ISOLATION AND CHARACTERISATION OF METAL-TOLERANT ACTINOMYCETES FROM A KARSTIC CAVE IN MALAYSIA

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ABSTRACT

The main objective of this study was to isolate actinomycetes from environmental samples collected from the Dark Caves, a karstic cave located in Batu Caves, Kuala Lumpur, Malaysia. The environmental samples were collected from different parts of the cave including cave ceilings, columns, soils and stalagmites. Results showed the number of actinomycetes isolated from cave soils was the highest among all sampling sites, followed by cave wall, cave columns and the lowest was from stalagmite. A few types of isolation media were used to selectively isolate actinomycetes and the results showed higher percentage of total actinomycete isolates were obtained from isolation media SM3 (a glucose peptone medium), humic-acid yeast-extract agar and tap water agar. A total of 113 actinomycete strains was isolated and characterized in terms of their morphological characteristics, ability to produce diffusible pigments and 16S rRNA gene restriction fragment patterns. Morphological observations showed many of the actinomycete strains belonged to the yellow colour series and was presumptively classified as *Streptomyces* spp. Analysis of 16S rRNA gene restriction fragment patterns revealed that the majority of actinomycete strains belonged to the genus *Streptomyces* but there were several strains belonging to the genera *Nocardia*, *Lentzea* and *Rhodococcus*. Additionally, the actinomycete strains were tested for tolerance towards copper and nickel at concentrations ranging from 50-200µg/mL. Results showed 18.6% and 51.3% of the isolated actinomycetes were able to grow on MSM supplemented with copper and nickel respectively ranging from concentration of 50µg/ml - 200µg/ml. This study reveals that a variety of actinomycetes could be found in karstic caves of Malaysia and may represent new sources of unreported genera and species.

ABSTRAK

Objektif utama projek ini adalah untuk mengisolasi aktinomiset sampel dari Dark Cave, gua karst yang terletak di Batu Caves, Kuala Lumpur, Malaysia. Sampel telah dikumpulkan dari bahagian gua termasuk siling-siling gua, kolum, tanah dan stalagmites. Pelbagai media pengasingan telah digunakan untuk mengisolasi actinomycetes dan keberkesanan media ini dibandingkan. Keputusan menunjukkan peratusan aktinomiset adalah lebih tinggi daripada media SM3 (glucose peptone medium), humic-acid yeast-extract agar and tap water agar. Sebanyak 113 strain aktinomiset telah dicirikan dari segi ciri-ciri morfologi dan keupayaan untuk menghasilkan diffusible pigmen dan corak gen restriction fragmen 16 rRNA. Pemerhatian visual menunjukkan aktinomiset yang dikategorasi dalam siri warna kuning mempunyai peratusan paling tinggi berbanding dengan siri warna yang lain, aktinomiset. Analisis corak gen restriction fragmen 16 rRNA menunjukkan majoriti strain actinomycete dikategorasi dalam genus *Streptomyces* dan beberapa strain merupakan genus *Nocardia*, *Lentzea* dan *Rhodococcus*. Di samping itu, toleransi strain aktinomiset terhadap copper dan nikel pada kontrantasi 50-200µg/mL telah diuji. Keputusan menunjukkan 18.6% dan 51.3% daripada aktinomiset isolat berupaya untuk menumbuh di MSM ditambah dengan copper dan nikel masing-masing yang terdiri daripada kontrantasi 50µg/ml - 200µg/ml. Keputusan menunjukkan 18.6% dan 51.3% daripada aktinomiset isolat dapat tumbuh di MSM ditambah dengan copper dan nikel masing-masing terdiri daripada kepekatan 50µg/ml - 200µg/ml. Kajian ini mendedahkan bahawa pelbagai aktinomiset boleh didapati di gua karstic Malaysia dan berupaya memberi maklumat tentang genera and spesies yang tidak dilaporkan sebelum ini.

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CHAPTER ONE: GENERAL INTRODUCTION

1.1 Isolation of actinomycetes from the Dark Cave, Malaysia

Karstic cave is the main target for sampling in this research as past studies suggested that actinomycetes are able to multiply to the highest degree with presence of organic matter in kastic caves (Jayasinghe & Parkinson, 2007). It is also reported that among heterotrophic bacteria, actinomycetes are the dominant population in caves (Groth and Saiz-Jimenez, 1999). Furthermore, growth of actinomycetes is greatly favored by high relative humidity and low temperature in karstic caves (Laiz *et al*., 2000). In addition, limitation of the environmental condition in the caves increases competitiveness among cave microbes for resources and thus increases likelihood to isolate microbes that are novel strains and are capable of producing beneficial substances in the pharmaceutical field.

Besides inhabiting in nutrient deprived condition, cave microbes are also devoid from sunlight. Studies carried out by Northup and colleagues demonstrated that cave microbes are more susceptible to ultraviolet radiation (UVR) than those surface microbes. As such, these bacteria that inhabit in the dark lose or inactivate traits that enable them to resist or tolerate UVR for survival when in attendance in surroundings with limited nutrients and resources (Snider *et al.*, 2009).

Such evolutional adaptations suggested that these microbes might acquire different pathways when it comes to physiological metabolisms which may lead to pharmaceutical drug discovery. The cave microorganisms are hence believed to have significant contribution to genetics studies and on the mechanisms at where their bioactive compounds are being produced.

In past studies, vast diversity of actinomycetes was isolated from Altamira cave, Cantabria, Spain (Laiz *et al*., 1999) and Grotta dei Cervi, Italy (Laiz *et al*., 2000). Nevertheless, studies of diversity of actinomycetes in Malaysia caves are relatively uncommon for their capability as potential sources of novel species and capacity on bioremediation of heavy metals. In addition, this study also provides a part of a larger study on the diversity of microbes in karstic cave in Malaysia.

1.2 Identification and characterisation of actinomycete isolates

Following isolation, the actinomycete isolates were subsequently characterized according to preliminary grouping system where isolates were first grouped based on their aerial mass and substrate mycelia color as well as their ability to produce diffusible pigmentation in ISP2 medium. Such classification scheme was originally used to systematically categorize streptomycetes into their respective colour series (Tresner & Backus, 1963). After grouping the actinomycete isolates based on colour grouping scheme, the isolates were further characterized up to genus level based on their 16S rRNA gene restriction fragment patterns. Such molecular approach provides a more comprehensive analysis for strain differentiation particularly among strains that have similar morphology on agar plates. In addition, the method was cost-effective compared to 16S rRNA gene sequencing and more practical when dealing with a large number of environmental isolates. Above and beyond, rare actinomycete strains were distinguished from common actinomycete strains and subsequently classified to different genera group. Thus, the 16S rRNA PCR-RFLP method was chosen in this study to investigate its effectiveness in characterizing actinomycete strains with combination of the colour grouping method.

1.3 Actinomycete isolates and their tolerance towards nickel (II) and copper (II)

Heavy metals play a vital role in the metabolic processes of the biota. However, they turn out to be toxic to living organisms when present at excessive concentration. The presence of metals in atmospheric, terrestrial settings and marine is ubiquitous, and geologists have found that heavy-metal rich minerals may be present in the karst environment such as speleotherm and cave deposits (White & Culver, 2012).

The contribution of toxic metals, metalloids, radionuclides and organometalloids to environmental contamination has turn out to be a major concern to the public health. A number of technologies have developed to cope with the presence and accumulation of these metals in the metal contaminated sites. However, these remediation approaches might not be commercially affordable. The interactions between metals and microorganisms have drawn much scientific attention and have lead to the discovery the biotechnological potential of using appropriate microorganisms to remediate metal contaminated sites (Szercyzk *et al.,* 2007; Villegas *et al.,* 2004). Studies revealed that toxicity of metals results from the displacement of essential metals from their native binding sites or ligand interactions. Heavy metals are listed as hazardous agent as they are capable of damaging cell membranes and DNA structure, altering enzymes specificity and disrupting cellular functions thus affecting the biochemical activities of a microorganism when appear at level where the microorganism cannot tolerate with. In that manner, microbial growth, morphology and biochemical activities are affected and consequently resulting in decreased diversity and biomass of the microorganism (Bruins *et al.,* 2000). Selective pressure arising from metals in growth environment has caused microorganisms to develop various mechanisms in order to tolerate with heavy metal stress. Previous investigations have identified some metal tolerance mechanisms including exclusion of metal ions by permeability barrier, intra- and extra-cellular

seizure of metal ions, enzymatic detoxification and reduction in cellular targets sensitivity to metal ions (Rathnayake *et al.*, 2009). The resistance mechanism applied by prokaryotes has been extensively studied and it was found that the efflux of divalent cationic metals is their preferential treatment to cope with metal contaminants (Nostrand *et al.*, 2007).

Copper and nickel tolerance/resistance mechanism(s) applied by actinomycetes are not well deliberated. For instance, extensive studies have been done on the two-component system utilized for copper resistance in Gram-negative bacteria such as Escherichia coli and *Pseudomonas* sp. and even the genetic determinants have been proposed (Munson *et al.*, 2000). Besides copper-resistance-based determinants, nickel resistance based minitransposons have been identified in Gram-negative bacterium *Ralstonia eutropha* (Taghavi *et al.*, 2001). In addition, studies have also reported the copper resistance mechanisms via ATPase operon in the Gram-positive bacterium *Enterococcus hirae* (Odermatt *et al.*, 1992).

In this study, the isolated strains were tested for their ability to tolerate copper and nickel. Preliminary screening was done by streaking plate method and results were compared with secondary screening by direct agar diffusion assay.

The objectives of this experiment were:

i) To isolate actinomycetes from environmental samples collected from the Dark Cave

ii) To characterize actinomycete strains using morphological and molecular method

iii) To screen for copper and nickel tolerant actinomycete strains

CHAPTER TWO: LITERATURE REVIEW

2.1 Actinomycetes

2.1.1 Characterisation, occurrence and nature of actinomycetes

Actinomycetes (actinobacteria) are a group of microorganism with more than 55% of G+C content in their genome and are characterised by their Gram positive and filamentous nature. The ability to inhabit all tolerable environments including hypogeal ones in the microbial community is well acknowledged particularly actinomycetes (Whitman *et al.*, 2012). They usually form spores in order to survive in harsh condition such as desiccation and starvation. However under favorable condition where sufficient moisture and nutrients are present, germination of spores by actinomycetes are possible leading to the formation of vegetative mycelium (Abdulla *et al.*, 2008).

It is reported that among heterothrophic bacteria, actinomycetes are the dominant population in caves (Groth & Saiz-Jimenez, 1999). They possess several characteristics that make them a suitable agent for bioremediation and organic compounds such as their mycelia formation, brisk colonization of selective substrates, metabolic diversity and growth characteristics (Polti *et al.*, 2006). In previous studies, (Amoroso *et al.*, 1998) have stated the possibilities of the wide spread of metal tolerance capability among actinomycetes that grow in metal contaminated environments. Besides, the actinomycetes are also well known for their role as the producers of biotechnologically significant substances such as antibiotics, vitamins and enzymes. For instance, the Streptomycetes are acknowledged for its role in producing an immense amount of antibiotics and other class of secondary metabolites that are biologically active (Raja *et al.*, 2010).

2.1.2 Industrial importance and applications of actinomycetes

The contribution of actinomycetes is indeed important in research and biotechnological field as they are known to be the main producers of beneficial substances notably the antibiotics (Gottleib, 1973). The isolation of antibiotics in systematic order began in the year of 1940 and actinomycin was the first antibiotic isolated in crystalline form, followed by isolation of streptothricin and streptomycin respectively in year 1942 and 1943 (Waksman, 1967).

In past studies, several protocols involving the traditional and unconventional methods have been developed for the characterisation and identification of filamentous actinomycetes. In most cases, the traditional methods used impose a series of specialised experiments to be conducted. Besides, it often involves the assessment of a range of biochemical reactions which requres the application of Thin Layer Chromatography (TLC). Such method has been used to differentiate the actinomycete genera into a wide chemotaxonomic group by analyzing the presence of the isomer of diaminopimelic acid (DAP) in the cell wall and the indicative sugar(s) in the whole-cell hydrolysate. Beyond just time-consuming and laborious, the results obtained have also not been always reproducible and may not be able to identify the isolate up to genus level (Cook & Meyers, 2003; Steingrube *et al.*, 1997).

2.1.3 Taxonomical classification and identification of actinomycetes

Taxanomic characterization of actinomycetes serves as an important step particularly in any screening programs for beneficial metabolites. Hence, rapid and precise identification of isolates provides significant clues to scientist on the type of metabolite produced, particularly whether the metabolite is a novel one if the isolate is identified up to species level. The classification of actinomycetes has always been largely dependent on morphological observations. In the first few editions of Bergey's manual, *Streptomyces* species was described based on morphological characteristics. However, this method alone is not sufficient to differentiate between many different genera and the outcome solely based on morphological observations might not be reliable at times. Molecular-based methods provide a faster and more accurate description on the identification of bacteria. In particular, the 16S rRNA gene restriction fragment patterns analysis allows isolates to be identified up to genus level. These fragment patterns are generated as an outcome of in silico digestions by specific restriction endonucleases on the 16S rRNA gene sequences of the isolates.

In a research done by Cook and Meyers (2003), they demonstrated that the genus *Streptomyces* could be differentiated from other actinomycete genera with only four restriction endonucleases in a week time. This molecular approach has been shown to be practical by a study conducted by Gurtler *et al.* (1991) when it comes to identifying and differentiating between different bacterial species within the *Clostridium* genus. In addition, a research done by Kohler Kohler *et al.* (1991)et al.showed that bacterial strains can be differentiated within the *Lactococcus* species based on this approach. Studies also demonstrated that the 16S rRNA gene restriction fragment patterns analysis provides useful

information in identifying numerous medically important species of aerobic actinomycetes that fall in the genera *Actinomadura, Gordonia, Nocardia, Rhodococcus, Saccharomonospora, Saccharopolyspora, Streptomyces* and *Tsukamurella* (Harvey *et al.*, 2001; Laurent *et al.*, 1999; Steingrube *et al.*, 1997; Wilson *et al.*, 1998).

2.2 Microbiological studies in karstic caves

2.2.1 Karstic cave

The process of karstification in caves has resulted in secondary mineral deposit which leads to the formation of numerous remarkable speleothems. These sedimentary rocks are mainly composed of mineral calcite and are formed through slow precipitation of calcium carbonate. Under natural circumstances, calcium carbonate precipitation is considered to arise from a series of chemical and biochemical processes. There are several possibilities for chemical precipitation to take place, such as changes in partial carbon dioxide pressure, hydrostatic pressure, temperature and pH level. As for biochemical precipitation, it happens mainly due to the production of carbonate by autotroph and heterotrophic bacteria and by fungi. There are three different polymorphic forms that calcium carbonates can be: calcite, aragonite and vaterite. Among all three forms, calcite appears to be the most stable form and is thereby the most commonly found carbonate in speleothems. Substantial investigations have suggested that the accumulation of carbonate in various natural habitats is a result of certain microorganisms where large quantities of carbonates are deposited. Nevertheless, researchers have yet to determine the exact role played

by microorganisms in speleothems formation in caves (Ercole *et al.*, 2001). A research done by Canaveras *et al.* (1999) has suggested the relationship of *Streptomyces* species with the formation of speleothems in Altamira cave. In addition, *Streptomyces* species was found to be the dominant species among the microbial communities in the Altamira cave. The finding has also suggested the presence of actinomycetes in other caves with similar environmental condition.

2.2.2 Environmental conditions in karstic caves

The environmental condition in caves and other subterranean habitats are well recognized by the limitation of nutrient supply and energy resources. However, various small aromatic compounds were found in some cave condensates that can serve as carbon and energy sources for cave microbes. Some nutrients make their way into the cave as atmospheric gases, soil-derived aromatic and polyaromatic compounds, percolating through water surface and reduce metal ions such as Mn^{2+} and Fe^{2+} (Barton & Jurado, 2007). Besides nutrient limitation, cave environment is also devoid of sunlight. In that manner, photosynthetic microbes are eliminated as primary productions derived from photosynthesis are not possible in dark condition. As a result, a complex phototrophic community known as the lampenflora was found existing in cave entrances and show caves where they obtain light sources from sunlight or cave lamps (Mulek, 2008).

The presence of cave microbes and their microbial activity can be identified through several features within the cave, such as spots occurring on surfaces, presence of biofilms and precipitation, distinctive colour formation of speleothems and structural changes (Barton, 2006). Cave microbes are able to adapt and grow at low nutrient concentrations in the cave environment; they are able to survive for long duration on various substrate. The existence of cave

microbes is commonly observed in guano, deceased animals and animal excrements where additional nutrients are present required for microbial survival. Based on a research done by Otoguro *et al.* (2001), appropriate amount of calcium carbonate and chitin were used to increase the population of the desired actinomycetes and the outcome was satisfying. Furthermore, another study has also proved that calcium carbonate treatment reduces the growth of other bacteria and fungi (El-Nakeeb & Lechevalier, 1962). For that reasons, karstic caves are the main target for sampling in this research as it is proven by researchers that actinomycetes are able to multiply to the highest degree with the presence of organic matter in karstic caves (Jayasinghe & Parkinson, 2007). Besides, the growth of actinomycetes is greatly favored by the high relative humidity and low temperature in karstic caves (Laiz *et al.*, 2000). In addition, the limitation of the environmental condition in caves increases the competitiveness among cave microbes for resources and thus increases the chance in searching for potential microbes that are capable of producing beneficial substances in the pharmaceutical field.

2.2.3 Formation and dissolution of minerals by microbial communities in caves

Previous investigations have proposed that microbial communities including cave microorganisms play important role in the concentration, dispersion and fractionation of certain substances. It is suggested that the dissolution of poorly soluble minerals like calcium carbonate, iron and manganese hydroxides into soluble compounds can be promoted by microorganisms. Besides, inorganic materials can also be acquired by microorganisms through several mechanisms

such as adsorption and cellular fixation, intracellular deposition and extracellular precipitation of insoluble compounds (Ben Omar *et al.*, 1997). On top of everything, some microorganisms play significant role in the formation of certain mineral deposits depending on the environmental condition where the microorganism is being exposed to. In this manner, different types of minerals can be produced by the same species of bacteria living in different environmental conditions. The formation of minerals by microorganisms happens via microbial metabolism at where metabolic products such as sugars, enzymes and organic acids are released. These microbial discharges are able to alter the physio-chemical environment such as the pH level. In addition, they are able to catalyze redox reactions causing a change in the redox state of metals thus promoting mineral deposition and dissolution (Ercole *et al.*, 2001). Microbial activity can also result in corrosion residues in caves and the microbes are further involved in the dissolving of host rock (Mulek, 2008).

2.3 Relation of actinomycetes and heavy metals

2.3.1 Toxicity of heavy metals and metal tolerant actinomycetes

Heavy metals are a group of elements with atomic density greater than 5g/cm^3. Metals have significant contribution to the metabolic processes of the biota. Besides being as trace elements (e.g. nickel, iron, zinc, etc.) that are essential and are required by some organisms as micro nutrients, they participate in redox processes that stabilize molecules via electrostatic interactions and act as catalyst in certain enzymatic reactions (Rathnayake *et al.*, 2009). In contrast, some heavy metals such as cadmium, mercury, lead, etc. are of no use in biological processes

and are harmful to organisms despite existing at very low concentration. Nevertheless, both of the essential and non-essential metals turn out to be toxic to the organisms when present at high concentration (Nies, 1999). The contribution of toxic metals, metalloids, radionuclides and organometalloids to environmental contamination has turn out to be a major concern to the public health. Studies revealed that toxicity of these heavy metals are resulted from the displacement of essential metals from their native binding sites or ligand interactions. Heavy metals are also listed as hazardous agent as they are capable of damaging cell membranes and DNA structure, altering enzymes specificity and disrupting cellular functions thus affecting the biochemical activities of a microorganism when appear at level where the microorganism cannot tolerate with. In that manner, microbial growth, morphology and biochemical activities are affected and consequently resulting in decreased diversity and biomass of the microorganism (Bruins *et al.*, 2000). Selective pressure arising from metals in growth environment has caused microorganisms to develop various mechanisms in order to tolerate with heavy metal stress. Previous investigations have identified some metal tolerance mechanisms including exclusion of metal ions by permeability barrier, intra- and extra-cellular seizure of metal ions, enzymatic detoxification and reduction in cellular targets sensitivity to metal ions (Rathnayake *et al.*, 2009). In past studies, the tolerance/resistance mechanism applied by prokaryotes has been broadly investigated and the efflux of divalent cationic metals was found to be their preferential resort to cope with metal contaminants (Nostrand *et al.*, 2007). Nevertheless, metal tolerance/resistance mechanism(s) applied by Gram-positive bacteria especially actinomycetes have not been well deliberated.

Bacterial tolerance/resistance towards heavy metal can also be related to the integration of prophage by phage into the bacterial cell. (Koch, 2007), have suggested that the resident prophage may benefit the bacterium host by conferring heavy metal resistance genes to the host. Therefore in this research, further studies will be carried out to detect presence of inducible prophage in the actinomycete isolates and study the effect of prophage upon strain ability to tolerate / utilize heavy metals.

The contribution of heavy metals to environmental contamination has turn out to be a major concern as heavy metals are increasingly discharged into the environment and became a threat to the public health. A number of technologies have developed to cope with the presence and accumulation of heavy metals in the heavy metal contaminated sites. However these remediation approaches might not be commercially affordable (Szercyzk *et al.*, 2007). A more practical and cost effective method using bioremediation involving the use of microorganisms to detoxify environmental contaminants should therefore be devised as an effective biotechnological approach to treat heavy metal polluted environment.

2.3.2 Heavy metals

2.3.2.1 Nickel (II) and Copper (II)

The element nickel (Ni) appears abundantly in almost every part of the biosphere. For survival purpose, microorganisms living together with the presence of nickel have evolved themselves to uptake the metal in necessary amount as trace element for the process of various metabolic pathways. Conversely, nickel will cause oxidative stress in cells when present at concentration that exceeds the microorganisms' tolerance level (Mirete *et al.*, 2007).

In most cases, Ni, with Z=28 and atomic weight 58.69 appear in either 0 or +II oxidation states as it readily loses 2 electrons, yielding Ni^{2+}. Ni can form strong complexes or organic ligands when dissolved organic matter exist. For instance, in environment where presence of calcareous water is abundant, Ni is stable as $NiHCO^{+3}$ and $NiCO_3$. Formation of these ligands may cause Ni to be quite mobile in soils with acidic pH condition, where fulvic and humic acid are formed by the decomposition of organic materials (Nieminen *et al.*, 2007). Such condition can commonly be found in karstic caves where humic acid and calcareous water are plentifully found.

Copper (Cu) exist as a compulsory element in soils and diets of all animals and plants. Studies have discovered survival and reproduction restriction in plants and/or animals species living in environment that are deficit of Cu (Albarracin *et al.*, 2010), as in the case where Cu is required as cofactor in enzymes in the electron transport system. Nevertheless, Cu is toxic when present at high concentration as it has the potential to destruct macromolecules synthesis and

other enzymatic reaction. Furthermore, Cu is capable in producing hydroxyl radicals in the presence of superoxide in its cuprous state (Rensing & Grass, 2003). The excess concentration of Cu were reported to be caused by building and construction materials, domestic products, mining, smelting, automotive parts, generation of power, burning of fossil fuels, Cu-based fungicides and fertilizer using Cu as ingredient for sewage sludge (Fairbrother *et al.*, 1999). In addition, Copper is not degradable in the environment and will ultimately mount up in soils, plants and animals (Lin *et al.*, 2008).

2.4 Phages of actinomycetes

Phages are known to be the most elementary microorganisms that depend on host cells to replicate. By way of explanation, phages are the smallest inhabitants of the soil that infect bacteria and actinomycetes. Due to its minute size, phages can only be observed in detail under an electron microscope (Burgess *et al.*, 1990). Like any other bacteria, actinomycetes are susceptible to actinophage attack and the interaction between them are highly specific (Waksman, 1967). Studies have found a symbiotic relationship between many phages and their hosts arising from the co-evolution between them especially under unfavorable environmental conditions such as presence of low nutrients level (Paul, 2008). In such circumstances, the host bacteria may undergo lysogenic conversion where the host bacteria are converted from non-virulent into virulent strains. Different from lytic process, this conversion involves a temperate or lysogenic bacteriophage infection and gives rise to a stable bacterial strain capable in expressing the genes acquired from the reside prophage. Reversely, the

prophages may be induced to undergo lytic processes either by chemical (e.g. mitomycin C) or physical factors (e.g. UV radiation) (Flegel *et al.*, 2002). The term "actinophage" is used to refer any viruses/phages that have the ability to infect and lyse members of the actinomycetes. Most phages of different bacterial genera can commonly be detected in soil where they are distinctively distributed while the rest were obtained from lysogenic wild-type strains (El-Sayed *et al.*, 2001). Bacterial tolerance/resistance towards heavy metal can also be related to the integration of prophage by phage into the bacterial cell. Koch (2007) suggested that the resident prophage might benefit the bacterium host by conferring heavy metal resistance genes to the host. Nevertheless, most studies on such molecular interaction have only been focused on coliphages and not much of research has been done on the actinophages (Koch, 2007).

2.4.1 Effects of soil condition on phages

The environmental condition of the soil in which the microorganisms inhabit, including the actinomycetes, has a profound effect on them. Indirectly, it is also affecting the phages that reside in these microorganisms. Several factors such as temperature, pH value, organic matter, presence of heavy metals, moisture content and acid pollutants significantly affect the inactivation and infectivity of phages towards their hosts. Generally, bacteriophages have longer latent period and survive longer at lower temperature. In addition, (Sykes *et al.*, 1981) reported that phages were not found in soils with pH value lower than 6.0 regardless of the existence of susceptible acidophilic hosts, which in his study was the actinomycete, in the soil as adsorption of the phages depends mainly on the electrostatic properties of the soil surface that are affected by pH condition.

Besides pH condition, parameter such as presence of organic matter weakens the electrostatic binding force of the phages to soil. In addition, divalent cations like magnesium and/or calcium, which are found in karstic caves, plays an important role on the multiplication of phages (Witzany *et al.*, 2011).

2.4.2 Replication strategies of bacteriophages

There are two different types of viral life cycles for bacteriophages: the lytic cycle and lysogenic cycle. Upon phage infection, virulent phages lyse or kill their hosts whereas temperate phages have the ability to replicate their genomes along with the host genomes without lysing their hosts. In other words, temperate phages have the option to undergo a different life cycle that forms a stable genetic relationship with their hosts at where a prophage is replicated in the occurrence with the host chromosome (Madigan & Martinko, 2006).

In lytic cycle, virulent phage infects a susceptible host and turns it into a phage factory, producing many more phage progeny. Lysis of the host bacterium that results in disruption of the infected host will eventually occur in the lytic cycle, releasing the phage progeny that have been replicated in the host bacterium into the environment (Malacinski, 2003).

Upon phage infection, temperate phages achieve the lysogenic state by replicating their genomes along with the host genomes without lysing their hosts. As mentioned above, temperate phages have the option to undergo a different life cycle that forms a stable genetic relationship with their hosts at where a prophage is replicated in the occurrence with the host chromosome (Madigan & Martinko, 2006). Studies have found a symbiotic relationship

between many phages and their hosts arising from the coevolution between them especially under unfavorable environmental conditions such as presence of low nutrients level (Paul, 2008). Nevertheless, most studies on such molecular interaction have only been focused on coliphages and not much of research has been done on the actinophages from karst caves. In this research, studies will be conducted to investigate the presence of prophages in actinomycete strains isolated from karstic cave and their relation to heavy metal tolerance.

2.4.3 Induction of lysogens from actinomycetes

Prophage induction was defined as the production of active phage in a host cell after the inactivation of its repressor. Often where virulence factors are directly acquired, host bacteria will undergo lysogenic conversion, where they are converted from non-virulent strains to virulent strains. Different from a lytic bacteriophage, the process involves a lysogenic or temperate bacteriophage infection at where a process called plasmid integration takes place. The integrated plasmid can either exist as a stable plasmid and replicate independently of host chromosome; or reside in the host as prophage at where the phage genetic material are incorporated into the host bacterial chromosome, allowing the prophage to be replicated in conjunction with the host genetic material without causing disruption to the host bacteria. The prophage may be induced into a lytic cycle via chemical method (i.e. Ethidium Bromide and mitomycin C) or physical method (i.e. UV light) (Flegel *et al.*, 2002).

Mitomycin C is an antibiotic that has been reported for its inhibitory action in a selective manner on the synthesis of DNA in bacteria cells (Crocket & Brownell,

1972). It acts as an alkylating agent that would cause direct DNA damage and activation of the recA repair system in cells, resulting in prophage induction. In a past study, it was observed that mitomycin C imposed a highly toxic and lethal effect on *actinomyces hygroscopicus* particularly when a 24-hour culture was exposed to mitomycin C with concentration of 50µg/ml. However, the toxic consequence of mitomycin C depended on several factors such as the concentration of mitomycin C, exposure time and the phase of the culture development (Pronina *et al.*, 1979). Even though mitomycin C has been categorized as a mutagenic and antineoplastic agent, it has been a common agent used in many prophage induction experiments (Iyer & Szybalski, 1963; Long *et al.*, 2007).

Ethidium bromide is well known for its application as fluorescent dye in biotechnology research. It is also recognized as a cationic stain dye for DNA where it intercalates between adjoining basepairs of double-stranded DNA to be brightly fluorescent and visible when exposed to UV light (Yonekura *et al.*, 2000). In a study done by (Kanda *et al.*, 1989), it appeared that phage in lysogenic strain was inducible by treatment with ethidium bromide. The impression behind the finding was similar to that of mitomycin C, as ethidium bromide could obstruct the synthesis of mitochondrial DNA and therefore inducing the phage from its host cell (Nass, 1972). Besides deterring mitochondrial DNA synthesis in cells, ethidium bromide was also used to induce production of antibiotics in inactive actinomycete strains as it was found that the intercalating agent was able to activate silent genes responsible for the biosynthesis of secondary metabolites (Malkina & Dudnik, 1993).

UV radiation has been commonly used as phage inducting agent in many

experiments. It is an electromagnetic radiation with wavelength of 40nm-400nm, arising between the shortest wavelength of light that is visible to human eyes and the longest wavelength of X-ray (MacDonald *et al.*, 1973). The radiation interacts directly with DNA and interferes with DNA synthesis when chromosomal replication takes place. In addition, it increases the persistence of nicks and gaps in DNA at non-permissive temperature. Treatment with UV radiation in some bacterial strains inactivates the repression system and results in the production of phage. Moreover, similar to mitomycin C treatment for phage induction, UV radiation requires protein synthesis to result in positive effect on repressor inactivation (Baluch & Sussman, 1978; Kirby *et al.*, 1967).

2.4.4 Phage induction in nickel(II) and copper(II) tolerant actinomycete isolates

The ability of the isolated actinomycete to tolerate nickel and/or copper lead to the studies of phage in actinomycetes. Like any other bacteria, actinomycetes are susceptible to actinophage attack and the interaction between them are highly specific (Waksman, 1967). Studies have found a symbiotic relationship between many phages and their hosts arising from the co-evolution between them especially under unfavorable environmental conditions such as presence of low nutrients level (Paul, 2008). In such circumstances, the host bacteria may undergo lysogenic conversion where the host bacteria are converted from non-virulent into virulent strains. Different from lytic process, this conversion involves a temperate or lysogenic bacteriophage infection and gives rise to a stable bacterial strain capable in expressing the genes acquired from the reside prophage. Reversely, the prophages may be induced to undergo lytic processes either by chemical (e.g. mitomycin C) or physical factors (e.g. UV radiation) (Flegel *et al.*, 2002). Bacterial tolerance/resistance towards heavy metal can also

be related to the integration of prophage by phage into the bacterial cell. Koch (2007) suggested that the resident prophage might benefit the bacterium host by conferring heavy metal resistance genes to the host. Nevertheless, most studies on such molecular interaction have only been focused on coliphages and not much of research has been done on the actinophages (Koch, 2007).

CHAPTER THREE: MATERIALS AND METHODS

3.1 Isolation of actinomycetes from karstic cave

3.1.1 Collection and processing of environmental samples

Environmental samples from the Dark Cave located in Kuala Lumpur, Malaysia (Figure 3.1 and Figure 3.2), were obtained from different sampling sites in the cave in March 2009 including cave soils separately from dry and wet sites, cave walls, cave column, stalagmites, dripping waters from stalactites and cave ceilings with at least two replication of sampling units within each sampling site. Pictures of sampling sites are shown in Figure 3.3 – Figure 3.6.

Sampling sites were selected based on their accessibility to researcher and location with minimal human contact and permission from Malaysian Nature Society. All samples were collected using 70% ethanol sterilized spatulas into sterile 50mL centrifuge tubes (ISC BioExpress, United States) wrapped with aluminium foils. The collected samples were then transported back to laboratory within three hours and the soil samples were then air dried for 24 hours in an incubator (Memmert, Germany) at $25^{o}C \pm 2^{o}C$. Subsequently, soil and water samples were stored at room temperature ($25^{o}C \pm 2^{o}C$) in darkness in laboratory cabinet until use for further studies.

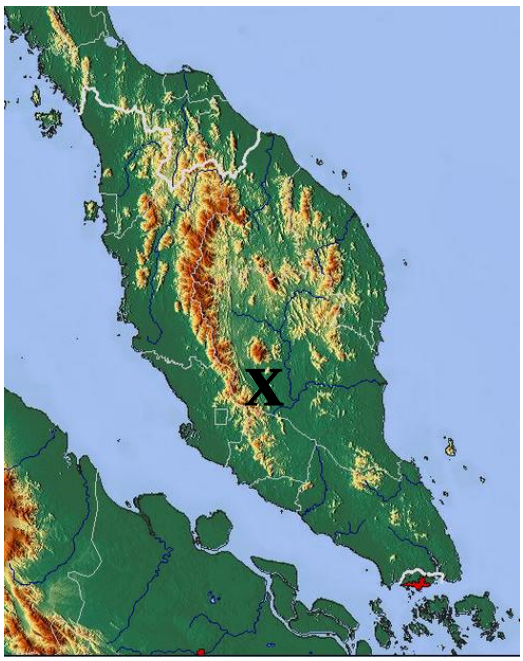

Figure 3.1 Collection of environmental samples from the Dark Cave of Batu Caves with the approximate location of the site marked with X on the map.

Figure 3.2 Exterior view of Batu Caves; arrow shows entrance into the Dark Cave.

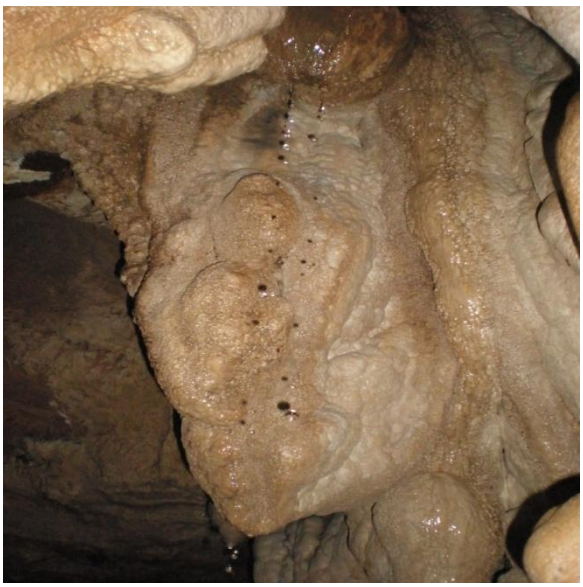

Figure 3.3 Sampling site: water drippings and cave wall

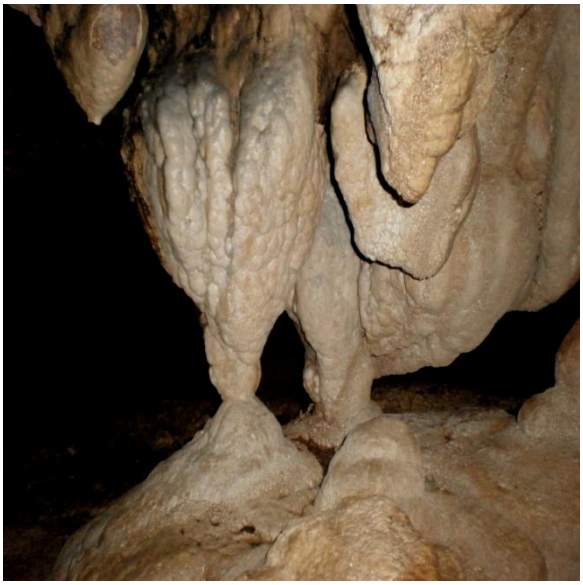

Figure 3.4 Sampling site: stalactites

Figure 3.5 Sampling site: cave ceilings

Figure 3.6 Sampling site: cave column and cave soil

3.1.2 Preparation of isolation media

A total of five isolation media were used for the isolation and enumeration of actinomycetes. These media were soil media 3 (SM3), starch casein agar (SCA), tap water agar (TWA), humic acid yeast extract agar (HYEA) and tryptic soy agar (TSA). All media were adjusted to pH 7.5. After autoclaving and cooling down media to approximately 50^{o}C, they were supplemented with filter sterilized cycloheximide (50µg/ml), nystatin (50µg/ml) and nalidixic acid (50µg/ml) to inhibit fungal contamination and minimize growth of undesired fast-growing bacteria in order to increase the actinomycete count. All antifungals and antibiotics used were purchased from Sigma-Aldrich, United Kingdom. All isolation plates were incubated at 28^{o}C for 14 days except for TWA isolation plates; they were incubated for 21 days as the growth rate of actinomycetes on TWA was slower due to the medium's poor nutrient content.

3.1.3 Serial dilution-plating of samples

Conventional dilution plate technique was applied for the isolation of actinomycetes. 1g of dried soil or 1mL of water sample (applicable only to dripping waters from stalactites) from each environmental was taken and suspended in quarter-strength 9mL of sterile Ringer solution (Thermo Scientific-Oxoid, United Kingdom). A series of dilution ranging from 10^{-1} to 10^{-6} from the suspension was done aseptically and aliquots of each dilution of the soil suspensions were spread using a sterile L-shaped glass rod on the isolation plates with two replicates per dilution. The procedure was followed by incubation for 7-21 days depending on the isolation media used at 28^{o}C. Colonies of putative

actinomycete strains that grown on the isolation plates were enumerated and regarded as mean value of Colony Forming Unit per gram dry soil weight (cfu/g) or Colony Forming Unit per milliliter water (cfu/mL) in the case of dripping waters obtained from stalactites.

3.1.4 Stock cultures

Pure colonies from 3-7 days old of each actinomycete isolates were transferred to ISP 2 agar slants in 30mL Sterilin bottles and maintained as spore suspension in 20% glycerol w/v in 1.5mL microcentrifuge tubes (ISC BioExpress, United States) to be used for subsequent investigation. The agar slants were stored in room temperature ($25^{o}C\pm$ 2 ^{o}C) and kept in plastic containers covered to preclude the incidence of light while strains in 20% (w/v) glycerol stock were kept in freezer at $-4^{o}C$ and $-70^{o}C$. All stock cultures were prepared in duplicates.

3.2 Characterisation and identification of actinomycete isolates

3.2.1 Color grouping of the actinomycete isolates

Actinomycete isolates were streaked on ISP2 medium adjusted to pH 7.3 and incubated at $28^{o}C\pm$ 2 ^{o}C for three to five days. The colour of matured or sporulating aerial mass, substrate mycelia viewed from inverted side of agar plates, and diffusible pigmentation was observed in line with method suggested by Shirling and Gottlieb (1966). Consequently, all colours were recorded using the National Bureau of Standard Color Name Charts as standard guide (National_Bureau_of_Standards, 1964) in order to minimize subjectivity and bias in naming and describing colour.

3.2.2 DNA extraction and PCR amplification of 16S rRNA genes

3.2.2.1 Genomic DNA extraction from actinomycete isolates

Actinomycete isolates were streaked on ISP2 medium adjusted to pH 7.3 and incubated at 28^{o}C± 2 ^{o}C. Subsequently, colonies of pure actinomycete isolates were picked before the occurrence of dense sporulation as presence of bacterial spores was reported to interfere or inhibit amplification of DNA. Genomic DNA of actinomycete isolates was then extracted using NucleoSpin® Extraction Kit from Macherey-Nagel, Germany. Isolation procedures were done as instructed in the user manual, at where samples were first pre-lysed with buffer T1 and proteinase K solution, followed by incubation for 12 hours at 56°C using a hot block (Grant Instruments). The pre-lysed samples were then mixed with buffer B3 and incubated for 70°C for 10 minutes followed by addition of 96-100% ethanol. Each sample were then transferred to NucleoSpin® Tissue Column attaching into a 1.5mL microcentrifuge tube and centrifuged at 11000rpm followed by discarding the flow-through. The silica membrane was washed by the addition of Buffer BW to the column and centrifuged followed by discarding the flow-through. For second wash, buffer BF was added to the column and centrifuged followed by discarding the flow through. Subsequently, silica membrane was dried by centrifugation at 11000rpm. Last of all, DNA of each sample were then eluted using pre-warmed buffer BE at 70°C along with incubation at room temperature (25^{o}C ± 2^{o}C) for 30 minutes and centrifuged. The final step was repeated twice for a higher yield of DNA as recommended in the user manual. The extracted DNA was then stored at -20°C in darkness for further studies (Appendix A, Figure A.1).

3.2.2.2 PCR reaction mixture

The extracted genomic DNA of actinomycete isolates was served as DNA template in colony PCR using PCR kit available from Fermentas (MBI Fermentas, Canada). All reaction mixtures were prepared in 1.0 ml PCR tubes (ISC BioExpress, United States). The total volume of reaction mixture was 50 µl, containing 1X PCR buffer, 0.2 mM dNTPs, 1.0 µM of each forward and reverse primers from 1st Base, 1.5 mM $MgCl_2$ and 0.25U *Taq* DNA polymerase.

3.2.2.3 PCR amplification procedure

PCR amplification was carried out using Swift™ Maxi Thermal Cycler (Esco, Malaysia). The 16S rRNA genes were amplified from genomic DNA of actinomycete isolates by PCR using universal primers 27f (5'-AGA GTT TGA TCM TGG CTC AG-3') and 1492r (5'-TAC GGY TAC CTT GTT ACG ACT T-3'). In addition, single reaction mixture without bacterial DNA was used as negative control to justify the procedure with the following conditions: initial DNA denaturation at 95 °C for 5 min, followed by 35 cycles of DNA denaturation for 30 seconds at 95°C, annealing of primers for 30 seconds at 53°C, and subsequently 90 seconds extension at 72 °C. After PCR amplification, PCR products were either analyzed instantaneously by gel electrophoresis stained with ethidium bromide (Sigma-Aldrich, United Kingdom) or stored at -20°C until use.

3.2.2.4 Agarose-gel electrophoresis of PCR products

A 2-µl of 6X gel loading buffer (Fermentas, Canada) was added to 5 µl of reaction mixture prior to electrophoresis. PCR products were subjected to electrophoresis through a 1.0% w/v agarose (ISC BioExpress, United States) dissolved in SB Buffer at 100 V for approximately 30 minutes or until the dye reached the bottom of the gel. Electrophoresis was conducted using a horizontal gel apparatus (Cleaver Scientific, United Kingdom). A 20X SB buffer was prepared by mixing 10 mL of 10N NaOH and 450 mL of sterile distilled water, followed by adjusting the pH of the mixture with boric acid to pH 8.5 at 25^{o}C. The working solution of 1.0X SB buffer was obtained by diluting the stock solution with sterile distilled water. Once electrophoresis was done, the gel was carefully placed in ethidium bromide solution at concentration of 0.5µg/ml and stained for 10 minutes. Subsequently, the gel was destained in distilled water for 20-30 minutes and PCR product bands were visualized by UV transillumination (Cleaver Scientific, United Kingdom) at 254nm and the image was captured (Canon, United States). Subsequently, PCR products were purified using QIAquick PCR purification kit (Qiagen, Germany) prior to 16S rRNA gene restriction fragment assay (Appendix A, Figure A.2).

3.2.3 Digestion of purified 16S rRNA PCR products using restriction endonucleases

Purified PCR products with volume of 0.2µg per 30µl were digested with FastDigest® endonuclease (Fermentas) at 37^{o}C *with Sau*3AI, *Kpn*I and *Hind*III, followed by thermal inactivation with respective incubation temperature and duration (Table 3.1). The DNA fragments were analyzed by gel electrophoresis stained with ethidium bromide according to procedures stated in 3.2.2.4.

Table 3.1: Restriction sties, digestion time and thermal inactivation of FastDigest® endonuclease

FastDigest® endonuclease	Recognition sequence	Digestion time with 1µl of FastDigest® endonuclease, min	Thermal inactivation
***Sau*3AI**	5'...^G A T C...3' 3'...C T A G^...5'	10	65°C, 20 min
***Kpn*I**	5'...G G T A C^C...3' 3'...C^C A T G G...5'	5	80°C, 5 min
***Hind*III**	5'...A^A G C T T...3' 3'...T T C G A^A...5'	20	80°C, 10 min

3.2.4 Identification of actinomycete strains based on 16S rRNA gene restriction fragment patterns

Restriction fragment patterns generated by each FastDigest® endonuclease was analyzed manually and compared with restriction fragment patterns listed in (Cook & Meyers, 2003)'s publication. Purified PCR products were first treated with *Sau3AI* followed by *Kpn*I digestion. Strains with similar restriction fragment patterns after treated with *Sau*3AI and *Kpn*I were classified under the same group and representative strains were selected from each group for *Hind*III digestion. The size of each restriction fragment formed was approximately determined to the nearest base pairs indicated by the DNA ladder (GeneRuler^{TM} Fermentas, Canada).

Finally, actinomycete strains were group according the restriction fragment patterns generated and representative strains were selected from each group for 16S rRNA partial sequencing for identification purpose.

3.3 Actinomycete isolates and their tolerance towards nickel (II) and copper (II)

3.3.1 Streaking-plate method

Actinomycete strains were tested for their tolerance towards nickel and copper. Strains were streaked on basal Mineral Salt Medium (MSM) supplemented with nickel (II) nitrate hexahydrate and copper (II) nitrate trihydrate (Sigma-Aldrich, United Kingdom) separately at concentration of 50µg/ml, 100µg/ml, 150µg/ml and 200µg/ml and incubated for 1-4 weeks at 28^{o}C. Growth of actinomycetes on MSM supplemented with heavy metals indicates positive results while observation of zero growth of actinomycete strains on MSM media supplemented with each heavy metal separately indicates negative result. The composition of the MSM used in this study was as follow: K_2HPO_4, 8g; KH_2PO_4, 1g; $(NH_4)_2.SO_4$, 0.5g; $MgSO_4.7H_2O$, 0.2g; $Ca(NO_3)_2$, 0.1g; $CaCl_2.2H_2O$, 20mg; $FeSO_4.7H_2O$, 0.5mg; $Na_2MoO_4.H2O$, 0.5mg; $MnSO_4$, 0.5mg; yeast extract, 25mg; agar, 20g; distilled water, 1L at pH 7.5.

3.3.2 Direct agar diffusion method

Ten microliter of each heavy metal solution (nickel and copper) of four different concentrations (50µg/ml, 100µg/ml, 150µg/ml and 200µg/ml) was placed on MSM lawned with actinomycete strains using Eppendorf (United Kingdom) micropipette and incubated for 1-4weeks at 28^{o}C. Sterile distilled water was used in this assay as negative control. The diameter of clearing zone exhibited was measured in millimeters and used in heavy metal tolerance assessment.

3.4 Phage induction in heavy metal tolerant actinomycete isolates

3.4.1 Bacteriophage induction via ultraviolet radiation

In ultraviolet induction experiment, one loopful of each young actinomycete culture strains was transferred to 1.5ml fresh ISP2 broth at pH7.2. Strains were then irradiated with ultraviolet light in an incubator with constant swirling using a tube rotator (Barloworld Scientific Ltd., United Kingdom) at 25^{o}C±2 ^{o}C. The absorbance of each actinomycete strains was measured at 600nm at one-hour interval of time, until a decrease of the optical density was observed with a spectrophotometer (Eppendorf Biophotometer, United Kingdom). Negative controls were included for each sample where strains were protected from ultraviolet light with aluminum foils. Each culture was then centrifuged at 3000 x g for 12 minutes and the supernatant was subsequently neutralize to pH7.0 with sterile 0.1N NaOH and filtered using a Minisart® syringe filter (Sigma-Aldrich, United Kingdom) with pore size of 0.45µm. The obtained sterile supernatants were kept at 4^{o}C in dark until use. The experiment was performed in duplicates for each strain.

3.4.2 Bacteriophage induction via mitomycin-C

The induction of bacteriophage with mitomycin-C (Sigma-Aldrich, United Kingdom) was performed by transferring one loopful of each young actinomycete culture strains into 1.5ml fresh ISP2 broth at where mitomycin-C was added to yield a final concentrations of 0 µg/mL (negative control), 5 µg/mL, 10 µg/mL and 20 µg/mL. The cultures in the mixtures were swirled at constant speed with a tube rotator at 25^{o}C±2 ^{o}C. The absorbance of each actinomycete strains was measured at 600nm at one-hour interval of time until a decrease of the optical density was observed. Each culture was then centrifuged at 3000 x g for 12 minutes and the supernatant was subsequently neutralize to pH7.0 with sterile 0.1N NaOH and filtered using a Minisart® syringe filter with pore size of 0.45µm. The obtained sterile supernatants were kept at 4^{o}C in dark until use. The experiment was performed in duplicates for each strain.

3.4.3 Bacteriophage induction via ethidium bromide

The induction of bacteriophage with ethidium bromide was performed by transferring one loopful of each young actinomycete culture strains into 1.5ml fresh ISP2 broth at where ethidium bromide was added to yield a final concentrations of 0.0 mM (negative control), 0.5 mM, 3.0 Mm and 5.0 mM. The cultures in the mixtures were swirled at constant speed with a tube rotator at 25^{o}C±2 ^{o}C. The absorbance of each actinomycete strains was measured at 600nm at one-hour interval of time until a decrease of the optical density was observed. Each culture was then centrifuged at 3000 x g for 12 minutes and the supernatant was subsequently neutralize to pH7.0 with sterile 0.1N NaOH and filtered using Minisart® syringe filter with pore size of 0.45µm. The obtained sterile supernatants were kept at 4^{o}C in dark until use. The experiment was

performed in duplicates for each strain.

3.4.4 Determination of the presence of induced bacteriophages in copper (II) and nickel (II) tolerant actinomycete strains by direct plating plaque assay

Actinomycete broth culture (300µL) was then incorporated into 3mL of ISP2 soft agar (0.7% agar) and poured evenly to petri dish with solidified ISP2 bottom agar. Subsequently, 3mL of sterile supernatant obtained from induction assay was poured over the solidified agar and incubated at 37^{o}C for 24-48 hours. Presence of bacteriophages was then observed in the form of clearing zones formed. The assay was performed in duplicates for each sample.

CHAPTER FOUR: RESULTS AND DISCUSSIONS

4.1 Isolation of actinomycetes from karstic cave

4.1.1 Assessment on distribution of actinomycetes in karstic cave

Table 4.1 Enumeration of actinomycetes from each sampling sites in the Dark Cave.

Sampling site	pH	Putative actinomycetes (cfu/g)	Total bacteria (cfu/g)
Cave ceiling	7.7		
HYEA		4.74×10^4	3.65×10^5
SCA		3.38×10^3	1.62×10^5
TSA		3.38×10^4	4.15×10^6
Stalagmite	7.7		
HYEA		7.36×10^3	2.26×10^5
TSA		3.86×10^4	2.93×10^6
Column	7.6		
HYEA		2.84×10^3	3.65×10^5
TWA		1.42×10^4	1.60×10^5
SM3		3.79×10^3	1.23×10^4
TSA		5.68×10^4	9.91×10^5
Cave soil (dry)	7.9		
HYEA		3.99×10^3	3.85×10^4
TWA		1.10×10^4	3.39×10^5
SCA		6.58×10^4	2.67×10^5
SM3		3.29×10^3	2.16×10^5
TSA		1.08×10^8	1.91×10^9
Cave soil (wet)	7.7		
TWA		4.19×10^3	1.54×10^5
TSA		1.60×10^5	6.41×10^6
SM3		4.01×10^3	4.86×10^4

Five isolation media were used for the isolation of actinomycetes including TWA, TSA, SCA, HYEA and SM3; and colony forming unit per one gram (cfu/g) of soil was determined (Table 4.1). Results showed concentration of

actinomycetes isolated from cave soils was the highest among all sampling sites, followed by cave wall, cave columns and the lowest was from stalagmite while no isolates were obtained from dripping waters from stalactites. The results may be explained based on the fact that soil has more organic matter and nutrient content compared to cave wall, cave columns and stalagmites, which mainly composed of carbonate minerals (Fischbeck & Müller, 1971). Furthermore, insignificant actinomycete counts from dripping water was supported by studies done by Laiz *et al*. (1999) and Schwabe *et al.* (2006) at where low proportions of Gram-positive bacteria and absence of culturable actinomycetes in dripping waters compared with sediments and soils from caves in Bahamas and Altamira Cave was observed. These findings suggested that water microbial communities were different from those of cave soil, sediments and deposits. In addition, both studies suggested that actinomycetes remained in the rocks and were not emancipated into water that travelled through the rocks and dripped downwards, explaining the significant low count of culturable isolates in dripping water.

In this study, cycloheximide, nystatin and nalidixic acid at concentration of 50ug/ml were used to inhibit growth of undesired fungus and fast-growing bacteria (Maier *et al.*, 2009). However, in this study, the incorporation of antimicrobial agents into isolation media have failed to minimized the growth of undesired bacteria effectively. The overgrew bacteria may have possibly inhibited the growth of desired actinomycete colonies on some of the isolation plates and affect the actual CFU count of actinomycetes in the environmental samples obtained. This incident often resulted in non-quantitative dilution, at where within the same series of dilution, actinomycetes counts of a higher dilution were equivalent or more than the actinomycetes counts of a lower

dilution (Jensen *et al.*, 1991).

4.1.2 Isolation and purification of actinomycete

Table 4.2: Number of isolates isolated from different types of isolation media.

Type of isolation medium	Source of isolation	Actinomycete isolates no.
SM3	Cave soil, cave ceiling, stalagmite and cave column	60
Humic acid yeast extract agar	Cave soil, cave column and stalagmite	25
Tap water agar	Cave soil and stalagmite	16
Starch casein agar	Cave soil, cave ceiling and cave column	8
Tryptone soy agar	Cave soil	4
	Total:	**113**

Higher percentage of total actinomycete isolates were obtained from isolation media SM3 (53.1%), HYEA (22.1%) and TWA (14.2%) followed by SCA (7.1%) and TSA (3.5%), and source of isolation was mainly from the cave soil (Table 4.2). SM3 medium was designed to selectively isolate members of the genus *Amycolatopsis* by (Tan *et al.*, 2006). However, in this study, a large number of streptomycete colonies ranging from pale yellow to grey were observed and isolated using this medium that were mainly composed of glucose, peptone and sodium chloride. The addition of peptone into SM3 was important as it serves as an important nitrogen source for actinomycete colonies on the isolation plates while glucose serving as the primary carbon source. In addition, sodium chloride was added to maintain the osmotic balance of the medium,

which is important to promote cell growth (Shabala, 2009). Similarly to SM3 medium, TSA which mainly composed of tryptone, soytone and sodium chloride yielded a significant amount of actinomycete isolates in this study.

HYEA was a modified medium from HV medium, which is known to be the selective medium for the isolation of soil actinomycetes formulated by (Hayakawa & Nonomura, 1987). However in this study, attempt to obtain actinomycete isolates from HV medium was unsuccessful as HV isolation plates were overgrew by fast-growing bacteria that could have possibly suppressed the growth of actinomycete colonies. In order to overcome the problem, yeast extract, serving as nitrogen source, was added into the isolation medium to support the growth of any potential actinomycete colonies (Khanna *et al.*, 2011). Consequently, an increased number of actinomycete colonies was observed and isolated.

4.2 Characterisation and identification of actinomycete isolates

4.2.1 Color grouping of actinomycete isolates

With reference to Colour-name Charts Illustrated with Centroid Colours (ISCC-NBS), actinomycete isolates were assigned to eight main colour series including yellow, orange, white, brown, grey, pink, red and green based on visual examination of the colour of their aerial mycelium. The actinomycete isolates were cultured on ISP2 medium that have been incubated at 28 ± 1 ^{o}C for three to seven days. For colour series of red, orange and grey, different colour shades of the same aerial mycelium were observed. Additionally, colour of substrate mycelium and diffusible pigmentation for each isolate was observed and recorded for chracterisation purpose, and microscopic observation for CY8, HY8, TWA12 and SM2G1 was done (Fig A.3 to A.6). A summary of the colour grouping of all isolates was tabulated in Table 4.3. (Details of color grouping of all isolates were summarized in Appendix A, Table A.1) Colour grouping of the test isolates into different main colour series based on the colour of aerial mycelium formed allows greater efficiency in managing all the isolates. Representative strains from each colour group were then randomly selected for further examination.

Table 4.3: Colour grouping of actinomycete isolates into eight general colour series based on visual examination of aerial mycelium colour cultured on ISP2 medium that have been incubated at 28 ± 1 ^{o}C for three to seven days.

Colour series	Number of isolates	Different colour shades of aerial mycelium	Colour of substrate mycelium	Diffusible pigmentation	Representa -tive strains
Yellow	35 (30.97%)	-	Pale/dark yellow; moderate olive	Moderate brown; moderate olive	SCA4, TWA17
Orange	22 (19.45%)	Moderate/dar k orange yellow; vivid reddish orange	Strong/moderat e orange; brilliant orange yellow	Orange	CY118, CY24
Grey	19 (16.84%)	Grayish-purple; grayish-pink; yellowish-gray; light greenish-gray	Dark yellow; dark olive, strong/dark/mo derate brown; deep/deep reddish orange	Moderate olive; deep/moderat e/brownish orange; blackish purple	CY16, CY22
White	18 (15.93%)	-	Moderate yellow	-	CY89, SCA2
Brown	8 (7.08%)	-	-	-	SCA3,
Pink	6 (5.31%)	-	Yellow; slight orange yellow	Deep orange yellow	CY138
Red	4 (3.54%)	Moderate/dar k red; reddish purple	-	Moderate/dar k red; deep orange yellow	CY22
Green	1 (0.88%)	-	Dark olive green	-	SCA6

In this study, visual observation showed majority of the test isolates belonged to the yellow and orange series where 30.97% and 19.45% of isolates were grouped respectively into the two major colour series, followed by grey and white colour series which each comprised of 16.84%. Both brown and pink colour series have 7.08% and 5.31% of isolates respectively and a minority (3.54%) of the isolates was observed to be in the red color series and only one (0.88%) out of 113 isolates appeared to produce green aerial mycelium. All isolates belonged to the yellow colour series which had the highest number of isolates were presumed to be *Streptomyces* in this study wherein similar to what had reported in past investigation on the diversity of actinomycetes in soils, *Streptomyces* was known to be the predominant isolates (Fischbeck & Müller, 1971).

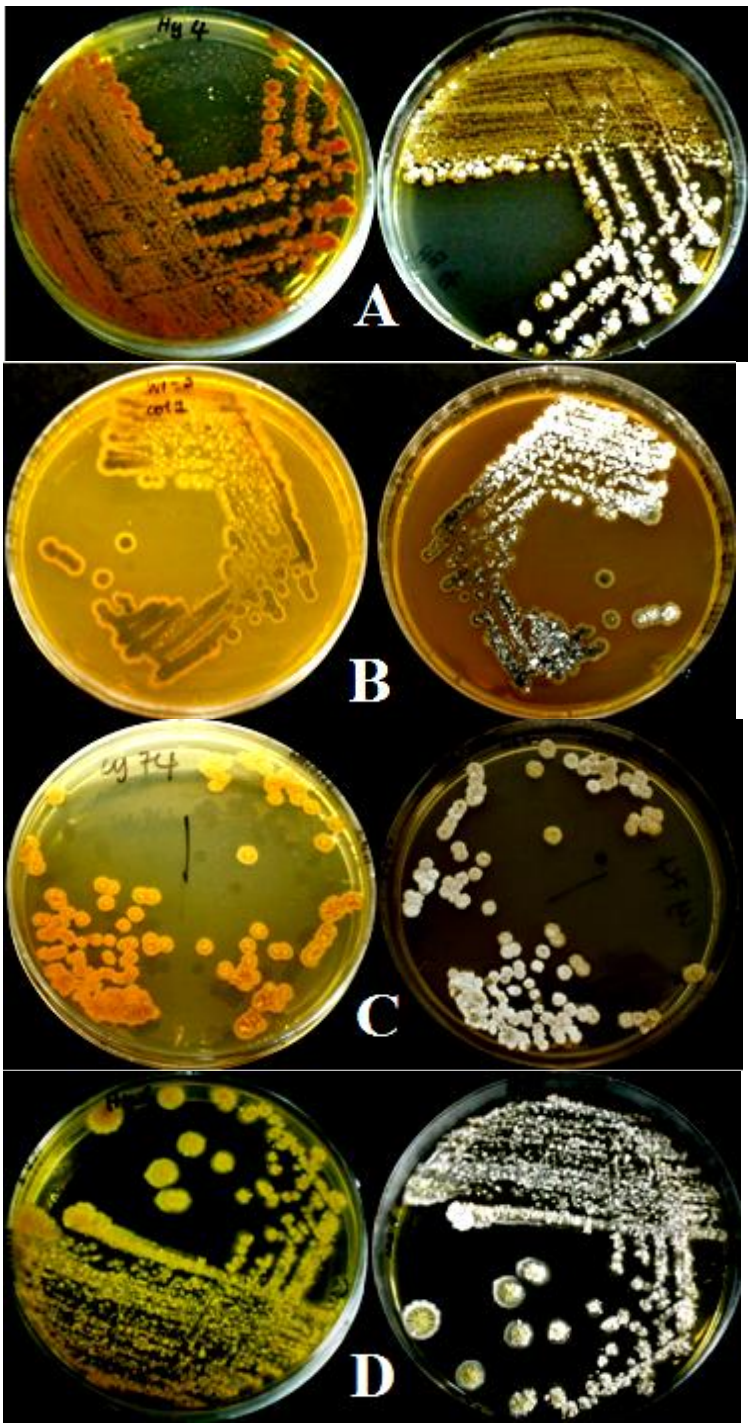

Figure 4.1: Colour of substrate mycelium, aerial mycelium and diffusible pigmentation exhibited by strain HY4 (A), SCA6 (B), CY74, and HY6 (D) on ISP2 medium.

DNA sequencing results revealed that representative strain selected from orange color series, CY24, belonged to the genus *Rhodococcus* while another representative strain from the same color series, CY118, belonged to the genus *Streptomyces*. The remaining representative strains from each color series were found to belong to the genus *Streptomyces*. To further examine the identity of actinomyetes, a more reliable approach was conducted based on molecular method (refer to 4.2.2), as the colour of aerial mycelium formed by actinomycetes may vary due to several factors especially after several subsequent sub-culturing on ISP2 medium. Furthermore, the isolates may undergo deterioration that may affect their aerial mycelium colour formation through autolysis, hygroscopic properties or dehydration (Snider *et al.*, 2009).

The genus *Rhodococcus* was first described by (Zopf) in 1981, and later reintroduced by Tsukamura in 1974 and redefined by Goodfellow & Alderson in 1977. They are described to be aerobic, non-motile, mycolate-containing, and are nocardioform which possess mycelia growth that fragment into rod-shaped or coccoid elements (Goodfellow, 1989; Lechevalier, 1989). Albeit being a member of the nocardioform actinomycetes, the genus *rhodococcus* can be differentiated from other closely related and mycolid acid containing genera based on the carbon atom numbers in the mycolic acids, where the *Rhodococcus* spp. are reported to have shorter carbon chains (Goodfellow, 1991). The aromatic rings are commonly used as carbon sources for bioremediation where studies have reported the utilization of *Rhodococcus* strain for degration of toxic aromatic compounds (Stoecker *et al.*, 1994).

4.2.2 16S rRNA gene restriction fragment patterns of actinomycete isolates

In this section, 63 representative strains were selected from a total of 113 actinomycete strains based on their colour groupings. Strains that were observed to have high similarity in terms of their colour morphology on ISP2 medium were grouped into the same group. Subsequently, strains with a slight colour difference were selected from each group and the identity of the representative strains was determined up to genus level using molecular approach. To conduct the experiment, concentration of each amplified and purified 16S rRNA sample, and amount of restriction endonuclease used were calculated accordingly. This is crucial to avoid the occurrence of insufficient initial concentration of each amplified and purified 16S rRNA sample/restriction endonuclease which could lead to the formation of faint restriction fragment bands. Moreover, the step is crucial to prevent incomplete or no digestion in each sample.

In this study, *Sau*3AI was the most crucial restriction endonuclease used as the digestion pattern generated by *Sau*3AI was able to differentiate actinomycetes into the genus *Streptomyces* and non-*Streptomyces* (Cook & Meyers, 2003). According to Cook and Meyers (2003), the largest size of the restriction fragment produced by *Sau*3AI that exceeds 750bp were presumed to be categorized as non-streptomycetes (Fig 4.2) and different types of restriction enzymes were required for further identification. Furthermore, among all restriction endonucleases used, a single digestion by *Sau*3AI was found to produce a higher number of restriction fragments patterns from the amplified and purified 16S rRNA of actinomycete isolates. The results indicated the presence of several recognition sequences of this restriction endonuclease in

most of the actinomycete strains. In other words, *Sau*3AI digestion allowed actinomycete isolates with similar restriction fragment patterns to be categorised under the same group as they were presumed to have a close relation.

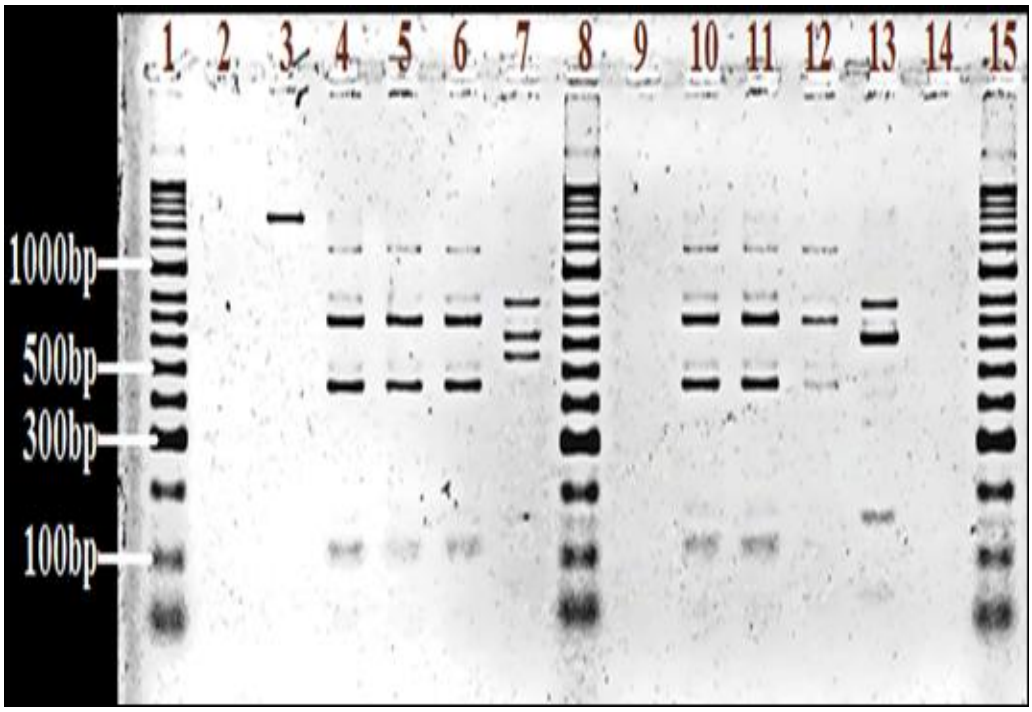

Figure 4.2: *Sau*3AI endonuclease restriction analysis of 8 actinomycete strains. Lanes: 1, 8 & 15, molecular size marker; 3, control (undigested 16s rRNA); 4, 5, 6, 10, 11 & 12 non-*Streptomyces* strains; 7 & 13, *Streptomyces* strains.

Subsequently, all samples that were treated with *Sau*3AI were treated with *Kpn*I for further identification, and the size of each restriction fragment formed was approximately determined to the nearest base pairs indicated by the DNA ladder. According to Cook and Meyer's (2003) publication, actinomycete strains were presumed to belong to the genus *Streptomyces* in the case where banding patterns with a fragment size less than 750bp produced by *Sau*3AI followed by the production of banding patterns of 410bp-470bp and 990bp-1100bp generated by *Kpn*I. In reference to Cook and Meyer's finding, it was

presumed that 32 representative strains subjected to *Sau*3AI with largest restriction fragment size smaller than 800bp, and with banding patterns of 410bp-470bp and 990bp-1100bp produced by *Kpn*I were streptomycetes, whereas the remaining 31 representative strains with dissimilar restriction fragment patterns were presumed to be non-streptomycetes and were subjected to *Hind*III for further identification. Actinomycete strains generating a similar pattern of restriction fragments after *Sau*3AI treatment and the approximate restriction fragment lengths generated were summarized in Table 4.2

Table 4.4: Summary of *Sau*3AI and the corresponding restriction fragment lengths produced in base pair.

Approximate restriction fragment lengths (bp)	**No. of strains**	**Origin of strains (sampling site; isolation medium)**
180, 280, 950, 1000, 1200, 1300	1	SCA2- cave soil; SCA
180, 300, 1000, 1300	1	TWA1- cave wall; TWA
180, 300, 1000, 1100, 1250	1	TWA5- cave wall; TWA
180, 300, 500, 700, 750, 1000, 1300	1	TWA2- cave wall; TWA
50, 100, 450, 500, 700, 800, 1200	1	CY41- cave soil; HYEA
500, 750, 850, 1200	1	CY69- cave column; SM3
100, 450, 500, 700, 800, 1200	4	CY49- cave soil; SM3 CY117A- cave soil; SM3 CY141- cave wall; SM3 CY5- cave soil; SM3
100, 150, 450, 500, 700, 800, 1200	1	CY142- cave wall; SM3
300, 750, 800, 900, 950, 1200	1	CY120- cave soil; SM3
480, 500, 700, 800, 1200	3	CY21- cave soil; SM3 CY15- cave soil; SM3 CY43- cave soil; HYEA
650, 800, 1200	1	CY22- cave soil; SM3
100, 180, 1200	1	CY50- cave soil; SM3
300, 600, 700, 1200	1	SM3A5- cave soil; SM3

Table 4.2, continued

Approximate restriction fragment lengths (bp)	**No. of strains**	**Origin of strains (sampling site; isolation medium)**
150, 250, 300, 400, 800, 1100	1	CY139- cave soil; SM3
150, 500, 800, 1100	3	CY69- cave column; SM3 CY76- cave column; HYEA CY79- cave column; SM3
700, 1100	1	CY110- cave soil; SM3
350, 450, 1000, 1100	1	HY2- cave soil; HYEA
50, 175, 200, 300, 400, 650, 800, 1000	1	CY3B- cave soil, SM3
850, 900, 950	3	CY38- cave soil; HYEA HY10- cave soil; HYEA SM3A6- cave soil; SM3
600, 800, 900	1	CY87- cave column; SM3
650, 700, 800, 900	1	CY89- cave column; SM3
450, 700, 850	1	CY53- cave soil; SM3
180, 450, 650, 800	1	TSA2- cave wall; TSA
180, 650, 800	2	HY6- stalagmite; HYEA SCA1- cave soil; SCA
700, 800	4	CY45- cave soil; SM3 CY52A- cave soil; SM3 CY63- cave soil; SM3 CY64- cave soil; SM3
550, 600, 800	1	TWA7- cave soil; TWA
500, 750, 800	1	CY28- cave soil; SM3
50, 500, 700, 800	1	CY5- cave soil; SM3
50, 500, 550, 750, 800	1	CY24- cave soil; SM3
50, 200, 350, 750, 800	1	CY23- cave soil; SM3
75, 150, 600, 700, 800	1	CY129- cave soil; HYEA
75, 150, 600, 650, 800	1	CY138- cave soil; SM3
175, 650, 700, 800	1	SM3A8- cave soil; SM3
400, 700, 800	1	SM3A4- cave soil; SM3
600, 700, 800	1	CY90- cave column; HYEA

Table 4.2, continued

Approximate restriction fragment lengths (bp)	**No. of strains**	**Origin of strains (sampling site; isolation medium)**
600, 800	5	CY137- cave soil; SM3 CY16- cave soil; SM3 CY22- cave soil; SM3 HY14- cave soil; HYEA HY3- cave soil; HYEA
500, 750	2	CY87- cave column; SM3 CY95- cave soil; SM3
450, 700	2	CY12- cave soil; SM3 CY121- cave soil; SM3
50, 150, 450, 700	1	CY96- cave soil; SM3
50, 500, 700	1	CY12- cave soil; SM3
500, 700	2	CY8- cave soil; SM3 TWA9- cave column; TWA
480, 700	1	CY101- cave soil; SM3
700	1	CY95- cave soil; SM3

In summary, 63 actinomycete strains were grouped into 32 putative streptomycete strains and 31 non-streptomycete strains after being treated with *Sau*3AI and *Kpn*I according to the restriction patterns formed.

In the case of putative streptomycete strains, CY89 and CY16 were then selected as representative strains for 16S rRNA partial sequencing. The analysis showed they both belonged to the genus *Streptomyces* (Figure 4.3 & Table 4.5).

As for the case of putative non-streptomycete strains, TWA 1, TWA 2 and TWA 5 were found to have rare and analogous restriction patterns after treated with *Sau*3AI. Representative strains from colour series of brown and yellow

respectively, TWA1 and TWA2 (Table 4.3) were then subjected to 16S rRNA partial sequencing analysis for identification purpose and results showed they belonged to the genus *Lentzea* and showed 99% similarity with *Violacea* species using NBCI nucleotide BLAST search. Consequencely, TWA5 with similar restriction fragment patterns was classified as *Lentzea*.

In the case of *Lentzea*, restriction patterns generated by *Sau*3AI ranged from four DNA bands to six bands, with four distinctive DNA fragments of the size of 180bp, 300bp, 1000-1100bp and 1250-1300bp.

The genus *Lentzea* was named after Friedrich A. *Lentze*, a German microbiologist who was a devoted researcher on pathogenic actinomycetes. The genus *Lentzea* represents a line of descent neighboring the genus *Actinosynnema* and is closely related to the genera *Saccharothrix* and *Kutzneria* phylogenetically. *Lentzea* was described to have vegetative mycelia branch and is also resistant to lysozyme apart of being catalase positive and aerobic. Its type III cell wall composed of meso-diaminopimelic acid with no characteristic sugar, and type PI1 phospholipid pattern showed high amount of phosphatidylethanolamine. The fatty acid profile was reported to have straight-chain saturated, unsaturated, and branched-chain saturated fatty acids (Yassin *et al.*, 1995).

Table 4.5: Restriction patterns generated by each *Sau*3AI on the amplified 16S rRNA actinomycete strains that belonged to the genus *Lentzea*.

No.	Colour series	Actinomycete strains	Restriction endonucleases	Approx. restriction fragment lengths (bp)
1	Yellow	TWA2, TWA5	*Sau*3AI	180; 300; 0-500; 0-700; 1000- 1100; 1250-1300
2	Brown	TWA1	*Sau*3AI	180; 300; 1000; 1300

Besides TWA2, TWA5 and TWA1, six other putative non-streptomycete strains were observed to have a different set of restriction patterns after treated with *Sau*3AI, *Kpn*I and *Hind*III. Representative strains from different colour group, CY121, CY12, CY69 and CY110 (Table 4.4) were subjected to 16S rRNA partial sequencing analysis and results showed they belonged to the genus *Nocardia*. The sequences were aligned, and neighbor-joining phylogenetic trees were constructed from Juke-Cantor-corrected using Mega4 (Tamura K. *et al.*, 2007).

Consequently, the other two actinomycete isolates with similar restriction patterns were classified as *Nocardia*.

Bacterial strains belonging to the genus *Nocardia* were first isolated by a French Veterinarian Edmond Nocard (Nocard, 1888). The colonial characteristics and cellular morphology of *Nocardia* strains may be misidentified with genera such as *Dietzia, Gordona, Mycobacterium, Rhodococcus, Tsukamurella* and *Streptomyces* as all these genera are closely related (Laurent *et al.*, 1999). The *Norcadia* strains are descrbed to have branched substrate hyphae that fragment into rod-shaped to coccoid elements. Strains of this genus are also characterized by the presence of meso-

diaminopimelic acid, arabinose, and galactose in their wall peptidoglycan, and with DNAs abundant in guanine and cytosine (Chun & Goodfellow, 1995).

In reference to past studies done on *Norcadia* species, the isolates were assigned to a particular species if the partial 16s rRNA sequence showed ≥99.3% similarity with the most closely related species in NCBI BLAST search (Conville et al., 2000; Joann *et al.*, 2004). In this study, CY121, CY12, CY69 and CY110 are presumed to be *Nocardia asteroids* with ≥99.9% similarity with their closest match (Figure 4.4 & Table 4.6).

In the case of *Nocardia*, restriction patterns produced by *Sau3AI* ranged one DNA bands to four bands with three distinctive DNA fragments of the size of 450-500bp, 700-800bp and 1000-1100bp were observed.

Table 4.6: Restriction patterns generated by each restriction endonuclease on the amplified 16S rRNA actinomycete strains that belonged to the genus *Nocardia*.

No.	**Colour series**	**Actinomycete strains**	**Restriction endonucleases**	**Approx. restriction fragment lengths (bp)**
1	Orange-Yellow-	CY12, CY121	*Sau3AI*	450; 700; 1100
			KpnI	450-500; 1000-1050
			HindIII	600; 900
2	Orange-	CY69, CY76	*Sau3AI*	150; 500; 800; 1100
			KpnI	500; 1000
			HindIII	600; 900
3	White-	CY79, CY110	*Sau3AI*	≤150; ≤500; 700-800; 1100
			KpnI	450-500; 1050-1000
			HindIII	600; 900

Based on the findings, actinomycete strains with similar morphology were found to be of different genus. In this case, restriction endonucleases serve as an important agent in the molecular approach to provide a more precise identification of putative actinomycete strains.

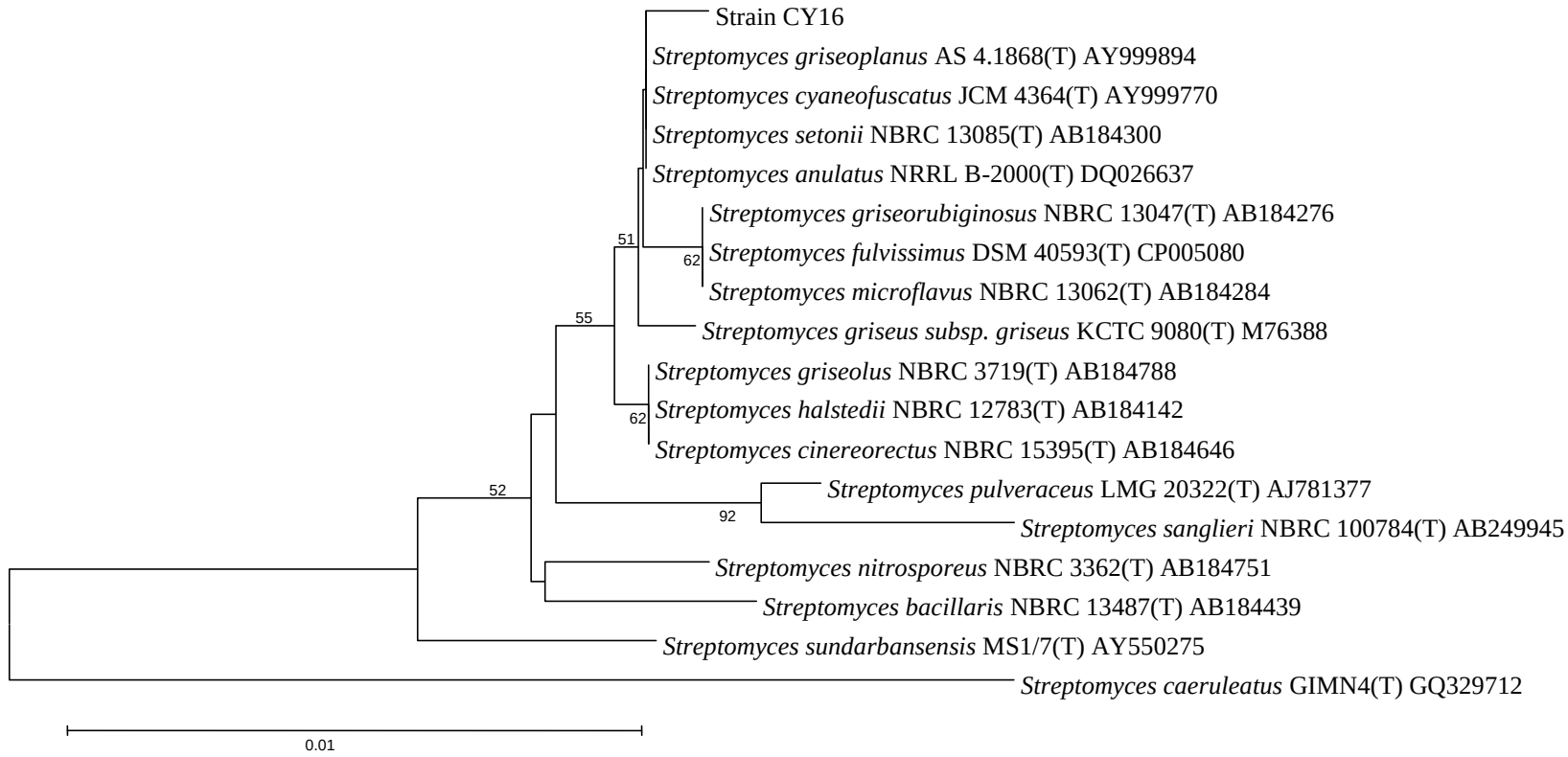

Figure 4.3: Bootstrap consensus neighbour-joining phylogenetic tree (Tamura 3-parameter method). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

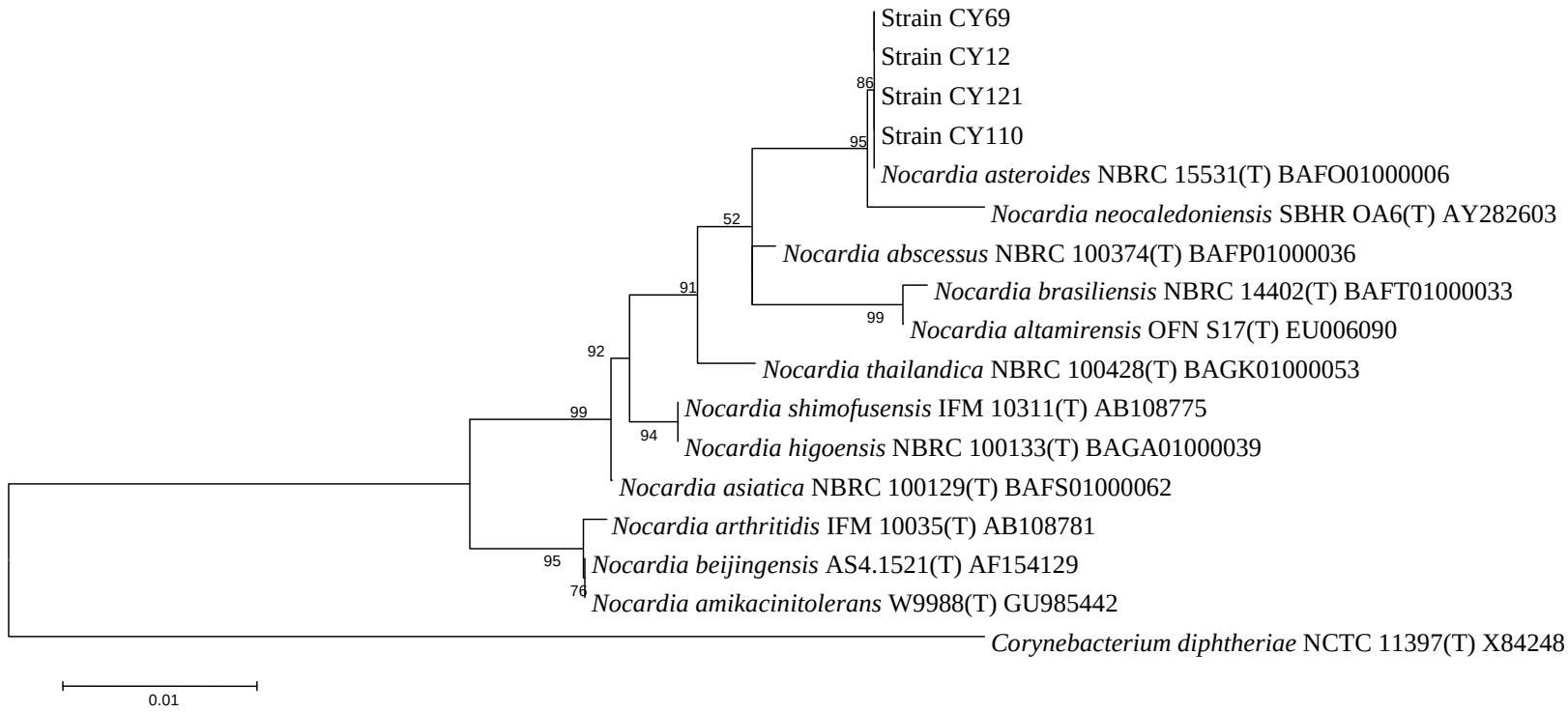

Figure 4.4: Phylogenetic tree showed 12 *Nocardia* species (type strain) closely related to the isolated strains (CY12, CY69, CY110 and CY121). Bootstrap consensus neighbour-joining phylogenetic tree (Tamura 3-parameter method). The analysis involved 12 *Nocardia* species (type strain) closely related to the isolated strains (CY12, CY69, CY110 and CY121). *Corynebacterium diptheriae* NCTC 11397 (T = type strain) (GenBank accession no.: X84248) was used as out-group.

Table 4.7 The analysis involved *Streptomyces* species closely related to the strain CY16 and *Nocardia* species closely related to the strains CY12, CY69, CY110 and CY121. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA5.

Query	Name	Strain	Accession	Pairwise Similarity (%)	Diff/Total nt	Completeness(%)
CY16	*Streptomyces cyaneofuscatus*	JCM 4364(T)	AY999770	99.9	1/962	98.7
CY12	*Nocardia asteroides*	NBRC 15531(T)	BAFO01000006	100	0/975	100
CY69	*Nocardia abscessus*	NBRC 100374(T)	BAFP01000036	98.47	15/979	100
CY110	*Nocardia neocaledoniensis*	SBHR OA6(T)	AY282603	98.59	13/920	98.6
CY121	*Nocardia thailandica*	NBRC 100428(T)	BAGK01000053	98.37	16/979	100

4.3 Actinomycete isolates and their tolerance towards nickel (II) and copper (II)

4.3.1 Screening of nickel and copper tolerant actinomycete strains via streaking plate and direct agar diffusion methods

As preliminary screening, 12 representative strains selected from each color group series were subjected to heavy metal tolerance assay based on streaking plate method, and the selected heavy metals in this assay were lead, molybdate, zinc, nickel and copper. Results showed all 12 actinomycete strains were able to grow on MSM supplement with lead, molybdate, zinc at concentration of 200µg/mL. However only 5 strains were able to grow on MSM supplemented with nickel and none of the 12 strains were able to grow on MSM supplemented with copper.

For further studies on the tolerance of the actinomycete strains towards nickel and copper, all 113 isolated strains were tested for their ability to tolerate copper and nickel based on streaking plate method. Results showed 4 strains, were able to grow on MSM supplemented with copper up to concentration of 50 µg/ml, 9 strains up to 100 µg/ml, 3 strains up to 150 µg/ml, and 6 strains up to 200 µg/ml (Table 4.7).

Table 4.8: Actinomycete strains on MSM supplemented with different concentration of copper

Concentration of copper	Actinomycete strains
50µg/ml	CY137, CY23, CY45*, CY69
100µg/ml	CY118, CY43*, CY8*, CY87, SCA7*, SCA8*, TWA2, TWA5*, TWA9
150µg/ml	HY1, HY15, TWA15
200µg/ml	CY79*, CY110*, HY12*, HY4, SM2G1*, TWA12*

Notes: *indicates strains that were also able to grow on MSM supplemented with nickel ranging from concentration of 50µg/ml - 200µg/ml

On the other hand, results showed 1 strain was able to grow on MSM supplemented with nickel up to concentration 50 µg/ml, 17 strains up to 100 µg/ml, 13 strains up to 150 µg/ml, and 27 strains up to 200 µg/ml. In addition, it was found that 10 actinomycete strains were able to tolerable both copper and nickel ranging from concentration of 50µg/ml - 200µg/ml (refer to table 4.8).

Table 4.9: Actinomycete strains that were able to grow on MSM supplemented with different concentration of nickel

Concentration of nickel	Actinomycete strains
50µg/ml	TSA4
100µg/ml	CY110*, CY115, CY139, CY28, CY31, CY43*, CY56, CY57, HV1, HY12*, HY2, HY3, HY7, HY9, SCA1, TSA2, TWA10
150µg/ml	CY121, CY142, CY143, CY24, CY45*, CY58, CY59, CY63, CY64, CY76, CY8*, HY6, SCA2
200µg/ml	CY101, CY112, CY12, CY125, CY137, CY16, CY21, CY25, CY46, CY50, CY52, CY53, CY61, CY79, CY95, HY14, HY8, SCA7*, SCA8*, SCA9, SM1A1, SM1A2, SM1G1, SM2G1*, TWA11, TWA12*, TWA5*

Notes: * indicates strains that were able to grow on MSM supplemented with copper and also nickel

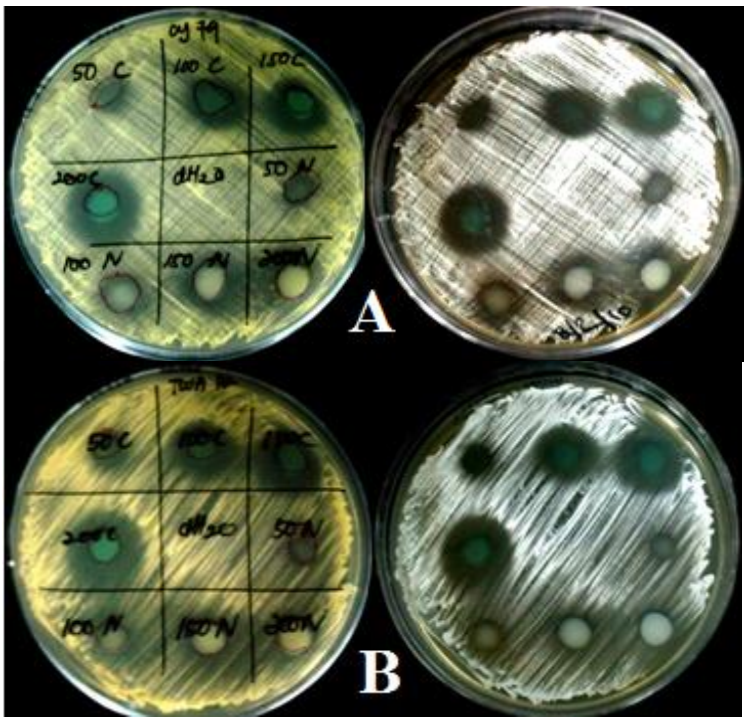

Figure 4.5: clearing zone exhibited by strain CY79 (A) and TWA12 (B) on Mineral Salt Medium against nickel and copper at concentration ranging from 50-200μg/mL.

Results were also compared with secondary screening by direct agar diffusion assay; at where copper and nickel at designated concentrations were dripped directly onto actinomycete lawn. Similar to a past experiment done on screening for heavy metal resistant actinomycetes, a clearing zone of 10mm is set as the arbitrary limit towards metal tolerance in this study (Labeda, 1987); as heavy metal dripped directly onto actinomycete lawn with diameter of around 10mm might affect the growth of actinomycete strains. Strain CY121 was the least susceptible strains among all strains against copper as no inhibition zone was showed on MSM dripped with copper; while strains CY117, CY12, CY 143, CY9, CY79, TWA 3, SM1A1, SM2G1 with clearing zones ranging from 0mm-10mm were observed.

By comparing both streaking plate method and direct agar diffusion assay, it was observed that most strains that were able to grow on MSM supplemented with

nickel or copper (at concentration up to 200µg/ml) exhibited clearing zone more than 10mm in direct agar diffusion assay; except for strains CY 125, CY 52, CY61, CY53, SCA8, SCA9, TWA2, TWA12 and TWA 19 that exhibited clearing zone less than 10mm against nickel up to 200µg/ml. This may suggest that the above mentioned strains were less susceptible to nickel when they had direct contact with the heavy metal and they might exhibit metal tolerance and/or metal consuming capability towards nickel. In other words, the finding suggested that the formation of clearing zone by actinobacteria strains against copper and nickel on MSM does not necessarily indicate the lack of tolerance towards the two heavy metal tested.

A representative strain, CY79, that was able to tolerate both nickel and copper at concentration up to 200µg/m was selected and subjected to partial 16s rRNA sequencing for identification purpose, and NCBI nucleotide BLAST search showed CY79 has 100% similarity with *Nocardia asteroids.*

The study on heavy metal tolerant actinomycete strains provides an opportunity for further investigation of metal tolerance mechanism in actinomycetes as genes encoding metal tolerance may contribute to bioremediation of contaminated sediments.

4.4 Phage induction in heavy metal tolerant actinomycete isolates

4.4.1 Induction of lysogens in copper (II) and/or nickel (II) tolerant actinomycete strains

Heavy metal tolerant isolates were tested for presence of potential phages in them to examine the correlation between presence of phages and tolerance to copper and nickel. The investigation was conducted in support of a suggestion by (Koch, 2007) that some genes in phages help bacterium host to have tolerant or resistant to harsh matters such as heavy metal.

To perform the experiment, 21 and 58 actinomycete isolates that were tolerant to copper and nickel respectively (with 10 among them were tolerant to both copper and nickel) were first subjected to phage induction assay, and were subjected to double layer assay afterward to detect presence of phages. Phage induction in heavy metal tolerant isolates were performed via mitomycin-C and/or ultraviolet ray with the intention of disrupting the cell wall of actinomycete isolates to release potential phages that reside in them (Crocket & Brownell, 1972; McKay & Baldwin, 1973). Upon induction, growth of each actinomycete isolate was followed until a decrease in absorbance value at 600 nm as a measure of cell lysis or when actinomycete isolates entered stationary growth phase. A slight increase in absorbance value in all samples besides CY125 (tolerant to nickel) and SCA8 (tolerant to both nickel and copper) was observed within the first few hours of the experiment due to continued cell growth before the lytic action took place. Subsequently, presence of phages was determined via double layer assay and results showed no presence of phages. It was then presumed that there were no phages residing in the actinomycete isolates; or the result could be explained by the possibility that the cell wall of

the actinomycete isolates were not disrupted and hence failed to induce phages that reside in them. In attempt to increase reliability of the result, the exposure time of actinomycete isolates to mitomycin-c/ultraviolet ray and concentration of the mitomycin-c were increased and had again showed absence of potential phages. The results were double confirmed by approaching another lytic agent: ethidium bromide, which is commonly used to stain dead cells economically for gel viewing purpose. Ehidium bromide has been reported in previous studies for its similar effect as mitomycin-c for the ability to induce phages from lysogenic strains as it can bind to DNA and inhibit the synthesis of DNA in vivo (Gurtler *et al.*, 1991).

CHAPTER 5: CONCLUSIONS

A total of 113 actinomycete strains were isolated from several sampling sites from Dark Cave, Malaysia and results showed number of actinomycetes isolated from cave soils was the highest among all mainly due to presence of higher organic matter and nutrient content. Five isolation media were used in this study for the isolation of actinomycetes, and results showed higher percentage of actinomycetes isolates were obtained from SM3, HYEA and TWA.

Following isolation, selected actinomycete strains were subjected to characterization and identification. Actinomycete strains with similar morphology were found based on molecular approach to be member of different genus. 16S rRNA sequencing revealed that representative strain from orange color series, CY24, belonged to the genus *Rhodococcus* while another representative strain from the same color series, CY118, belonged to the genus *Streptomyces*. 16S rRNA gene fragment pattern restriction endonucleases serve a rapid molecular approach to screen putative actinomycete strains. The analysis revealed that 55.75% of the actinomycete strains belonged to the genus *Streptomyces* and minority belonged to the genera *Nocardia* (5.31%) and *Lentzea* (2.65%).

In this study, 21 and 58 actinomycete strains were able to grow on MSM supplemented with copper and nickel respectively ranging from concentration of 50µg/ml - 200µg/ml; and 10 strains were able to grow on both MSM supplemented with coper and nickel (2 strains were Nocardia spp., 1 strain was Lentzea spp. and the remaining were Streptomyces spp.). This provides an opportunity for further investigation of metal tolerance mechanism in actinomycetes.

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APPENDIX A

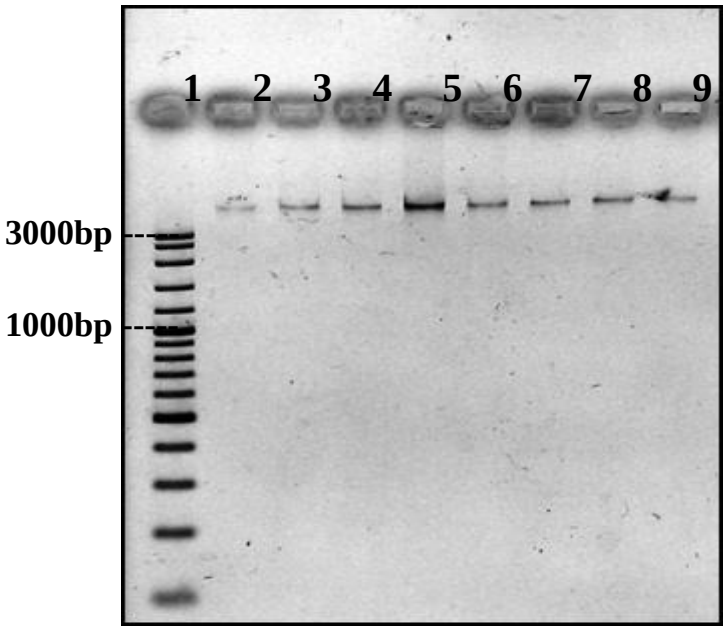


Figure A.1: Genomic DNA extraction of actinomycete isolates. Lanes: 1- molecular size marker; 2- Strain CY121; 3-Strain CY12; 4-Strain CY69; 5-Strain CY110; 6-Strain CY89; 7-Strain CY16; 8-Strain TWA1; 9-Strain TWA2.

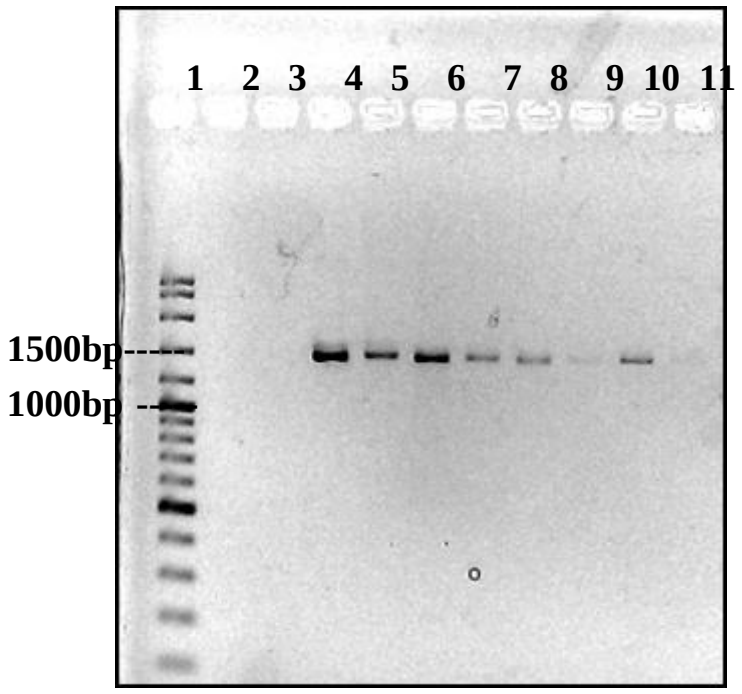


Figure A.2: Agarose-gel electrophoresis of PCR products. Lanes: 1- molecular size marker; 2, 3-negative control without DNA; 4, 5-strain CY16; 6, 7-strain TWA1; 8, 9-strain CY12; 10, 11-strain CY69.

Table A.1 showed actinomycete strains grouped according to aerial mycelium colour, substrate mycelium colour and diffusible pigment colour.

Colour Series	Aerial mycelium colour	Substrate mycelium colour	Diffusible pigment colour	Number of strains	Strains
Yellow	Pale yellow	Pale yellow	None	31	SCA4, HY9, HY10, HY15, TSA 1, TSA2, TSA3, TWA2, TWA3, TWA4, TWA5, TWA6, TWA7, TWA8, TWA17, TWA18, TWA19, CY9, CY11, CY15, CY28, CY38, CY61, CY64, CY121, SM3A1, SM3A2, SM3A3, SM3A4, SM3A6, SM3A9
	Moderate yellow	Moderate olive	Moderate olive	2	CY56, CY74
	Pale yellow	Dark yellow	Moderate brown	2	CY140, HY8
Orange	Light orange	Moderate orange	None	17	CY4, CY5, CY8, CY12, CY23, CY24, CY41, CY43, CY49,CY58, CY69, CY76, CY81, CY95; CY96, CY117, SM3A5
	Orange	Orange	Moderate orange yellow	1	HY6
	Vivid reddish orange	Strong orange	None	1	CY101
	Moderate orange yellow	Brilliant orange yellow	None	1	TWA12
	Dark orange yellow	Strong orange	Orange	2	HY4, CY118

Table A.1, continued

Colour Series	Aerial mycelium colour	Substrate mycelium colour	Diffusible pigment colour	Number of strains	Strains
Grey	Light greenish grey	Dark yellow	Moderate olive	5	CY16, CY19, CY22, CY31, CY37
	Yellowish gray	Dark yellow	None	5	CY63, CY66, CY137, SCA1, HY7
	Grayish pink	Dark yellow	None	2	CY52, CY138
	Grayish pink	Moderate brown	Moderate olive	1	CY115
	Grayish pink	Deep reddish orange	Moderate orange	2	CY18, CY112
	Grayish purple	Strong brown	Brownish orange	1	TSA4
	Grayish pink	Dark brown	Moderate olive	1	CY45
	Light greenish gray	Dark olive	Blackish purple	1	CY46
	Yellowish gray	Dark yellow	Moderate olive	1	CY25
White	White	Moderate yellow	None	18	CY34, CY42, CY53, CY57, CY79, CY87,CY89, CY90, CY110, CY129, CY128, CY141, CY142, CY143, TWA11 , SCA2, SM1A1, SM1A2
Brown	Brown	Brown	None	8	TWA1, TWA9, TWA10, TWA15, SCA3, SCA8, SCA9, SM3A8

Table A.1, continued

Colour Series	Aerial mycelium colour	Substrate mycelium colour	Diffusible pigment colour	Number of strains	Strains
Pink	Pale pink	Light orange yellow	Deep orange yellow	1	HY1
	Pale pink	Yellow	None	3	HY3, HY14, CY125
	Pale pink	Pale pink	None	2	HY12, HY5
Red	Moderate red	Dark red	Moderate red	2	CY50, CY62
	Reddish purple	Dark red	Dark red	1	CY1
	Dark red	Dark red	Deep orange yellow	1	SCA7
Green	Dark green	Dark green	Dark olive green	1	SCA6

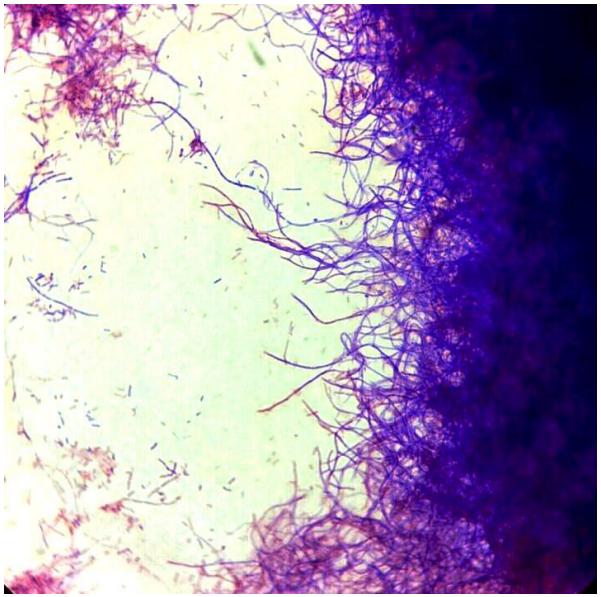


Figure A.3 shows strain CY8 filamentous after 5 days of growth using light microscope.

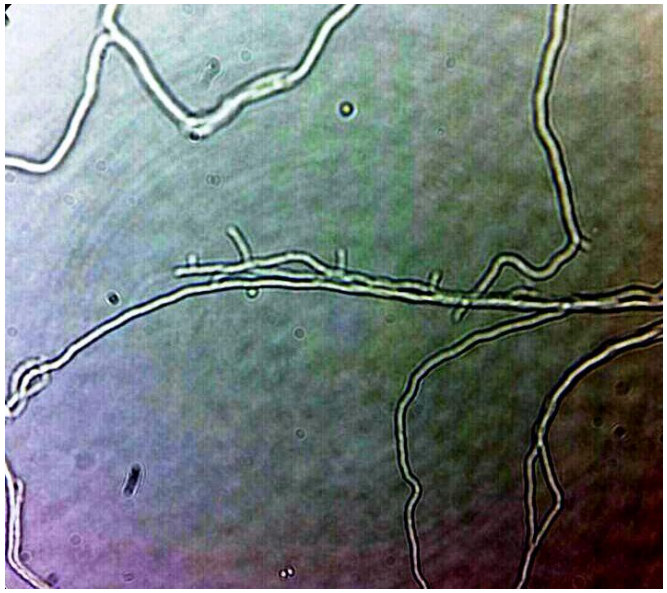


Figure A.4 shows strain HY8 filamentous after 5 days of growth using light microscope (coverslip method).

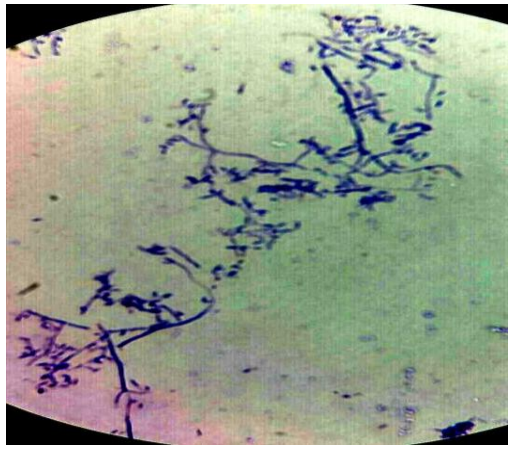
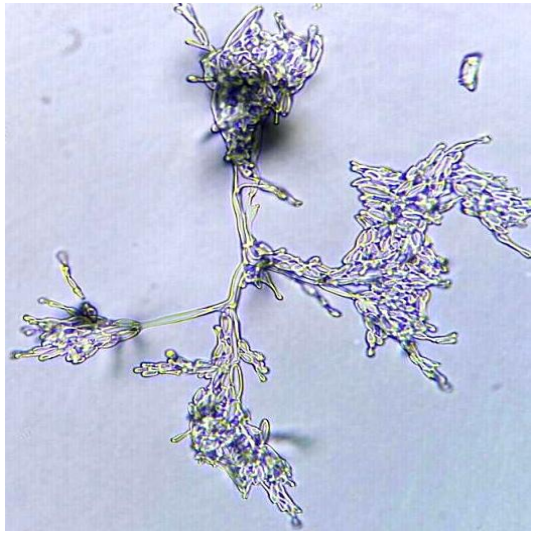


Figure A.5 shows strain TWA12 filamentous after 5 days of growth using light microscope (coverslip method).



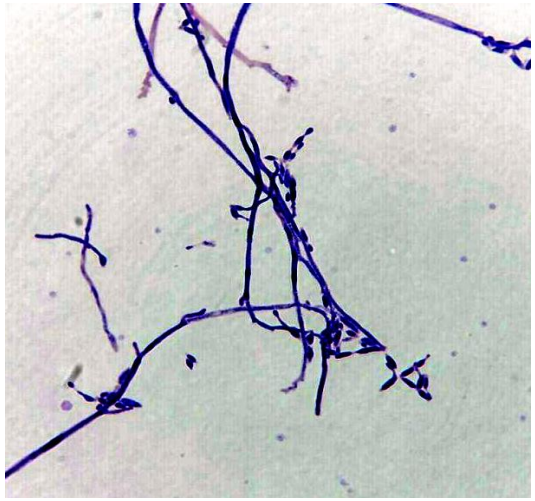


Figure A.6 shows strain SM2G1 filamentous after 5 days of growth using light microscope (coverslip method).