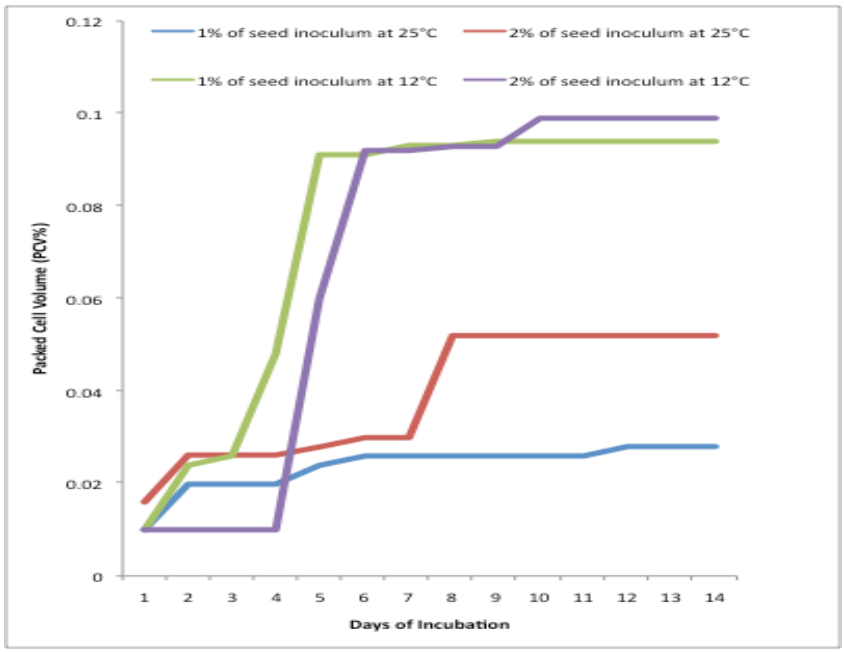


Figure 14: Effect of temperature on the growth of the RA004 isolate.

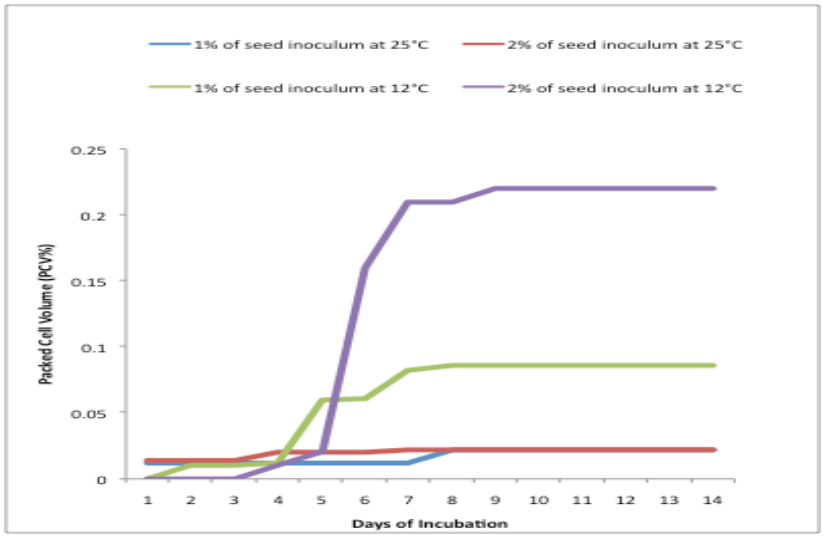

1% of seed inoculum at 25°C
2% of seed inoculum at 25°C
1% of seed inoculum at 12°C
2% of seed inoculum at 12°C
Packed Cell Volume (PCV%)
0.25
0.2
0.15
0.1
0.05
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Days of Incubation

Table 8: Best culture conditions for the antimicrobial activity of each isolate.

Isolate	pH	Temperature	Seed Inoculum	Days
RA001	8.3-8.5	12˚C	2%	3-13
RA003	8.5-8.6	12˚C	2%	4-11
RA004	8.3	12˚C	2%	4-11
PM58B-RA	7.8-8.0	25˚C	2%	3-8

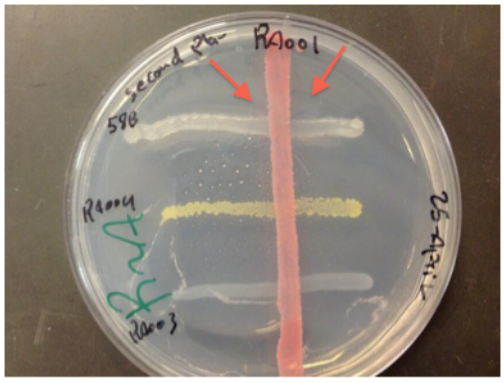
The best temperature and pH for the antimicrobial activity of the PM58B-RA, RA001, RA004 and RA003 isolates ranged from 12 and 25˚C and 7.8-8.6, and each isolate had different optimal number of days of culture (Table 8).

3.4 Test of the antimicrobial activity of the four isolates against each other using the cross-streak assay and paper disc diffusion method

The antimicrobial activity of each of the four isolates (RA001, RA003, RA004, PM58B-RA) against the other isolates was used to determine how cave isolates communicate through their metabolites by using the cross-streak assay method and paper disc diffusion method. No inhibition or interruption of the growth of any of the four isolates was observed using the cross-streak assay, and additionally, and some colonies even spread across the plates (Figure 16). However, using the paper disc diffusion assay, clear inhibition zones were observed on day six (Figure 17). In particular, PM58B-RA inhibited the growth of RA001, RA003, RA004 and the positive control *Streptomyces griseus*, while *Streptomyces griseus* inhibited the growth of RA001, RA003 and RA004 (Table 9). Thus, the paper disc diffusion method was a much more sensitive way of testing the antimicrobial activity of the isolates against one another, and indicated that the PM58B-RA isolate and *Streptomyces griseus* possess antimicrobial activity and factors that do not exist in RA001, RA003 and RA004.

Table 9: Inhibition zones (in mm) for each the four isolates against one another and *Streptomyces griseus* in the antimicrobial activity assay.

Tested isolates	**RA001**	**RA003**	**RA004**	**PM58B-RA**	**Streptomyces griseus**
RA001	0	0	0	11.02	12.03
RA003	0	0	0	11.05	11.32
RA004	0	0	0	10.02	11.96
PM58B-RA	0	0	0	0	0
Streptomyces griseus	0	0	0	14.96	0

A) 

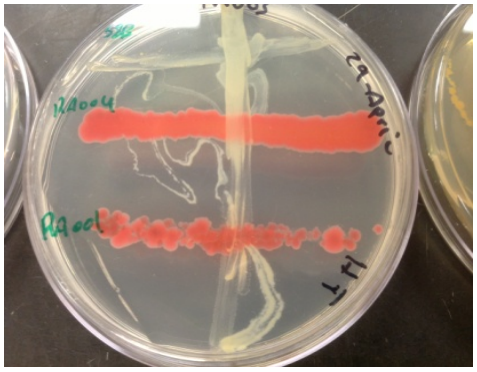
B) 

Figure 16: Test of the antimicrobial activity of each of the four isolates against the other isolates using the cross-streak assay. (A) RA003, RA004 and PM58B-RA did not inhibit the RA001 isolate on R_2A medium. (B) RA001, RA004 and PM58B-RA did not inhibit the RA003 isolate and spread over the entire plate on HT and R_2A medium.

A) 

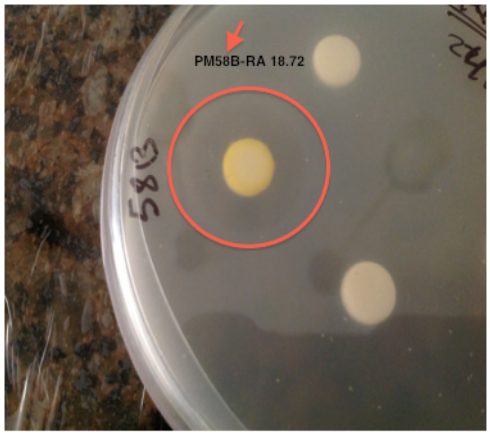
B) 

Figure 17: Test of the antimicrobial activity of each of the four isolates against the other isolates using the paper disc diffusion assay. (A) Clear zones of inhibition were observed for PM58B-RA (14.37 mm) and *Streptomyces griseus* (19.04 mm) against RA001 and (B) PM58B-RA against *Streptomyces griseus* (18.72 mm) in R_2A media.

3.4 Identification of the four isolates with antimicrobial activity

3.4.1 Morphological observations and chemotaxonomic study

Primary classification and identification of the four isolates was based on their microscopic/ macroscopic morphological and chemotaxonomic characteristics. The four cave isolates were identified as Gram-positive bacteria on basis of their morphological characteristics under the light microscope, such as Gram staining. However, each of the four isolates also possessed morphological characteristics that supported their identification as non-actinomycete bacteria (Table 10). For example, RA001 and RA004 were rod-shaped and RA003 and PM58B-RA were cocci. Under the light microscope, PM58B-RA appeared filamentous; however, RA001, RA003 and RA004 appeared non-filamentous. Furthermore, the colonies of all four isolates shared some similar features. RA001 and RA004 had similar circular colonies but different pigmentations, and RA003 had circular colonies and differed from PM58B-RA in both colony shape and pigmentation (Figure 18). PM58B-RA had filamentous colonies. Cell wall analysis was performed using thin layer chromatography. Notably, LL-diaminopimelic acid, a vital component of peptidoglycan in *S. griseus,* was not detected in the whole cell hydrolsates or sonicated cells samples from the RA001, RA003, RA004 or PM58B-RA isolates. All four isolates results in the plate observed as streaking and the silica gel plate surface were pricking, this experiment repeated two times with the same results (Figures 19, 20, 21 and 22).

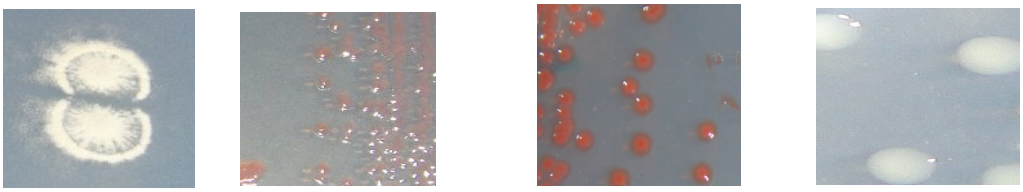


Figure 18: Morphology of the colonies of the PM58B-RA, RA001, RA004 and RA003 isolates in R_2A medium at 25˚C after about 5 days (All images are 50x magnifications).

Table 10: Microscopic morphological features of the four cave isolates possessing antimicrobialactivity.

Isolate	Gram reaction	Cell shape	Microscopic morphology	Classification
RA001	+	Cocci	Non-filamentous	Non-actinomycetes
RA003	-	Rod	Non-filamentous	Non-actinomycetes
RA004	+	Cocci	Non-filamentous	Non-actinomycetes
PM58B-RA	+	Rod	Filamentous	Non-actinomycetes

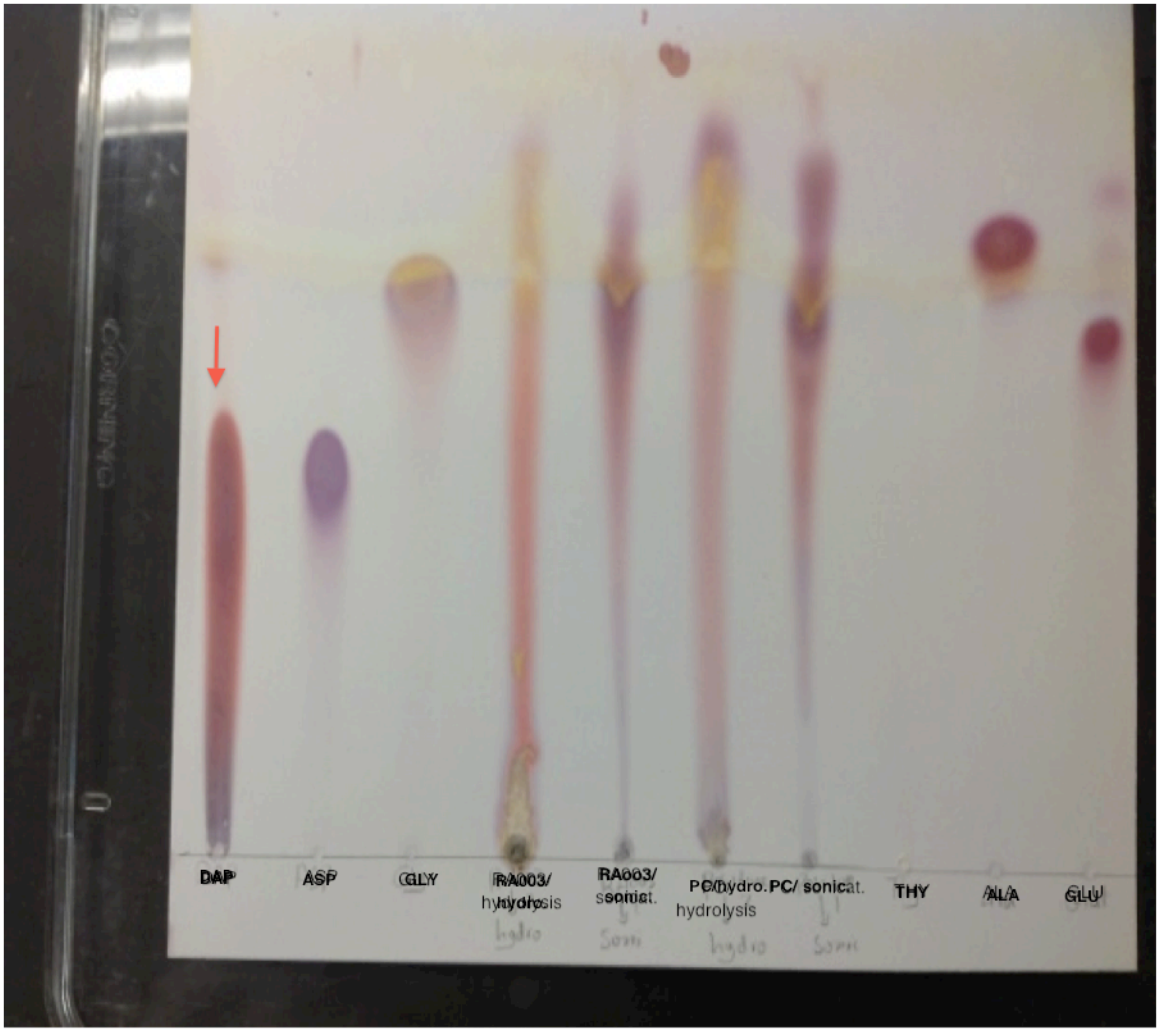


Figure 19: Thin layer chromatography analysis of the amino acid composition of whole cell hydrolysates of the RA003 isolate. The arrow indicates the LL-diaminopimelic acid (DAP) spot.

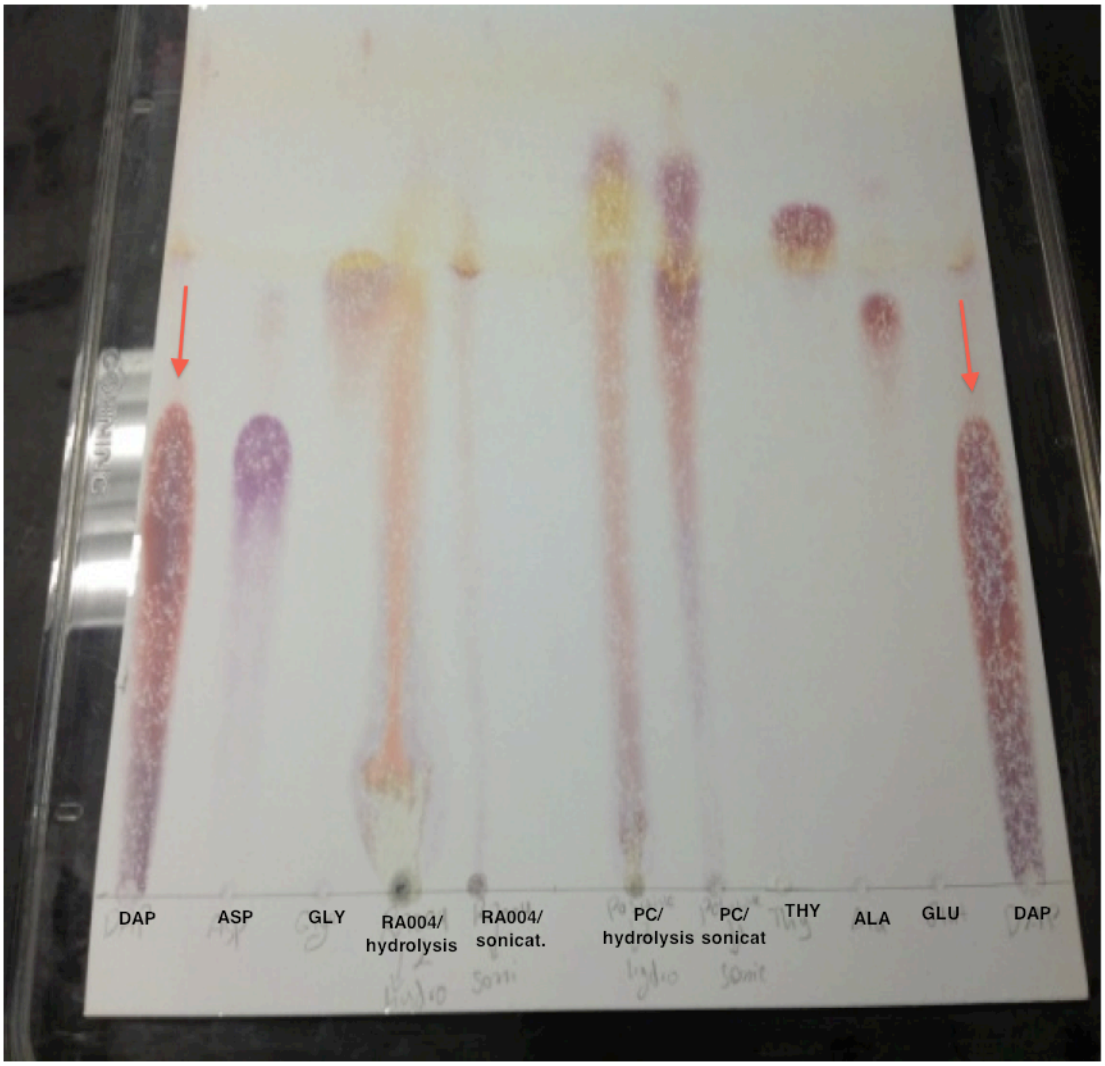


Figure 20: Thin layer chromatography analysis of the amino acid composition of whole cell hydrolysates of the RA004 isolate. The arrows indicate the LL-diaminopimelic acid (DAP) spots.

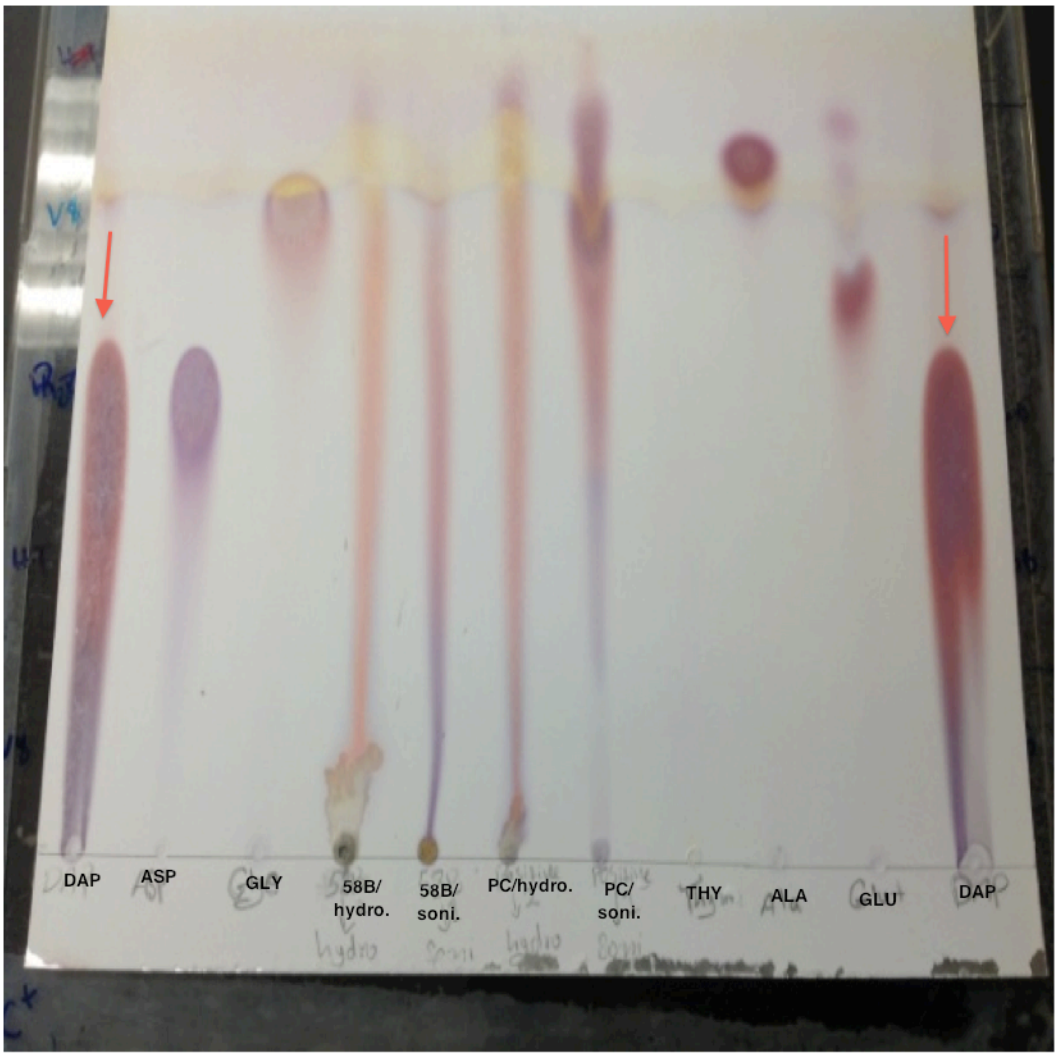


Figure 21: Thin layer chromatography analysis of the amino acid composition of whole cell hydrolysates of the PM58B-RA isolate. The arrows indicate the LL-diaminopimelic acid (DAP) spots.

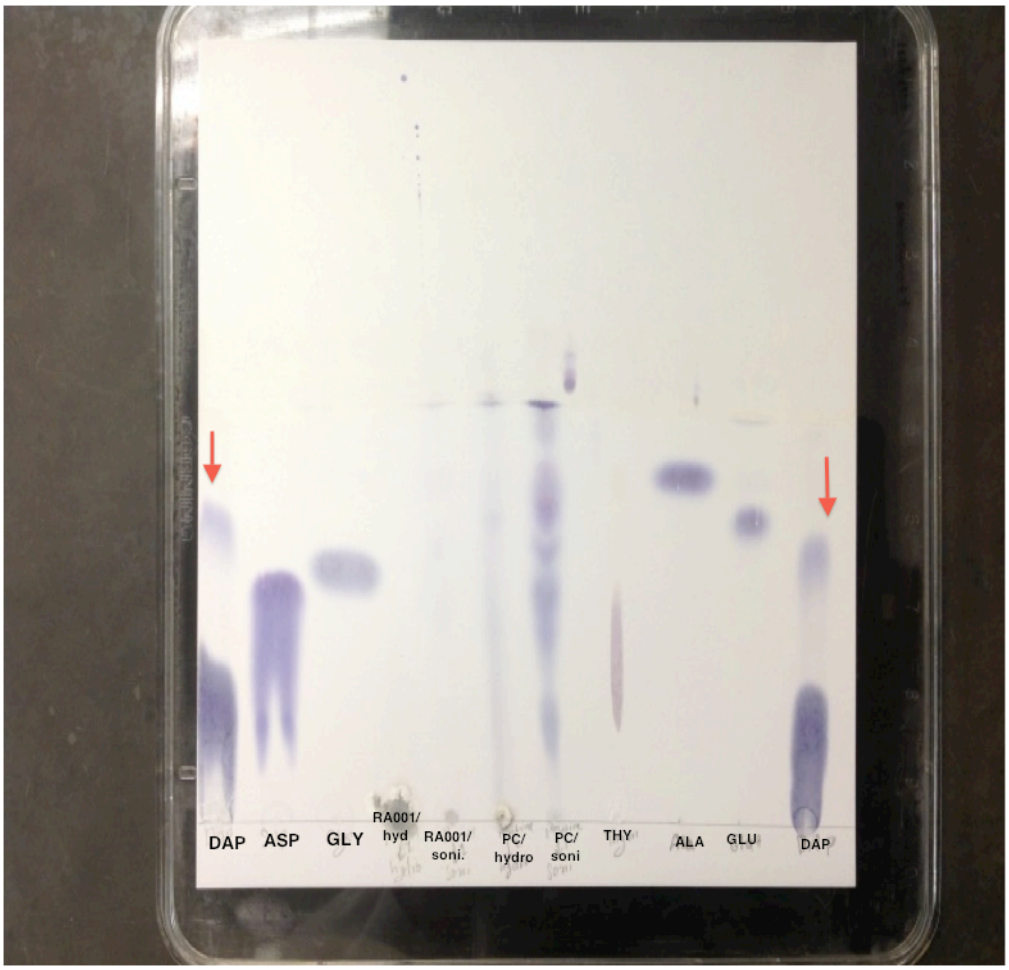


Figure 22: Thin layer chromatography analysis of the amino acid composition of whole cell hydrolysates of the RA001 isolate. The arrows indicate the LL-diaminopimelic acid (DAP) spots.

3.4.2 Production of pigments by the isolates

Pigments can be found in various natural sources including fruits, vegetables, seed roots and microbes. Pigments extracted from microbes are called "biocolors" because of their biological source, and many have been proven to be safe for human use (Goswami et al., 2010). It was particularly noteworthy that PM58B-RA and RA003 produced a white pigment, while RA001 and RA004 produced a pink pigment after about 5 days of culture at 25˚C in R_2A medium. However, PM58B-RA and RA003 isolates produced white pigments at 12 and 25˚C; the pink pigment was only observed after 14 days of fermentation days.

3.4.3 Molecular identification of the four antimicrobial cave isolates by 16S rRNA gene sequencing

The taxonomy of the four antimicrobial-producing isolates was determined by 16S rRNA gene sequencing. The results revealed that all four isolates are non-streptomycete bacteria. Specifically, the RA003 isolate belongs to the *Sphingomonadaceae* family, RA001 and RA004 belong to the *Arthrobacteria agilis* species, and PM58B-RA belongs to the species *Bacillus licheniformis*. All four isolates shared 99% similarity to their most closely-related ribosomal RNA sequences in the Genbank database (Table 11; see sequences in the Appendix C).

Table 11: Taxonomic status of the four isolates according to 16S rRNA gene sequencing.

Bacterial isolates	Family/Species	% Similarity
RA001	*Arthrobacter agilis*	99
RAOO3	*Sphingomonadaceae*	99
RA004	*Arthrobacter agilis*	99
PM58B-RA	*Bacillus licheniformis*	99

Table 12: Identification of the four cave isolates using MALDI-TOF MS.

Isolate	Classification status	Taxonomy level
RA001	*Arthrobacter agilis*	Species
RA003	*Sphingopyxis terrae*	Species
RA004	*Arthrobacter agilis*	Species
PM-58B-RA	*Bacillus licheniformis*	Species

3.4.4 Identification of the four antimicrobial cave isolates by MALDI-TOF MS

MALDI-TOF mass spectrometry was used to further confirm the identity of the RA001, RA003, RA004 and PM58B-RA isolates. Two methods were used to prepare the isolates for analysis: formic acid extraction and the direct spotting method, and the spectra of the isolates were compared to MALDI-TOF mass spectrometry protein or peptide profiles in the Biotyper 2.0 database.

None of the isolates could be identified using the direct spotting method, and culture on R_2A agar media combined with formic acid extraction led to higher identification scores for the isolates than culture in R_2A broth combined with formic acid extraction (see Figures 21, 22, 23 and 24). There were differences in result interpretations, for example, the direct spotting method showed inconsistent results which may due to contamination while the results with formic acid extraction was more sensitive which showed consistent results.

The mass spectra of the four cave isolates were compared to the MALDI-TOF mass spectra database ((see Figures 21, 22, 23 and 24).). The RA003 isolate obtained the highest identification score, indicating that RA003 can most likely be confirmed as *Sphingopyxis terrae* DSM 12444T HAM (Appendix D 1-1, 1-2). For the PM58B-RA, RA001 and RA004 isolates, MALDI-TOF MS helped to confirm their identity in combination with the 16S rRNA gene sequencing analysis; RA001 and RA004 was confirmed to belong to the species *Arthrobacteria agilis* and PM58B-RA was confirmed to belong to the species *Bacillus licheniformis*. However, a low identification score was obtained for RA003 using MALDI-TOF MS (data not shown).

The identification of the RA001, RA003, RA004 and PM58B-RA isolates is summarized in Table 11. MALDI-TOF MS was found to be a complementary technique for identification of the isolates in conjunction with 16S rRNA sequence analysis. Using this combination of techniques, the RA003 isolate was identified to the species level and PM58B-RA, RA001 and RA004 were identified to the genus level. Thus, MALDI-TOF

MS could be a rapid, complementary and accurate method for identifying bacteria compared to other traditional microbiology methods.

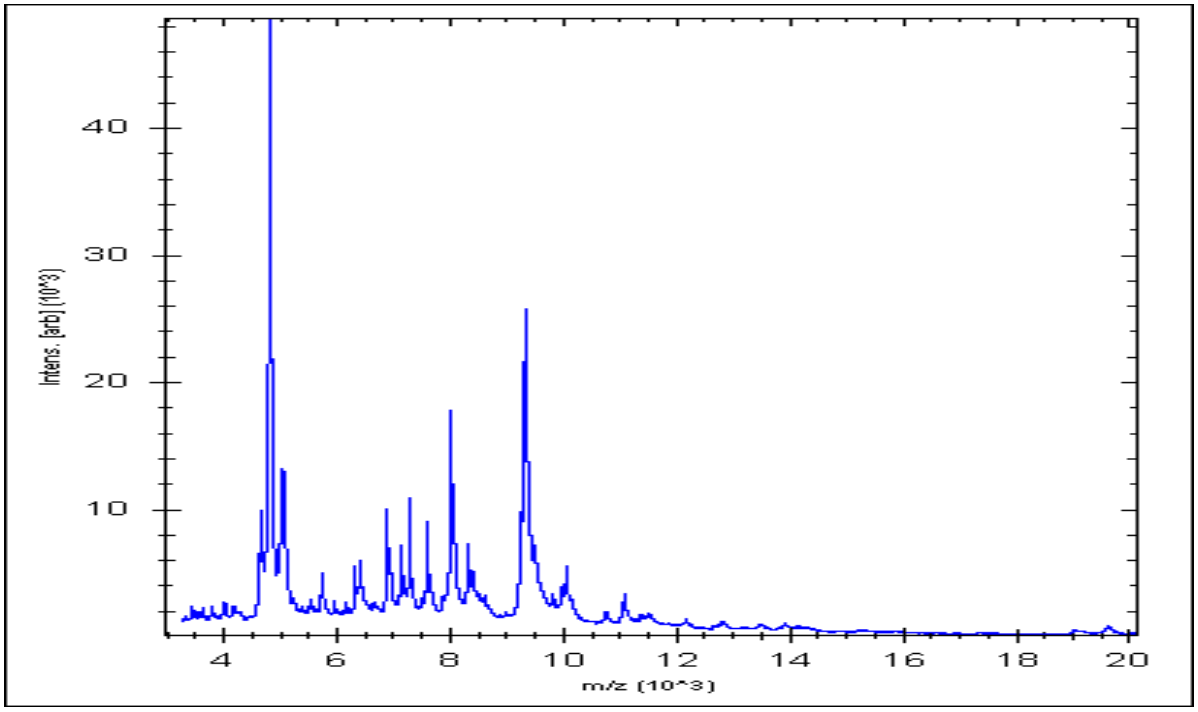


Figure 23: MALDI-TOF MS spectra for the RA003 isolate

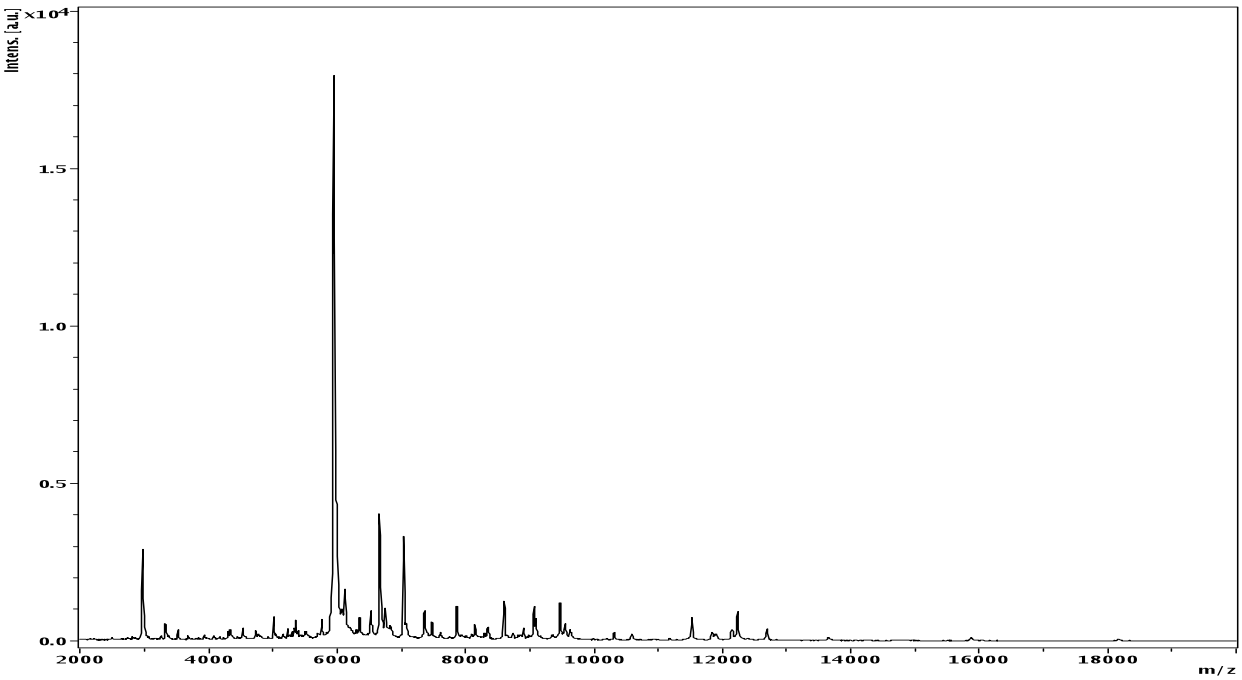


Figure 24: MALDI-TOF MS spectra for the RA001 isolate

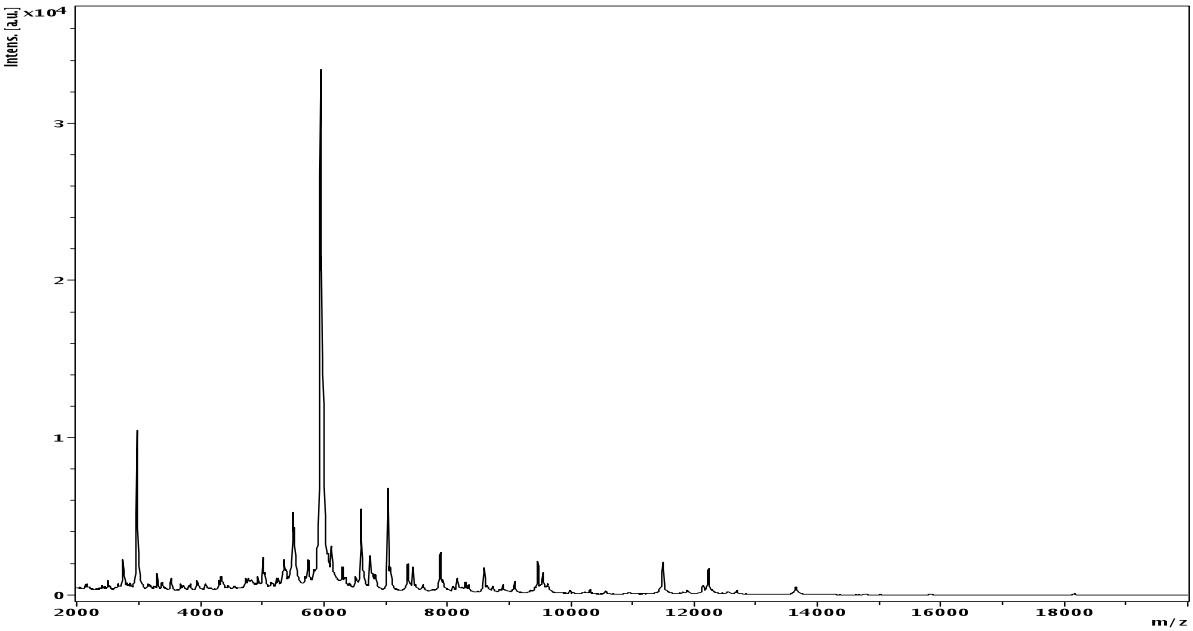


Figure 25: MALDI-TOF MS spectra for the RA004 isolate

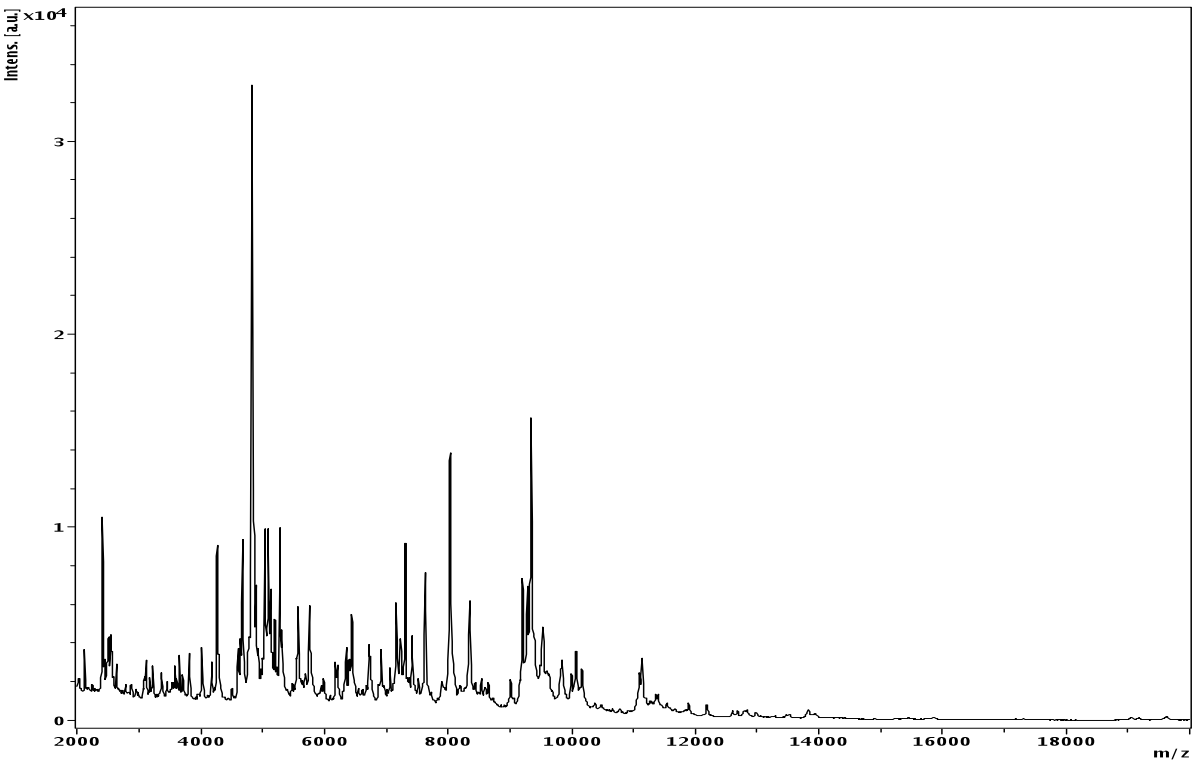


Figure 26: MALDI-TOF MS spectra for the PM58B-RA isolate.

3.5 Extraction and purification of the bioactive antimicrobial compounds produced by the four isolates

Dry mycelia from RA001 (260 mg), RA003 (470 mg), RA004 (450 mg) and PM58B-RA (460 mg) were extracted twice with MeOH (25.0 mL x 2), and the combined MeOH extracts for each isolate were dried under reduced pressure yielding MeOH extracts (PM58B-RA, 50.0 mg; RA003, 20.0 mg; RA004, 90.0 mg; RA001, 120 mg). The MeOH extracts were further fractionated by solid phase extraction using Discovery DIC-18 cartilages and elution with a H_2O/MeOH/EtOAc gradient into several fractions (see Appendix B). The fractions from each isolate were subjected to antimicrobial activity assays against tested microorganisms which they previously demonstrated antimicrobial activity: *M. smegmatis* and *M. luteus* for PM58B-RA, *M. luteus* for RA001, *A. baumannii* for RA004, and MDR-MRSA and *M. luteus* for RA003.

The ^{1}H NMR spectra of all of the fractions from the RA001, RA003, RA004 and PM58B-RA isolates were recorded; none of the fractions were found to be pure. Unfortunately, none of the fractions demonstrated antimicrobial activity against any of the tested microorganisms tested, except for fraction 2 from isolate RA003 (RA003E0102). This active fraction inhibited the growth of MDR-MRSA (by 12.38 mm) and was subjected to further analysis to identify the active compound (Figure 23).

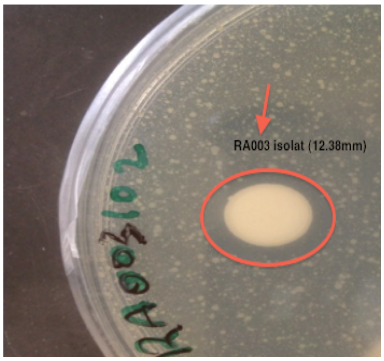


Figure 27: Inhibition zone (12.38 mm) of the active fraction RA003E0102 from the RA003 isolate against MDR-MRSA.

The proton NMR and LC/MS data for the active RA003E0102 fraction are provided in Appendix E. The NMR signals represent the hydrogen atoms of the compounds present in RA003E0102. No interesting signal was observed using LC/MS, and all of the compounds eluted between 1-2 min (UV – lower chromatogram) indicating the fraction mostly contained water soluble chemicals from the culture media. After removing water-soluble chemicals from the culture media, lyophilized dry mycelia (580 mg) were extracted twice with MeOH (25.0 mL x 2), the combined extract was dried under reduced pressure yielding MeOH extract (160 mg), further fractionated by solid phase extraction using a Discovery DIC-18 cartilage and eluted using a H_2O/MeOH/EtOAc gradient into two fractions: fraction 1 (44.2 mg, H_2O 50 mL) and fraction 2 (5.0 mg, H_2O/MeOH, 50 mL). Further LC/MS and NMR study (awaiting the results of further anti-bacterial activity tests).

4.0 Discussion

4.1 Sample collection and initial selection

The objective of this study was to isolate cave bacteria from a volcanic cave in Wells Gray Provincial Park, BC, and to determine if they produce metabolites with antimicrobial activity against various microorganisms including multi-drug resistant pathogens. The present study demonstrated that cave bacteria are promising sources of antimicrobial compounds. This finding is supported by Kay et al. (2013), Cheeptham et al., (2013) who previously showed that cave bacteria isolated from the Helmcken Falls cave, BC, possessed significant antimicrobial activity against Gram positive and negative bacteria. Of the total of 317 microorganisms isolated, 16 bacterial isolates were identified based on their growth features and selected for further antimicrobial activity assays on the basis of their viability and antimicrobial activity in initial screening tests.

4.2 Antimicrobial activity of the 16 bacterial isolates in different media

Sixteen isolates were cultured separately in four different fermentation broths (Hickey Tresner, R_2A, ISP#2, V-8 juice), and the antimicrobial activity of the isolates was examined using the disc-diffusion (Kirby-Bauer) method. These 16 isolates were also grown in the same fermentation broth that had been diluted ten-fold in order to replicate the environmental conditions of the cave. Caves are considered to be an oligotrophic environment with low amounts of nutrients available, in which the organisms such as cave bacteria grow slowly (Gabriel and Northup, 2013).

Therefore, diluted media have been shown to be appropriate for bacteria that are adapted to an oligotrophic environment (Vartoukian et al., 2010). When incubated in the diluted broths, none of 16 isolates demonstrated the ability to inhibit or kill any of the seven microorganisms (*Mycobacterium smegmatis*, *Micrococcus luteus*, MDR-MRSA, *Acinetobacter baumannii*, ESBL *E. coli*, *Candida albicans*), even though some of the

isolates demonstrated antimicrobial activity when cultured in the undiluted fermentation broths. This result may be explained by the fact that the production of antimicrobial compounds can be influenced by the concentrations of the carbon, nitrogen and phosphorus available (Yegneswaran et al., 1988; El-Refai et al., 2011).

The four media contain different concentrations of nutrients; however, ten-fold dilution may have decreased the concentration of the nutrients to a level lower than that required for the production of antimicrobial secondary metabolites. The nutrients in the media enable microbes to gain energy, build cellular structures and grow. In general, variations in the nutrient concentrations of the culture media, including carbon, nitrogen, ammonia and phosphate, can prevent or enhance the production of antibiotics by microorganisms (EL-Refai et al., 2011).

The presence of the optimum carbon and nitrogen sources are also important for their antimicrobial activity (Northup at el., 2011). The glucose concentration of the culture media also affects antibiotic production (Himabindu and Jetty, 2006; Awais et al., 2007; Muhammad et al., 2009; Usta and Demirkan, 2013). Hasan et al. (2009) reported that maximum zones of inhibition (32 mm and 24 mm) were produced by *B*. *pumilus* against *M*. *luteus* and *S*. *aureus* when cultured in media containing 3% glucose. Awais et al. (2007) and Muhammad et al. (2009) found that B. pumilus produced the maximum zone of inhibition (26 mm) against *M*. *luteus* in 5% glucose, and *B*. *subtilis* produced a maximum zone of inhibition (19 mm) in 1% or 5% glucose. Awais et al. (2007) demonstrated that the antimicrobial activity of *B*. *pumilus* against *M*. *luteus* increased with the glucose concentration, with 2% glucose the optimal concentration. Haavik (1974) reported that high concentrations of carbon decreased or inhibited the enzymatic synthesis of a polypeptide antibiotic by *Bacillus licheniformis*. The results of Haavik (1974) support our findings, as the maximum inhibition zones were obtained when the isolates were cultured in R_2A broth, which contains 0.05% glucose and 0.05% wt/vol soluble starch. R_2A broth was also best for the growth of the PM58B-RA, RA001, RA004 and RA003 isolates. R_2A broth contains lower concentrations of carbon than other broths such as HT, ISP#2 and V-8 juice. However, these isolates did not show any antibiotic activity when grown in ten-fold diluted R_2A broth, possibly due to the very low

concentration of carbon and nitrogen sources in this media. Therefore, bacteria can not gain energy, build their cell structure or grow in diluted media. Another possibility is that these 16 isolates may need more than 14 days incubation in diluted media to adapt to diluted media.

In this study, the lowest rates of antimicrobial activity were observed in ISP#2 and V-8 juice. On the contrary, Cheeptham et al. (2013) cultured cave actinomycetes in ISP#2, V-8 juice and HT broths (within 1% variation for each chemical), and found ISP#2 and V-8 juice were the best broths for the production of secondary metabolites by actinomycetes. However, HT led to half the antimicrobial activity of ISP#2 and V-8 juice. Kay et al. (2012) found that strain E9, which was also isolated from the Helmcken Falls cave, showed significant antimicrobial activity against *P. larvae* when grown in V-8 juice. Badji et al. (2006) found that ISP#2 was the best media for the antifungal and antibacterial activity of actinomycetes isolated from soil. ISP#2 and V-8 juice contain higher concentrations of glucose and nitrogen compared to R_2A and HT. For example, ISP#2 contains glucose (0.4% wt/vol) and yeast extract (0.7% wt/vol) as a nitrogen source. V-8 juice contains D-glucose (1.4% wt/vol) and D-fructose (1.4% wt/vol), but contains a lower concentration (0.07%) of nitrogen from organic and inorganic sources (Cheeptham et al., 2013); V-8 juice also contains yeast extract (0.01%) as a source of nitrogen. However, Haavik (1974) supported the use of low concentrations of carbon as high concentrations of carbon decreased or inhibited the enzymatic synthesis of a polypeptide antibiotic. However, in the present study, some of the 16 isolates were identified as non–actinomycetes, in contrast to the studies of Cheeptham et al. (2013) and Kay et al. (2012) in which all isolates were identified as actinomycetes. Cheeptham et al. (2013) reported that actinomycetes produce secondary metabolites under less than ideal conditions.

HT media contains dextrin (1% wt/vol) as a carbon source and led to higher antibiotic production than ISP#2 and V-8 juice in this study. Awais et al. (2007) and Muhammad et al. (2009) found that B. subtilis produced a maximum zone of inhibition (19 mm) in 1% glucose. In the present study, a maximum zone of inhibition (18 mm) against M. luteus was found with HT broth, which contains a lower concentration of glucose than ISP#2 and V-8 juice. Ten-fold dilution decreased the concentrations of

carbon and nitrogen in ISP#2 and V-8 juice and inhibited the antimicrobial activity of the 16 isolates.

Another possible explanation for our results could be that ISP2# and V-8 juice are too rich for the cave isolates. Northup et al. (2011) reported that cave microorganisms often survive in very low nutrient concentrations and may not be able to live in rich media. This supports our results that ISP2# and V-8 juice broths may not sufficiently replicate the actual environment for growth of most of the 16 isolates, except for the RA003 and PM184 isolate which demonstrated antimicrobial activity in ISP#2 broth.

Seven of these isolates (NC18, PM184, PM58B-RA, PMA1A3, RA003, RA001 and RA004; Table 2), inhibited or killed some of the selected microorganisms (*Mycobacterium smegmatis*, *Micrococcus luteus*, MDR-MRSA, *Acinetobacter baumannii*, and *Candida albicans*) when they grew in non-diluted broth. However, they did not show growth and antimicrobial activity in diluted version of the different broths used in the primary screening. Vartoukian et al. (2010) and Giraffa and Neviani (2001) reported that when viable cells are exposed to stresses such as low pH, low temperature and limited nutrients, the cells may shift to a state in which they remain viable but non-cultivable (VBNC) (Vartoukian et al., 2010). In addition, the low nutrient conditions in our study could cause a stress on the cave isolates, replicating the process known as selective pressure (Gabriel & Northup, 2013). A poor medium that contains limited nutrients may stimulate the cells to become resistant instead of undergoing slow growth (Cheeptham, 2013). In addition, VBNC isolates have specific growth requirements, such as specific nutrients, pH conditions, incubation temperatures or oxygen levels (Cladera et al., 2004; Vartoukian et al., 2010). This may explain the fact that the seven isolates showed antimicrobial activity in R_2A and HT broth, but may have shifted to VBNC when cultured in diluted or non-diluted ISP#2 and V-8 juice.

4.3 Selection of best media for further antimicrobial activity assays

Different media have been examined for culture of the cave and soil community (Rule, 2013; Cheeptham, 2013; Ababutain et al., 2013). However, it is well recognized that only a small proportion of bacteria can be cultured in vitro (Davis, 2005; Vartoukian, 2010). Molecular ecological techniques have revealed the presence of a wide variety of novel microbes in cave and soil communities, demonstrating our inability to culture and study soil bacteria. This problem limits our understanding of the variety of species in soil communities (Dayal et al., 2013**;** Hugenholtz et al., 1998). Therefore, in this study, we attempted to improve the fermentation process for cave microorganisms by using non-traditional media.

Many studies have demonstrated the effects of different media on fermentation and antibiotic production by microbes (Dayal et al., 2013; Al-Judaibi, 2011). In the current study, the composition of the fermentation broth was found to enhance antimicrobial production. This was confirmed by the fact that PM58B-RA, RA001, RA004, RA003, NC18, 46A, A1A3 and 245 produced the maximum inhibition zones when grown in R_2A (6/16 isolates demonstrated antimicrobial activity), compared to Hickey Tresner (5/16) and ISP#2 (2/16). However, the other nine isolates (Table 1) did not demonstrate antimicrobial activity when grown in V-8 juice.

RA003 inhibited *M. luteus* (15.62 mm) when cultured in R_2A broth, and inhibited MDR-MRSA (13.45 mm) when cultured in HT broth. PM-58B-RA inhibited *M. luteus* (18 mm) when cultured in HT broth, and inhibited *M. smegmatis* (16 mm) when cultured in R_2A broth. It is clear that the R_2A and HT broths enabled these isolates to produce antimicrobial compounds; however, these media have different nutrient compositions.

HT and R_2A broths were prepared according to standard methods (within 1% for each chemical; Appendix A). HT broth contains beef and yeast extract (0.1% wt/vol) as a nitrogen source, $CaCl_2$ (0.002% wt/vol) as a mineral source and dextrin (1% wt/vol) as a carbohydrate source. Dextrin contains mixture of substances including glucose, lactose, sucrose and cornstarch, which are all sources of carbon. However, R_2A broth contains yeast extract (0.05% wt/vol) and proteose peptone (0.05% wt/vol) as nitrogen sources,

Casamino Acids (0.05% wt/vol) as a source of amino acids, and glucose (0.05% wt/vol) and soluble starch (0.05% wt/vol) as carbon sources. R_2A broth also contains minerals such as K_2HPO_4 (0.3% wt/vol) and $MgSO_4.7H_2O$ (0.005% wt/vol), and sodium pyruvate (0.3% wt/vol), which is a source of energy for dividing cells. R_2A broth contains most of the essential nutrients such as carbon, nitrogen and salts, while HT broth lacks some of these minerals. Starch was previously found to be the most suitable sole carbon source for optimum growth and antibiotic production, with peptone and $NaNO_3$ the most suitable sole nitrogen source for antibiotic production and optimum growth, respectively (Ababutain et al., 2003). The maximum antibiotic production by *Streptomyces* sp. was obtained in a medium that included soluble starch, peptone, K_2HPO_4, $MgSO_4.7H_2O$, KCl and trace $FeSO_4.5H_2O$ (Ababutain et al., 2003).

PM58B-RA (*Bacillus licheniformis*) and RA003 (*Sphingopyxis terrae*) preferred R_2A broth for the production of antimicrobial activity, while RA001 (*Arthrobacter agilis*) and RA004 (*Arthrobacter agilis*) preferred R_2A broth for pigment production. In addition, PM58B-RA, RA001, RA004 and RA003 started growing after 5 days on R_2A plates while they only began to grow after 7 days on HT. R_2A medium contains low concentrations of carbon and nitrogen and higher levels of nutrients than HT, V-8 juice and ISP#2. Cheeptham et al. (2013) identified a Helmcken Falls cave isolate that grew in HT medium as *Streptomyces microflavus*. In addition, many studies have indicated the advantages of R_2A medium, especially for promoting the growth of bacteria and pigment production (Reasoner and Geldreich, 1984). R_2A medium contains a variety of minerals such as K_2HPO_4 (0.3% wt/vol) and $MgSO_4.7H_2O$ (0.005%) which are essential for bacterial growth and antimicrobial activity (Ueda et al., 1997; Atta, 1999; Aman, 2001). Ripa et al. (2009) and Mangamuri et al. (2014) stated that K_2HPO_4 and $MgSO_4.7H_2O$ enhanced antimicrobial production by *Streptomyces* sp. compared to HT medium, which does not contain these minerals. Therefore, R_2A medium was the best medium for the antimicrobial activity and production of pigments by the isolates compared to HT, ISP#2 and V-8 juice. In the end, the PM58B-RA and RA003 isolates were selected for further study due to their high antimicrobial activity, while RA001 and RA004 were selected as they produced pink pigmentation in R_2A medium in addition to their high and consistent antimicrobial activity.

4.3.1 Production of pigments by the isolates

Reasoner and Geldreich (1984) reported that a long incubation time (up to 14 days) increased bacterial yield as well as pigment production on R_2A medium. The results of our study support the observations of RA001 and RA004 produced pink pigmentation after 5 days of incubation, especially on agar plates rather than broth. However, R_2A medium was only suitable for the production of pigmentation by RA001 and RA004 at 25°C.

Young *et al*. (1985) reported that no parallelism existed between pigment production and the production of antibiotics. However, a number of studies have reported the effects of bacterial pigmentation on antimicrobial activity (Gauteier and Flatau, 1976; Nakamura *et al*., 2003). Nakamura *et al*. (2003) studied the antibacterial activity of a violet pigment produced by the psychrotrpic bacterium RT102 strain, and found that high concentrations of the violet pigment led to growth inhibition or caused cell death of a number of pathogens. In addition, pigmentation in microorganisms has recently been suggested to be an indicator of the production of active compounds. For example, *Streptomyces* are known produce different pigments such as red, blue and violet. These colours are associated with compounds with antimicrobial, antiviral, antitumor, antiprotozoal, antioxidant, anticancer and other activities (Ferreira *et al*., 2004; Matz *et al*., 2004; Deorukhkar *et al*., 2007; Kim *et al*., 2010). Further experiments are necessary to investigate the relationship between these cave bacterial pigmentation and the production of bioactive compounds.

4.4 Study of fermentation conditions for the four selected isolates

The optimal fermentation conditions for various caves bacterial isolates have been examined in a number of previous studies (Lueschow et al., 2013; Bhullar, 2011). Different physiochemical conditions such as the growth media, seed inoculum volume,

pH, temperature and fermentation time were studied with the aim of enhancing the antimicrobial activity of the cave isolates.

4.4.1 Temperature

Three of the four isolates selected for further analysis in this study (RA001, RA004, RA003) produced maximum antimicrobial activity when incubated at 12°C and are therefore adapted to the cold. Many other studies have provided similar results. Laiz et al. (2003) studied different caves in Spain at different temperatures (5, 13, 20, 28 and 40°C), and reported that most cave microorganisms could grow at temperatures between 13-40°C; however, a low temperature (13°C) was optimal for the growth of a higher diversity of species. Khizhnyak et al. (2011) isolated bacteria from water in a cave where the air temperature varied from 0 to 5°C, and found that the bacteria could grow at a low temperature (7°C) but not at a higher temperature (35°C); the bacteria altered their morphology and subsequently died at the higher temperature.

However, PM58B-RA produced maximum antimicrobial activity at 25°C. This result is in agreement with Rule and Cheeptham (2013), who reported that 100 actinomycete strains, which were collected from a volcanic cave, showed the highest metabolite production when fermented at 25°C.

The actual temperature range in the Helmcken Falls cave is 7.5-12.2°C (Cheeptham, 2012). The differences in the optimum temperatures for antimicrobial activity may be due to variations in the precise habitats of the microorganisms in the cave. This may be explained by that fact that the temperature also affected the growth of the microorganisms. For example, RA001, RA004 and RA003 grew faster at lower temperatures (Figure 12, 13, 14 and 15), leading to maximal antimicrobial activity (Table 7).

4.4.2 pH

Many studies have reported that various pH values are optimal for antimicrobial production and growth by bacteria from a different habitat. Ababutain et al. (2013) found that *Streptomyces* sp. demonstrated maximal production of antimicrobial agent in media with an initial pH of 6.0. Three actinomycete isolates had a broader spectrum of antibacterial activity and showed production of potential antibiotics under mildly alkaline conditions at pH 7.2 (Arifuzzaman et al., 2010). Crawford et al. (1993) reported that the highest growth of actinomycete strains occurred between pH 6.5-8; some strains could not grow at pH 6.0, and pH 5.5 inhibited the growth of a large number of strains. On the contrary, Höltzel et al. (1998) reported that pH 5.5 was optimal for the production of antimicrobial compounds.

Previously, it was shown that the region of the cave from where the samples were collected has a pH ranging from 6.42-8.38 (Cheeptham, 2013). Alkaline pH values ranging from 7.8 to 8.6 were found to effectively promote the antimicrobial activity and growth just for all the four isolates in the current study. In general, the pH changes during the fermentation process. Variations in the antimicrobial activity of each isolate were observed between pH 7.8 and 8.5, with pH values of 7.8, 8.3, 8.3 and 8.5 best for PM58B-RA, RA001, RA004 and RA003, respectively. Our results indicate that, not surprisingly, the bacteria have maximal antibiotic producing activity at the same pH as their natural conditions.

4.4.3 Fermentation period

In the current study, the maximum production of antimicrobial agents and isolate growth occurred between 3 to 11 days of a 14 day incubation period on R_2A broth. Figures 11, 12, 13, and 14 illustrate the effect of fermentation days on the antimicrobial activity of the isolates. The maximum inhibition zones for the PM58B-RA isolate (15 mm and 11.5 mm) against *M. luteus* and *M. smegmatis*, respectively, were observed on days 4 and 11, (Figure 11).

The maximum inhibition zone for the RA001 isolate (9.92 mm) against *M. luteus* was observed on day 4 (Figure 12). The maximum inhibition zone for the RA004 isolate (12.85 mm) against *A. baumannii* occurred on day 9. The maximum inhibition zone for the RA003 isolate (19.17 and 17.19 mm) against *M. luteus* and MDR-MRSA was observed on day 7. After these times, the antimicrobial activity of the four isolates decreased. However, there were obvious variations in the antimicrobial activity of the PM58B-RA, RA001, RA004 and RA003 isolates between days 4 and 11, as the inhibition zones of these isolates varied over time. For example, the inhibition zone of the PM58B-RA isolate against *M. luteus* was 9 mm on day 8, decreased to 7.93 mm on day 9 and then increased to 8.87 mm on day 10; similar trends were also observed for RA001, RA004 and RA003.

The antimicrobial activity of the PM58B-RA, RA001, RA004 and RA003 isolates was examined over a 14 day fermentation period. Reasoner and Geldreich (1985) observed that a longer incubation time (up to 14 days) led to higher bacterial counts and pigment production for slow-growing bacteria, especially in R_2A media. However, 7-12 days were reported to be best in other studies.

For example, Kay et al. (2013) found that actinomycete strains isolated from the Helmcken Falls cave displayed the maximal production of antimicrobial agents on day 4 of a 7-day incubation. On the contrary, Ripa et al. (2009) found that *Streptomyces* sp. began producing bioactive metabolites after 7 days of incubation in Czapek-Dox (alkaline) broth; however, maximal production occurred after 10 days and slowly decreased thereafter. El-Refai et al. (2011) found that maximal growth and antibiotic production by *Nocardioides luteus* were observed up to 12 days of incubation in five different media: starch casein medium, arginine glycerol salts medium, starch nitrate medium, M3 medium and ISP medium. Rule and Cheeptham (2013) reported that 100 actinomycete strains, which were collected from the Helmcken Falls cave, showed highest metabolite production when incubated in Hickey Tresner (HT) at 25°C for 7 to 10 days.

Cave microorganisms are usually slow-growing (Vartouukian et al., 2010), so they may require more time to grow in vitro. This supports the low PCV (%) values

obtained in this study for all four isolates compared to other studies. For example, Kay et al. (2012) found that strain E9, isolated from the Helmcken Falls cave, showed high PCV (%) values ranging from 0.5 to 2.5%. In this study, the maximal PCV (%) values for PM58B-RA, RA001, RA004 and RA003 were 0.12, 0.22, 0.08 and 0.07%, respectively. Even though these isolates showed low PCV (%), they demonstrated effective antimicrobial activity, especially, the RA003 isolate against MDR-MRSA.

This indicates that the ability to produce antibiotics is not fixed for microbes; some isolates were capable of producing antimicrobial agents after only a short duration of culture (8 days for RA004) whereas others kept producing antimicrobial agents up to 11 days of culture (RA003). As shown in Figures 8, 9, 10 and 11, alternating patterns of antibiotic activity were observed over time for the PM58B-RA, RA001, RA004 and RA003 isolates, which indicates that antibiotic production may increase or decrease with time without stabilizing. This may be due to alterations in the metabolic activity of the bacteria during growth in the stationary phase (Bibb, 2005).

4.4.4 Inoculation volume

Previous studies reported that smaller inoculum sizes resulted in higher viable cell counts (Casida, 1969; James and Sutherland, 1940). In our study, the 2% seed inoculum volumes led to higher rates of growth and antimicrobial activities than 1% seed inoculum volumes for all four isolates (Figures 8, 9, 10 and 11). The results of the present study show that when the volume of inoculum increased, the production of secondary metabolites and bacteria growth increased. This result is supported by Carraturo et al. (2014), who observed a positive linear correlation between the volume of inoculum and the size of the inhibition zone when studying the effect of serial dilations of phenolic compounds derived from the fleshy seed coat of Ginkgo biloba in different volumes of inoculum against different bacteria.

The results of present study are in agreement with Grag and Neelakantan (1981) and Hassan et al. (2001), which demonstrated that the size of the inoculum was an important

factor in bacterial fermentation processes. Use of 2% inoculum may decrease the time in the lag phase, during which time the bacterial cells need to adapt to the growing environment. Therefore, increasing the inoculum size will allow the cells to enter the exponential phase or stationary phase more rapidly. Further experiments could be performed using a variety of inoculum volumes, such as 0.5%, 2% and 5%, to investigate this suggestion. In our study, it appeared that the active compounds from the cave bacteria were produced at the earlier stages of fermentation when using 2% inoculum; this may be due to the rapid growth associated with a reduced lag phase. In contrast to this study, antibacterial production was directly proportional to inoculum size for *Streptomyces griseus* (Nader, 2009), and increase in cell number lead to higher production of antibacterial compounds by *Streptomyces clavuligerus* and *Aspergillus nigar* (Demain and Jermini, 1989; Berovic and Loger, 1993).

4.5 Classification and identification of the cave isolates

Classification and identification of the four isolates were based on their morphology, 16S rRNA gene sequencing and MALDI-TOF MS. A chemotaxonomic method was also employed to identify the amino acids of the four isolates; however, RA001, RA003, RA004 and PM58B-RA were difficult to classify using thin layer chromatography. LL-diaminopimelic acid, a vital part of *S. griseus* peptidoglycan, can be used to determine the similarities to other bacteria; however, none of the isolates exhibited separated spots of the whole cell hydrolsates or sonicated cell samples (Figures 17, 18, 19 and 20). As a result, the morphological and chemotaxonomic analysis demonstrated that the four isolates were not actinomycetes; they could be typical bacteria or new species. RA001, RA004 and PM58B-RA isolate are gram positive bacteria and have a thick peptidoglycan layer whereas RA003 is a gram negative bacteria. TLC may be an important method for determining the cell wall structure of these isolates but this method was not accurate and was time consuming; however, TLC may be used successfully in combination with other analytical methods such as electrophoresis or ion-exchange chromatography (Grushka and Grinberg, 2009).

The resolution of the isolate spots may be affected by humidity and temperature in the lab as this technique is an open procedure (Fair and Komos, 2008). The silica gel plate may be affected by the high humidity in the lab which could lead to pricking of the surface of the gel, even though the silica was covered with a clean plastic plate to avoid the effects of high humidity (Fair and Komos, 2008). For example, this technique should be performed at less than 60% relative humidity; however, the atmosphere of Kamloops reaches 66% relative humidity. The RA003, RA004 and PM58B-RA spots also streaked and did not separate into single spots, possibly due to the use of highly concentrated samples. On the contrary, the RA001 spot was not visible, possibly due to the low concentration of the sample for this isolate. In future experiments, dilution of the isolate solutions may help the substances to move and separate, and solve this problem (Fair and Komos, 2008). Classification using TLC is based on some chemical properties of bacterial cell walls; in this case, we used a limited TLC protocol that will reveal cell wall components specific to steptomycete and actinomycete bacteria. This may be a reason why this specific TLC is not a good technique for classifying isolated cave microorganisms other than actinomycetes.

In previous studies, genus *Arthrobacter agilis* and *Bacillus licheniformis* were found in the Cave of Bats in Zuheros, Spain, where the average temperature ranges between 8 to 14°C (Leo et al., 2012). Kampfer et al. (2002) found *Sphingopyxis terrae* in a wastewater treatment plant. Cheeptham et al. (2013) suggest that the communities of microorganisms in each cave are unique; however, researchers have attempted to find common features shared by the bacterial communities of many caves. Northup et al. (2011) suggested that caves could be a rich source of microbial phyla such as ctinobacteria, acidobacteria, and proteobacteria. Microorganisms from the same phyla are frequently discovered in different caves. Cheeptham et al. (2013) and Northup et al. (2011) suggested that a combination of culture-dependent and molecular based approaches should be employed to better understand the bacterial community of caves. It is clear that the presence of certain types of microbes is not limited in to certain types of cave, and it is possible that the isolates in this study could also be found in other caves throughout the world.

MALDI-TOF MS confirmed the identification of isolates obtained using 16S rRNA gene sequencing, demonstrating that that MALDI-TOF MS is a useful complementary technique for bacterial identification. For example, RA003 was identified at the genus level by 16S rRNA gene sequencing; however, RA003 was identified to the species level with MALDI-TOF MS.

In this study, MALDI-TOF MS proved to be a rapid technique for identification of the four isolates. The preparation of samples from the four isolates for MALDI-TOF MS using direct transfer and by formic acid method was generally complete within one hour. In contrast, 16S rRNA gene sequencing sample preparation usually takes several hours to one day. The 16S rRNA gene sequencing in this study was performed by the Biotyper 2.0 database in Seoul, Korea and it took more than two weeks to obtain the results. However one major drawback of MALDI-TOF MS is that the certainty level in the identification is lower than that of 16S rRNA sequencing. Another drawback is the lack of reference spectra for environmental isolates in the database; therefore, 16S rRNA was found to be more reliable for the identification of bacterial species.

In conclusion, use of a combination of molecular and software techniques to identify bacteria may lead to more reliable results. This study indicates that MALDI-TOF MS may be a useful tool for bacterial identification, especially for bacteria isolated from cave habitats. In addition, MALDI-TOF MS is a rapid method for identifying microorganisms grown on agar media (but not necessarily liquid media).

4.6 Assay of the antimicrobial activity of the isolates against each other

To survive in such environments, bacteria may produce antimicrobial compounds that inhibit the growth of other microorganisms, which enables them to survive and thrive in such a extreme habitat in a cooperative manner. The antimicrobial activity of the four isolates against the other three isolates was tested using the paper disc diffusion assay and cross-streak assay.

The aim of this study was to determine how cave isolates communicate through their metabolites and to preliminarily screen bacterial cave isolates to see if they produced known or novel compounds. Different methods can be employed to detect antimicrobial activity, such as diffusion methods through solid or semi-solid media (Lertcanawanichakul and Sawangnop, 2008). The cross-streak assay technique is a simple and rapid method for screening and searching for new antimicrobial compounds (Pereira and Kamat, 2011).

Montano and Henderson, (2013) reported that the cross-streak assay method was used to examine the antimicrobial activity of cave isolates against various tested microorganisms. The cave isolates were streaked in single vertical lines onto HT agar and R_2A agar plates and incubated at 25˚C for 48 h, in order to obtain a thin line of growth. Subsequently, another layer of R_2A and HT medium was added over the first layer of the medium on the original R_2A and HT plates, and the media were solidified. Then, each cave isolate was horizontally inoculated onto its vertical streak, the plates were incubated at 25˚C for 48 h and the zones of inhibition were evaluated. In this study, several problems occurred with the cross plate assay. Firstly, the bacteria grew in swirl formations on the second layer of agar, making it difficult to obtain a conclusive result. Changing the incubation time may help to overcome this issue, as cave bacteria are generally slow growing and the cross-streak assay requires a short incubation time (personal communication with Elizabeth Montano). Secondly, it was found that the thickness of second layer was crucial for accurately assaying antibacterial activity by agar diffusion. Studies have demonstrated that it is difficult to obtain quantitative data using the cross streak assay, as it produces fuzzy and unclear inhibition zones (Pereira and Kamat, 2011). Pereira and Kamat (2011) used a ‘modified cross streak method’ to quantitatively evaluate the effect of actinobacterial isolates against a number of pathogens. In the present study, the paper disc diffusion assay was more sensitive for assaying the antimicrobial activity of the isolates against each other than the cross-streak assay. In the paper disc diffusion assay, the PM58B-RA isolate inhibited the RA001, RA003 and RA004 isolates (Table 9).

Table 9 shows the inhibition zones for each of the four isolates against one another and also against *Streptomyces griseus*, which was used as our positive control standard (this isolate was purchased from the ATCC). PM58B-RA killed RA001, RA003 RA004 and *Streptomyces griseus*, suggesting that RA001, RA003 and RA004 had not been exposed to PM58B-RA and *Streptomyces griseus* before, so RA001, RA003 and RA004 may not possess any possible protection mechanisms against the active compounds that PM58B-RA produces. However, RA001, RA003 and RA004 did not inhibit the growth of PM58B-RA and *Streptomyces griseus*, suggesting these isolates may have been exposed to each other in the cave habitat and developed some protection mechanisms that prevent them from being rid of by these strains. These possible protection mechanisms must have been developed overtime and may lead to possessing of antibiotic resistant genes in those bacteria. This observation is in agreement with Forsberg and colleagues (2012) who described natural environments as reservoirs of antibiotic-resistant genes in which the genes can be transferred between soil bacteria and clinical pathogens. However, in the cave, where the microorganisms used for the screening in this study are not present, the antibiotic-resistant genes may only be transferred between the soil bacteria present in the cave, enabling them to become resistant to each other.

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Appendix

Appendix A- 1- Growth media

Hickey Tresner (HT)

1.0g Yeast Extract

1.0g Beef Extract

0.02g $CaCl_2$

2.0g N-Z Amine

15.0g Bacto Agar

pH 9.0

1L De-ionized Water

20µg/mL Nalidixic Acid

V-8 Juice

10.0g Dextrin	V8 supernatant	200.0 ml
$CaCO_3$	3.0g	
dH_2O	To 1L	
pH	6.0	
Yeast extract	0.1g/L	

Source of V-8 juice; Western Family Low Sodium vegetable juice was centrifuged at 10.000 rpm for 10 minutes to isolate the supernatant

Actinomycete Agar (Act)

2.0g Sodium Caseinate

0.1g L-Asgargine

4.0g Sodium Propionate

0.5g K_2HPO_4

0.1g Magnesium Sulfalte

0.001g Ferrous Sulfate

15.0g Bacto Agar

5mL Glycerol

pH 8.0

1L De-ionized Water

20µg/mL Nalidixic Acid

Starch Casein Nitrate Agar (SCNA)

10.0g Soluble Starch

0.3g Vitamin Free Casein

2.0g Sodium Nitrate

2.0g Sodium Chloride

2.0g K_2HPO_4

0.05g Magnesium Sulfate

0.02g Calcium Carbonate

0.01g Ferrous Sulfate

15.0g Bacto Agar

pH 8.0

1L De-ionized Water

20µg/mL Nalidixic Acid

Humic Acid Vitamin

1g Humic Acid

1.0g Yeast Extract

0.5g NaH_2PO_4

1.7g KCl

0.5g $MgSO_4$

0.02g $CaCO_3$

15.0g Bacto Agar

pH 7.0

1L De-ionized Water

20µg/mL Nalidixic Acid

International Streptomyces Project #2 (Yeast-Malt

Extract)

Yeast Extract1 4.0 g

Glucose 4.0 g

Malt Extract3 10.0 g

dH_20 To 1 L

pH 7.3

Hickey- Tresner Media

(HT)

Yeast Extract[1] 1.0 g

Beef Extract[2] 1.0 g

N-Z Amine A[3] 2.0 g

Dextrin[3] 10.0 g

pH 7.3

R_2A Media

0.5 g of yeast extract,

0.5 g of Difco Proteose Peptone no. 3 (Difco Laboratories),

0.5 g of Casamino Acids (Difco),

0.5 g of glucose,

0.5 g of soluble starch,

0.3 g of K_2HPO_4,

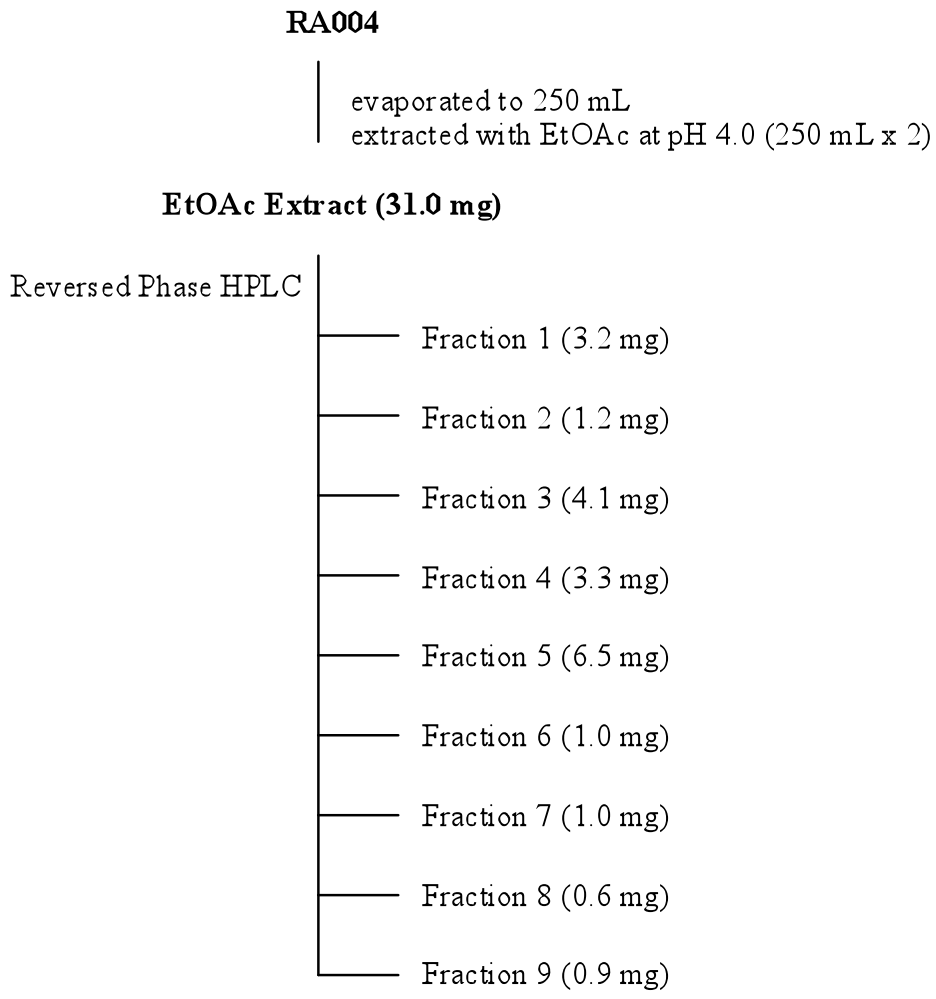
0.05 g of $MgSO_4 \times 7H_2O$,

0.3 g of sodium pyruvate,

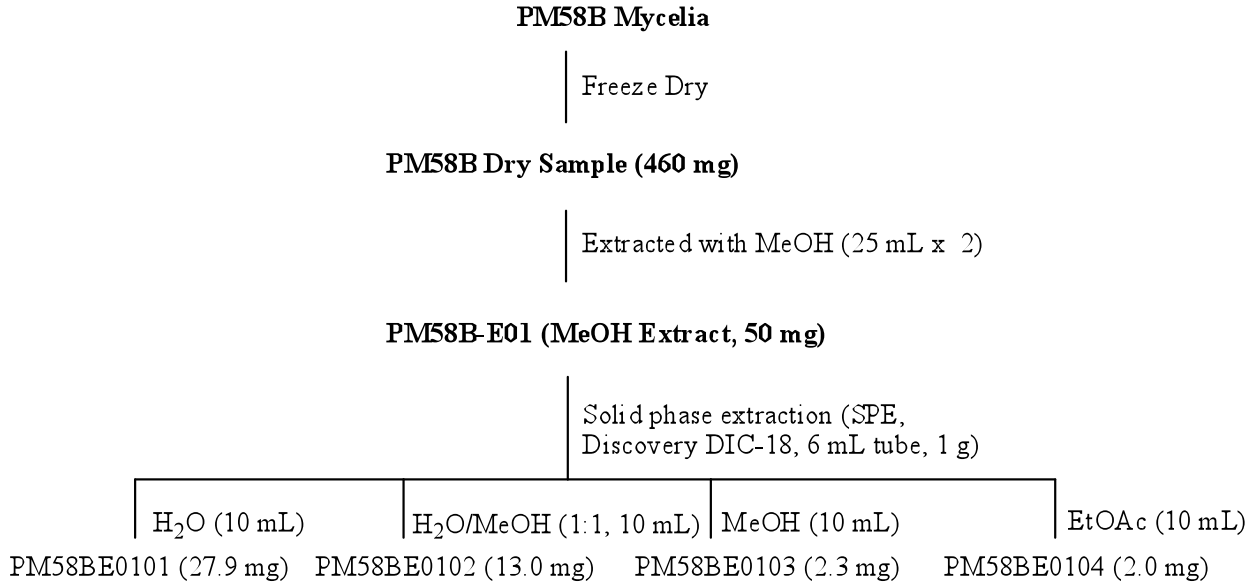
and 15 g of agar per liter of

laboratory quality water

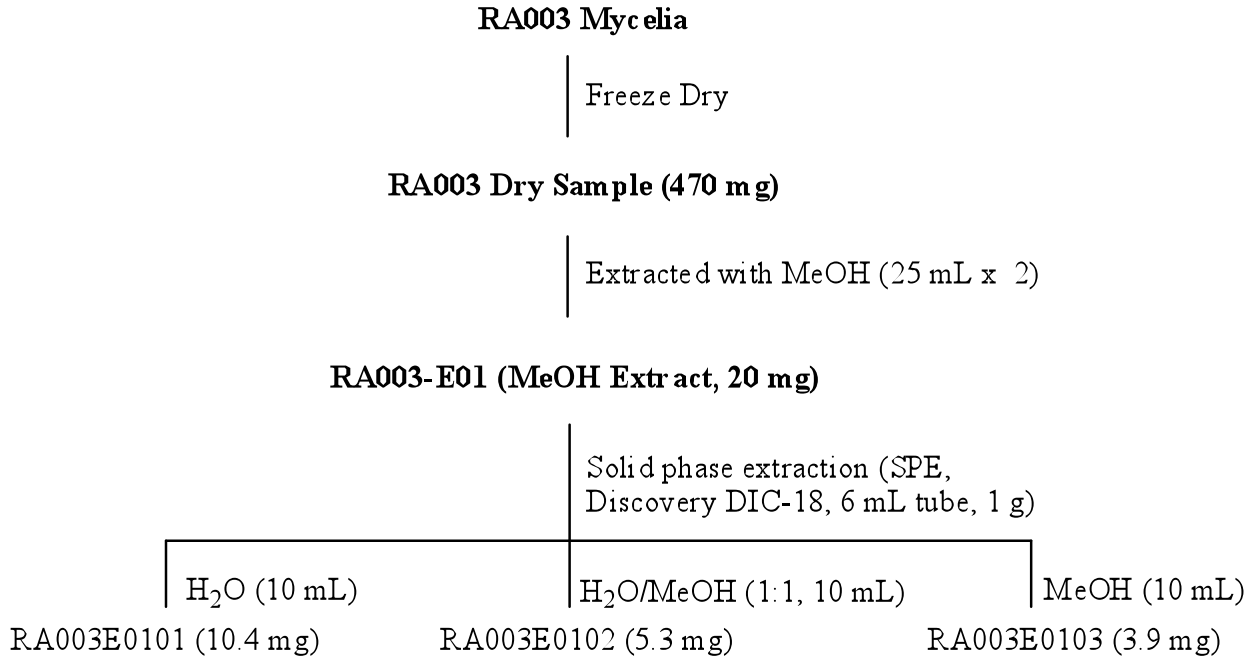
Appendix B



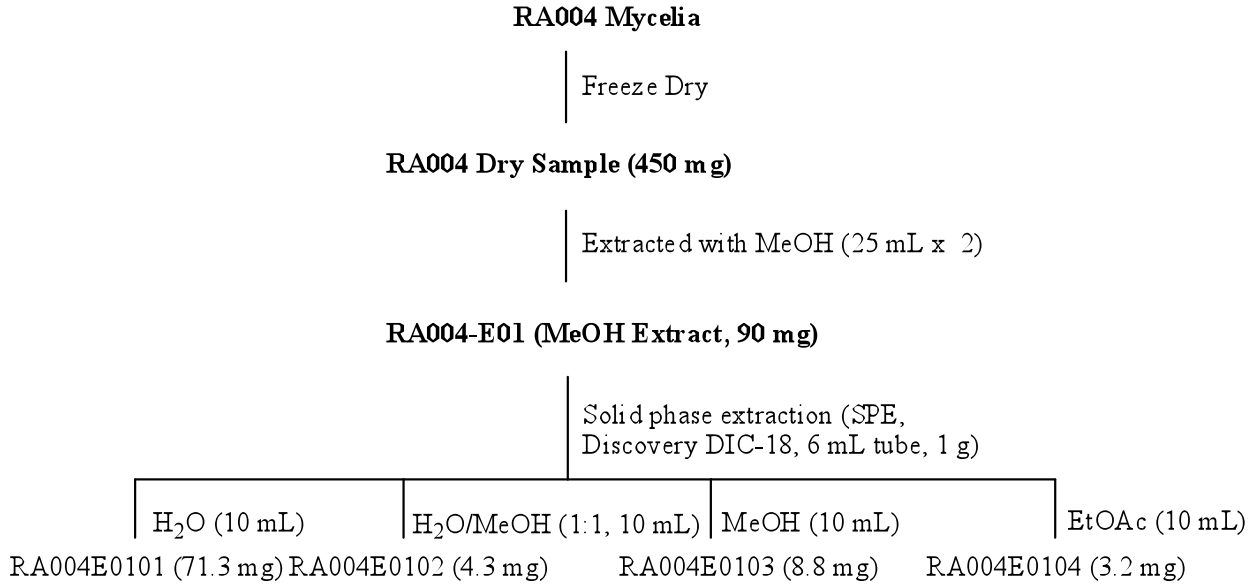
Flow chart 1: Extraction and isolation of RA004 strain using Revesed Phase HPLC.



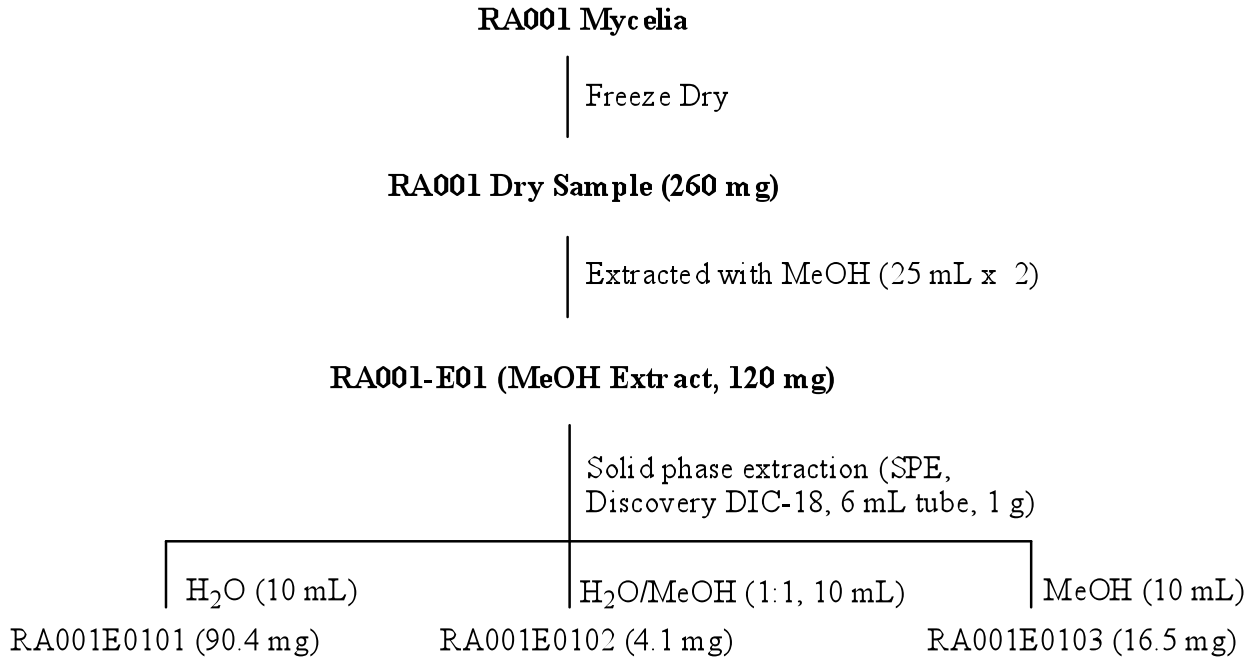
Flow chart 2: Extraction and isolation of PM58B-RA strain using Solid Phase Extraction (SPE).



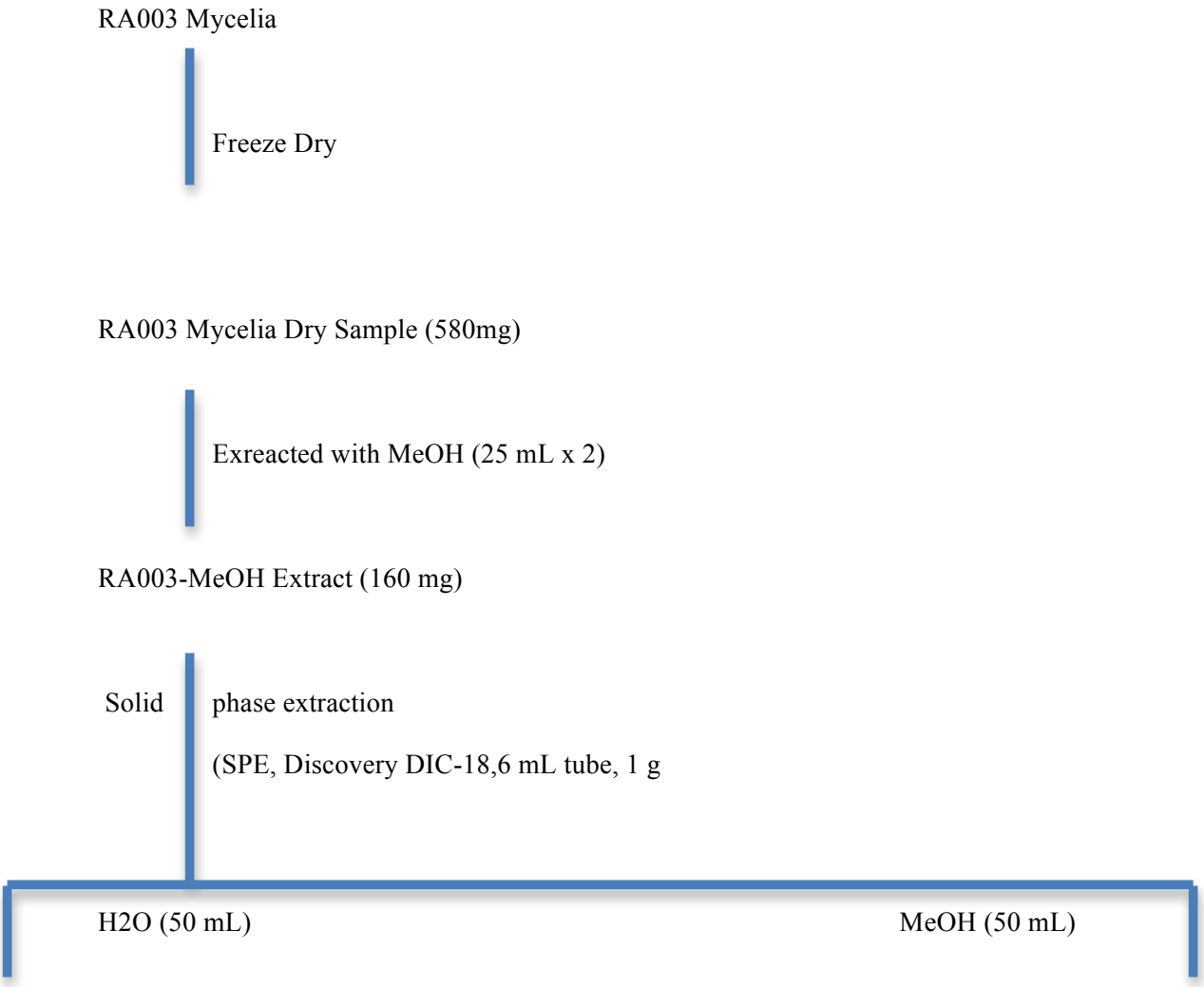
Flow chart 3: Extraction and isolation of RA003 strain using Solid Phase Extraction (SPE).



Flow chart 4: Extraction and isolation of RA004 strain using Solid Phase Extraction (SPE).



Flow chart 5: Extraction and isolation of RA001 strain using Solid Phase Extraction (SPE).



Flow chart 6: Two fractions of secondary metabolite of RA003 isolate After removing water-soluble chemicals from the culture media.

Appendix C- Isolates sequences from using 800R and 518F primer.

PM58B sequences from using 800R primer

>130423-29_C05_PM58B_RA_800R.ab1 787

CGCGGCCCTCAGCGGTCAGTTACAGACCAGAGAGTCGCCTTCGCCACTGG

TGTTCCTCCACATCTCTACGCATTTCACCGCTACACGTGGAATTCCACTC

TCCTCTTCTGCACTCAAGTTCCCCAGTTTCCAATGACCCTCCCCGGTTGA

GCCGGGGGCTTTCACATCAGACTTAAGAAACCGCCTGCGCGCGCTTTACG

CCCAATAATTCCGGACAACGCTTGCCACCTACGTATTACCGCGGCTGCTG

GCACGTAGTTAGCCGTGGCTTTCTGGTTAGGTACCGTCAAGGTACCGCCC

TATTCGAACGGTACTTGTTCTTCCCTAACAACAGAGTTTTACGATCCGAA

AACCTTCATCACTCACGCGGCGTTGCTCCGTCAGACTTTCGTCCATTGCG

GAAGATTCCCTACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTC

CCAGTGTGGCCGATCACCCTCTCAGGTCGGCTACGCATCGTCGCCTTGGT

GAGCCGTTACCTCACCAACTAGCTAATGCGCCGCGGGTCCATCTGTAAGT

GGTAGCTAAAAGCCACCTTTTATGATTGAACCATGCGGTTCAATCAAGCA

TCCGGTATTAGCCCCGGTTTCCCGGAGTTATCCCAGTCTTACAGGCAGGT

TACCCACGTGTTACTCACCCGTCCGCCGCTGACCTAAGGGAGCAAGCTCC

CGTCGGTCCGCTCGACTTGCATGTATTAGGCACGCCGCCAGCGTTCGTCC

TGACGGGGAAAACACCATATATATAAATTTCCCCCCC

PM58B sequences from using 518F primer

>130423-29_A05_PM58B_RA_518F.ab1 964

CGGGGAATTATTGGGGCGTAAAGCGCGCGCAGGCGGTTTCTTAAGTCTGA

TGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGGAACT

TGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTA

GAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTG

ACGCTGAGGCGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTA

GTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTT

AGTGCTGCAGCAAACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAA

GACTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATG

TGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTC

TGACAACCCTAGAGATAGGGCTTCCCCTTCGGGGGCAGAGTGACAGGTGG

TGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCA

ACGAGCGCAACCCTTGATCTTAGTTGCCAGCATTCAGTTGGGCACTCTAA

GGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCAT

CATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGGCAGAACAAA

GGGCAGCGAAGCCGCGAGGCTAAGCCAATCCCACAAATCTGTTCTCAGTT

CGGATCGCAGTCTGCAACTCGACTGCGTGAAGCTGGAATCGCTAGTAATC

GCGGATCACATGCCGCGGGTTGAATACGTTCCCGGGCCTTGTACACACCG

CCCGTCACACCACGAAGAGTTTGTAACACCCGAAGTCCGGTGAGGTAACC

TTTTTGGAGCCAGCCGCCGAAGGTGGGACAGATGATTGGGTGATAGAAAA

GGGGGGGGGGGGGA

RA001 sequences from using 800R primer

>130423-29_G03_RA001_800R.ab1 763

GGGCTTTTCGCTTCTCAGCGTCAGTTACAGCCCAGAGACCTGCCTTCGCC

ATCGGTGTTCCTCCTGATATCTGCGCATTTCACCGCTACACCAGGAATTC

CAGTCTCCCCTACTGCACTCTAGTCTGCCCGTACCCACCGCAGATCCGGA

GTTAAGCCCCGGACTTTCACGGCAGACGCGACAAACCGCCTACGAGCTCT

TTACGCCCAATAATTCCGGATAACGCTTGCGCCCTACGTATTACCGCGGC

TGCTGGCACGTAGTTAGCCGGCGCTTCTTCTGCAGGTACCGTCACCCACC

CAAAAGACAGGCTTCTTCCCTACTGAAAGAGGTTTACAACCCGAAGGCCT

TCATCCCTCACGCGGCGTCGCTGCATCAGGCTTGCGCCCATTGTGCAATA

TTCCCCACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGT

GTGGCCGGTCACCCTCTCAGGCCGGCTACCCGTCGTCGCCTTGGTAGGCC

ATTACCCCACCAACAAGCTGATAGGCCGCGAGTCCATCCAAAACCACAAA

AGCTTTCCACCAACATGGCATGCGCCAGAAGGTCGTATCCAGTATTAGAC

CCGGTTTCCCAGGCTTATCCCAGAGTCAAGGGCAGGTTACTCACGTGTTA

CTCACCCGTTCGCCACTAATCCCCCCCACAAGTGAGGTTCATCGTTCGACT

TGCATGTGTTAAGCACGCCGCCAGCGTTCATCCTGCGAAAAAAAAAAAAA

AAAAAAAAAANNN

RA001 sequences from using 518F primer
>130423-29_E03_RA001_518F.ab1 968

TCCGGGGAATTATTGGGGCGTAAAGAGCTCGTAGGCGGTTTGTCGCGTCT

GCCGTGAAAGTCCGGGGCTTAACTCCGGATCTGCGGTGGGTACGGGCAGA

CTAGAGTGCAGTAGGGGAGACTGGAATTCCTGGTGTAGCGGTGAAATGCG

CAGATATCAGGAGGAACACCGATGGCGAAGGCAGGTCTCTGGGCTGTAAC

TGACGCTGAGGAGCGAAAGCATGGGGAGCGAACAGGATTAGATACCCTGG

TAGTCCATGCCGTAAACGTTGGGCACTAGGTGTGGGGGACATTCCACGTT

TTCCGCGCCGTAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCG

CAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGC

ATGCGGATTAATTCGATGCAACGCGAAGAACCTTACCAAGGCTTGACATG

AACCGGAATGATGCAGAGATGTGTCAGCCACTTGTGGCCGGTTTACAGGT

GGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCG

CAACGAGCGCAACCCTCGTTCCATGTTGCCAGCGGGTTATGCCGGGGACT

CATGGGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAA

TCATCATGCCCCTTATGTCTTGGGCTTCACGCATGCTACAATGGCCGGTA

CAAAGGGTTGCGATACTGTGAGGTGGAGCTAATCCCAAAAAGCCGGTCTC

AGTTCGGATTGAGGTCTGCAACTCGACCTCATGAAGTTGGAGTCGCTAGT

AATCGCAGATCAGCAACGCTGCGGTGAATACGTTCCCGGGCCTTGTACAC

ACCGCCCGTCAAGTCACGAAAGTTGGTAACACCCGGAAGCCGGTGGCCTA

ACCCCTTGTGGGAGGGAGCCGTCGAAGGTGGGACCGGCGATTGGATATAG

AAGGAAGAGGGGGGGAAA

RA003 sequencing from using 518F primer
>130423-29_I03_RA003_518F.ab1 1006

TTCGGGGATTTACTGGGCGCGTAGAGCGCACGTAGGCGGCTTTGTAAGTC

AGAGGTGAAAGCCTGGAGCTCAACTCCAGAACTGCCTTTGAGACTGCATC

GCTTGAATCCAGGAGAGGTGAGTGGAATTCCGAGTGTAGAGGTGAAATTC

GTAGATATTCGGAAGAACACCAGTGGCGAAGGCGGCTCACTGGACTGGTA

TTGACGCTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTG

GTAGTCCACGCCGTAAACGATGATAACTAGCTGTCCGGGCACTTGGTGCT

TGGGTGGCGCAGCTAACGCATTAAGTTATCCGCCTGGGGAGTACGGTCGC

AAGATTAAAACTCAAAGGAATTGACGGGGGCCTGCACAAGCGGTGGAGCA

TGTGGTTTAATTCGAAGCAACGCGCAGAACCTTACCAGCGTTTGACATGT

CCGGACGATTTCCAGAGATGGATCTCTTCCCTTCGGGGACTGGAACACAG

GTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCC

CGCAACGAGCGCAACCCTCGCCTTTAGTTACCATCATTTAGTTGGGGACT

CTAAAGGAACCGCCGGTGATAAGCCGGAGGAAGGTGGGGATGACGTCAAG

TCCTCATGGCCCTTACGCGCTGGGCTACACACGTGCTACAATGGCGGTGA

CAGTGGGCAGCAAACTCGCGAGAGTGCGCTAATCTCCAAAAGCCGTCTCA

GTTCGGATTGTTCTCTGCAACTCGAGAGCATGAAGGCGGAATCGCTAGTA

ATCGCGGATCACCATGCCGCGGGTGAATACGTTCCCAGGCCTTGTACACA

CCGCCCGTCACACCATGGGGAGTTGGGTTCACCCGAAGGCGTTGCGCTAA

CTCGCAAGAGAGGCAGGCGACCACGGTGGGCTTAGCGACTGGGGTGAATC

TACAAAAGGAGGACCCCACATATATAAAGGGGAAGGAACGCGCGGGGGGG

TGTATC

RA003 sequencing from using 800R primer
>130423-29_K03_RA003_800R.ab1 707

AGGCCGGTTCATTACCAGGTCCAAGTGAGCCGCCTTCGCCACTGGTGTTC

TTCCGAATATCTACGAATTTCACCTCTACACTCGGAATTCCACTCACCTC

TCCTGGATTCAAGCGATGCAGTCTCAAAGGCAGTTCTGGAGTTGAGCTCC

AGGCTTTCACCTCTGACTTACAAAGCCGCCTACGTGCGCTTTACGCCCAG

TAATTCCGAACAACGCTAGCTCCCTCCGTATTACCGCGGCTGCTGGCACG

GAGTTAGCCGGAGCTTATTCTCCCGGTACTGTCATTATCATCCCGGGTAA

AAGAGCTTTACAACCCTAAGGCCTTCATCACTCACGCGGCATTGCTGGAT

CAGGCTTTCGCCCATTGTCCAATATTCCCCACTGCTGCCTCCCGTAGGAG

TCTGGGCCGTGTCTCAGTCCCAGTGTGGCTGATCATCCTCTCAGACCAGC

TAAAGATCGTCGCCTTGGTGAGCCTTTACCTCACCAACTAGCTAATCTTA

CGCGGGCTCATCCCTGGGCGATAAATCTTTGGTCTTACGACATCATCCGG

TATTAGCACGCCTTTCGGCGAGTTATTCCGAACCCAAGGGCAGATTCCCA

CGCGTTACGCACCCGTGCGCCACTAGACCCGAAGGTCTCGTTCGACTTGC

ATGTGTTAGGCATGCCGCCAGCGTTCGTTCTGAGAGAAAAAAAAAAATTA

AAAAAAN

RA004 sequencing from using 518F
>130423-29_M03_RA004_518F.ab1 978

GCGGTTTTCGGGATTATTGGGGCGTAAAGAGCTCGTAGGCGGTTTGTCGC

GTCTGCCGTGAAAGTCCGGGGCTTAACTCCGGATCTGCGGTGGGTACGGG

CAGACTAGAGTGCAGTAGGGGAGACTGGAATTCCTGGTGTAGCGGTGAAA

TGCGCAGATATCAGGAGGAACACCGATGGCGAAGGCAGGTCTCTGGGCTG

TAACTGACGCTGAGGAGCGAAAGCATGGGGAGCGAACAGGATTAGATACC

CTGGTAGTCCATGCCGTAAACGTTGGGCACTAGGTGTGGGGGACATTCCA

CGTTTTCCGCGCCGTAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACG

GCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGCG

GAGCATGCGGATTAATTCGATGCAACGCGAAGAACCTTACCAAGGCTTGA

CATGAACCGGAATGATGCAGAGATGTGTCAGCCACTTGTGGCCGGTTTAC

AGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGT

CCCGCAACGAGCGCAACCCTCGTTCCATGTTGCCAGCGGGTTATGCCGGG

GACTCATGGGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGT

CAAATCATCATGCCCCTTATGTCTTGGGCTTCACGCATGCTACAATGGCC

GGTACAAAGGGTTGCGATACTGTGAGGTGGAGCTAATCCCAAAAAGCCGG

TCTCAGTTCGGATTGAGGTCTGCAACTCGACCTCATGAAGTTGGAGTCGC

TAGTAATCGCAGATCAGCAACGCTGCGGTGAATACGTTCCCGGGCCTTGT

ACACACCGCCCGTCAAGTCACGAAAGTTGGTAACACCCGGAAGCCGGTGG

CCTAACCCCTTGTGGGAGGGAGCCGTCGAAGGTGGGACCGGCAATGGATA

TAGAAAAAAAAGGGGGAGGGGGAAAAAA

RA004 sequencing from using 800R primer
>130423-29_O03_RA004_800R.ab1 764

GCCTTCCTCCGCGGTCAGTTACAGCCCAGAGACCTGCCTTCGCCATCGGT

GTTCCTCCTGATATCTGCGCATTTCACCGCTACACCAGGAATTCCAGTCT

CCCCTACTGCACTCTAGTCTGCCCGTACCCACCGCAGATCCGGAGTTAAG

CCCCGGACTTTCACGGCAGACGCGACAAACCGCCTACGAGCTCTTTACGC

CCAATAATTCCGGATAACGCTTGCGCCCTACGTATTACCGCGGCTGCTGG

CACGTAGTTAGCCGGCGCTTCTTCTGCAGGTACCGTCACCCACCCAAAAG

ACAGGCTTCTTCCCTACTGAAAGAGGTTTACAACCCGAAGGCCTTCATCC

CTCACGCGGCGTCGCTGCATCAGGCTTGCGCCCATTGTGCAATATTCCCC

ACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCC

GGTCACCCTCTCAGGCCGGCTACCCGTCGTCGCCTTGGTAGGCCATTACC

CCACCAACAAGCTGATAGGCCGCGAGTCCATCCAAAACCACAAAAGCTTT

CCACCAACATGGCATGCGCCAGAAGGTCGTATCCAGTATTAGACCCGGTT

TCCCAGGCTTATCCCAGAGTCAAGGGCAGGTTACTCACGTGTTACTCACC

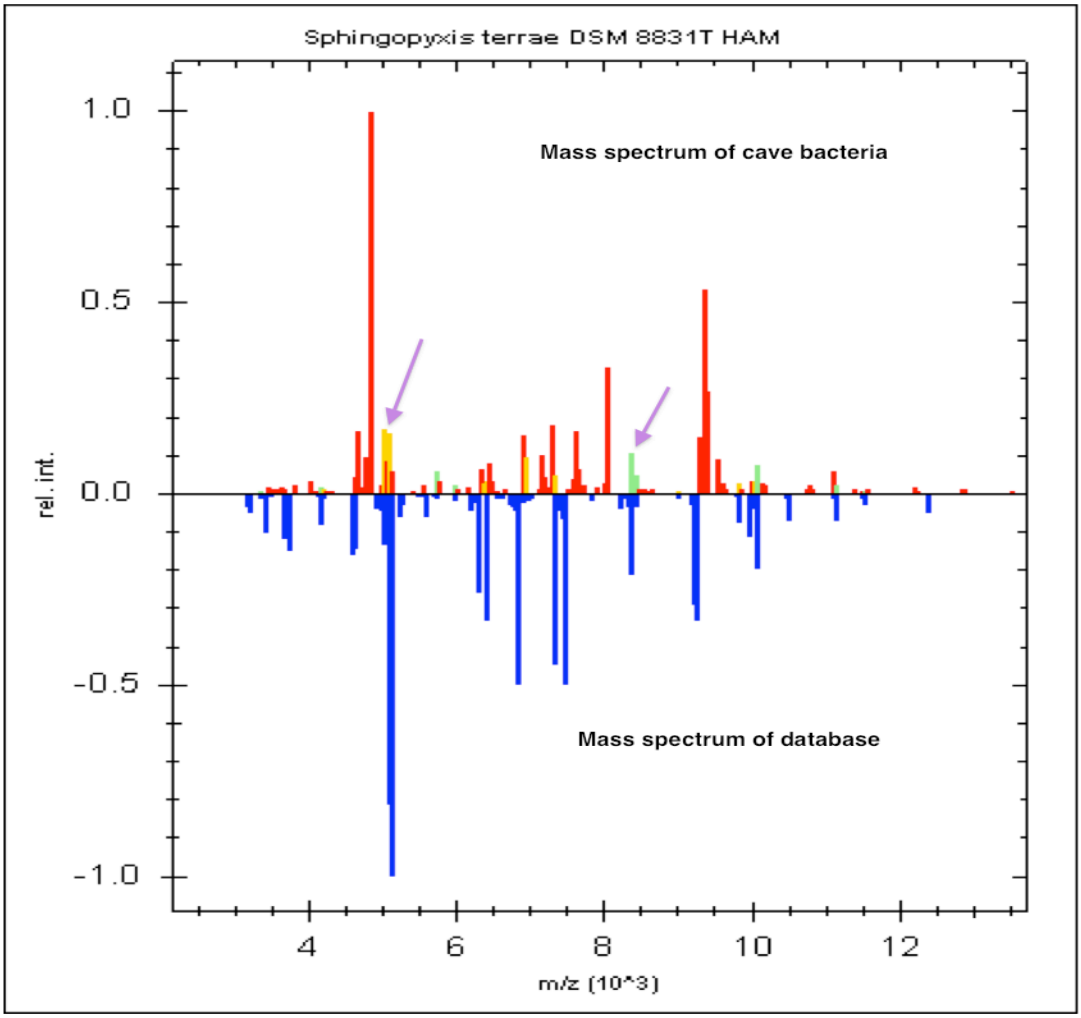
CGTTCGCCACTAATCCCCCCCACAAGTGAGGTTCATCGTTCGACTTGCATG

TGTTAAGCACGCCGCCAGCGTTCATCCTGAGCCTGACCCAAAAATTCAAA

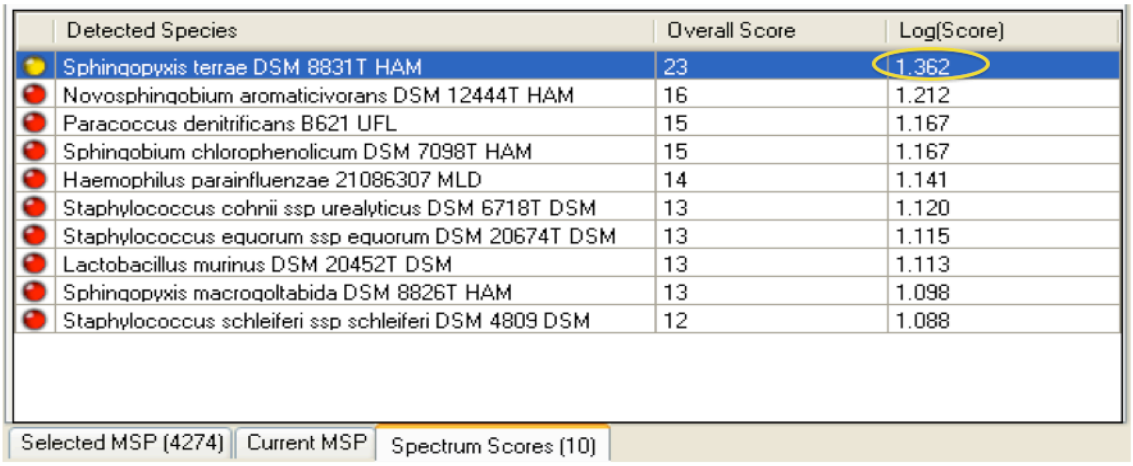
AAAGACCGCAACCC

Appendix D- Actual mass spectrum of RA003, RA001, RA004, PM58B-RA isolate

1-1: Similarity of mass spectrum of database with RA003 isolates.



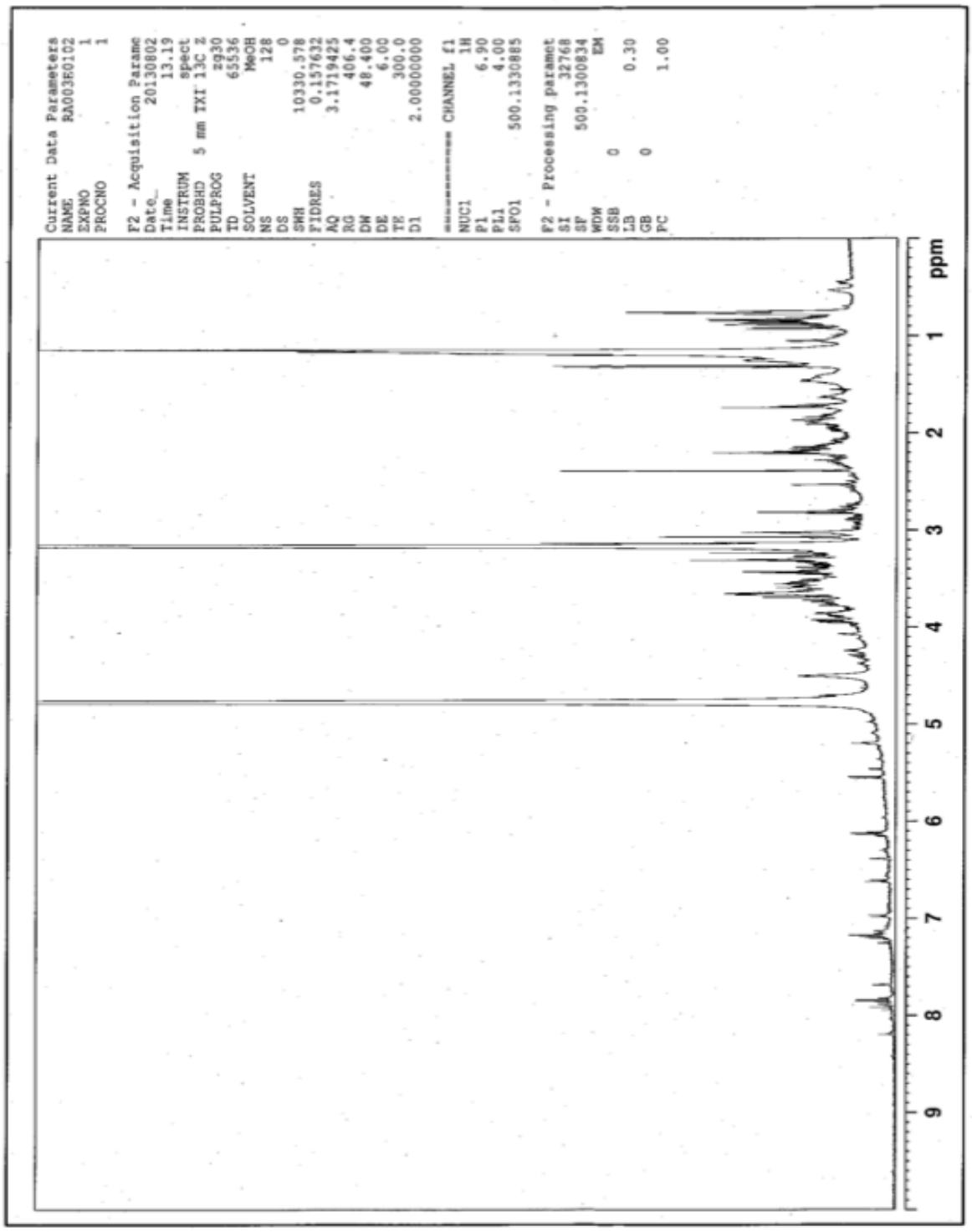
1-2: MALDI-TOF MS detected RA003 isolate at species level that had the high log score of identification (yellow circle).



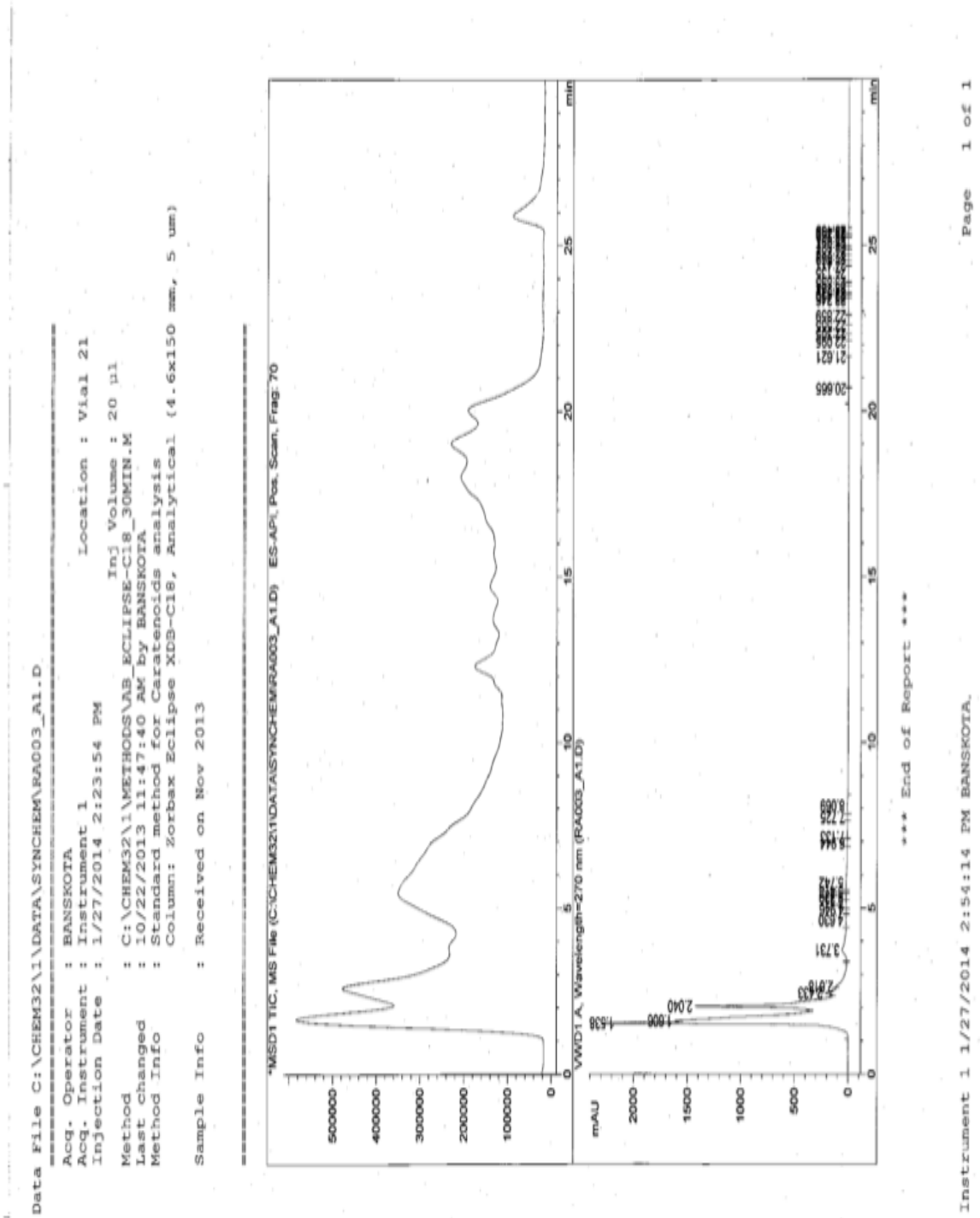
Detected Species	Overall Score	Log(Score)
Sphingopyxis terrae DSM 8831T HAM	23	1.362
Novosphingobium aromaticivorans DSM 12444T HAM	16	1.212
Paracoccus denitrificans B621 UFL	15	1.167
Sphingobium chlorophenolicum DSM 7098T HAM	15	1.167
Haemophilus parainfluenzae 21086307 MLD	14	1.141
Staphylococcus cohnii ssp urealyticus DSM 6718T DSM	13	1.120
Staphylococcus equorum ssp equorum DSM 20674T DSM	13	1.115
Lactobacillus murinus DSM 20452T DSM	13	1.113
Sphingopyxis macrogoltabida DSM 8826T HAM	13	1.098
Staphylococcus schleiferi ssp schleiferi DSM 4809 DSM	12	1.088

Selected MSP (4274) | Current MSP | Spectrum Scores (10)

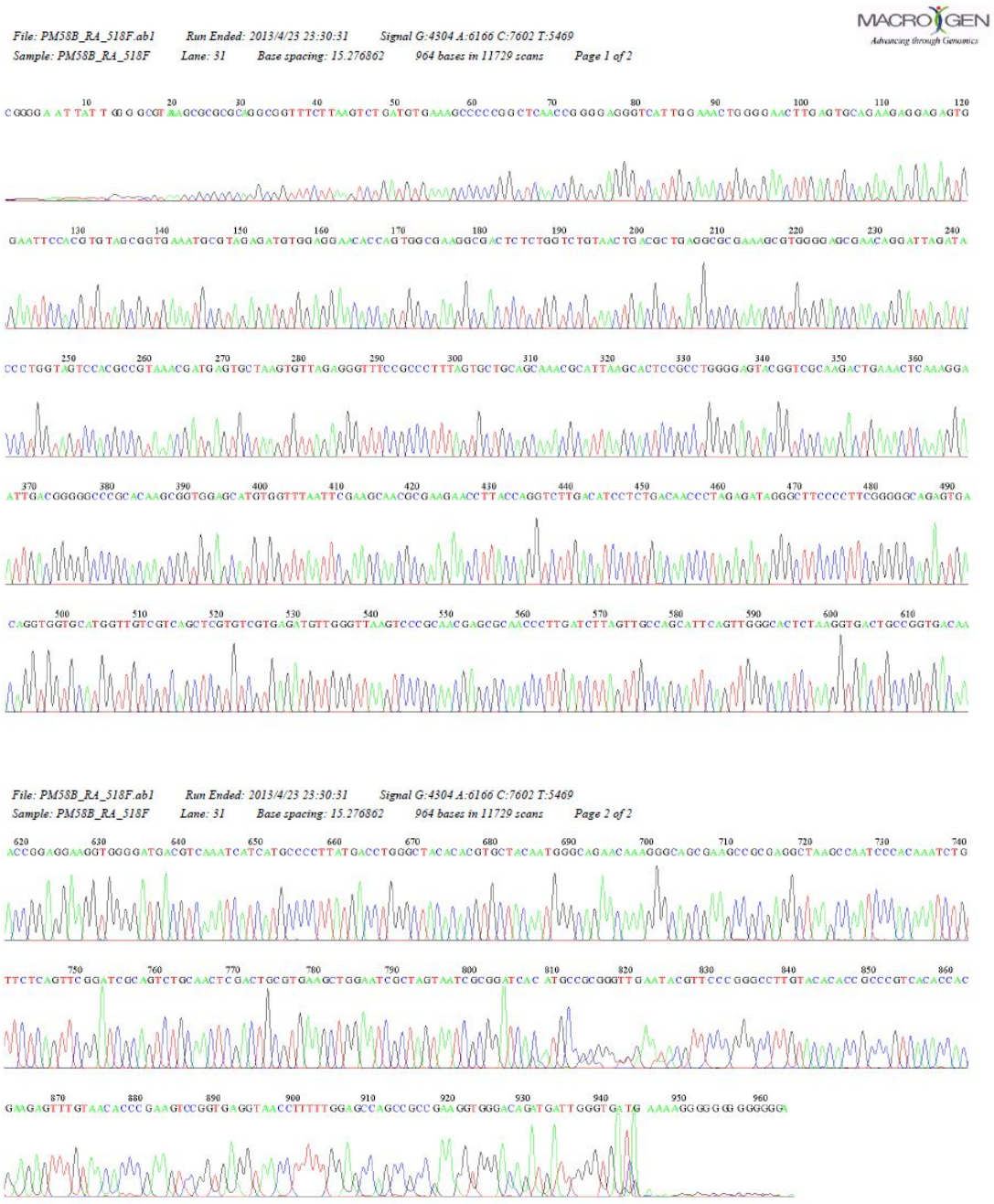
Appendix E: 1- 1H NMR of active fraction RA003E0102

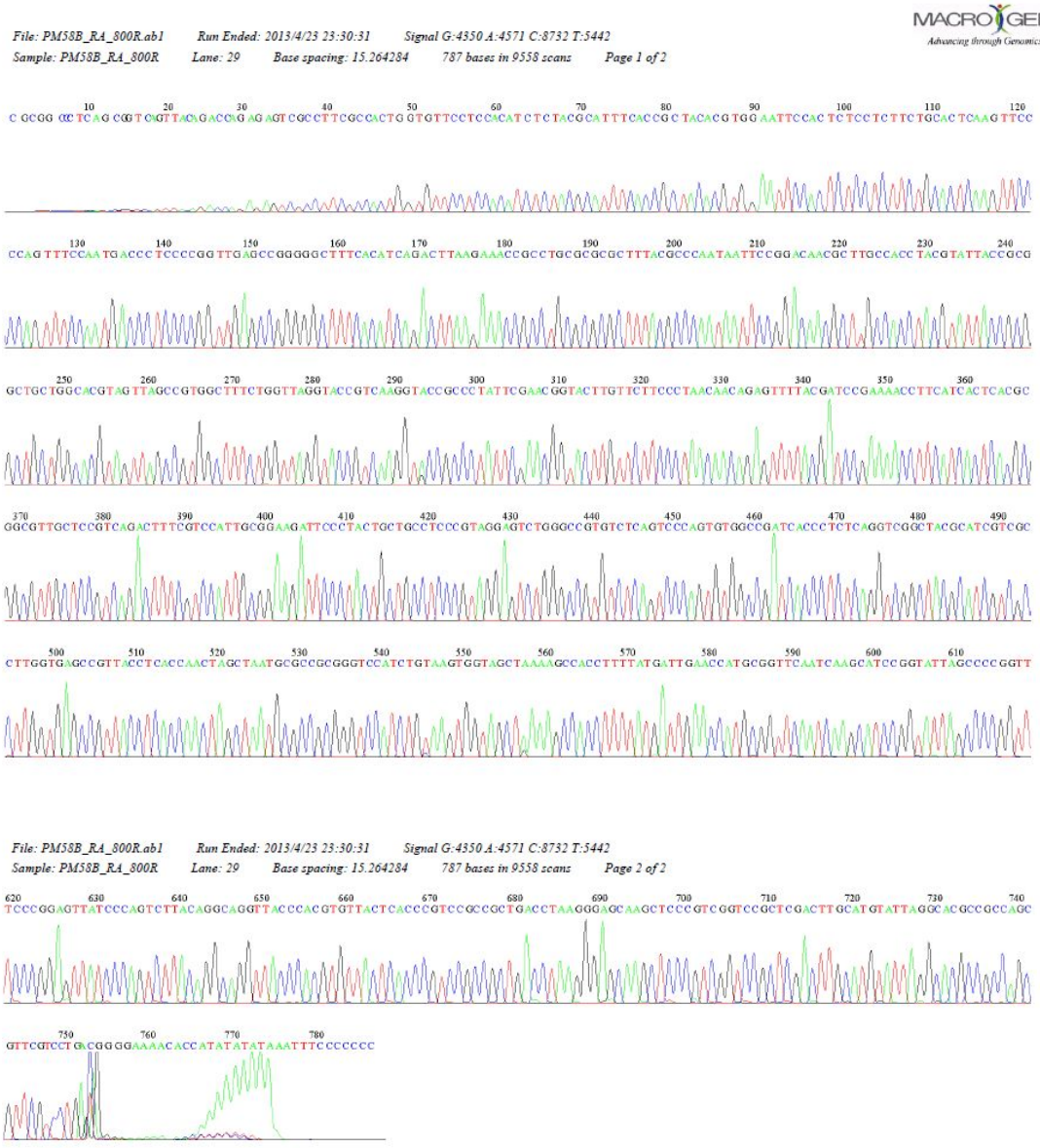


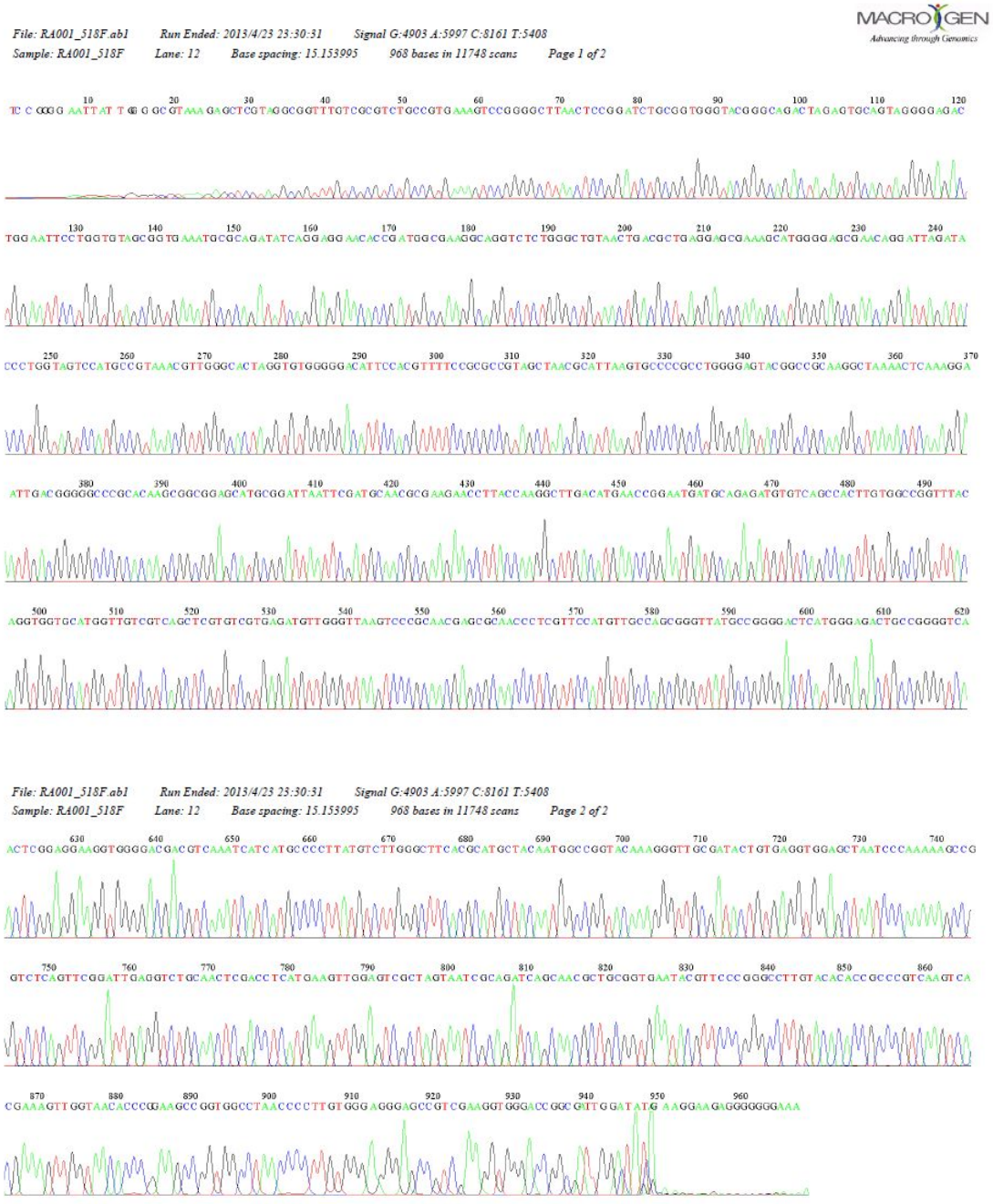
2- LC/ MS of active fraction RA003E0102



Appendix C- (continued) isolates' sequencing results.


MACROGEN
Advancing through Genomics
File: PM58B_RA_518F.ab1 Run Ended: 2013/4/23 23:30:31 Signal G:4304 A:6166 C:7602 T:5469
Sample: PM58B_RA_518F Lane: 31 Base spacing: 15.276862 964 bases in 11729 scans Page 1 of 2
File: PM58B_RA_518F.ab1 Run Ended: 2013/4/23 23:30:31 Signal G:4304 A:6166 C:7602 T:5469
Sample: PM58B_RA_518F Lane: 31 Base spacing: 15.276862 964 bases in 11729 scans Page 2 of 2

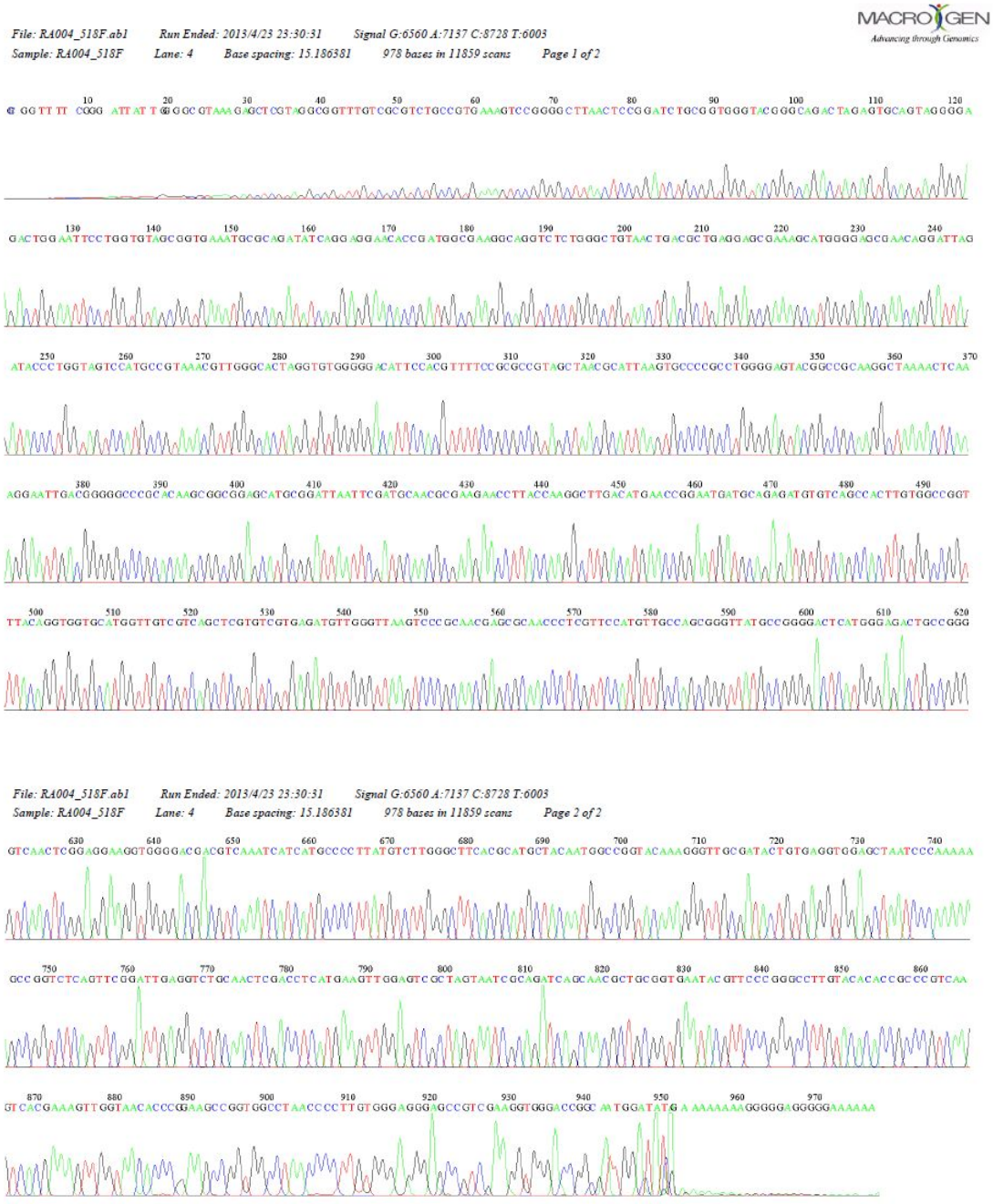

File: PM58B_RA_800R.ab1 Run Ended: 2013/4/23 23:30:31 Signal G:4350 A:4571 C:8732 T:5442
Sample: PM58B_RA_800R Lane: 29 Base spacing: 15.264284 787 bases in 9558 scans Page 1 of 2
File: PM58B_RA_800R.ab1 Run Ended: 2013/4/23 23:30:31 Signal G:4350 A:4571 C:8732 T:5442
Sample: PM58B_RA_800R Lane: 29 Base spacing: 15.264284 787 bases in 9558 scans Page 2 of 2

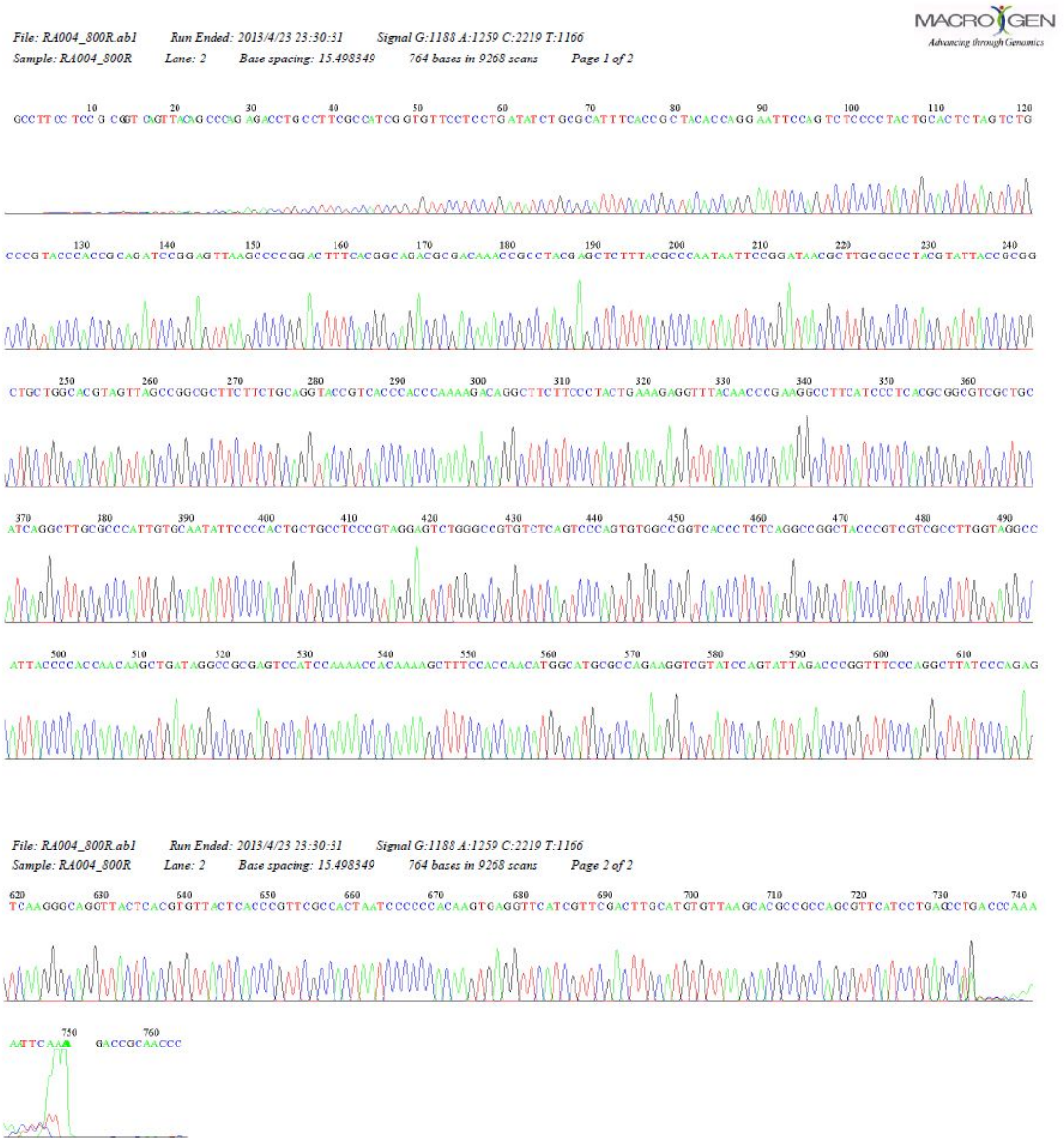

File: RA001_518F.ab1 Run Ended: 2013/4/23 23:30:31 Signal G:4903 A:5997 C:8161 T:5408
Sample: RA001_518F Lane: 12 Base spacing: 15.153995 968 bases in 11748 scans Page 1 of 2
MACROGEN
Advancing through Genomics
File: RA001_518F.ab1 Run Ended: 2013/4/23 23:30:31 Signal G:4903 A:5997 C:8161 T:5408
Sample: RA001_518F Lane: 12 Base spacing: 15.153995 968 bases in 11748 scans Page 2 of 2


File: RA001_800R.ab1 Run Ended: 2013/4/23 23:30:31 Signal G:6604 A:6979 C:14216 T:7102
Sample: RA001_800R Lane: 10 Base spacing: 15.175862 763 bases in 9066 scans Page 1 of 2
File: RA001_800R.ab1 Run Ended: 2013/4/23 23:30:31 Signal G:6604 A:6979 C:14216 T:7102
Sample: RA001_800R Lane: 10 Base spacing: 15.175862 763 bases in 9066 scans Page 2 of 2


File: RA003_518F.ab1 Run Ended: 2013/4/23 23:30:31 Signal G:1525 A:2866 C:3657 T:3362
Sample: RA003_518F Lane: 8 Base spacing: 15.153963 1006 bases in 12173 scans Page 1 of 2
MACROGEN
Advancing through Genomics
File: RA003_518F.ab1 Run Ended: 2013/4/23 23:30:31 Signal G:1525 A:2866 C:3657 T:3362
Sample: RA003_518F Lane: 8 Base spacing: 15.153963 1006 bases in 12173 scans Page 2 of 2


File: RA003_800R.ab1 Run Ended: 2013/4/23 23:30:31 Signal G:2866 A:4349 C:8700 T:6062
Sample: RA003_800R Lane: 6 Base spacing: 15.286138 707 bases in 8447 scans Page 1 of 2
File: RA003_800R.ab1 Run Ended: 2013/4/23 23:30:31 Signal G:2866 A:4349 C:8700 T:6062
Sample: RA003_800R Lane: 6 Base spacing: 15.286138 707 bases in 8447 scans Page 2 of 2


MACROGEN
Advancing through Genomics
File: RA004_518F.ab1 Run Ended: 2013/4/23 23:30:31 Signal G:6560 A:7137 C:8728 T:6003
Sample: RA004_518F Lane: 4 Base spacing: 15.186381 978 bases in 11859 scans Page 1 of 2
File: RA004_518F.ab1 Run Ended: 2013/4/23 23:30:31 Signal G:6560 A:7137 C:8728 T:6003
Sample: RA004_518F Lane: 4 Base spacing: 15.186381 978 bases in 11859 scans Page 2 of 2


File: RA004_800R.ab1 Run Ended: 2013/4/23 23:30:31 Signal G:1188 A:1259 C:2219 T:1166
Sample: RA004_800R Lane: 2 Base spacing: 15.498549 764 bases in 9268 scans Page 1 of 2
File: RA004_800R.ab1 Run Ended: 2013/4/23 23:30:31 Signal G:1188 A:1259 C:2219 T:1166
Sample: RA004_800R Lane: 2 Base spacing: 15.498549 764 bases in 9268 scans Page 2 of 2