# HEAVY METAL TOLERANCE AND ANTIBIOTIC RESISTANCE OF ACTINOMYCETES ISOLATED FROM TIN TAILINGS AND FOREST SOIL

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**FACULTY OF SCIENCE**
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**ABSTRACT**

A collection of 421 actinomycetes, isolated from an ex-tin mining area in Bidor (238 isolates) and soil samples collected from Pasoh forest (183 isolates), was successfully revived from FRIM Actinobacteria Culture Collection. Rapid metal tolerance screening procedures that involved the use of solid synthetic media containing $Cd^{2+}$, $Cu^{2+}$, $Hg^{2+}$, $Ni^{2+}$, $Pb^{2+}$, $Zn^{2+}$ and $As^{3+}$ allowed the selection of 29 metal tolerant isolates. The selected isolates were further assayed for antibiotic resistance. Pearson Correlation analysis showed that a positive correlation (r = 0.359) exist between multiple antibiotic resistance and heavy metal tolerance in the actinomycetes isolated from Bidor while no correlation was observed among the Pasoh isolates (r = - 0.192). Further, all 29 isolates were assayed for metal toxicity in liquid minimal media (MM). Based on the metal toxicity assay, 13 potential metal tolerant isolates were selected. Eight of these isolates were able to tolerate at least one metal ion at the highest test concentration while three isolates able to tolerate $Ni^{2+}$ at the second highest concentration and another two isolates showed moderate tolerance to $Cu^{2+}$ and $Hg^{2+}$, respectively. All the isolates were de-replicated based on their DNA fingerprint using BOX-PCR. Out of these isolates, TY049-057 from Bidor and TY028-047 from Pasoh were analysed for their ability to uptake $Cd^{2+}$ into the cell biomass. Isolates TY028-047 and TY049-057 were identified as *Streptomyces lannensis* (100% similarity) and *Streptomyces malaysiensis* (100% similarity), respectively, based on 16S rRNA gene sequence analysis. Both isolates showed maximum $Cd^{2+}$ uptake at day 8 when grown in liquid MM media containing an initial concentration of 1mM $Cd^{2+}$. The maximum uptake of $Cd^{2+}$ in isolate *S. lannensis* was 4.66 umol $Cd^{2+}$/mg dry cell mass while 3.17 umol $Cd^{2+}$/mg dry cell mass was recorded in isolate *S. malaysiensis*. The findings from this study showed that maximum $Cd^{2+}$ uptake was observed in both tolerant isolates during the stationary phase of growth profile. A significant increase in $Cd^{2+}$ uptake after 8 days of growth were observed in

*S. lannensis* from Pasoh and not in *S. malaysiensis* from Bidor. This may be explained by the metal tolerance screening and metal toxicity assay results where strains isolated from Pasoh showed higher ability to tolerate $Cd^{2+}$ compared to Bidor. Further, many other aspects influence the level of $Cd^{2+}$ uptake such as nutrients, metal concentration in the media and growth viability rate of bacteria. The two $Cd^{2+}$ tolerant isolates represent potential candidates to be used as agents in bioremediation of $Cd^{2+}$ containing wastewaters and soil.

# ABSTRAK

Koleksi aktinomiset sebanyak 421 yang telah dipencil dari kawasan bekas lombong timah di Bidor (238 pencilan) dan sampel tanah hutan semulajadi Pasoh (183 pencilan), telah berjaya dihidupkan semula daripada FRIM Actinobacteria Culture Collection. Cara kerja yang digunakan untuk saringan pantas toleransi logam telah melibatkan penggunaan media pepejal sintetik yang mengandungi $Cd^{2+}$, $Cu^{2+}$, $Hg^{2+}$, $Ni^{2+}$, $Pb^{2+}$, $Zn^{2+}$ dan $As^{3+}$ dan membolehkan pemilihan 29 pencilan toleran kepada logam. Pencilan-pencilan yang terpilih ini, telah dikaji untuk kehadiran rintangan antibiotik. Analisis korelasi Pearson menunjukkan korelasi positif (r = 0.359) wujud antara rintangan antibiotik berganda dan toleransi logam berat pada aktinomiset yang telah dipencil dari Bidor manakala tiada korelasi wujud diantara pencilan dari Pasoh (r = - 0.192). Seterusnya, kesemua 29 pencilan ini dinilai untuk ketoksikan logam dalam cecair media minimum (MM). Berdasarkan asai ketoksikan logam, 13 pencilan yang berpotensi toleran kepada logam telah dipilih. Lapan daripada pencilan ini dapat toleran kepada sekurang-kurangnya satu ion logam pada kepekatan yang paling tinggi manakala tiga dapat toleran kepada $Ni^{2+}$ pada kepekatan yang kedua tertinggi dan dua pencilan lagi, masing-masing menunjukkan toleran sederhana pada $Cu^{2+}$ dan $Hg^{2+}$. Semua pencilan ini de- replika berdasarkan cap jari DNA mereka menggunakan BOX- PCR. Daripada jumlah pencilan ini, TY049-057 daripada Bidor dan TY028-047 daripada Pasoh dianalisis untuk keupayaan pengambilan $Cd^{2+}$ ke dalam biojisim sel. Pencilan TY028-047 dan TY049-057, masing-masing telah dikenal pasti sebagai *Streptomyces lannensis* (100% persamaan) dan *Streptomyces malaysiensis* (100% persamaan) berdasarkan analisis jujukan 16S rRNA gen. Kedua-dua pencilan menunjukkan pengambilan $Cd^{2+}$ yang maksimum pada hari ke-8 apabila ditumbuhkan dalam cecair media MM yang mempunyai kepekatan awal sebanyak 1mM $Cd^{2+}$. Jumlah maksimum pengambilan $Cd^{2+}$ oleh pencilan *S. lannensis* adalah 4.66 umol $Cd^{2+}$/mg jisim sel kering manakala 3.17

umol $Cd^{2+}$/ mg jisim sel kering dicatatkan oleh pencilan *S. malaysiensis*. Hasil kajian ini menunjukkan bahawa pengambilan $Cd^{2+}$ yang maksimum diperhatikan pada kedua-dua pencilan toleran ini semasa fasa pegun dalam profil pertumbuhan. Peningkatan bererti dalam pengambilan $Cd^{2+}$ selepas 8 hari telah diperhatikan pada *S. lannensis* dari Pasoh dan tidak pada *S. malaysiensis* dari Bidor. Terlebih dahulu, ini telah dijelaskan oleh keputusan saringan toleransi logam dan asai ketoksikan logam di mana pencilan daripada Pasoh menunjukkan keupayaan yang lebih tinggi untuk toleran dengan $Cd^{2+}$ berbanding Bidor. Selanjutnya, banyak aspek lain mempengaruhi tahap pengambilan $Cd^{2+}$ seperti nutrien, kepekatan logam dalam media dan kadar pertumbuhan bakteria. Kedua-dua pencilan toleran kepada $Cd^{2+}$ ini, merupakan pencilan yang berpotensi untuk digunakan sebagai agen biopemulihan air sisa dan tanah yang mengandungi $Cd^{2+}$.

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# List of abbreviations

| | |
|---|---|
| % | percentage |
| v/v | volume per volume |
| w/v | weigh per volume |
| cm | centimeter |
| $^{o}$C | degree Celcius |
| Ni | Nickel |
| Zn | Zinc |
| µl | microliter |
| ml | mililiter |
| L | liter |
| N | nanomolar |
| µM | micromolar |
| mmol/L (mM) | milimolar |
| µg | microgram |
| mg | miligram |
| g | gram |
| kg | kilogram |

| | |
|---|---|
| rpm | revolutions per minute |
| ppm | parts per million |
| V | Voltan |
| BC | Before Christ (dates before the year zero) |
| CFU | Colony-forming unit |
| EDTA | Ethylenediaminetetraacetic acid |
| DMSO | Dimethylsulfoxide |
| $MgCl_2$ | Magnesium Cloride |
| NaCl | Natrium Cloride |
| dNTPs | Deoxyribonucleotide triphosphate |
| TBE | Tris/Borate/EDTA |
| $H_2O$ | Water |
| PBS | Phosphate buffered saline |
| rRNA | Ribosomal Ribonucleic Acid |
| DNA | Deoxyribonucleic acid |
| PCR | Polymerase Chain Reaction |
| bp | base pair |
| ISP | International *Streptomyces* Project |

| | |
|---|---|
| MM | Minimal Media |
| spp. | Species |
| *et al.* | et alia (~ and others) |
| e.g. | example |
| $HgCl_2$ ($Hg^{2+}$) | Mercury (II) Chloride |
| $As_2O_3$ ($As^{3+}$) | Arsenic (III) Oxide |
| $PbCl_2$ ($Pb^{2+}$) | Plumbum (II) Cloride |
| $Cu_2SO_4$ ($Cu^{2+}$) | Cuprum (II) Sulfate |
| $CdCl_2$ ($Cd^{2+}$) | Cadmium (II) Cloride |
| $NiCl_2$ ($Ni^{2+}$) | Nickel (II) Chloride |
| $ZnCl_2$ ($Zn^{2+}$) | Zinc (II) Cloride |
| Hg | Mercury |
| Pb | Plumbum |
| As | Arsenic |
| Cu | Cuprum |
| Cd | Cadmium |
| P | Penicillin |
| Am | Ampicilin |

| | |
|---|---|
| Na | Nalidixic Asid |
| E | Erythomycin |
| Gn | Gentamycin |
| Ch | Chloramphenicol |
| An | Amikacin |
| K | Kanamycin |
| Va | Vancomycin |

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# CHAPTER 1

# INTRODUCTION

## 1.0     General Introduction

Soil contains a vast array of life forms ranging from submicroscopic (the viruses), to earthworms, to large burrowing animals such as gophers and ground squirrels. Microscopic life forms in the soil are generally called the soil microflora and the larger animals are called macrofauna. Soil microorganisms occur in huge numbers and display an enormous diversity of forms and functions. Major microbial groups in soil are fungi, algae, protozoa and bacteria which include actinomycetes (Rao, 1999).

Soil contaminated by heavy metals has become a serious problem in areas of intensive industrial and agricultural activities. Heavy metal contamination in soil is mostly due to use of fertilizer and agrochemicals, mining activities, industrial wastewater irrigation and sewage irrigation (Luo, 2009). Even forest soils which are close to industrial emission sources are contaminated with heavy metal deposits (Shparyk and Parpan, 2004). Most heavy metals are toxic to plants, animals and humans. Metals such as cadmium, manganese, arsenic, lead, zinc, nickel, copper and antimony are easily accumulated in vital organs and therefore threaten human health (Shivika, 2011). For example, itai-itai disease caused by cadmium poisoning due to mining activity at Japan (Masanori, 2006) and minamata disease caused by mercury poisoning (Toshihide and Takashi, 2011). While in plants, toxic levels of heavy metal ions induce several cellular stress responses and damages different cellular components such as membranes, proteins and deoxyribonucleic acid (DNA) (Waisberg *et al.*, 2003; Jimi *et al.* 2004). Thus, heavy metal pollution in soil is a growing environmental and problem which requires immediate attention as soil is the habitat of many microflora

macrofauna and it produce a large amount of food source for human.

In Malaysia, it has been reported that fruits from trees grown on ex-mining land in Bidor, a famous tin mining town in the 1940s, located in the northern state of Perak contains high levels of heavy metals exceeding the permissible limits (Ang *et al*., 2000). Many timber species such as *Acacia mangium*, *Hopea odorata*, *Intsia palembanica* and *Swietenia macrophylla* have been used as phytoremediation agents to extract heavy metals from the contaminated soil in order to ensure safe food chain between plants, animals and humans and to maximize the usage of metal contaminated land for agriculture and forest plantation (Ang *et al*., 2010). Microorganisms such as *Aspegillus* spp*., Bacillus* spp., *Staphylococcus* spp., *Pseudomonas* spp. (Kumar *et al*., 2010), *Trichoderma* spp. (Sen and Charaya, 2010) and *Streptomyces* spp. (Sineriz *et al*., 2009) have been reported to be able to tolerate high concentration of various heavy metals (Shivika, 2011). Bioremediation using microorganisms as biological agents have been used to degrade toxic wastes from the environment (Milic *et al*., 2009).

Studies have shown that the filamentous Gram positive actinomycetes with a highly diversed metabolic capability has been widely studied for bioremediation of heavy metals such as copper, chromium, cadmium (Amoroso and Abate, 2012), plumbum (Kumar *et al*., 2011a) and zinc (Lin *et al*., 2012). This is because many genera of actinomycetes are able to survive in extreme conditions such as high temperature, low moisture and nutrient starvation to produce biosurfactants which increase pollutant biodisponibility and facilitate biodegradation process (Alvarez *et al*., 2011). In high metal concentration environments, homeostasis within the bacteria cell is maintained to keep the reactive heavy metals at an optimal, sub-toxic level. Thus, bacteria possess resistance mechanisms assisted by proteins such as superoxide dismutases (Kim *et al*., 2003), efflux transporters (Anton *et al*., 1999; Mergeay *et al*., 2003) and metal-binding

proteins (Silver and Phung, 2005) in order to survive.

Besides that, actinomycetes produce metabolites which are important source of antibiotics, enzyme, and bioactive products (Goodfellow *et al.*, 1988; Bull *et al.*, 1992). It produces antibiotics that inhibit bacterial growth (Reyes *et al.*, 1997) and possess resistance genes for the antimicrobial molecules they produce. A correlation exists between metal tolerance and antibiotic resistance in bacteria because of the likelihood that resistance genes to both (antibiotics and heavy metals) may be located closely together on the same plasmid in bacteria and are thus more likely to be transferred together in the environment (Lawrence, 2000). Thus, heavy metal contamination in the environment which represents a long-standing, widespread and recalcitrant selection pressure contributes to the maintenance and spread of antibiotic resistance genes in bacteria.

Since actinomycetes have become a focus in heavy metal tolerance studies, the current work was undertaken with the aim to select and identify potential heavy metal tolerant actinomycetes from heavy metal contaminated and forest soil as an early exploration into their bioremediation capabilities.

A total of 238 isolates previously collected from an ex-mining area in Bidor, Perak and 183 isolates from forest soil collected in Pasoh, Negeri Sembilan were screened for their heavy metal tolerance ability. The ex-mining area is a substation of Forest Research Research Institute of Malaysia (FRIM). It is located at $4^06$'N latitude and $101^016$E longitude, and had been a famous mining town during 1940's. Previous studies showed that the tin tailings in Bidor can be classified into slime, sandy and sandy slime tailings. The tailings contain few potentially toxic elements (PTEs) such as arsenic, mercury, lead, copper, cadmium, nickel and zinc (Ang *et al.*, 2000; Ang and Ng, 2000). Actinomycetes isolated from forest soil collected in Pasoh were also studied for their

heavy metal tolerance as a comparison with the Bidor isolates.

The objectives of this study are:

i.  to select potential heavy metal tolerant actinomycetes using direct agar diffusion assay, metal toxicity assay and metal uptake assay.

ii.  to determine the correlation between heavy metal tolerance and antibiotic resistance of actinomycetes obtained from two different environments (heavy metal contaminated tin tailings in Bidor, Perak and forest soil in Pasoh, Negeri Sembilan).

iii.  to differentiate the potential heavy metal tolerant actinomycetes by using BOX-PCR method

iv.  to identify the selected isolates based on morphological, physiological, and genotypic characteristics.

**CHAPTER 2**

**LITERATURE REVIEW**

## 2.1    Introduction to Actinomycetes

Actinomycetes are a group of prokaryotic organisms and it comprises gram-positive bacteria with a high G+C content in their deoxyribonucleic acid (DNA), ranging from 51% in some corynebacteria to more than 70% in *Streptomyces* and *Frankia* (Marco *et al*., 2007). The existence of actinomycetes has been recognized for over a hundred years. In fact, they were long thought to be fungi and were called actinomycetes. Fungi are eukaryotes, but the actinomycetes cell structure and antimicrobial susceptibility patterns are typical of bacteria. The earliest bacterial phylogenies that included actinomycetes was proposed by Kluyver and Van Niel (1936), but in contrast Krasilnikov (1949) still believed the class '*Actinomycetes*' to have evolved from a common ancestor that also led to the fungi. Later, Woese (1987) divided the gram positive bacteria into two subdivisions, low G+C and high G+C based on his 16S ribosomal ribonucleic acid (rRNA) sequence-derived phylogeny. Species with high-G+C were confirmed to a general actinomycete phenotype with tendency to form branched filaments. Further, the class *'Actinomycetes'* represent a collection of phylogenetically diverse families and genera based on its traditional shape (Krasilnikov, 1949).

In the year 1997, Stackebrandt and coworkers proposed a novel hierarchic structure for the high-G+C Gram positive actinomycetes, which was solely delineated from 16S rRNA sequence-based phylogenetic clustering and the presence of taxon specific signature nucleotides in these sequences. Actinomycetes were recognized as a distinct class, *Actinobacteria*, within the Gram positive bacteria. Thus, actinomycetes

are also known as actinobacteria due to nomenclature. Within 16 years after its publication, large numbers of newly described members have been assigned to the *Actinobacteria*. At the same time, the number of 16S rRNA gene sequences deposited in public databases has increased tremendously. Therefore, the recently proposed actinobacteria taxonomy in *Bergey's Manual of Systematic Bacteriology Volume 5* (Whitman *et al*., 2012) has been used in this thesis to describe actinomycetes (Figure 2.1). The taxanomic assignment based the 16S rRNA gene sequencing has led to the recognition of 6 classes and with 15 orders and 43 families for the class of Actinobacteria.

Among actinomycetes, the genus *Streptomyces* are the dominant (Vijayakumar *et al*., 2007; Peela *et al*., 2005). Whereas other genera such as *Actinoplanes, Amycolatopsis, Catenuloplanes, Dactylosporangium, Kineospora, Microbispora, Micromonospora* and *Nonomuraea* are often difficult to isolate from the environment due to their slow growth. Members of these genera are known as rare actinomycetes or non-*Streptomyces* (Hayakawa, 2008).

### 2.1.1 Life Cycle of Actinomycetes

Actinomycetes reproduce through special sporulating bodies. Actinomycetes have filamentous hyphae that do not normally undergo fragmentation but produce asexual spores. The hyphae growth is followed by fragmentation and release of spores (Prescott *et al.,* 2005) (Figure 2.2). The spore deportment hyphae are formed on the mycelium either singly and monopodially, or in broom-like or cluster-like formations, or in verticilliate-like tufts or whorls upon the mycelium. The mycelium is either vegetative and growing in the substrate, or aerial, where a special mycelium is produced above the vegetative growth (Waksman, 1943). For an example, the life cycle of *Streptomyces* (Figure 2.3) begins with germination of a single spore. This spore

**Family**

a:*Actinomycetaceae*,
b:*Actinopolysporaceae*,
c:*Bifidobacteriaceae*,
d:*Catenulisporaceae*,
d:*Actinospicaceae*,
e:*Corynebacteriaceae*,
e:*Dietziaceae*,
e:*Mycobacteriaceae*,
e:*Nocardiaceae*,
e:*Segniliparaceae*,
e:*Tsukamurellaceae*,
f:*Frankiaceae*,
f:*Acidothermaceae*,
f:*Cryptosporangiaceae*,
f:*Geodermatophilaceae*,
f:*Nakamurellaceae*,
f:*Sporichthyaceae*,
g:*Glycomycetaceae*,
h:*Jiangellaceae*,
i:*Kineosporiaceae*,
j:*Micrococcaceae*,
j:*Beutenbergiaceae*,
j:*Bogoriellaceae*,
j:*Brevibacteriaceae*,
j:*Cellulomonadaceae*,
j:*Dermabacteraceae*,
j:*Dermacoccaceae*,
j:*Dermatophilaceae*,
j:*Intrasporangiaceae*,
j:*Jonesiaceae*,
j:*Microbacteriaceae*,
j:*Promicromonosporaceae*,
j:*Rarobacteraceae*,
j:*Ruaniaceae*,
j:*Sanguibacteraceae*,
k:*Micromonosporaceae*,
l:*Nocardioidaceae*,
l:*Propionibacteriaceae*,
m:*Pseudonocardiaceae*,
n:*Streptomycetaceae*,
o:*Streptosporangiaceae*,
o:*Nocardiopsaceae*,
o:*Thermomonosporaceae*

**Orders**

a:*Actinomycetales*,
b:*Actinopolysporales*,
c:*Bifidobacteriales*,
d:*Catenulisporales*,
e:*Corynebacteriales*,
f:*Frankiales*,
g:*Glycomycetales*,
h:*Jiangellales*,
i:*Kineosporiales*,
j:*Micrococcales*,
k:*Micromonosporales*,,
l:*Propionibacteriales*,
m:*Pseudonocardiales*,
n:*Streptomycetales*,
o:*Streptosporangiales*
p:*Incertaesedis*

**Class** *Actinobacteria*

**Phylum (Actinobacteria)**

**Class** *Acidimicrobiia* — **Orders** *Acidimicrobiales* — **Family** *Acidimicrobiaceae, Iamiaceae*

**Class** *Coriobacteriia* — **Orders** *Coriobacteriales* — **Family** *Coriobacteriaceae*

**Class** *Nitriliruptoria* — **Orders** a:*Nitriliruptorales*, b:*Euzebyales* — **Family** a:*Nitriliruptoraceae*, b:*Euzebyaceae*

**Class** *Rubrobacteria* — **Orders** *Rubrobacterales* — **Family** *Rubrobacteraceae*

**Class** *Thermoleophilia* — **Orders** a:*Thermoleophilales*, b:*Solirubrobacterale* — **Family** a:*Thermoleophilaceae*, b:*Solirubrobacteraceae*, b:*Conexibacteraceae*, b:*Patulibacteraceae*

**Figure 2.1**    Taxonomic outline for the phylum *Actinobacteria* based upon taxonomy for *Actinobacteria* in the *Bergey's Manual of Systematic Bacteriology Volume 5. edition 2*: The Actinobacteria (Whitman *et al*., 2012).

**Figure 2.2**     Actinomycetes hyphae fragmentation (Prescott *et al.,* 2005).



**Figure 2.3**     Life cycle of *Streptomyces* (Kieser *et al*., 2000).

produces one or more mold consists of long, branched, thread like filaments of cells called hypae that form a vegetative mycelium (Figure 2.4) followed by aerial growth with the production of aerial hyphae. When the growth of the aerial hyphae stops, the hyphae will undergo septation to produce unigenomic spore compartments which will disperse and thus commence a new cycle (McGregor, 1954).

## 2.1.2 Morphological, Physiological and Biochemical Characterization of Actinomycetes

Morphology, physiology and biochemical characters have been an important characteristic to identify actinomycete isolates. It was this characteristic that was described first as identification key by Nonomura (1974) and Bergey's Manual of Determinative Bacteriology (Buchanan and Gibbons, 1974) which was very useful in the identification of *Streptomyces* species. Morphological observations including aerial mass colour, reverse side colour, soluble pigments and spore chain morphology have been used to identify actinomycetes (Sathiyaseelan and Stella, 2012; Gurung *et al*., 2009; Holt *et al.,* 1994).

Actinomycete strains are grouped based on the colour of the mature sporulating aerial mycelium and reverse side colony colour which is normally recorded in a simple way (white, grey, red, green, blue and violet). When the aerial mass colour falls between two colours series, both the colours are recorded. If the aerial mass colour of a strain to be studied shows intermediate tints, then also, both the colour series are noted. Either that, the strains are divided into two groups by their ability to produce soluble pigments other than melanin: namely, produced (+) and not produced (-). The colour is recorded red, orange, green, yellow, blue and violet (Nonomura, 1974; Buchanan and Gibbons, 1974).
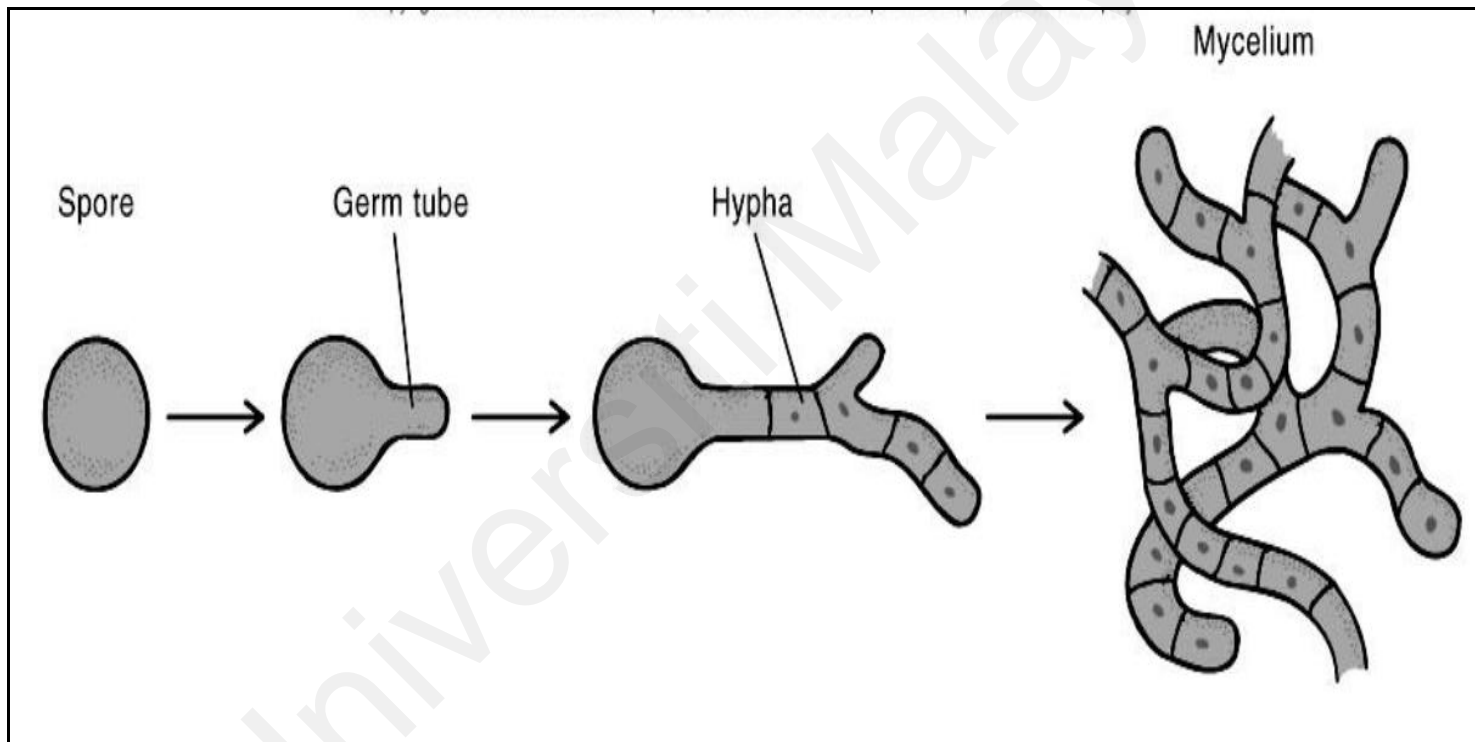
**Figure 2.4**    Growth of *Streptomyces* filaments (Prescott *et al*., 2005).

Actinomycetes are higher organisms than bacteria, according to their structure and they are known to form a well-developed mycelium. Formation of aerial mycelium, substrate mycelium and spores were studied by light microscopy and the spore surface and spore structure by scanning electron microcopy. The mycelia threads are thin, 0.5-1.0 µm diameter, without septa. The mycelial threads are thinner than in fungi, more fragile, are easily broken and destroyed, forming shreds and splinters (Krasilnikov, 1961). Further, spores of many actinomycetes have different forms - spherical, oval, and rod like. Thus, actinomycetes exhibit a wide variety of morphologies, from coccoid (*Micrococcus*) or rod-coccoid (e.g., *Arthrobacter*) to fragmenting hyphal forms (e.g., *Nocardia* spp.) or permanent and highly differentiated branched mycelium (e.g., *Streptomyces* spp.) (Marco *et al*., 2007) (Figure 2.5). Based on Shirling and Gottlieb (1966), the species belonging to the genus *Streptomyces* can be grouped into three different categories namely rectiflexibiles (RF), retinaculiaperti (RA) and spirals (S). Meanwhile, when a strain forms two types of spore chains, both are noted (e.g., SRA) (Figure 2.6).

In order to observe colony morphology of actinomycetes, the study is best made by using a variety of standard culture media, especially described in the International *Streptomyces* Project (ISP) (Shirling and Gottlieb, 1966). Actinomycetes grow on such solid nutrient media in the form of compact, dense, gristle like leathery colonies. The latter have a smooth, granular, rough or plicate surface. The colonies grow into the medium with their threads, and have a flat or convex form. Examples of bacterial colony morphology are shown in Figure 2.7.

The physiological property of actinomycetes is their capacity to utilize many organic substrates which is an indispensable tool for classification and identification of actinomycetes and influencing the growth rate of actinomycetes (Shimizu *et al*., 2000;
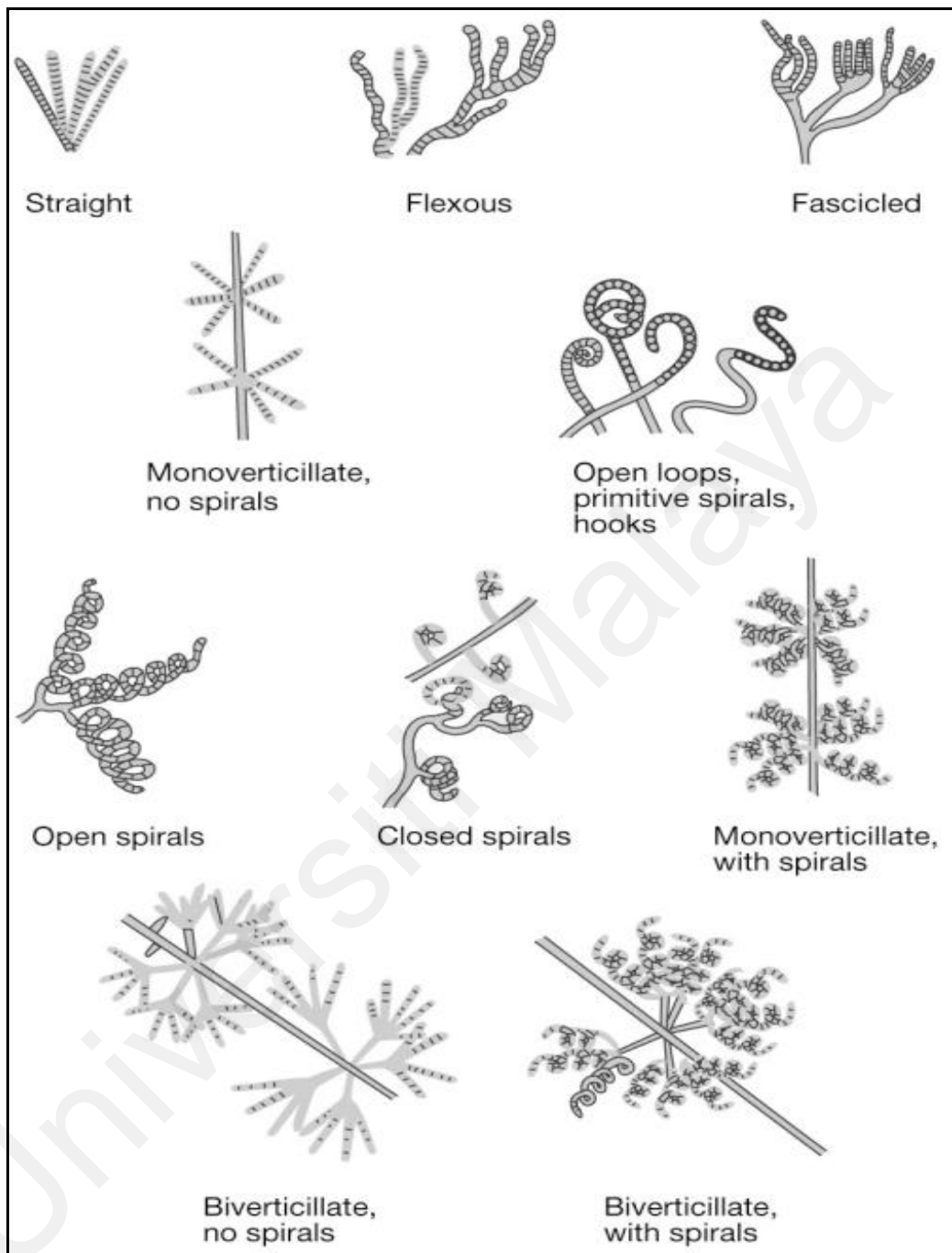
**Figure 2.5** Various type of spore-bearing structure in *Streptomyces* species (Madigan and Martinko, 2006).

RF Spore chains (400X)

Spiral Spore chains (400X)

RA Spore chains (400X)
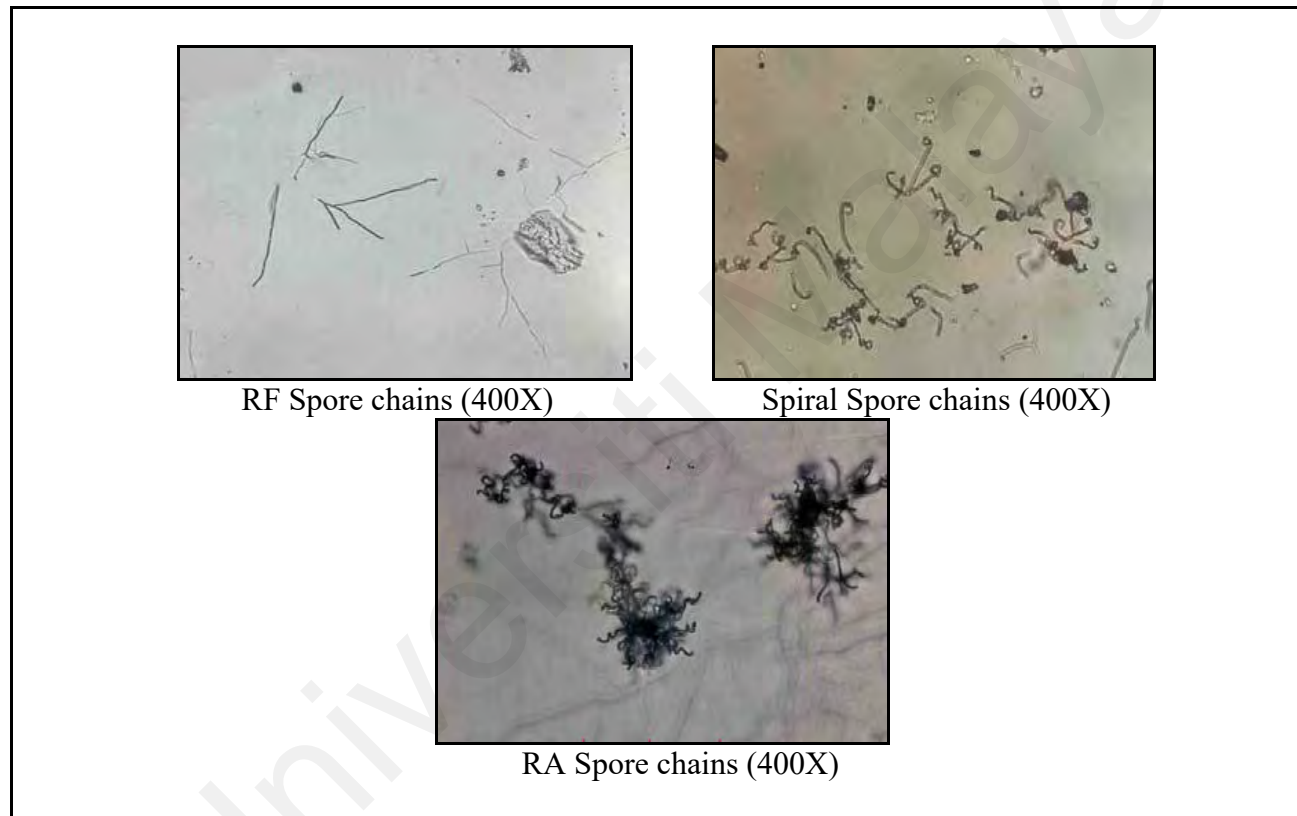
**Figure 2.6**    Three different categories of spore chain morphology of *Streptomyce*s under microscopic observation (Shirling and Gottlieb, 1966).
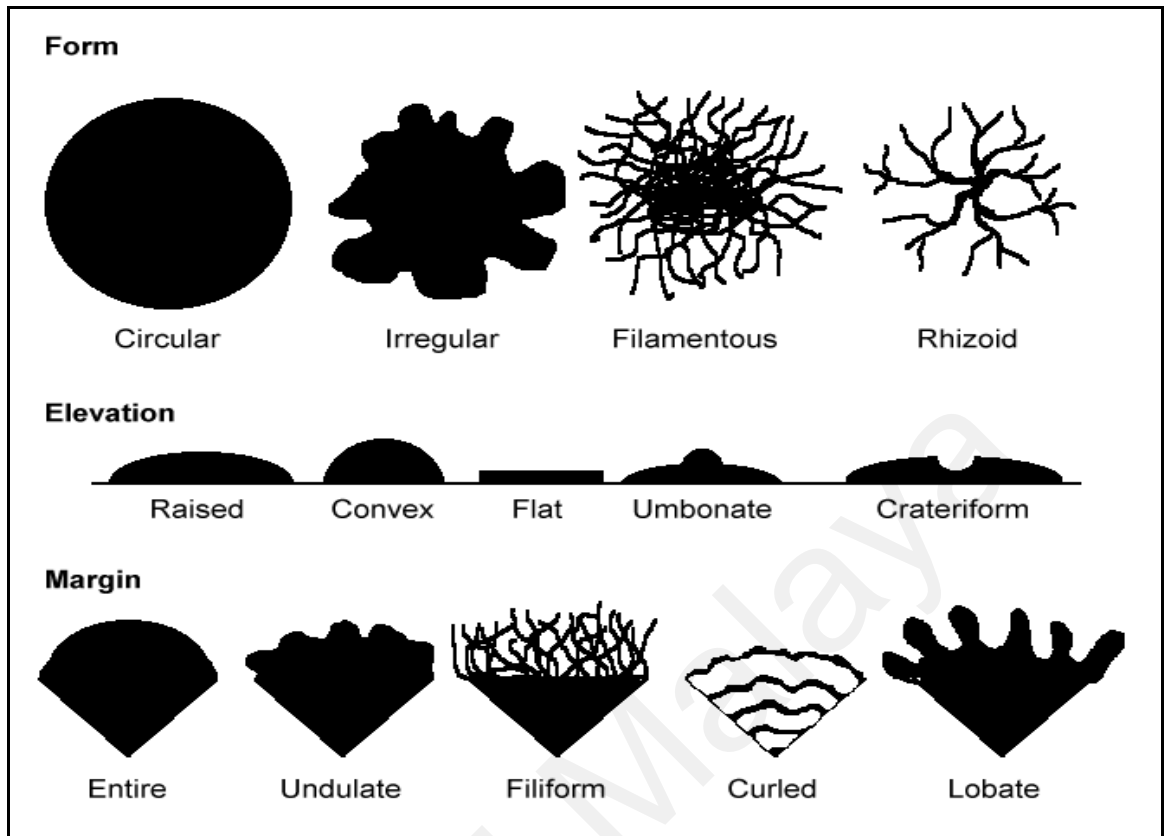
**Figure 2.7**     Example of actinomycetes colony morphology [Bergey's Manual of Determinative Bacteriology; (Holt *et al*., 1994)].

Kim *et al.,* 1999). The most common physiology characteristics carried out are optimum pH, optimum temperature and optimum salt tolerance for the growth of actinomycetes.

Meanwhile, the ability of different actinomycete strains in utilizing various carbon compounds as described in ISP (Shirling and Gottlieb, 1966) could be used as an aid for species determination (Pridham and Gottlieb, 1948). Pure carbon sources which are free from admixture with other carbohydrates or contaminating materials such as arabinose, xylose, inositol, mannitol, fructose, rhamnose, sucrose and raffinose were used for this purpose. These carbon sources should be sterilized by ether sterilization without heating. Comparing the properties of the isolated strain with the representative species found in the key of Nonomura (1974) and Bergey's Manual of Determinative Bacteriology (Buchanan and Gibbons, 1974) can help in the species level identification.

Biochemical or chemotaxonomy is the study of chemical variation in cell compositions in microorganisms. It is one of the valuable methods to identify the genera of actinomycetes. Cell walls of most actinomycetes contain anionic polymers including teichoic acids. The major feature of these polymers is that they impart a negative charge to the bacterial cell surface, which is important for its physiological functions. Teichoic acids regulate the activity of autolytic enzymes which are required for the growth and division of bacterial cells; secondary functions of these polymers include their involvement in phage reception and immunogenicity (Alexander *et al*., 1999). Anaerobic actinomycetes could be separated into four types of cell wall based on the characteristics of peptidoglycan composition and structure (Willey *et al*., 2010). They possess cell wall type I,II, or IV, depending on the presence of L-diaminopimedic acid(DAP) and glycine (type I), meso-DAP and glycine (type II), meso-DAP (type III), or meso-DAP, arabinose and galactose (type IV) (Goodfellow and O'Donnell, 1989). The presence of Diaminopimelic Acid (DAP) isomers is one of most important cell-wall

properties of actinomycetes and gram-positive bacteria. These amino acids mostly located in the peptidoglycan of bacterial wall envelope are generally contained as one of the isomers, LL-form or *meso*-form. Cells of the actinomycetes contain glucosamine and muramic acid, the monomers of peptidoglycan. Most components of the cell wall and whole-cell of actinomycetes are shown in Table 2.1.

The sugar composition often provides valuable information on the classification and identification of actinomycetes. Actinomycete cells contain few kinds of sugars, in addition to the glucosamine and muramic acid of peptidoglycan. The sugar pattern plays a key role in the identification of sporulating actinomycetes which have meso‑DAP in their cell walls. However, the actinomycetes which have LL‑DAP along with glycine (wall chemo type‑I) have no characteristic pattern of sugars (Lechevalier and Lechevalier, 1970) and hence the whole cell sugar test has not received much attention here. Actinomycete whole cell sugar patterns are shown in Table 2.2.

## 2.1.3 Molecular Characterization of Actinomycetes

### 2.1.3.1 Bacterial Identification using rRNA Gene Sequence

Investigation of microbial composition and diversity in natural and anthropogenically impacted or created habitats is important in the characterization of such habitats, since microbes are key players in many environmental processes. Molecular biological tool have become interestingly popular to overcome several limitations of culture dependent methods in discovering bacterial diversity (Rastogi and Sani, 2011).

Ribosomes occur in very cellular organism and rRNA molecules are functionally and structurally conserved. Sequence conservation of rRNAs was early recognized from

**Table 2.1** Actinomycete cell wall types

| Cell wall type | Diaminopimelic acid isomer | Glycine in interpeptide bridge | Characteristic sugars[a] | Representative genera |
|---|---|---|---|---|
| I | L,L | + | NA | *Nocardioides*, *Streptomyces* |
| II | *meso* | + | NA | *Micromonospora*, *Pilimelia*, *Actinoplanes* |
| III | *meso* | - | NA | *Actinomadura*, *Frankia* |
| IV | *meso* | - | Arabinose, Galactose | *Saccharomonospora*, *Nocardia* |

[a]NA, either not applicable or no diagnostic sugars
(Source adapted from Lechevalier and Lechevalier, 1970)

**Table 2.2** Actinomycete whole cell sugar patterns

| Sugar pattern types[a] | Characteristic sugars | Representative genera |
|---|---|---|
| A | Arabinose, galactose | *Nocardia*, *Rhodococcus*, *Saccharomonospora* |
| B | Madurose[b] | *Actinomadura*, *Streptosporangium*, *Dermatopilus* |
| C | None | *Thermomonospora*, *Actinosynnema*, *Geodermatophilus* |
| D | Arabinose, xylose | *Micromonospora*, *Actinoplanes* |

[a]Characteristic sugar patterns are present only in wall cell type II-IV, those actinomycetes with meso-diaminopimelic acid
[b]Madurose is 3-*O*-methyl-D-galactose
(Source adapted from Prescott *et al*., 2005)

studies of heterologous rRNA/DNA hybridization (Pace and Campbell, 1971) and now it has become the most common housekeeping genetic marker and a powerful technology to study the bacterial phylogeny and taxonomy. It is commonly used for a number of reasons. These reasons include (i) its presence in almost all bacteria, often existing as a multi gene family, or operons; (ii) the function of the 16S rRNA gene over time has not changed, suggesting that random sequence changes are a more accurate measure of time (evolution); and (iii) the 16S rRNA gene (1,500 bp) is large enough for informatics purposes (Patel, 2001). Therefore, it is ideal for primer design, polymerase chain reaction (PCR) or sequencing, and sequence alignment. It is possible to design universal primers for most of the bacteria. It also contains specific variable regions that allow species identification (Hugh *et al*., 2003).

Specific identification of the metabolically active microorganisms is important, since they are responsible for the microbially driven environmental processes. For an example, knowledge of the active microorganisms in polluted habitats is relevant to the development of optimal in situ bioremediation strategies, as well as contributing to the identification of yet undescribed bacteria which may play important roles in pollutant degradation or other community process (Nogales *et al*., 2001). Several reports on the analysis of actinomycete communities using 16S rRNA have been published (Khucharoenphaisan *et al*., 2012; Jose and Jebakumar, 2012; Nithya *et al*., 2012)

Gram positive bacteria with a high G+C content are currently recognized as a distinct phylum, actinomycete, on the basis of their branching in 16S rRNA or 16S ribosomal DNA (16S rDNA) tress. Except for an insert in the 23S ribosomal RNA (23S rRNA), there are no unique biochemicals or molecular characteristics known at present that can distinguish this group from all other bacteria (Gao and Gupta, 2005). The gene encoding the small subunit ribosomal (16S rRNA in prokaryotes and 18S rRNA in

eukaryotes) has been sequenced most often and is particularly useful for defining phylogenies at the genus and higher taxanomic levels (Rossello-Mora and Amann, 2002). Therefore, rRNA sequencing is a successful means for the identification of actinomycete which are complex to identify by conventional techniques.

Ribosomal sequences are generally submitted to and can be retrieved from the European Molecular Biology Laboratory (EMBL) Heidelberg, Germany, Genbank (NCBI), Bethesda, MD, USA and the DNA Database of Japan (DDBJ) Mishima, Japan. While, sequences can be retrieved via the World Wide Web and new sequences compared with those held in the databases by using the basic local alignment search tool (BLAST).

### 2.1.3.2 Bacterial De-replication using BOX-PCR

Differentiation of bacteria genera was traditionally performed by physiological and biochemical descriptions, but this approach had some limits such as time consuming and laborious. The biochemical tests used rely on the expression of phenotypes that may not always differentiation between species or strains within a species (Shuhaimi *et al*., 2001). Further, characterization of microorganism is becoming a crucial step during the screening assay especially when it comes to handling a large number of test organism. Thus, detection of duplicates is necessary to reduce redundancy in screening assays and help in the discovery of novel compounds (Zhao *et al*., 2004).

A number of analytical methods for differentiating between strains of bacteria have been evaluated. These methods include pulsed-field gel electrophoresis, PCR-based locus-specific RFLP, repetitive element PCR fingerprinting (rep-PCR), random amplified polymorphic DNA (RAPD), and sequencing. A perfect method to

differentiate between strains must have high discrimination power combined with good to moderate inter- and intra-laboratory reproducibility. In addition, it should be easy to set up, to use and to interpret. All of these requirements are fulfilled by the rep-PCR (Olive and Bean, 1999). There are three main sets of repetitive DNA elements used for typing purposes. The repetitive extragenic palindromic (REP), ERIC, and BOX elements which consist of differentially conserved subunits, namely boxA, boxB, and boxC.

BOX-PCR is the most commonly used technique due to its simplicity, efficiency, low cost and BOX-PCR patterns are not affected by the culture age of the strain to be analyzed (Kang and Dunne, 2003) and fingerprinting output can be easily analyzed by computer assisted studies in environmental microbiology (Oda *et al*., 2003; Cherif *et al*., 2003; Singh *et al*., 2001). Only the boxA-like subunit sequences (BOX-A1R primer) appear highly conserved among diverse bacteria and it was the first repetitive sequences identified in a Gram-positive organism (*Streptococcus pneumoniae*) meanwhile REP- and ERIC-sequences were originally identified in Gram-negative bacteria and then found to be conserved in all related Gram-negative enteric bacteria and in many diverse, unrelated bacteria from multiple phyla (Elke and Christoph, 2003).

BOX-PCR as a unique, stable, and reproducible method has proved a relationship above the species level in differentiating *Streptomyces* strains (Rai *et al*., 2014 and Bouizgarne *et al*., 2009). Further, it revealed the possibility of delineating *P. syringae* genomospecies (Marques *et al.*, 2000), as well as for typing *Aeromonas* spp. strains (Tacao *et al.*, 2005) and for identification of races and biovars of *Ralstonia solanacearum* (Galal *et al.*, 2003). The technique has also been used to investigate bacterial inoculum sources (Greco *et al.*, 2004), as a tool for unequivocal identification

of strains belonging to a unique pathovar (El Tassa *et al.*, 1999) or to define new species, as a part of a polyphasic approach (Catara *et al.*, 2002). Since the BOX repetitive sequences are interspersed throughout the genome, BOX-PCR is a method potentially capable of simultaneously surveying many DNA regions scattered in the bacterial genome. It has been shown to have similar or even better strain differentiation power, as well as to be easier to perform, than ribosomal intergenic spacer analysis (RISA), restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), random amplified polymorphic DNA (RAPD) and other techniques (Chmielewki *et al.*, 2002; Niemann *et al.*, 1997; Olive and Bean, 1999).

## 2.2    Habitats of Actinomycetes

Actinomycetes are primarily saprophytic and widely distributed in nature (Babalola *et al.*, 2009; Gurung *et al.*, 2009) but best known from soils (Williams *et.al.*, 1984). They grow abundantly on semi-rotten residues. When a lump of peat or humus is inoculated with actinomycetes, it will soon be penetrated by the threads of the latter, and its surface will be covered with a white coat of the aerial mycelium. The soil actinomycetes produce a volatile compound called geosmin, which literally translates to "earth smell" (Gust *et al.*, 2003). This organic compound is responsible for a contributor to the strong odor that occurs in the air when rain falls after a dry spell of weather. The most commonly found actinomycetes in soil and in composts are the genus *Streptomyces* eventhough *Norcardia, Microbispora*, *Micromonospora*, *Actinomyces*, *Actinoplanes* and *Streptosporangium* have also been isolated from the soil (Adegboye and Babalola, 2012).

Actinomycetes have been isolated from diverse soil types and locations such as tropical forest, mining, cave, and desert. The important factor controlling the abundance of variety actinomycetes in the soil are geographical location, soil temperature, soil

type, soil pH, organic matter content, agricultural activities, aeration, nutrient availability, moisture content and soil vegetation (Arifuzzaman *et al*., 2010). Actinomycetes grow better in well aerated, slightly alkaline soils rich in organic matters (Adegboye and Babalola, 2012) and at relatively low moisture, even at 15 to 20 % of the moisture-holding capacity of the soil (El-Tarabily and Sivavithamparam, 2006).

As a soil microbe, they grow in close association with the plant organs. Such environment is referred as rhizosphere while the bacteria which colonize the plant roots are called as rhizobacteria (Adegboye and Babalola, 2012). Few studies done by previous researches have shown that actinomycetes are one of the important groups of root-colonizing micro-organisms (Franco-Correa *et al*., 2010; Nimnoi *et al*., 2010). The high nutrient content from plant to root cell in rhizosphere makes the microbial load higher at the surrounding bulk soil.

Actinomycetes as a rhizobacteria able to inhabit the internal part of the plants and causes symbiotic relationship. For an example, they able to produce antibiotics that inhibit plant pathogens in exchange for nutrients which referred as plant growth promoting actinomycetes (Compant *et al*., 2010). Either that, as a rhizobacteria they have ability to produce other varieties of bioactive metabolites including plant growth inhibitors and hydrolytic cell wall-degrading enzymes such as cellulase, hemicellulase, chitinase and other complex polysaccharides (Getha and Vikineswary, 2002; Taechowisan *et al*., 2003; Hasegawa *et al*., 2006). Such hydrolictic enzyme produced by actinomycetes play an important ecological role in recycling and mineralization of nutrients in soil through decomposition of organic materials (Macagnan *et al*., 2008). This hydrolytic enzyme replenishes the supply of nutrients in the soil and is an important part of humus formation (Marsh and Wellington, 2007). Further, as a plant growth promoter they also help in solubilization of nutrients, immobilization of

nutrients, siderophores production, biological control and soil structure maintenance (Kekuda *et al*., 2010; Macagnan *et al*., 2008; Rascio *et al*., 2008; Vargas Gil *et al*., 2009). Therefore, actinomycetes as a rhizobacteria influences plant growth antagonize plant pathogens and makes nutrients available for the plants (Maheshwari and Shimizu, 2011). Besides this, approximately 10,000 antibiotics have also been found, and almost half of them are produced by *Streptomyces* that originated in the soil (Lazzarini *et al*., 2000).

Further, nitrogen fixation by actinomycetes is not done from the air, but instead from ammonia discharges of organic matter where nitrites are reduced to nitrates. They are less vigorous in acid or waterlogged soils but are extremely vital to the decay of organic matter in dry regions. They are visible as the white, fungus-like threads on which give them an advantage in colonizing the rhizosphere effectively and decaying organic matter (Adegboye and Babalola, 2012). Actinomycetes may work near surface or many feet below the ground. Thus, they are not fastidious, they are widespread in nature and can be detected everywhere in the extreme north and in the tropic, on barren rocks in fertile chernozems (Krasilnikov, 1961) and in aquatic environments, freshwater and marine habitats (Fenical and Jensen, 2006; Pathom-aree *et al*., 2006).

Recent data from culture-dependent studies have shown that indigenous marine actinomycetes indeed exist in the oceans. These include members of the genera *Dietzia, Rhodococcus, Streptomyces, Salinispora, Marinophilus, Solwaraspora, Salinibacterium, Aeromicrobium marinum, Williamsia maris and Verrucosispora* (Lam, 2006). The marine environment contains several habitats, from the sea surface microlayer, down through the bulk water column (containing marine organisms and marine snow), which extends from a few millimeters below the surface to >10000 meters depth, and further down to the habitats on and under the sea floor (Alan *et al*.,

2006). Thus, such marine organisms exhibit unique physiological and structural characteristics that enable them to survive in extremes of pressure, salinity and temperature, with the potential production of novel secondary metabolites not observed in terrestrial microorganism (Radajewski *et al*., 2002).

Therefore, actinomycetes have great importance in nature and seem to be ultimately involved in soil ecology (Van Hop *et al*., 2011).

## 2.3    Secondary Metabolites from Actinomycetes

Actinomycetes have an unparalleled ability to produce diverse secondary metabolites (Mincer *et al*., 2002) (Figure 2.8). Thus, they have been widely used for the production of secondary metabolites which are useful to human (Dhanasekaran *et al*., 2005).

Soil actinomyctes, especially *Streptomyces* spp. represent an important source of biologically active compounds (Watve *et al*., 2001; Berdy, 2005) for a variety of reasons, including defense and communication. The biological active compounds produced by actinomycetes are antibiotics, immunosuppressant, extracellular hydrolytic enzymes, plant growth promoters and siderophores. Approximately 80% of world antibiotics are produced by actinomycetes, mostly by the genus *Streptomyces* and *Micromonospora* (Pandey *et al*., 2004). *Streptomyces* spp. produce tetracyclines, aminoglycosides (streptomycin and its relatives), macrolides (erythromycin and its relatives), chloramphenicol, rifamycins, and most other clinically-useful antibiotics that are not beta-lactams (Todar, 2009). Besides this, a range of useful actinomycete antibiotics were reported as well (Table 2.3).

Actinomycetes are the main stay of the antibiotics industry. For example, Streptomycin proved to be highly effective in the treatment of a large number of
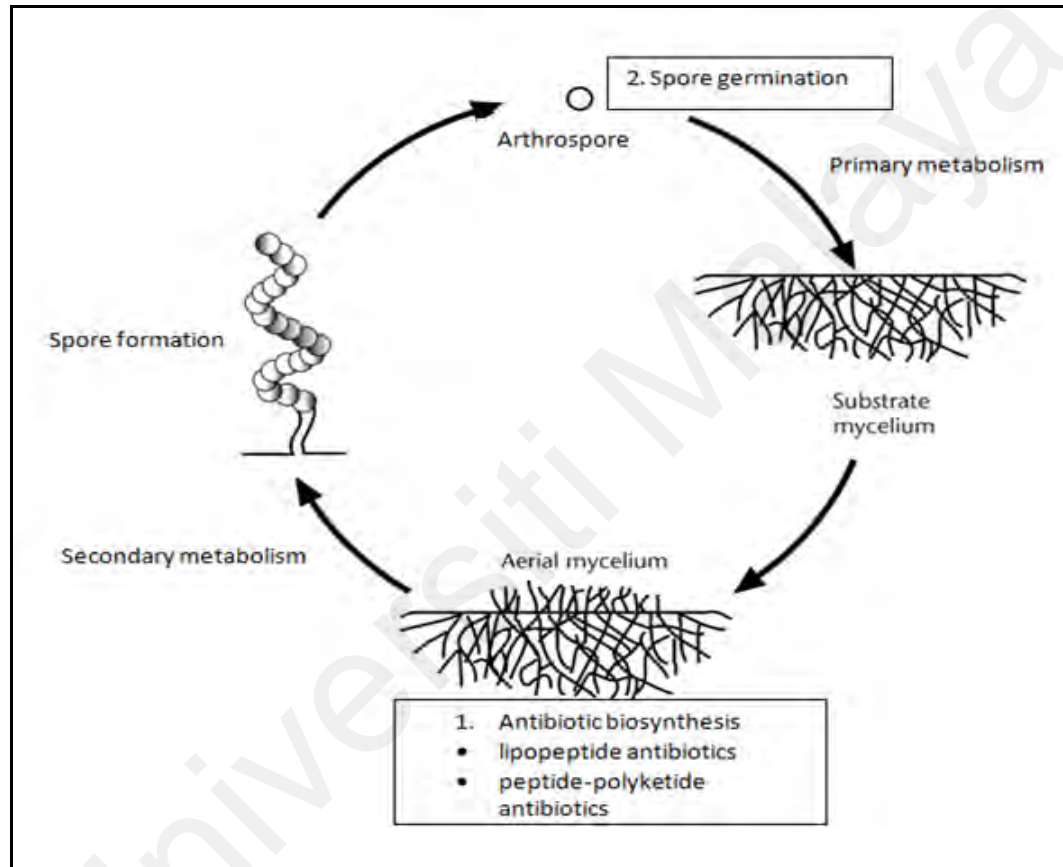
**Figure 2.8** Secodary metabolite production by actinomycetes (Mincer et al., 2000)

**Table 2.3**    Actinomycete antibiotics for medical applications

| Antibiotic | Producer | Application |
|---|---|---|
| Erythromycin | *Saccharopolyspora erythraea* | Antibacterial against gram positive bacteria, gram negative bacteria not enterics, *Neisseria, Legionella* and *Mycoplasma* |
| Gentamicin | *Micromonospora* sp. | Antibacterial against gram negative bacteria |
| Nocardicin A | *Nocardia uniformis* | Antibacterial against gram negative bacteria |
| Nystatin | *Streptomyces noursei* | Antifungal especially *Candida* sp |
| Rifamycin | *Amycolatopsis mediterranei* | Antibacterial against *M. tuberculosis* |
| Ristocetin | *Amycolatopsis lurida* *Streptococcus* sp. | Antibacterial against gram positive bacteria |
| Spinosyns | *Saccharopolyspora spinosa* | Insecticidal |
| Streptomycin | *Streptomyces griseus* | Antibacterial against gram positive and gram negative bacteria |
| Teicoplanin | *Actinoplanes teichomyceticus* | Antibacterial against gram positive bacteria |
| Vancomycin | *Amycolatopsis orientalis* *Streptococcus* sp. | Antibacterial against gram positive bacteria |

(Source adapted from Kieser *et al.,* 2000; Todar 2009)

infectious diseases brought about in experimental animals by various bacteria. This was first brought in a comprehensive study by Waksman (1952) using a group of diseases (tularemia, urinary tract infections, Klebsiella and Hemophilus infections, various forms of meningitis and whooping cough) which could be considered to be definitely controlled by streptomycin or to give promise that they would respond favorably.

Apart from that, Erythromycin A, the first macrolide, was introduced in the 1950's and after years of clinical experience it still remains a commonly relied upon antibiotic. Erythromycin is a macrolide antibiotic which has an antimicrobial spectrum similar to or slightly wider than that of penicillin, and is often used for people who have an allergy to penicillins. The function of erythromycin as a prokinetic agent has also been investigated for a range of gastrointestinal motility disorders and more recently within the context of critically ill patients. Prokinetic agents are drugs that increase contractile force and accelerate intraluminal transit. It is also used to treat outbreaks of chlamydia, syphilis, acne, and gonorrhea. Erythromycin is produced from a strain of actinomycetes *Saccharopolyspora erythraea*, formerly known as *Streptomyces erythraeus*. Erythromycin prevents bacteria from growing by interfering with their protein synthesis (Catherine *et al*., 2007).

Other secondry metabolites that act as an antibiotic are Abyssomicin C and Diazepinomicin. Abyssomicin C is a novel polycyclic polyketide antibiotic produced by marine *Verrucosispora* strain. It is a potent inhibitor of para-aminobenzoic acid biosynthesis and, therefore, inhibits the folic acid biosyhthesis at an earlier stage than the well-known synthetic sulfa drugs. While, Diazepinomicin is a unique farnesylated dibenzodiazepinone produced by a *Micromonospora* strain. It possesses antibacterial, anti-inflammatory and antitumor activity (Lam, 2006).

## 2.4    Actinomycetes in Bioremediation

Bioremediation can be defined as removal of toxic and noxious compounds by the action of microorganisms which degrades pollutants to harmless substances hence making the environment cleaner and better (Collin, 2001). Meanwhile, the actinomycetes are well known to posses many properties that make them potential bioremediation candidates. They play an important role in disintegrating high doses of herbicides, pesticides and other xenobiotics in the environment (Praseetha *et al*., 2012). The pesticides that they manage to degrade are widely in different chemical structures such as organochlorines, *s*-triazines, triazinones, carbamates, organophosphates, organophosphonates, acetanilides, and sulfonylureas (de Schrijver *et al*., 1999). Either that, actinomycetes especially the *Streptomyces* flora plays an effective role in degradation of hydrocarbons (Radwan *et al*., 1998; Barabas *et al*., 2001) especially petroleum hydrocarbons. The *Streptomyces plicatus* is one of the important actinomycetes which are known for degradation of petrol (Syed Amir Manzoor, 2011). They utilize petrol as carbon sources and in turn synthesize commercially viable enzymes and proteins (Praseetha *et al*., 2012) which able to degrade complex polymers (Goodfellow and Williams, 1983). Actinomycete strains were also found having ability to solubilise lignin and degrade lignin-related compounds by producing cellulose- and hemicellulose-degrading enzymes and extracellular peroxidases (Mason *et al*., 2001). Further, there are studies reported on actinomycetes as a potential 2,4,6-trinitrotoluene (TNT) transforming microorganism (Funk *et al*.,1993; Kaplan and Kaplan, 1982; Klausmeier *et al*., 1973). The highly inflammable and toxic TNT can be transformed in to its less toxic intermediates which further form polymers by actinomycetes in both aerobic and anaerobic conditions (Syed Amir Manzoor, 2011).

This shows that actinomycetes are with multiple physico-chemical and biological mechanisms effecting transformations between soluble and insoluble phases and produces significant levels of biosurfactants (Subhajit, 2012). Thus, in some contaminated sites actinomycetes represent the dominant group among the degraders (Johnsen, 2002).

## 2.5    Heavy metal pollution

### 2.5.1    Heavy metal in the environment

Based on Lide (1992) "heavy metals" are chemical elements with a specific gravity at least 5 times that of water which is 1 at 4°C (39°F) while Weast (1984) refers "heavy metal" as metals with a density beyond 5 $g/cm^{-3}$. There are 90 naturally occurring elements where 21 are non metals, 16 are light metals and the remaining 53 (with As included) are heavy metals (Weast, 1984). Although they comprise the major part of the elements, the understanding of the metabolism of heavy metals and the biotechnological use of these metabolic functions are in their infancy.

Heavy metals occurs naturally (Nies, 1999; Mighall *et al.*, 2002) and can be found in the earth crust (Malle, 1992), but rarely at toxic levels. Natural erosion processes such as weathering and abrasion of rocks, soils and sediments by wind and water, a small but significant fraction of natural metals are continuously being mobilized and transported in the environment. Further, volcanic eruptions, forest fires and aerosol formation above seas also contribute to the natural transport of metals. Therefore, recycling of these processes causes the presence of heavy metals naturally in the air, surface waters and soil (Mighal *et al.*, 2002; Raab and Fieldman, 2003).

Meanwhile, the anthropogenic sources of environmental metal contamination are metalliferous mining and smelting, industry, atmospheric deposition, agriculture and

waste disposal (Ross, 1994). Thus, places around mining waste piles and tailings, industrial areas where chemicals may have been dumped on the ground or in areas downwind from industrial sites old landfill sites particularly those that accepted industrial wastes, old orchards that used insecticides containing arsenic as an active ingredient and fields that had past applications of waste water or municipal sludge might highly contaminated with heavy metals.

In Malaysia, Department of Environment (DOE), 1997 identifies industrial and municipal wastes effluent discharges as the main reasons for high level of trace metal pollution in Malaysia groundwater. The current industrialization and waste disposal causes a significant quantity of heavy metal in soil being contributed by the metal-contaminated wastewater runoff derived from sources including atmospherically deposited metals, residues from pesticide usage, metal waste components such as food cans and scrap metal and the indiscriminate dumping of household hazardous waste and electronic waste such as batteries and old computer (Agamuthu and Fauziah, 2010).

However, this wasn't the start of environmental pollution in Malaysia. According to Malaysian Environmental Quality Act, 1998, Malaysian soil and rivers have been polluted about 100 years ago during the rapid development of tin mining. In Peninsular Malaysia itself, the ex-mining land covers approximately 113,700 ha which consist of 14.4% of water bodies and 85.6% of tin tailings (Chan, 1990). However, large tracts of these tin tailings are still remaining unproductive. Many research have been done in order to green the ex-mining land in Malaysia. An example of the tin tailing is the Bidor tin tailings at Perak. Bidor tin tailing is a substation of Forest Research Institute of Malaysia (FRIM). FRIM initiated afforestation trials in the 1950s and the effort has been renewed since the 1980s. Based on their researchers study, there are many problems to grow trees here and one of them is the presence of toxic compounds. A

range of heavy metal contaminant has been discovered here and below is some basic information of seven different heavy metal which represent the main contaminants at the Bidor tin-tailings (Ang and Ang, 1997).

a) Arsenic

Arsenic is a chemical element with symbol As. Its atomic number is 33 and atomic weight is 74.922 in the periodic table. Arsenic is a metalloid and it can exist in various allotropes. The three most common arsenic allotropes are metallic gray, yellow and black arsenic. However, only the gray form has important use in industry. Arsenic was first discovered by Early Bronze Age during 2500 BC (Accessed from Wikipedia, Arsenic).

b) Cadmium

Cadmium is a chemical element with the symbol Cd. Its atomic number is 48 and atomic weight is 112.411 in the periodic table. It is soft and bluish-white in colour with low melting point (321.07 °C) compared to other transition metals. It was first discovered by Karl Samuel Leberecht Hermann and Friedrich Stromeyer in the year 1817(Accessed from Wikipedia, Cadmium).

c) Plumbum

Plumbum or known as lead is a chemical element in the carbon group with symbol Pb. Its atomic number is 82 and atomic weight is 207.2 in the periodic table. Lead is a soft and malleable metal, with a melting and boiling point of 327.46 °C and 1749 °C, respectively. It is regarded as a heavy metal and poor metal. Lead was first discovered by Middle Easterns during the 7th millennium BC (Accessed from Wikipedia, Lead).

d) Mercury

Mercury is a chemical element with the symbol Hg. Its atomic number is 80 and atomic weight is 200.59 in the periodic table. Mercury is commonly known as quicksilver and was formerly named as hydrargyrum. It is a heavy, silvery-white metal with melting and boiling point of −38.8290 °C and 356.73 °C, respectively. Mercury was first discovered by ancient chinese and indians before 2000 BC (Accessed from Wikipedia, Mercury).

e) Zinc

Zinc is a metallic chemical element with symbol Zn. Its atomic number is 30, atomic weight is 65.39 and it's the first element of group 12 of the periodic table. Zinc is the 24th most abundant element in the Earth's crust with melting and boiling point of 419.53 °C and 907 °C, respectively. It was first discovered by Indian metallurgists before 1000 BC (Accessed from Wikipedia, Zinc).

f) Cuprum

Cuprum or also known as copper is a chemical element with the symbol Cu. Its atomic number is 29 and atomic weight is 63.546 in the periodic table. Cuprum is a ductile metal with very high thermal and electrical conductivity. Its melting and boiling point are 1084.62 °C and 2562 °C, respectively. Pure copper is soft and malleable while a freshly exposed surface has a reddish-orange color. It was first discovered by Middle Easterns during 9000 BC (Accessed from Wikipedia, Copper).

g) Nickel

Nickel is a chemical element with the chemical symbol Ni. Its atomic number is 28 and atomic weight is 58.69 in the periodic table. Nickel belongs to the transition metals

and is hard and ductile. It is a silvery-white lustrous metal with a slight golden tinge. Nickel was first discovered by Axel Fredrik Cronstedt in the year 1751 (Accessed from Wikipedia, Nickel).

The source of heavy metals and their permissible limit in soil are shown in Table 2.4.

## 2.5.2 Heavy Metal and Toxicity

Once in the soil, heavy metals are redistributed into different chemical forms with varying bioavailability, mobility, and toxicity through initial fast adsorption reactions followed by slow adsorption reactions (Shiowatana *et al*., 2001; Buekers, 2007). This reaction includes mineral precipitation and dissolution, ion exchange, adsorption, and desorption, aqueous complexation, biological immobilization and mobilization, and plant uptake which is believed to control the distribution of heavy metal in soil (Levy *et al*., 1992).

Living organisms require varying amounts of heavy metals. Most heavy metals are transition elements with incompletely filled d-orbitals. These d-orbitals provide heavy metal cations with the ability to form complex compounds which may be redox-active or not. Thus, heavy metal cations plays an important role in sophisticated biochemical reactions such as nitrogen fixation, water cleavage during oxygenic photosynthesis, respiration with oxygen or nitrate, one-electron catalysis, re-arrangement of C-C bonds, hydrogen assimilation, cleavage of urea, transcription of genes into mRNA, and programmed development of a single cell to a human being. These are all based on the formation of or catalysis by biochemical heavy metal complex compounds (Andre *et al*., 2005).

**Table 2.4**      Source of heavy metals and their permissible limit in the soil

| Pollutants | Major source of heavy metal[@] | Permissible level (mg/kg$^{-1}$) |
|---|---|---|
| Arsenic | Pesticides, fungicides, metal smelters | 50* |
| Cadmium | Welding, electroplating, pesticide, fertilizer, Cd and Ni batteries, nuclear fission plant | 3[#] |
| Plumbum | Paint, pesticide, smoking, automobile emission, mining, burning of coal | 300[#] |
| Mercury | Pesticides, batteries, paper industry | 1* |
| Zinc | Refineries, brass manufacture, metal plating, plumbing | 200[#] |
| Cuprum | Mining, pesticide production, chemical industry, metal piping | 50[#] |
| Nickel | Hydrogenated vegetable oil, nickel-plated jewelry, nickel plating on metallic objects, cigarette smoking, manufacture of steel batteries, machine parts, wire, electrical parts | 50[#] |

@ Source adapted from Singh *et al*., 2011
# Source adapted from USEPA Standards (1997)
*Source adapted from Ang *et al*., 2000

Thus, low concentration of heavy metal such as copper, iron and zinc is necessary and not harmful for organisms. These metal ions are essential because of their incorporation in enzymes or cofactors (Andre *et al*., 2005). However, all metals are toxic at higher concentrations (Chronopoulos *et al*., 1997). Excessive levels can be damaging to the organism.

Serious attention is taken worldwide to health hazards which are caused by the increase of heavy metal contamination in the environment. This is because, accumulation of heavy metal over time in living tissues of human and animals throughout the food chain, poses serious illnesses (Zouboulis *et al*., 2004). The types of heavy metals and their effect on human health with their permissible limits are enumerated in Table 2.5.

On the other hand, plants experience oxidative stress upon exposure to heavy metals that leads to cellular damage and disturbance of cellular ionic homeostasis. In order to survive, they minimize the detrimental effects of heavy metal exposure and their accumulation by evolving detoxification mechanisms mainly based on chelation and subcellular compartmentalization (Singh *et al*., 2011). However, consumption of heavy metal by plants is still poisonous to human and animals especially when the particular plant is a food crops. The types of heavy metals and their effect on plant tissue with their permissible limits are enumerated in Table 2.6.

Heavy metals are toxic at higher concentrations in microorganisms because of adversary binding to enzymes and DNA, and by production of oxygen radicals through the Fenton reaction (Lopez-Maury *et al*., 2002). Homeostasis within the cell must be maintained to keep the reactive heavy metals at an optimal, sub-toxic level. Therefore, in order to survive, microorganisms such as bacteria possess resistance mechanisms through selection pressure and adaption (Figure 2.9). Mutations are the source of

**Table 2.5**     Heavy metal effects and permissible level of heavy metal in human drinking water

| Pollutants | Effect on human health[@] | Permissible level (mg/l) |
|---|---|---|
| Arsenic | Bronchitis, dermatitis, poisoning | 0.01[#] |
| Cadmium | Renal dysfunction, lung disease, lung cancer, bone defects, increased blood pressure, kidney damage, bronchitis, gastrointestinal disorder, bone marrow, cancer | 0.005[#] |
| Plumbum | Mental retardation in children, fetal infant encephalopathy, congenital paralysis, sensor neural deafness, acute or chronic damage to the nervous system, epilepticus, gastrointestinal damage | 0.01[#] |
| Mercury | Tremors, gingivitis, minor psychological changes, acrodynia characterized by pink hands and feet, spontaneous abortion, damage to nervous system, protoplasm poisoning | 0.001[#] |
| Zinc | Zinc fumes have corrosive effect on skin, cause damage to nervous membrane | 5.0* |
| Cuprum | Anemia, liver and kidney damage, stomach and intestinal irritation | 2.0[#] |
| Nickel | Dermatitis; nickel carbonyl: myocarditis, encephalopathy; Occupational (inhaled): pulmonary fibrosis, reduced sperm count, nasopharyngeal tumors | 0.02[#] |

@Source adapted from Singh *et al*., 2011 #Source adapted from European Union, EPA
* Source adapted from Canadian Health Act Safe Drinking Water Regulation, 2001

**Table 2.6**     Heavy metal effects and permissible level of heavy metal to plant tissue

| Pollutants | Effect on plants tissue | Permissible level (ug/g) |
|---|---|---|
| Arsenic | Not needed | 0.1-5 |
| Cadmium | Not needed, poisonous | 0.2-0.8 |
| Plumbum | Not needed, a little poisonous | 10 |
| Mercury | Not needed, poisonous | 1 |
| Zinc | Needed, more than 200 ppm is poisonous | 15-200 |
| Cuprum | 2-4 ppm is needed, more than that is poisonous | 2-15 |
| Nickel | Not needed, more than 50 ppm is poisonous | 1-10 |

(Source adapted from Dastjerdi *et al*., 2013)
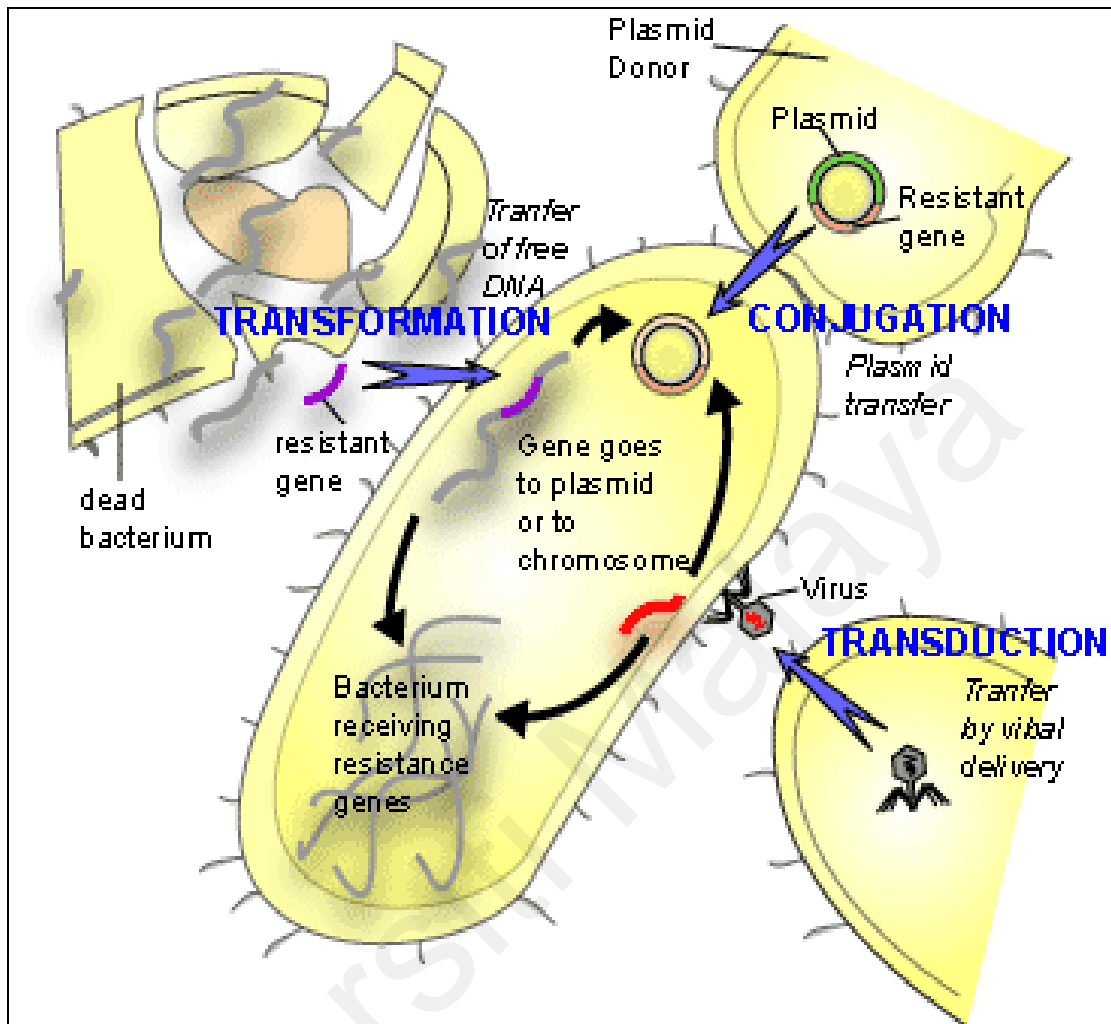
**Figure 2.9**    Example of pathways on how bacteria becomes resistant (Todar, 2012)

genetic variability in cells. Natural selection acts upon that variability to generate adaptations such as antibiotic and/or metal resistance (Timoney *et al*., 1978). The bacterial cells also exchange metal and/or antibiotic resistance genes by transfer of genetic material between bacteria through direct cell-to-cell contact which is known as horizontal gene transfer in plasmids. Up to 1000 plasmid copies may exist in a cell and each one may carry as many as 300 different genes (Zajicek, 2013). Plasmids spread easily from one bacterium to even across species.

### 2.5.3 Soil Remediation for Removal of Heavy Metal

Soil remediation is done to find a solution in order to protect human health and the environment (Martim and Ruby, 2004). An appropriate remediation treatment is strongly associated with the physical and chemical form of the heavy metal contaminant in soil. The remediation technologies can be classified into three categories: (i) gentle *in situ* remediation, (ii) *in situ* harsh soil restrictive measures, and (iii) *in situ* or *ex situ* harsh soil destructive measures (Gupta *et al*., 2000). *In situ* means that the contaminated soil is treated in its original while *ex situ* means that the contaminated soil is moved or excavated from the site or subsurface (Raymond and Felix, 2011).

Various studies conducted on heavy metal removal indicated the severity of heavy metal pollution and its danger to environment and human. Currently available commercial remediation methods for reducing the harmful effects at heavy metal contaminated sites include those involving technical inputs (Table 2.7). However, these new technologies of remediation has many factors such as cost, long term effectiveness/ permanence, commercial availability, general acceptance, applicability to high metal concentrations, applicability to mixed wastes (heavy metals and organics), toxicity reduction, mobility reduction, and volume reduction (Raymond and Felix, 2011) which are still disrupting the usage of this methods world widely. Therefore, bio-adsorbents

**Table 2.7**     Technologies for remediation of heavy metal-contaminated soils

| Category | Remediation technologies |
| --- | --- |
| Isolation | 1) capping<br>2) subsurface barriers. |
| Immobilization | 1) solidification/stabilization<br>2) vitrification<br>3) chemical treatment |
| Toxicity and/or mobility reduction | 1) chemical treatment<br>2) permeable treatment walls<br>3) biological treatment bioaccumulation, phytoremediation (phytoextraction, phytostabilization, and rhizofiltration), bioleaching, biochemical processes. |
| Physical separation | |
| Extraction | 1) soil washing, pyrometallurgical extraction, in situ soil flushing, and electrokinetic treatment. |

(Source adapted from Raymond and Felix, 2011)

such as bacteria, fungi, algae and some other agricultural wastes that emerged as an eco-friendly, effective and low cost material are choosen as potential inexpensive alternatives to the conventional adsorbents (Valls and Lorenzo, 2002) and physic-chemical methods. Further, metal remediation using soil microorganism can minimize the bioavailability and biotoxicity of heavy metals (Rajendran *et al*., 2003). Congeevaram (2007) have reported that different species of *Aspergillus*, *Pseudomonas*, *Sporophyticus*, *Bacillus*, and *Phanerochaete* as efficient for chromium and nickel reducers. Meanwhile, actinomycetes have been used in biotransformation, biodegradation and various other purposes. They cause degradation of herbicides, pesticides, chromium (IV), petrochemicals, nitroaromatic and 2,4,6 –trinitrotoluene (TNT) compounds (Syed Amir, 2011).

## 2.6    Bioremediation of Heavy Metals

### 2.6.1    Microbial Resistance Mechanisms to Heavy Metal Ions

Microorganism posses' different defense system towards heavy metal stress (Figure 2.10) such as metal exclusion by permeability barriers, enzymatic detoxification of the metal to a less toxic form, reduction in metal sensitivity of cellular targets (Bruins *et al*., 2000; Nies and Silver, 1995; Silver, 1996), efflux trasporters (Anton *et al*., 1999; Mergeay *et al*., 2003), compartmentalization and synthesis of binding proteins like metallothioneins (Olafson *et al*., 1988; Robinson *et al*., 2001). Among this, the two general protective mechanisms used by microorganism's are the avoidance, restriction of metal entry into the cell by reduced uptake/active efflux or by formation of complexes outside the cell and sequestration, reduction of free ions in the cytosol by synthesis of ligands to achieve intracellular chelation or by compartmentalization (Table 2.8).
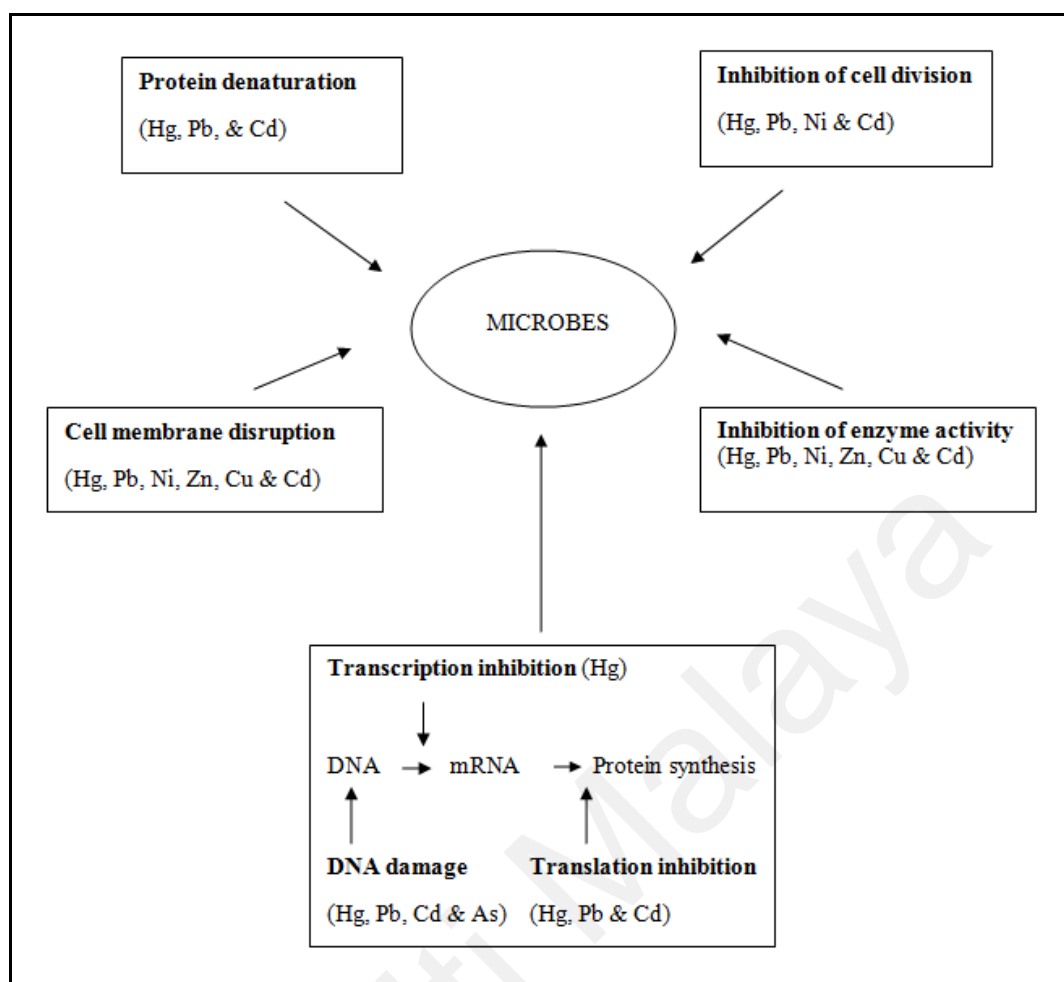
**Figure 2.10** Heavy metal toxicity mechanisms to microbes (Rajendran *et al.*, 2003).

**Table 2.8** Microorganisms metal tolerance mechanism

| Metal | Tolerance mechanism |
|---|---|
| $AsO_2^-$, $AsO_4^{3-}$ & $Sb^{3+}$ | Anion efflux (ATPase) |
| $Cd^{2+}$ & $Zn^{2+}$ | Efflux (ATPase) |
| $Co^{2+}$, $Pb^{2+}$ & $Ni^{2+}$ | Efflux |
| $Hg^{2+}$ | Reduction |
| $Cu^{2+}$ | DNA damage |

(Source adapted from Rajendran *et al.*, 2003; Silver and Phung, 2005)

The detoxification mechanisms may be directed against one metal or a group of chemically related metals (Nies and Silver, 1995). This is because, most bacterial cells are known to have specific genes which normally code for specific proteins and enzymes (Table 2.9/Figure 2.11) that perform specific functions either to protect the bacterial cell, or block or alter the incoming toxic metal, or both (Cavicchioli and Thomas, 2002). Furthermore, the detoxification mechanisms may vary depending on the type of microorganism (Nies and Silver, 1995). The mentioned resistance genes are mercury. These plasmids have also genes for resistances to many toxic ions of heavy metal such as $Ag^+$, $Cd^{2+}$, $Co^{2+}$, $Cu^{2+}$, $Ni^{2+}$, $Pb^{2+}$ and $Zn^{2+}$. Thus, this explains why some microorganisms are able to survive in metal contaminated environments.

## 2.6.2 Bioremediation of Heavy Metal using Microbes

A preliminary screening is done on the isolated microbes in order to find a potential candidate for bioremediation purpose.While, rapid screening of heavy metal tolerance is important to identify and select tolerant isolates quickly and to eliminate the weak ones which then would help to narrow down the number of selected isolates from a large group. This in return, helps in the reduction of cost and time of study. Many preliminary screening to determine heavy metal tolerance microbes have been established in previous studies. Chandy (1998) have placed different concentration of heavy metals directly on agar surface seeded with the non-filamentous bacteria. In the current study, this assay was modified to screen the filamentous actinomycetes. Minimal medium (MM) agar was used to avoid or minimize the complexation of heavy metals with ingredients of the medium (Schmidt *et al*., 2009). In this assay, different concentrations of metal salt solution were placed directly on agar surface seeded with the bacteria to allow maximum interaction between metal and the microbes.

**Table 2.9**     Gene coding for specific bacterial heavy metal resistance system

| Metal | Gene mnemonic |
|-------|---------------|
| $AsO_2^-$, $AsO_4^{3-}$ | *ars* & *aso* |
| $Cd^{2+}$ & $Ni^{2+}$ | *ncc* |
| $Cd^{2+}$ & $Zn^{2+}$ | *czc* |
| $Pb^{2+}$ | *pbr* |
| $Hg^{2+}$ | *mer* |
| $Cu^{2+}$ | *cop* & *pco* |
| $Co^{2+}$ & $Ni^{2+}$ | *cnr* |
| $Ni^{2+}$ | *nre* |

(Source adapted from Silver and Phung, 2005)
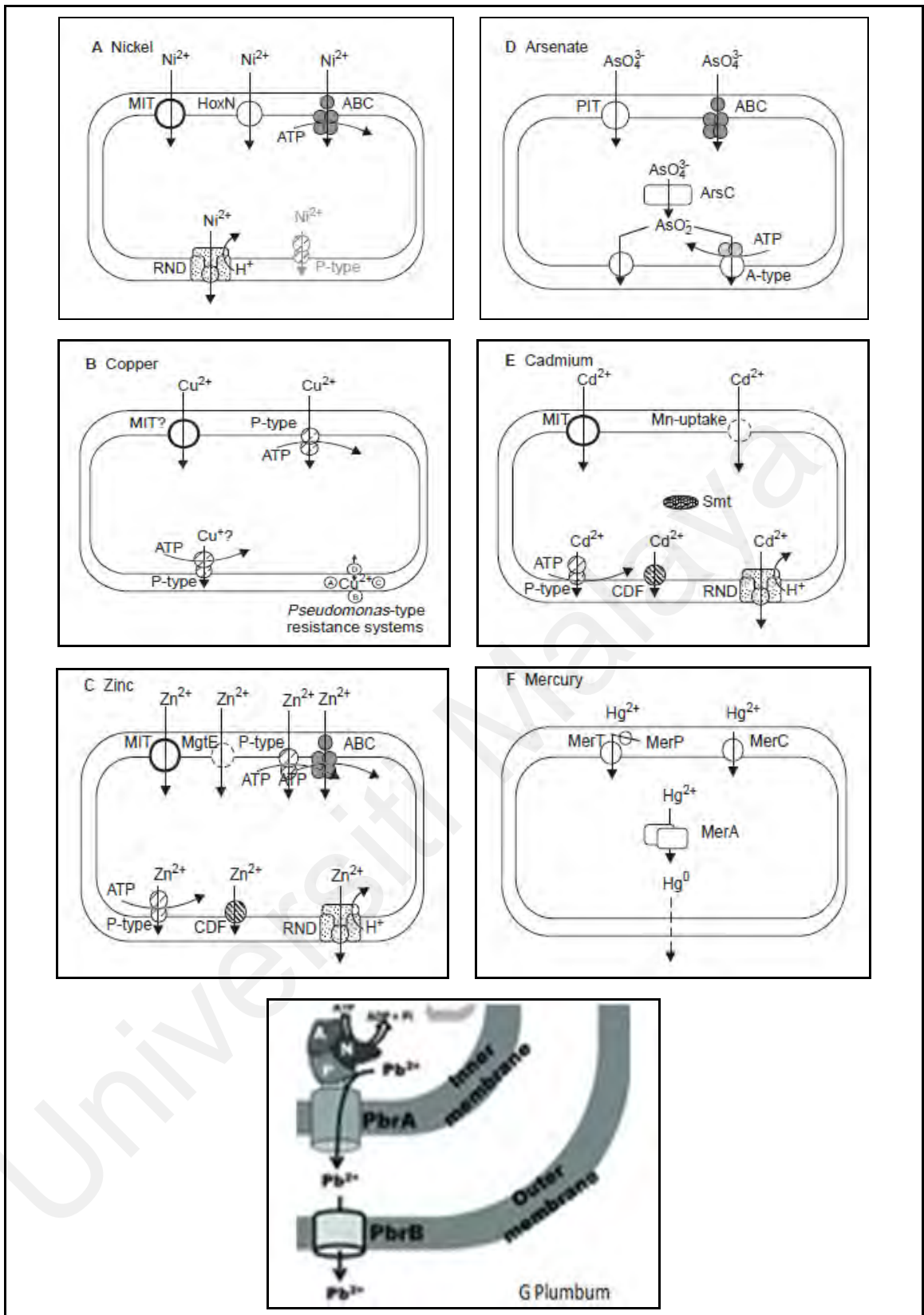
**Figure 2.11** Bacterial heavy metal mechanism involving different protein families (Nies, 1999)

According to Chandy (1998), this method is better than the method reported in previous studies by Amoroso (1998). Here, the authors used paper discs saturated with heavy metal salts which were placed on seeded agar surface, or filled up troughs with metal of interest in the centre of seeded plates in order to determine heavy metal tolerance in actinomycetes. It is also better than the agar dilution method by Albarracin (2005) where the heavy metals were incorporated into MM agar before pouring it into plates for metal tolerance screening. Heavy metals have a better chance to interact with the isolates inoculated on agar when metal solutions are placed directly onto the cultures. This is because metal ion absorbs directly into the agar allowing maximum interaction with the actinomycete cultures (Chandy, 1998). The assays conducted by Amoroso (1998) and Albarracin (2005) are expected to lead to overestimation of the resistance level due to metal ion interactions with inorganic and organic components in the nutrient media (Kanopka and Zakharova, 1999).

Once a potential candidate is obtained, the cost-effective innovative bioremediation technique such as bioaccumulation and biosorption (Rajendran *et al*, 2003) are conducted. Bioaccumulation uses live cells to uptake toxicants. The toxicant can transport into the cell, accumulate intracellularly across the cell membrane and through the cell metabolic cycle (Malik, 2004). While, biosorption is defined as the passive uptake of toxicants by dead microbial biomass. Biosorption happens because of number of metabolism-independent processes that essentially take place in the cell wall where the mechanisms responsible for the pollutant uptake. However, the mechanism will differ according to the biomass type (Vijayaraghavan and Yun, 2008). The comparison between bioaccumulation and biosorption are shown in Table 2.10. Even though both techniques has different biological methods, bioaccumulation and biosorption have been demonstrated to possess good potential to replace conventional

**Table 2.10**     Comparison of the features of biosorption and bioaccumulation

| Features | Biosorption | Bioaccumulation |
|---|---|---|
| Cost | Usually low. Most biosorbents used were industrial, agricultural and other type of waste biomass. Cost involves mainly transportation and other simple processing charges | Usually high. The process involves living cells and; hence, cell maintenance is cost prone. |
| pH | The solution pH strongly influences the uptake capacity of biomass. However, the process can be operated under a wide range of pH conditions. | In addition to uptake, the living cells themselves are strongly affected under extreme pH conditions. |
| Temperature | Since the biomass is inactive, temperature does not influence the process. In fact, several investigators reported uptake enhancement with temperature rise. | In addition to uptake, the living cells themselves are strongly affected under extreme pH conditions. Temperature severely affects the process. |
| Maintenance/storage | Easy to store and use | External metabolic energy is needed for maintenance of the culture. |
| Selectivity | Poor. However, selectivity can be improved by modification/processing of biomass | Better than biosorption |
| Versatility | Reasonably good. The binding sites can accommodate a variety of ions. | Not very flexible. Prone to be affected by highmetal/salt conditions. |
| Degree of uptake | Very high. Some biomasses are reported to accommodate an amount of toxicantnearly as high as their dry weight. | Because living cells are sensitive to hightoxicant concentration, uptake is usually low. |
| Rate of uptake | Usually rapid. Most biosorption mechanisms are rapid. | Usually slower than biosorption. Since intracellular accumulation is time consuming. |

"Table 2.10, continued"

| | | |
|---|---|---|
| Toxicant affinity | High under favorable conditions. | Depends on the toxicity of the pollutant. |
| Regeneration and reuse | High possibility of biosorbent regeneration, with possible reuse over a number of cycles. | Since most toxicants are intracellularly accumulated, the chances are very limited. |
| Toxicant recovery | With proper selection of elutant, toxicant recovery is possible. In many instances, acidic or alkaline solutions proved an efficient medium to recover toxicants. | Even if possible, the biomass cannot be utilized for next cycle. |

(Source adapted from Vijayaraghavan and Yun, 2008)

and physic-chemical methods for the removal of metals (Volesky and Holan, 1995; Malik, 2004).

## 2.7    Heavy Metal Ion Removal using Actinomycetes

Actinomycetes are natural inhabitants of soil, water and manure, continuously exposed to different metal present in these habitats. Thus, they adapt or acquire various tolerance or resistance mechanisms to these metals in order to survive in the polluted environment.

Resistance or tolerance factors due to the metal stress environment allow the organisms to maintain intracellular low levels of heavy metals or intracellular fractionation of the metal in non-harmful complexes (Eitinger and Mandrand-Berthelot, 2000). Therefore, adapted microbial populations are prone to show higher resistance to heavy metals compared to populations of non-contaminated sites. Further, selection pressures such as metal contamination functions as a selective agent in the proliferation of metal/antibiotic resistance in bacteria (Baker-Austin *et al.*, 2006). Production of new bioactive compounds as metabolites may be expected if adaptation of the actinomycetes to such environments is due to metabolic changes in the microorganism (Imada *et al.*, 2007). Apart from that, metal resistance and biosorption capability may be widespread among microbes growing in contaminated environments (Amoroso *et al.*, 1998). Therefore, many previous study have preferred to study actinomycete isolates from metal contaminated area such as former uranium mining area, heavy metal contaminated river and soil (Schmidt *et al.*, 2009; Amoroso *et al.*, 1998; Amoroso and Abate, 2012).

Actinomycetes play an important role in the recycling of organic carbon and are able to degrade complex polymers (Goodfellow and Wiliams, 1983) which make them

to be good candidates for application in bioremediation of soils contaminated with heavy metals. Some reports indicates that actinomycetes are the most suitable agents for bioremediation of metal and organic compounds due to their metabolic diversity and particular growth characteristics, mycelial form and relatively rapid colonization of selective substrates (Poopal *et al.*, 2009).

It was proven that, actinomycetes have potential capacity in Chromium (IV) bioremediation. In previous studies, Polti (2007) has reported on Cr (IV) resistance and removal by actinomycetes strains isolated from sediment while Laxman and More (2002) and Pattanapipitpaisal (2001) reported on *Streptomyces* spp. showing Cr (VI) reduction ability. *Streptomyces* was also known for possessing two superoxide dismutases which are iron- and nickel- containing enzyme, regulated by nickel (Kim *et al.*, 1998).

## 2.8    Relationship between Heavy Metal Tolerance and Antibiotic Resistance in Microbes

Antibiotics are a group of diverse chemicals or secondary metabolites of microorganisms that inhibit the growth or kill other microbes at low concentrations. The first antibiotic was penicillin which inhibits the growth of *Staphylococcus* on agar plate. After penicillin, many other antibiotics were discovered including the one produced by actinomycetes. These antibiotics have broad spectrum of action and were more effective than penicillin but after many years of antibiotic usage, antibiotic resistance a growing problem are being faced by the world (Dominic *et al.*, 2007).

There are a variety of ways for bacteria to tolerate the uptake and effects of antibiotics. They decrease the intracellular concentrations of the drug, inactivation of the drug, changes in the binding sites for the drug, and adaptations that bypass the need

for the binding site targeted by the antibiotic, efflux pump where it pump antibiotics out of cells, production of hydrolytic enzymes which destroy inhibitory capacity of the antibiotics and production of protective bio-films in cells (Figure 2.12) (Kaye *et al.,* 2000)

Methods to decrease intracellular concentrations of the drug include changes in the cell wall to increase the efflux of the antibiotic from the cell. This is seen in tetracycline and quinolone resistance. Another method is decreasing the cell membrane permeability, seen as a bacterial defense in beta-lactam antibiotic and quinolone resistance. In addition, bacteria can prevent influx of the antibiotics by decreasing cytoplasmic membrane transport as seen with the use of aminoglycosides.

Examples of enzymes that deactivate the drug are the lactamases that deactivate beta-lactams, and the phosphotransferases and actyltransferases that deactivate aminoglycosides. There are numerous methods for altering or bypassing the binding site of antibiotics. The target of the antibiotic can be altered in such a way that the antibiotic can no longer bind to and inactivate it. Examples include alterations in the DNA gyrase that prevent the binding of quinolones and methylation of rRNA so macrolides cannot bind to them. An example of an adaptation that bypasses a binding site is the ability of some bacteria to use an alternate metabolic route in folate synthesis, avoiding the effects of trimethoprim (Neu *et al.,* 1989).

These resistances can be acquired through mutations in the genes that encode for the target or affected transport proteins. As the bacterial cells without the adaptive mutations are killed as a result of an antibiotic, the cells that have the mutation continue to replicate, replacing the original population with a resistant one.
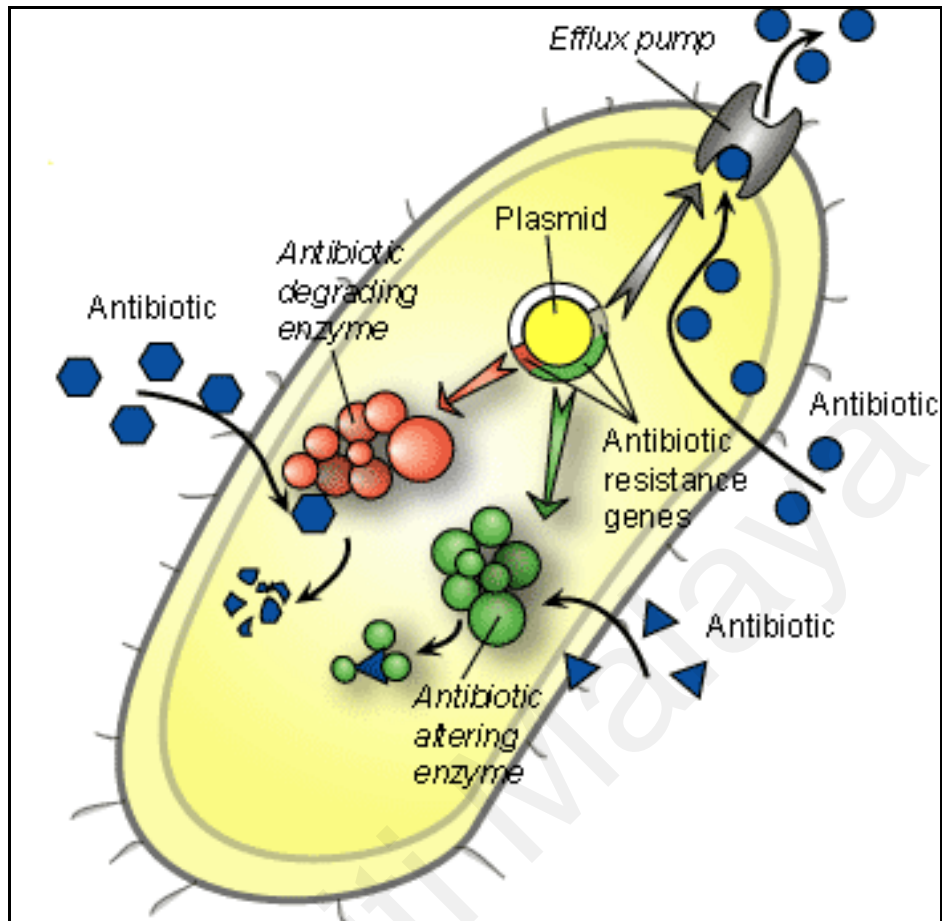
**Figure 2.12**    Example of pathways on how bacteria tolerate uptake of antibiotics (Todar, 2012)

The resistances can also be acquired as a result of the transfer of plasmids or transposons and similar agents. These are small segments of DNA that are readily exchanged between bacteria. A plasmid that contains a gene for an adaptive mutation can be shared with a large number of nearby bacteria, which may or may not be the same species. In this manner, resistance can quickly spread from species to species (Normark and Normark, 2002).

Many strategies have been used in an attempt to circumvent the multiple mechanisms of resistance that have developed in bacteria. Adding beta-lactamase inhibitors to penicillin drugs, combining sulfa drugs with pyrimethamine, trimethoprim, and erythromycin, and chemically altering cephalosporins to create the additional generations of the drugs, are examples of these strategies.

Bacteria develop heavy- metal resistance mostly for their survivals, especially a significant portion of the resistant phenomena was found in the environmental strains (with or without the presence of heavy metals). They are frequently associated with bacterial antibiotic resistances, some even with multiple antibiotic resistances because many antibiotic resistant genes are located on mobile genetic mobile elements (e.g., plasmids, transposons, and integrons), some of which are easily exchanged among phylogenetically distant bacteria. Many of these mobile genetic elements encode resistance to multiple antibiotics, heavy metals, and other toxic compounds. Studies have proved, *Escherichia coli* with multiple-antibiotic and heavy metal resistance are common at one site in sediment of the sewage sludge dump area due to selective pressure. Resistant genes are grouped on the same plasmid, thus it is reasonable to assume that either heavy metal or antibiotic serve as selection pressures for the population of bacteria hosting these types of plasmids (Timoney *et al*., 1978). Bacterial antibiotic-plasmids (sometime these plasmids are very big and called megaplasmid)

existed in bacteria before the antibiotic era but their presence was brought into prominence by the use of antibiotics, which selected for antibiotic resistant strains. Subsequently, the range of genes carried on these plasmids (frequently associated with these heavy metal resistant determinants) was shown to extend far beyond those coding for antibiotic resistance. Similarly, heavy metals are also widespread in the environment; exert a selective pressure; for the population of these plasmid-harboring bacteria. Therefore, it is proposed that selective pressure by one such compound indirectly selects for the whole set of pound resistances (Tsai, 2006).

In conclusion, studying resistance of actinomycete strains to heavy metals and antibiotics as well as testing for their potentials in bioremediation could be of great significance since the results could provide some information on their tolerance capability and the possible use of these bacterial strains for bioremediation of heavy metals in metal contaminated environments and other industrial purposes.

# CHAPTER 3

## MATERIALS AND METHODS

### 3.1    Research Outline

In this study, heavy metal tolerance in actinomycete isolates was screened using primary (rapid heavy metal tolerance screening) and secondary screening (metal toxicity assay). Further, the tolerant isolates were studied for their antibiotic resistance pattern. The heavy metal tolerant isolates were then dereplicated using BOX-PCR method to choose potential actinomycetes isolates for heavy metal accumulation study. De-replication is an important step in delineating/grouping unknown strains into taxonomically meaningful groups and manageable clusters from which representatives are selected for further studies. BOX-PCR analyses using BOX A1R primers have been successfully used for determining and clustering intra- species and inter- species relationship (Lee and Wong, 2009).  Finally, the isolates that showed heavy metal uptake ability were characterized and identified based on morphological, physiological, and genotypic characteristics. The complete research flowchart is shown in Figure 3.1.

### 3.2    Actinomycete Isolates

### 3.2.1   Revival of Cultures

A total of 421 actinomycete isolates were used for this study. These isolates were previously isolated by FRIM researchers from soil samples collected at Bidor tin tailings (238 strains) and Pasoh forest (183 strains) using the dilution technique. Soil samples were pre-heated using either chemical or physical treatments and serially diluted with sterile 0.9% (w/v) saline solution. The soil suspensions were then spread onto notably starch-casein agar and humic acid-vitamins agar containing various
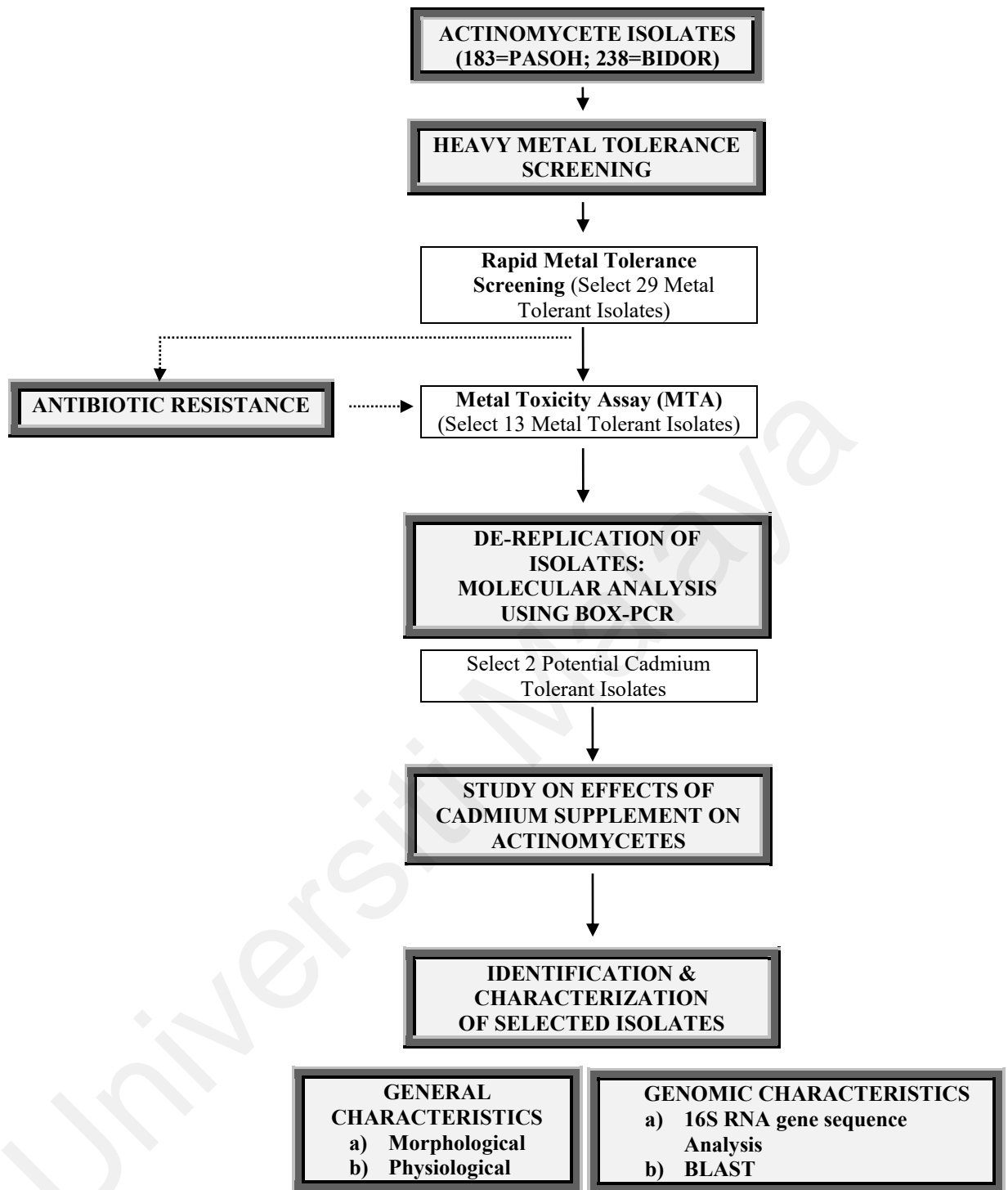
**Figure 3.1**          Flowchart of the studies conducted for this thesis

chemicals and antibiotics in order to increase the possibility of isolating actinomycetes (Getha *et al*., 2008). Successfully produced cultures were stored at -80$^{o}$C in cryovials containing 20% (v/v) glycerol and maintained in FRIM Actinobacteria Culture Collection (FACC). The cryovials were thawed at 36$^{o}$C in water bath. An aliquot of 50µl of the culture was inoculated onto sterile International *Streptomyces* Project medium 2 (ISP2) agar (Appendix A-1) (Shirling and Gottlieb, 1966). Streaked agar plates were incubated at 28±2$^{o}$C for 7 - 14 days.

## 3.2.2 Preliminary Grouping of Isolates based on Macromorphological Characteristics

A preliminary grouping of the isolates into *Streptomyces*-like (S) and non-*Streptomyces* (NS) groups was carried out on 7-14 days old cultures. Isolates were tentatively placed into either group based on their macromorphological characteristics according to Table 3.1 (Getha *et al*., 2004). Colour grouping of the isolates was done based on NBS/ISCC Colour Systems (Mundie, 1995).

## 3.3 Determination of Heavy Metal Tolerance in Actinomycetes

### 3.3.1 Heavy Metal Solution

Seven different types of heavy metal salts as mentioned in Table 3.2 were used for the metal tolerance assay. Metal solutions were prepared in Phosphate Buffer Saline (PBS) pH 6.8 (Appendix A-5) to maintain the pH of the metal solutions (Selvin *et al*., 2007) and were stored at 4$^{o}$C for no longer than 1 month. Three different concentrations (C1, C2, and C3) for each metal salt were selected for this experiment based on Chandy (1998) with slight modification as in Table 3.2. The used glasswares were leached in 2N nitric acid (HNO$_3$) and rinsed with distilled water for few times before washing to avoid metal contamination during the next usage of the same glassware.

**Table 3.1** Criteria for classification of *Streptomyces*-like (S) and Non-*Streptomyces* (NS)

| Macromorphological Characteristics | Grouping of Actinomycetes | |
|---|---|---|
| | *Streptomyces*-like (S) | Non-*Streptomyces* (NS) |
| Presence of aerial mycelia | Abundant with powdery spore mass | Sparse/ None |
| Colour grouping | Spore mass, substrate mycelium and diffusible pigment colour was observed | Surface growth, substrate mycelium and diffusible pigment colour was observed |

**Table 3.2** List of heavy metal salts and its concentration used in heavy metal tolerance test

| Heavy Metal Salts | Product manufacturer | Concentration (mmol/L) | | |
|---|---|---|---|---|
| | | C1 | C2 | C3 |
| $HgCl_2$ | Acros Organic, New Jersey, USA | 0.04 | 0.4 | 4.0 |
| $As_2O_3$ | Sigma-Aldrich INC., USA | 5.0 | 25.0 | 51.0 |
| $PbCl_2$ | Sigma-Aldrich INC., USA | 0.4 | 4.0 | 18.0 |
| $Cu_2SO_4$ | Fisher Scientific, UK Ltd. | 6.0 | 31.0 | 63.0 |
| $CdCl_2$ | Fisher Scientific, UK Ltd. | 0.06 | 0.6 | 6.0 |
| $NiCl_2$ | Sigma-Aldrich INC., USA | 8.0 | 36.0 | 77.0 |
| $ZnCl_2$ | Acros Organic, New Jersey, USA | 7.0 | 37.0 | 73.0 |

### 3.3.2 Heavy Metal Tolerance Screening

Heavy metal tolerance screening was carried out based on direct agar diffusion assay modified from Chandy (1998). Minimal Medium (MM) Agar (Appendix A-6) was prepared in petri dish and inoculated with different isolates of actinomycetes. The aerial growth of 7-10 days old culture was suspended in 5 ml sterile 20% (v/v) glycerol. An aliquot of 50 µl of the prepared inoculum was lawned on MM agar and test plates were left to dry for 30 minutes before use. Surface of the lawned test plates were divided into four quadrants, three areas corresponding to different concentration of metal solutions and one area for the control sterile PBS solution without metal salt. Ten microliter of heavy metal and control solutions were spotted on the test plates using a micropipette. Plates were incubated at 28°C for 7days and examined for the appearance of clear zone after 7 days. The diameter of inhibition zone (cm) at the spotted area was measured. Each test was done in replicate plates and mean of the two readings was calculated and used in heavy metal tolerance assessment. Means are categorized as "+" for inhibition zones bigger than 0.6 cm and "-" if inhibition zone is absent. Presence of an inhibition zone indicates the isolates being sensitive towards the heavy metal, whereas absence indicates heavy metal tolerance.

## 3.4 Determination of Antibiotic Resistance in Selected Heavy Metal Tolerant Actinomycetes

Disc diffusion assay was used to screen antibiotic resistance in 20 tolerant isolates (Bauer *et al*., 1966) selected from the rapid metal tolerance screening. A total of nine antibiotics representing different chemical classes were chosen for this study. Antibiotic discs [BD BBL™ Sensi-Disc™ - kanamycin (K-30 µg), ampicilin (Am-10 µg), nalidixic acid (Na-30 µg), chloramphenicol (Ch-30 µg), amikacin (An-30 µg), penicillin (P-10 µg), gentamycin (G-10 µg), vancomycin (Va-30 µg) and erythromycin

(E-15 µg)] were placed on the surfaces of ISP2 agar inoculated with inoculums of selected isolates prepared as in section 3.1. A blank disc was used as control. The diameter of growth inhibition zone around the discs was measured after incubation at 28ºC for 7 days. The average of four readings was calculated and scored as mentioned in Table 3.3.

**Table 3.3**     Criteria and its scoring for average reading of antibiotic resistant assessment

| Criteria | Score | Diameter Inhibition Zone (DIZ) |
|---|---|---|
| Extremely susceptible | ++ | DIZ $\geq$ 3 cm |
| Susceptible | + | 1 cm $\leq$ DIZ < 3cm |
| Weakly susceptible | $\pm$ | DIZ < 1 cm |
| Resistant | - | no inhibition zone |

The Multiple Antibiotic Resistance (MAR) index for each isolates was calculated based on Krumpermann (1983): MAR index = a/b where a = number of isolates resistant to antibiotics, b = total number of antibiotic tested. Statistical analysis was done using the SPSS version 16.0 program. Pearson Correlation was carried out where average of the total multiple antibiotic resistances isolates with its total average of metal tolerance was calculated in order to determine the correlation between multiple antibiotics resistance and heavy metal tolerance.

## 3.5 Determination of Minimum Tolerable Concentration (MTC) Levels of Heavy Metals in Actinomycetes

### 3.5.1 Metal Toxicity Assay

Metal toxicity assay based on a modified method of Sabry *et al* (1997) was carried out on the selected 29 isolates that showed tolerance to determine the MTC

levels. Seven days old ISP2 cultures of actinomycetes were used as inoculums prepared as described in section 3.2.1. Test tubes containing 5 ml MM medium supplemented with different concentrations of metal salt were inoculated with 0.5 ml actinomycete inoculums. Six different types of metal salt at five different concentrations labeled as C1 (highest test concentration) - C5 (lowest test concentration) were studied as shown in Table 3.4. Two sets of control tubes were prepared for this study: (a) inoculums in tubes without metal salt, and (b) different concentration of metal salt in tubes without inoculum. All test tubes were incubated in an orbital incubator shaker at $28\pm2^{\circ}C$ and 220rpm. After 10 days of incubation, 10µl culture broth from each test tube was inoculated on fresh ISP2 agar plates. Metal concentration in culture broth samples which showed microbial growth was used as a qualitative parameter of heavy metal resistance to determine the MTC levels. Growth of isolates on ISP2 was categorized as good "++", moderate "+", weak "±" and sensitive "-" by comparing with the growth of isolates from negative control tubes without metal salt. Statistical analysis was done using the Mann Whitney test to analyze the significant difference between sampling sites with percentage of heavy metal tolerant isolates and also with percentage of heavy metal tolerant isolates belonging to S and NS groups.

**Table 3.4** List of heavy metal salts and the concentration used in metal toxicity assay

| Heavy Metal Salts | Concentration (mmol/L) | | | | |
|---|---|---|---|---|---|
| | C1 | C2 | C3 | C4 | C5 |
| $HgCl_2$ | 6.0 | 3.0 | 1.5 | 0.8 | 0.4 |
| $As_2O_3$ | 182 | 91.0 | 45.5 | 22.8 | 11.4 |
| $PbCl_2$ | 43.2 | 21.6 | 10.8 | 5.4 | 2.7 |
| $Cu_2SO_4$ | 225.6 | 112.8 | 56.4 | 28.2 | 14.1 |
| $CdCl_2$ | 21.8 | 10.9 | 5.45 | 2.7 | 1.4 |
| $NiCl_2$ | 277.8 | 138.9 | 69.45 | 34.7 | 17.4 |

## 3.6 BOX-PCR Amplification to Differentiate Potential Heavy Metal Tolerant Isolates

### 3.6.1 DNA Extraction

A total of 13 potential heavy metal tolerant isolates selected from the metal toxicity assay was grown on ISP2 agar at $28^{o}C$ for 3-5 days. The genomic deoxyribonucleic acid (DNA) from actinomycetes was extracted using *NucleoSpin Tissue Kit* (Macherey-Nagel, Germany). Isolation protocols are according to the manufacturer's instruction manual. The concentration of the extracted DNA was then measured using a nano spectrophotometer (Nano 2000, Thermo Scientific Ltd.)

### 3.6.2 Polymerase Chain Reaction (PCR) Amplification using BOXA1R Primer

BOX-PCR was conducted on the extracted DNA samples using BOXA1R primer, 5'-CTACGGCAAGGCGACGCTGACG-3' (Clark *et al.*, 1998). The normal length of amplified DNA using BOX-PCR is ranged approximately from 0.5kb to 3.7kb. However, most intense bands ranged from 0.5 to 1.6kb. BOX- PCR amplification for all the potential heavy metal tolerant isolates was carried out in 25 μl reaction mixtures containing components as listed in Table 3.5. Dimethyl sulfoxide (DMSO) was additionally added in order to improve the denaturation of the DNA. In each experiment control without template DNA was included as well.

After PCR amplification, 2.5 μl of 5X DNA loading dye (Fermentas Corp.) was mixed well with 13 μl of the PCR product and then the mixture was loaded into a 1.5% agarose gel (Appendix A-7) and electrophoresis in 0.75X TBE buffer at room temperature for 1h 30 min at 5 V/cm. The gel was stained with 0.5 μg/ml of ethidium bromide for 10 to 15 minutes, destained with distilled water, and photographed using a computerized ultraviolet (UV) transilluminator (Cleaver Scientific Ltd.). The 100bp

DNA ladder (Fermentas Corp.) was used as reference to estimate the size of bands (Sambrook *et al*., 1989).

**Table 3.5**     List of components added in 25µl reaction mixture of BOX-PCR amplification

| Components | Volume of single reaction (µl) | Final concentration |
|---|---|---|
| Sterile distilled $H_2O$ | 14.4 | |
| 10X PCR buffer | 2.5 | 1X |
| 5U *Taq* DNA polymerase | 0.075 | 1.5U |
| 10mM dNTP mix | 0.5 | 200 µm |
| 20µM BOXA1R primer | 2.5 | 2 µM |
| 25mM $MgCl_2$ | 1.5 | 1.5 mM |
| 10% DMSO | 2.5 | 0.1% |
| Template DNA | 1.0 | |
| Total | 25 | |

PCR amplification was performed with a Perkin-Elmer thermal cycler (Perkin-Elmer Corp.,Norwalk, CT) based on Marques *et al*. (2008) with minor modification as follows:

Initial denaturation: 95°C for 7 minutes

Denaturation       : 94°C for 1 minute

Annealing          : 53°C for 1 minute          30 cycles

Extension          : 65°C for 8 minutes

Final extension    : 65°C for 16 minutes

### 3.6.3 Gel Compar II Software Analysis

The comparisons of DNA band patterns were performed by measurement of band positions of PCR products using computer-assisted cluster analyses with the biostatistical analysis program Gel Compar Software II (Applied Maths, Inc., Belgium) to derive the similarity groups using the measured qualitative data.

### 3.7 Identification of Selected Tolerant Isolates

Isolates TY028-047 and TY049-057 were characterized by cultural, micromorphological, physiological, and molecular characteristics.

### 3.7.1 Cultural Characteristics

The cultural characteristics of isolates were studied on different ISP media [ISP2, ISP3 (Appendix A-2), and ISP4 (Appendix A-3)] (Shirling and Gottlieb, 1966). Based on Mundie (1995), the colours of spore mass, substrate mycelium and diffusible soluble pigment was determined.

### 3.7.2 Micromorphological Characteristics

Microscopic characterization was done by cover-slip method (Cross and Williams, 1971). A loop full of isolates was taken from 7 days old culture media and inoculated onto ISP2 agar plates. Sterile cover slips (2 cm diameter) were inserted at an angle of 45°C in the ISP2 agar and incubated at 28°C for 14 days. The cover slip was carefully removed by using sterile forceps and placed downward on a clean glass slide. The bacterial growth on the cover slip was adhered to the glass slide with few drops of 0.9% NaCl saline and the spore chain arrangement in 14 days culture was examined under a light microscopic magnifications 150X and 400X.

### 3.7.3 Physiological Characteristics

Physiological characterization of actinomycetes was carried out by performing the growth at different salinity (NaCl), temperature and pH (Reddy *et al.*, 2011). Effect of salinity was determined by growing the isolates on Modified Benett's Agar (MBA) medium (Appendix A-4) incorporated with different concentrations of NaCl from 0-10% w/v. Maximum NaCl concentration in the medium allowing growth was recorded.

Further, to determine the optimal temperature for growth, cultures were inoculated on ISP4 plates. The plates were sealed with parafilm and incubated at different temperature ($28^{o}$C, $45^{o}$C, $10^{o}$C, $4^{o}$C). Plates were then inspected for growth weekly for 6 weeks. Formation of macroscopically visible colonies was recorded as growth.

On the other hand, the same medium ISP4 was used to test different level of pH from 4-10. However, sealed plates were only incubated for 14 days and then the visible growth was documented.

### 3.7.4 Phylogenetic Analysis

### 3.7.4.1 DNA Extraction

As mentioned in section 3.6.1.

### 3.7.4.2 PCR Amplification of 16S rRNA Gene

The 16S ribosomal ribonucleic acid (rRNA) gene is highly conserved between different species of bacteria and archaea. Thus, the universal primer pair devised by Weisburg *et al* (1991) which is currently referred to as 27F (5'AGAGTTTGATCCTGGCTCAG 3') and 1492R (5' GGCTACCTTGTTACGACTT

3') were used in this study. PCR was performed in a 50 µl reaction mixture containing components as in Table 3.6. Control without template DNA was also included in each experiment.

The amplified DNA fragments were subjected to 1% (w/v) agarose gel (Appendix A-7) electrophoresis. Gels were submerged in 0.5X TBE buffer. 5 µl of the PCR product were mixed with 1µl 5X DNA loading dye (Fermentas Corp.) and loaded into the gel. The gel was electrophoresis at 100V for 40 minutes. The gel was stained, destained and photographed as mentioned in section 3.5.2. 100bp DNA ladder (Fermentas Corp.) was used as a reference to estimate the size of bands (Sambrook *et al.*, 1989). The 16S rRNA gene products were then purified using PCR Clean up Gel Extraction (Macherey-Nagel, Germany). Purification protocols are according to the manufacturer's instruction manual. Successfully purified PCR product was sent for sequencing. Sequencing of the purified PCR products were conducted at facilities of First Base Laboratories Sdn. Bhd., Shah Alam using ABI PRISM ® 377 DNA Sequencer (Applied Biosystems).

### 3.7.4.3 Phylogenetic Tree Analysis

The 16S rRNA sequences were compared to corresponding sequences in the NCBI gene bank database with the Basic Alignment Search Tool (BLAST) (Altschul *et al.*, 1990). Twenty sequences showing the closest match were selected for phylogenetic analysis using Molecular Evolutionary Genetics Analysis (MEGA) software Version 4 (MEGA4) (Tamura *et al.*, 2007). The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 19871). The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which

**Table 3.6**    List of components added in 50 µl reaction mixture of 16S rRNA PCR amplification

| Components | Volume of single reaction (µl) | Final concentration |
|---|---|---|
| Sterile distilled water | 32.75 | |
| 10X PCR buffer | 10 | 1X |
| 5U *Taq* DNA polymerase | 0.25 | 1.5 U |
| 10mM dNTP mix | 1 | 200µM |
| 10µM 27f primer | 1 | 0.5µM |
| 10 µM 1492r primer | 1 | 0.5µM |
| 25mM MgCl$_2$ | 3 | 1.5mM |
| Template DNA | 1 | |
| Total | 50 | |

PCR amplification was performed with a Perkin-Elmer thermal cycler (Perkin-Elmer Corp., Norwalk, CT) based on Savic and Vasiljevic (2006) with s slight modification as following cycles:

Initial denaturation: 95°C for 2 minutes

Denaturation      : 94°C for 30 seconds

Annealing      : 53°C for 30 seconds          } 30 cycles

Extension      : 72°C for 1 minute 30 seconds

Final extension      : 72°C for 10 minute

the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980) and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option).

## 3.8 The Effect of Cadmium Supplement on Metal Uptake and Growth of Selected Tolerant Isolates

### 3.8.1 Metal Uptake

Method modified from Albarracin *et al* (2005) was used for this study. Spore suspensions of the two cadmium tolerant isolates representing Pasoh (TY028-047) and Bidor (TY049-057) selected based on BOX-PCR study, and one metal sensitive isolate TY049-044, were inoculated into 50 ml MM medium supplemented with 1.0 mM $CdCl_2$ in 500 ml Erlenmeyer flasks. Actinomycete cultures grown in MM media without $Cd^{2+}$ supplement were used as controls. Duplicate flasks were harvested at 0, 2, 4, 6, 8 and 10 days after incubation at $28^{o}C$ with agitation of 200 rpm in an orbital shaker. Aliquots of 1ml culture broth were removed from each flask for growth viability study using serial dilution plate method (Benson, 2002) and the remaining cells were harvested by centrifugation at 10 000 rpm for 15 minutes. Cell pellets were rinsed three times with sterilized distilled water in order to remove $Cd^{2+}$ adsorbed to the cell surface. The cell pellets were then dried at $100^{o}C$ until constant weight. The $Cd^{2+}$ concentration in culture biomass was determined by Inductively Coupled Plasma (ICP) analysis. Total biomass was first digested with concentrated nitric acid before the analysis and the average metal concentration readings obtained from duplicate flasks were calculated.

### 3.8.2 Determination of Cell Viability

Growth viability of the isolates was determined using serial dilution plate method (Figure 3.2). Aliquots of 1ml inoculum were mixed into 9 ml saline (NaCl) to produce a $10^{-1}$ dilution. The 10-fold dilution is repeated sequentially to produce $10^{-2}$, $10^{-3}$ and $10^{-4}$ dilutions. A total of 50 ul of serially diluted culture broth samples from the $10^{-2}$, $10^{-3}$ and $10^{-4}$ dilutions were plated on ISP2 agar and incubated at 28°C for 5-7 days. Actinomycetes colonies on ISP2 agar were counted to calculate the CFU (Colony Forming Units). The average CFU readings obtained from duplicate flasks were calculated to determine the effect of cadmium supplement on the selected isolates. The CFU was calculated as described below:

$$\frac{\text{Number of colonies formed on test plate}}{\text{Amount of serially diluted sample plated on test plate (ml)}} \quad X \quad \text{Dilution Factor} = \text{CFU/ml}$$
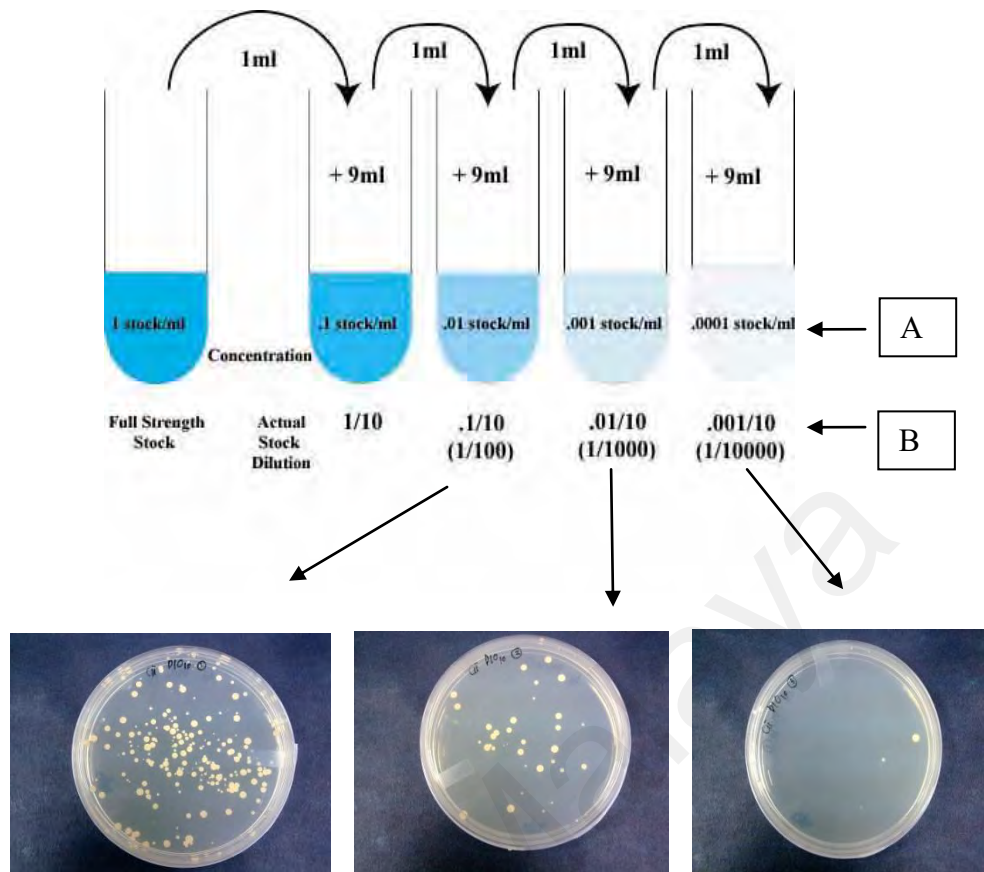
**A-** Amount (in ml) of the stock solution present in each ml of the dilute solution
**B-** Dilution of the original stock solution is shown below the tubes (Dilution factor)

**Figure 3.2**: Serial dilution method to determine growth viability of actinomycetes culture

# CHAPTER 4

# RESULTS AND DISCUSSIONS

## 4.1 Actinomycete Isolates from Bidor and Pasoh

In this study, actinomycete isolates from tin tailings and natural forest soil were dereplicated by grouping them into *Streptomyces*-like and non-*Streptomyces* groups in order to compare the level of heavy metal tolerance in isolates belonging to both groups from different environments. A total of 238 isolates from Bidor and 183 isolates from Pasoh were revived from cryovials stored at -80$^{\circ}$C. All isolates showed good and moderate growth on International *Streptomyces* Project medium 2 (ISP2) plate. These isolates were morphologically characterized.

*Streptomyces*-like group of isolates were distinguished from the non-*Streptomyces* group by the presence of abundant aerial mycelium with powdery spore mass on culture surface of agar plates (Getha *et al*., 2004) (Figure 4.1a). Isolates classified under the non-*Streptomyces* group might belong to different genera such as *Micromonospora, Actinoplanes*, *Norcardia* and other rare genera characterized by colonies having none or sparse aerial mycelium, with or without the presence of diffusible pigments in agar cultures (Figure 4.1b).

More than 50% of the isolates from Bidor and Pasoh belonged to the *Streptomyces* group (Table 4.1). Spore mass colours of *Streptomyces*-like isolates from both sites were mainly grey, white and brown in colour. While the non-*Streptomyces* isolates showed a variety of surface growth colours with sparse or no aerial mycelium. A higher number of *Streptomyces*- like group with grey spore mass and green substrate mycelia colour produce green and light green diffusible pigments in both study sites (Table 4.1). Interestingly, one isolate with white spore mass and orange substrate

(a) Example of *Streptomyces*-like



(b) Example of non-*Streptomyces*
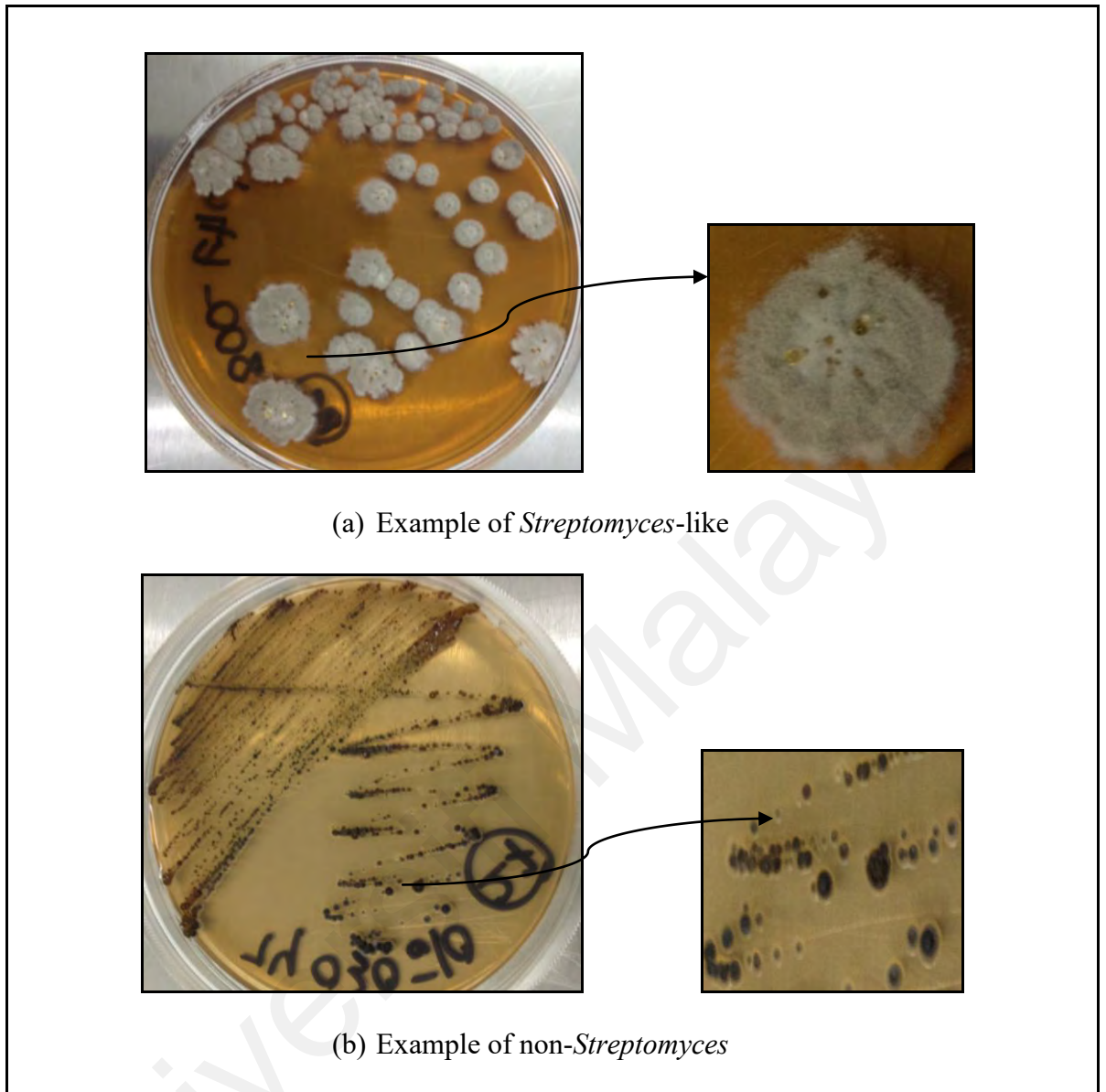
**Figure 4.1**     Macromorphological characteristics of *Streptomyces*-like and non-*Streptomyces* isolates.

**Table 4.1**　　　Grouping of actinomycete isolates into *Streptomyces*-like (S) and non-*Streptomyces* (NS) groups based on spore mass colour for S group and surface growth colour for NS group, substrate mycelium colour and presence of diffusible pigments on ISP2 media

| Sampling Sites | Spore mass/ Surface growth colour | Substrate mycelium colour | Number of Isolates | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | *Streptomyces*-like | | | Non-*Streptomyces* | | |
| | | | Pigment Producer (colour) | Non-Pigment Producer | Total Isolates | Pigment Producer (colour) | Non-Pigment Producer | Total Isolates |
| Bidor (BD) | Grey | Green | 4 (green) 18 (light green) | 18 | 40 | - - | - | - |
| | | Dark Brown | 5 (brown) | - | 5 | - | - | - |
| | | Brown | 6 (light brown) - | 17 | 23 | 2 (light yellow) | 1 | 3 |
| | | Light Brown | - | 9 | 9 | - | 1 | 1 |
| | | Grey | 1 (yellow) - | 8 | 9 | 2 (dark orange) | 1 | 3 |
| | | Black | 1 (light yellow) | 2 | 3 | - | 5 | 5 |
| | | Yellow | 1 (light yellow) | - | 1 | - | - | - |
| | | White | - | 1 | 1 | - | - | - |
| | White | Brown | 3 (light yellow) | 6 | 9 | - | 3 | 3 |
| | | Light Brown | - | 13 | 13 | 1 (light brown) | 9 | 10 |
| | | Grey | - | 2 | 2 | - | - | - |
| | | Yellow | - | 1 | 1 | - | - | - |
| | | White | - | 2 | 2 | - | 5 | 5 |
| | | Pink | - | 1 | 1 | - | 33 | 33 |
| | | Orange | - | - | - | 1 (orange) | 4 | 5 |
| | | Red | - | - | - | - | 3 | 3 |

"Table 4.1, continued"

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Dark Brown | Dark Brown | 1 (dark brown) | - | 1 | - | 1 | 1 |
| Brown | Brown | 1 (brown) | - | 1 | 2 (brown) | 8 | 10 |
| | Black | 1 (brown) | - | 1 | - | - | - |
| | Orange | - | - | - | - | 1 | 1 |
| Light Brown | Light Brown | - | 2 | 2 | 2 (light yellow) | 8 | 10 |
| Orange | Orange | - | - | - | - | 9 | 9 |
| | Black | - | - | - | - | 3 | 3 |
| Black | Black | - | - | - | 1 (brown) | 5 | 6 |
| Colorless | Colorless | - | - | - | - | 3 | 3 |
| **Total Isolates** | | 42 | 82 | 124 52.1% | 11 | 103 | 114 47.9% |
| Pasoh (PA) | Grey | Green | 22 (light green) | 4 | 26 | - | - | - |
| | | Dark Brown | - | - | 4 | - | - | - |
| | | | 4 (brown) | - | | - | | - |
| | | Brown | 1 (light yellow) | 10 | 11 | - | 1 | 3 |
| | | | - | | | 2 (dark brown) | |
| | | Grey | 1 (green) | 13 | 15 | - | 3 | 4 |
| | | | 1 (brown) | | | - | |
| | | | - | | | 1 (yellow) | |
| | | Black | 1 (black) | 8 | 9 | - | - | - |
| | | Yellow | 2 (yellow) | - | 2 | - | - | - |
| | White | Dark Brown | 2 (brown) | - | 2 | - | - | - |
| | | Brown | - | 16 | 16 | - | 5 | 5 |
| | | Light Brown | - | 5 | 5 | - | 2 | 2 |

|  |  |  |  |  |  |  |  |
|---|---|---|---|---|---|---|---|
|  | Black | - | 1 | 1 | - | - | - |
|  | Yellow | - | 1 | 1 | - | - | - |
|  | White | - | 7 | 7 | - | 1 | 1 |
|  | Pink | - | 2 | 2 | 1 (brown) | 4 | 5 |
|  | Orange | - | 1 | 1 | - | - | - |
|  | Maroon | - | 1 | 1 | - | 1 | 1 |
|  | Cream | - | - | - | - | 2 | 2 |
| Dark Brown | Dark brown | 1 (brown) | - | 1 | 2 (brown) | - | 2 |
| Brown | Brown | - | 1 | 1 | 3 (light brown) 2 (brown) | 20 | 25 |
|  | Maroon | - | - | - | - | 1 | 1 |
| Light Brown | Light Brown | - | - | - | - | 2 | 2 |
| Orange | Orange | - | 1 | 1 | 1 (light orange) | 4 | 7 |
|  | Black | - | - |  | 1 (light brown | 1 |  |
| Black | Black | - | 1 | 1 | 1 (brown) | 1 | 2 |
| Maroon | Maroon | - | - | - | - | 5 | 5 |
| Yellow | Yellow | - | - | - | - | 1 | 1 |
| Colorless | Colorless | - | - | - | - | 8 | 8 |
| Total Isolates |  | 35 | 72 | 107 58.5% | 14 | 62 | 76 41.5% |

Legend : Grey- 4D4234, White- F9DFCF, Dark Brown- 35170C, Brown- 673923, Light Brown- A86540, Dark Orange- FF6800, Orange- FF6F1A, Light Orange- FFA161, Black- 121910, Maroon- 4F0014, Yellow- FFDC33, Light Yellow- FFDE5A, Colorless- FFE2B7, Green- 7F8F18, Light Green- DCD36A, Pink- FF7E93, Red- BF2233, Cream- FFCA86 (NBS/ISCC Colour Systems: Mundie, 1995)

mycelia producing orange pigment and two isolates with grey spore mass and grey substrate mycelia producing dark orange pigment belonged to the non-*Streptomyces* group were obtained from Bidor. Only one isolate from the non-*Streptomyces* group obtained from Pasoh produced orange spore mass and orange substrate mycelia, with light orange pigment. Orange coloured diffusible pigment which was not observed in the *Streptomyces*-like group, was the only colour different from the other common diffusible pigments such as green, brown and yellow produced by both groups of actinomycetes in this study (Table 4.1).

Microbes are largely identified based on their morphological characterization as a preliminary determination of the genus. Thus, bacterial systematic still depends on the morphological and behavioral properties of microorganisms, as well as molecular approaches. The conventional macromorphology characterization conducted on selected agar is able to provide useful and rapid clues for identification of their respective genus (Seong *et al*., 2001 and Khanna *et al*., 2011). Actinomycetes can be classified by observing the coloration of aerial mycelium, substrate mycelium and diffusible pigment (Lo *et al*., 2002). According to Shirling and Gottlieb (1966), the four standard media to determine colours of sporigenous aerial mycelium, reverse and diffusible pigment are ISP Media 2, 3, 4 and 5. However, in this study actinomycetes were dereplicated by tentatively grouping them into *Streptomyces*-like or non- *Streptomyces* groups based on their macromorphology on ISP Media 2 (yeast extract-malt extract agar). Previous study by Numata and Nimura (2003) had clearly distinguished more than 900 actinomycete isolates into *Streptomyces*-like and non- *Streptomyces* groups before selecting the hit isolates via bioassays and identifying them based on 16S ribosomal deoxyribonucleic acid (rDNA) sequence analysis. Thus, dereplication based on macromorphology acts as preliminary grouping method which is useful, economical and rapid in order to look at

the diversity and potentials of a large group of isolates.

Actinomycetes, especially *Streptomyces* spp., are known to produce a range of diffusible pigment colours such as blue, violet, red, rose, yellow, green, brown and black. They are also well known as a rich source of antibiotics and bioactive molecules (Seong *et al*., 2001; Qin *et al*., 2009). Some of the antibiotics produced by actinomycetes include many of these pigments (Shaaban, 2013). According to Selvameenal (2009), pigments produced by *Streptomyces hygroscopicus* showed antibacterial activity against drug resistant pathogens such as methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Staphylococcus aureus* (VRSA) and extended-spectrum beta lactamase (ESBL) strains.

Recent studies have shown that to discover novel bioactive molecules from actinomycetes, it is recommended to study the non-*Streptomyces* groups or rare actinomycetes from uncommon environments (Seong *et al*., 2001; Qin *et al*., 2009; Khanna *et al*., 2011). Rare actinomycetes are usually described as genera or species which are often difficult to be isolated and cultivated using the conventional methods (Seong *et al*., 2001; Qin *et al*., 2009). Novel isolates belonging to the rare actinomycetes group have been isolated from the surface of acidic and heavy metal galleries of mining areas (Routh *et al*., 2007; Carlson *et al*., 2008). Thus, it is interesting to isolate and study actinomycetes from different environments.

## 4.2    Heavy Metal Tolerance Screening in Actinomycete Isolates

All 421 actinomycete isolates obtained from Bidor and Pasoh were screened for heavy metal tolerance using agar diffusion method (Figure 4.2). Seven different types of heavy metal selected in this study represented the contaminants that may be present in Bidor (And and Ang, 1997). These are nickel (Ni), plumbum (Pb), zinc (Zn), cadmium

(a) Sensitive isolate



(b) Tolerant isolate
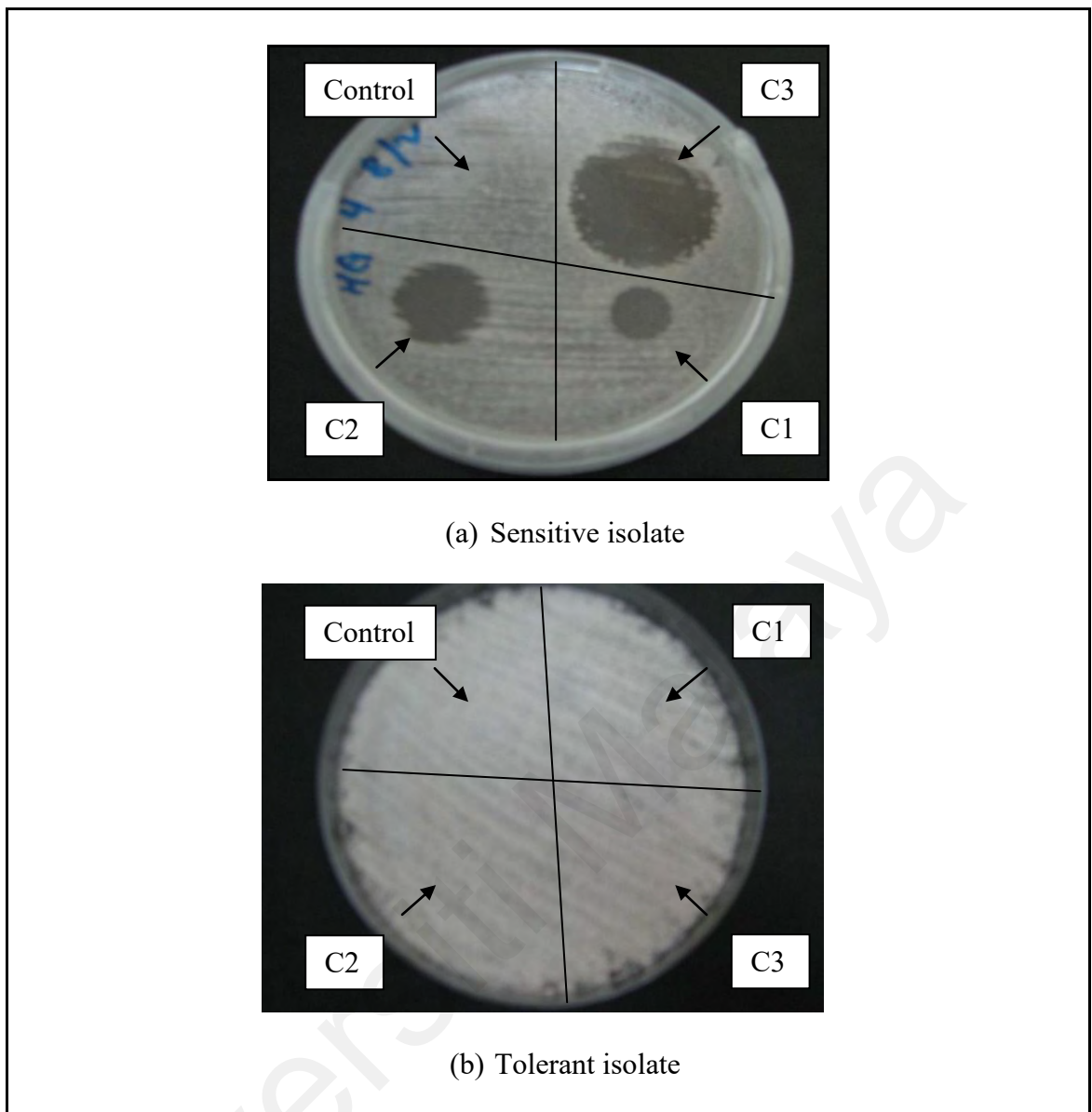
**Figure 4.2**    Heavy metal tolerance screening of actinomycetes based on direct agar diffusion assay.

*C1: Highest test concentration, C2: Moderate test concentration, C3: Lowest test concentration

(Cd), cuprum (Cu), mercury (Hg) and arsenic (As).

Figure 4.3/Appendix-B1 shows the tolerance of isolates from the two different study sites towards different types of heavy metals tested at three different concentrations. The order of metal toxicity observed at the highest test concentration among the actinomycete isolates can be arranged as: $Hg > Cd = Cu > Ni = Pb > As = Zn$ (for isolates obtained from Bidor tin tailings) and $Hg > Cu > Cd = Ni > Pb > As = Zn$ (for isolates obtained from Pasoh forest soil). Less than 1% of the isolates tested from both Bidor and Pasoh, showed tolerance to 4 mmol/L $Hg^{2+}$ (Figure 4.3a). Only 3-5% of isolates from both sites tolerated $Cd^{2+}$ at 6 mmol/L (Figure 4.3b) and 2-4% of isolates tolerated $Cu^{2+}$ at 63 mmol/L (Figure 4.3c). The number of $Ni^{2+}$ tolerant isolates was significantly higher for Bidor (12.6%) compared to Pasoh (6.6 %) (Table 4.2/Appendix-B2). While, more than 10% of isolates from both sites can tolerate $As^{3+}$, $Pb^{2+}$ and $Zn^{2+}$ at the highest test concentrations (Figure 4.3d, 4.3e, 4.3f). $Zn^{2+}$ was the least toxic among the tested metals.

Mann Whitney test (Table 4.3/Appendix-B3) showed that a significantly higher number of Bidor isolates from the *Streptomyces*-like group showed tolerance towards $As^{3+}$, $Pb^{2+}$, $Ni^{2+}$ and $Zn^{2+}$. On the other hand, Pasoh showed significantly higher percentage of non-*Streptomyces* isolates being tolerant towards $Cd^{2+}$.

Heavy metals are elements having atomic weights between 63.546 to 200.590 which forms almost two thirds of the chemical elements listed in the periodic table. Low concentration of heavy metals such as copper (Cu), iron (Fe) and zinc (Zn) are necessary and not harmful for microorganisms (Andre *et al.,* 2005). At higher concentration, they are toxic to these microbes (Lopez-Maury *et al.,* 2002). Heavy metals have been increasingly found in microbial habitats due to natural and industrial processes (Anne, 2003). Actinomycetes, as natural inhabitants of soil, water and manure
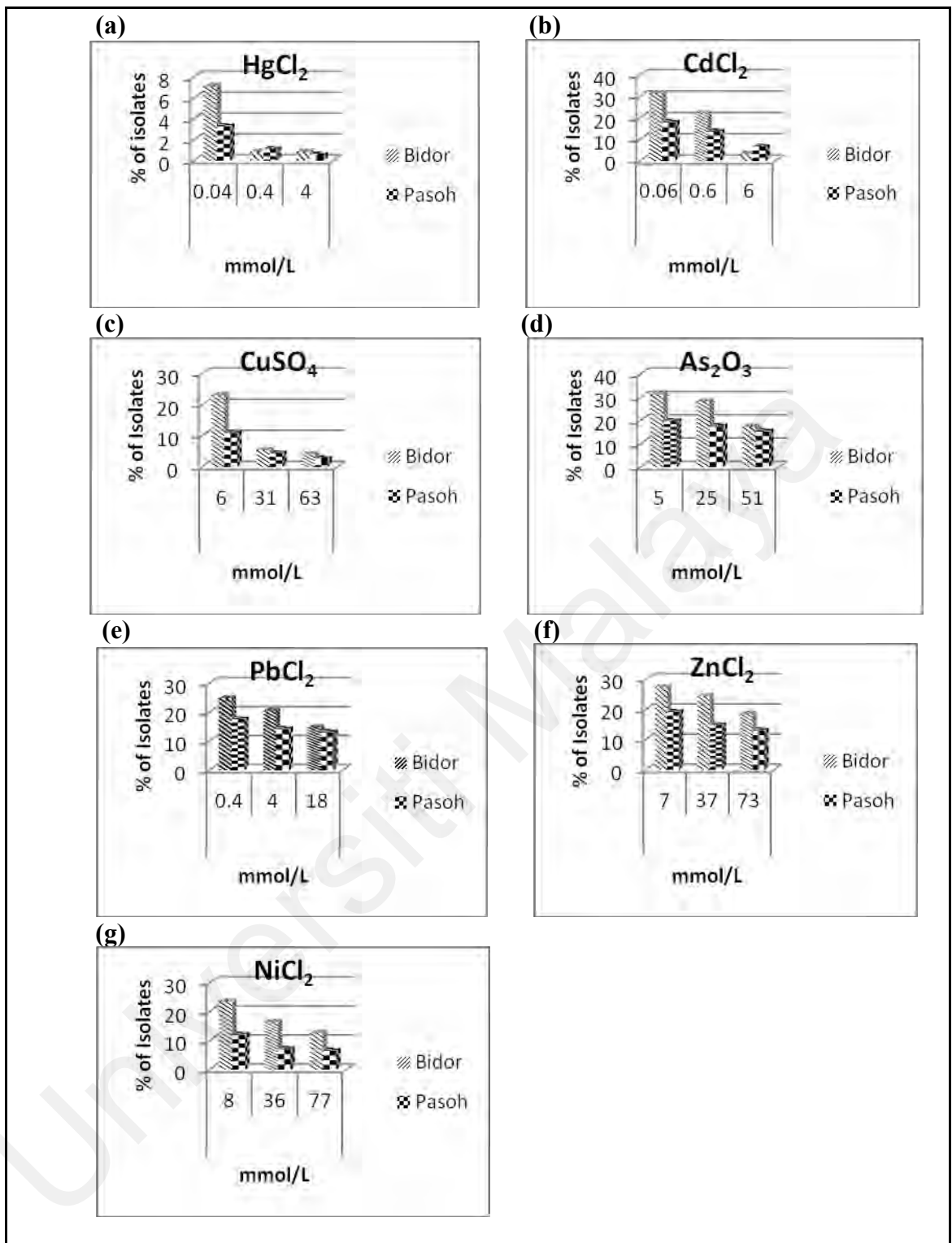
**Figure 4.3** Percentage of isolates from Bidor and Pasoh showing tolerance to different types of heavy metal salts tested at three different concentrations (mmol/L).

**Table 4.2**  Percentage of isolates from Bidor and Pasoh tolerant towards different metal

| Sampling Sites | % of tolerant isolates towards different metal (highest test concentration*) | | | | | | |
|---|---|---|---|---|---|---|---|
| | $Hg^{2+}$ | $Cd^{2+}$ | $Cu^{2+}$ | $As^{3+}$ | $Pb^{2+}$ | $Zn^{2+}$ | $Ni^{2+}$ |
| Bidor | 0.8 | 3.4 | 4.2 | 17.2 | 14.7 | 18.5 | 12.6** |
| Pasoh | 0.5 | 6.6 | 2.7 | 15.3 | 13.1 | 13.1 | 6.6 |

Concentrations: $Hg^{2+}$ = 4 mmol/L, $Cd^{2+}$ = 6 mmol/L, $Cu^{2+}$ = 63 mmol/L, $As3^{+}$ = 51 mmol/L, $Pb2^{+}$ = 18 mmol/L, $Zn2^{+}$ = 73 mmol/L, $Ni2^{+}$ = 77 mmol/L.

**p value less than the 5% level of significance (Mann Whitney test)

**Table 4.3**  Percentages of *Streptomyces*-like and non-*Streptomyces* isolates from Bidor and Pasoh tolerant towards different metal (highest test concentration)

| Sampling Sites | Actinomycetes groups | % of tolerant isolates towards different metal (highest test concentration*) | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | $Hg^{2+}$ | $Cd^{2+}$ | $Cu^{2+}$ | $As^{3+}$ | $Pb^{2+}$ | $Zn^{2+}$ | $Ni^{2+}$ |
| Bidor | S | 1.59 | 2.38 | 4.76 | 23.02** | 20.63** | 26.19** | 20.63** |
| | NS | 0 | 4.46 | 3.57 | 10.71 | 7.14 | 7.14 | 3.57 |
| Pasoh | S | 0.93 | 3.74 | 1.87 | 18.69 | 13.08 | 10.28 | 4.44 |
| | NS | 0 | 10.52** | 3.95 | 10.53 | 13.16 | 17.11 | 10.13 |

Concentrations: $Hg^{2+}$ = 4 mmol/L, $Cd^{2+}$ = 6 mmol/L, $Cu^{2+}$ = 63 mmol/L, $As^{3+}$ = 51 mmol/L, $Pb^{2+}$ = 18 mmol/L, $Zn2^{+}$ = 73 mmol/L, $Ni^{2+}$ = 77 mmol/L.

** p value less than the 5% level of significance (Mann Whitney test), S = *Streptomyces-like* & NS = non-*Streptomyces*

are continuously exposed to different metals present in these habitats. According to Hiroki (1992), actinomycetes can immobilize metals. They adapt or acquire tolerance or resistance in order to survive in the permanent exposure of exceedingly high concentrations of heavy metals (Poopal and Laxman, 2009).

In this study, a total of 40% and 33% of $Ni^{2+}$ tolerant isolates obtained from Bidor and Pasoh, respectively showed multi metal tolerant (tolerant to four and more metals) (Appendix B-1). Thus, metal phenotype of co-tolerance was much easily seen among the $Ni^{2+}$ tolerance isolates from Bidor compared to Pasoh. In Bidor, nickel was reported to be in high concentration in sand tailings (0.29-8.78 mg/g Ni at 0-20 cm depth of tin tailings) and slime tailings (3.64-29.31 mg/g Ni at 0-20 cm depth of tin tailings) (Ang and Ang, 1997). Therefore, high nickel contamination in Bidor may have created a selective pressure for soil microbes and this can be widespread among actinomycetes growing in tin tailings. In the case of $Zn^{2+}$, the highest test concentration of 73 mmol/L may still be lower than the inhibitory concentration for actinomycetes since this metal is essential for their growth as cofactors for enzymes (Schmidt *et al.*, 2005).

A significantly higher percentage of *Streptomyces*-like isolates from Bidor showed tolerance to $As^{3+}$, $Pb^{2+}$, $Ni^{2+}$ and $Zn^{2+}$ and a significantly higher number of non-*Streptomyces* isolates showed tolerance to cadmium (Table 4.3). This indicates that metal tolerance in actinomycetes may not directly relate to a particular genera or group of actinomycetes but is widely distributed in different genera. Heavy metal tolerance among bacteria was reported to differ depending on factors such as heavy metal pollution, type of heavy metal and soil characters (Nemeth and Kadar, 2005).

Further, the direct agar diffusion assay used for this study was found to be useful to screen a large number of isolates of heavy metal tolerance in a rapid manner. The

assay meets all the required factors such as cheap and time effective in order to be a potential method for rapid heavy metal tolerance screening of filamentous actinomycetes.

Final selection of potential isolates for the next studies were according to the following criterias: (1) Actinomycetes tolerant to multi-metals, (2) Actinomycetes tolerant to highly toxic metals without biological function and becomes toxic to living organism and environment at a very low concentration and (3) Actinomycetes tolerant to trace minerals which are essential to all living organisms but toxic when comes to a certain concentration.

As shown in Table 4.4, actinomycetes tolerant to four and more metals, and those tolerant to $Cd^{2+}$, $Hg^{2+}$ and $Cu^{2+}$ were selected for further studies. Metals such as $Cd^{2+}$, $Hg^{2+}$, $As^{3+}$ and $Pb^{2+}$ fall under the toxic group. However, only isolates showing tolerance to $Cd^{2+}$ and $Hg^{2+}$ were selected even if they are not multi-metal tolerant. Both cadmium and mercury are interesting for further studies compared to $As^{3+}$ and $Pb^{2+}$ as they have no biological function to any organism and toxic to the soil at very low concentrations meanwhile $As^{3+}$ were found in phospholipids of algae and fungi which was later converted into variety of organoarsenic chemicals (Benson and Summons, 1981) and the toxicity range of $Pb^{2+}$ in soil is very much similar to the trace minerals even though they are listed under the toxic group.

On the other hand, $Ni^{2+}$, $Cu^{2+}$ and $Zn^{2+}$ fall under the trace mineral group. However, $Ni^{2+}$ does not clearly explains a trace metal group characteristics because it is only possibly required trace mineral and not essential to all organism. A clear characteristic of the heavy metal is important, in order to study the different functions of heavy metal groups with their antibiotic resistance pattern. Thus, $Ni^{2+}$ tolerant isolates were not specifically selected. Only $Cu^{2+}$ and $Zn^{2+}$ are essential to all organisms and

**Table 4.4**     Actinomycetes selection criteria for antibiotic resistance and metal toxicity assay

| Selection criteria | Heavy metals | | | | | | |
|---|---|---|---|---|---|---|---|
| | Cd | Hg | As | Pb | Ni | Cu | Zn |
| Multi metal | ←———————— Able to tolerate more than 4 metals (any of these metals) ————————→ | | | | | | |
| Toxic metals* | / | / | / | / | | | |
| Trace minerals** | | | | | Possibly required | / | / |
| Biological function^ | none | none | in phospho-lipid of algae and fungi | none | essential to plants | essential to all organisms | essential to all organisms |
| Soil toxicity range^ (mg/kg) | 0.06- 1.1 | 0.04-0.28 | 3.6 - 8.8 | 17-26 | 13 - 30 | 14 - 29 | 34 - 84 |

* Listed as top 20 hazardous substance in ATSDR, (2011)
**Souce adapted from Wilson, (2012)
^ Source adapted from Hagedorn, (1996)

considered as macronutrients which are required in large amounts by living organisms (Wilson, 2012). Compared to $Cu^{2+}$, $Zn^{2+}$ generally shows low toxicity in living organism and environment. Results obtained from the heavy metal tolerance screening also showed that $Zn^{2+}$ was the least toxic metal to the isolates among the tested metals. Thus, $Zn^{2+}$ was totally dropped for further studies even though it is one of the metals in multi metal tolerant isolate meanwhile all $Cu^{2+}$ tolerating actinomycetes were selected even if they do not show multi-metal tolerance.

A total of 44 isolates were selected from the heavy metal tolerance screening based on the selection criteria given in Table 4.4. Out of these, a total of 15 isolates from both Bidor and Pasoh showed poor growth on agar plates during sub-culturing from the cryovials. Twelve of these isolates belonged to the non-*Streptomyces* group which is known for their slow growth pattern after repeated sub-culturing. These isolates were not studied further. Thus, a total of 29 tolerant actinomycetes showing good growth were successfully grown from cryovials and used for antibiotic resistance study.

## 4.3     Antibiotic Resistance Pattern of Selected Metal Tolerant Actinomycetes

Antibiotic resistant pattern of the selected isolates is presented in Table 4.5 and Figure 4.4. Values calculated for multiple antibiotic resistance (MAR index) showed that 59% of the selected isolates showed MAR index greater than 0.2. The MAR index is a tool used to identify the distribution of resistance in a given population of bacteria (Krumpermann, 1983). A MAR index greater than 0.2 indicates the bacteria are originated from an environment where several antibiotics are used (Jain *et al*., 2012).

In this study, isolates from both the *Streptomyces*-like and non-*Streptomyces* groups exhibited varied degree of susceptibility and resistance to different antibiotics.

**Table 4.5**     Antibiotic resistance pattern and heavy metal tolerance profile (at highest test concentration) of selected isolates

| Heavy Metal Tolerance Groups | Isolates | Actinomycetes Group | Location | MAR Index | Antibiotics | | | | | | | | | Heavy Metals | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | P | Am | Na | E | Gm | Ch | An | K | Va | Hg | As | Pb | Cu | Cd | Ni | Zn |
| Multi metal | TY046-078 | S | Bidor | 0.3 | - | - | - | + | + | + | + | + | ++ | + | - | - | + | + | - | - |
| | TY046-018 | S | Bidor | 0.4 | - | - | - | - | + | + | + | + | ++ | + | | - | + | + | - | - |
| | TY046-073 | S | Bidor | 0.3 | - | - | - | ± | + | + | + | + | ++ | + | - | - | + | + | - | - |
| | TY046-071 | S | Bidor | 0.3 | - | - | - | ± | + | + | + | + | ++ | + | - | - | + | - | - | - |
| | TY047-019 | S | Bidor | 0.2 | - | - | + | ± | + | + | + | + | + | + | - | - | - | + | - | - |
| | TY046-027 | S | Bidor | 0.2 | - | - | + | ++ | + | + | + | + | ++ | + | - | - | + | + | - | - |
| | TY046-004 | S | Bidor | 0.1 | - | + | + | ++ | + | + | + | + | + | + | - | - | + | + | - | - |
| | TY046-021 | S | Bidor | 0 | + | ++ | + | + | + | + | + | + | + | + | - | - | + | - | - | - |
| | TY046-016 | S | Bidor | 0 | ++ | ++ | + | ++ | + | + | + | + | + | + | - | - | + | + | - | - |
| | TY047-062 | NS | Bidor | 0 | ++ | ++ | + | + | + | + | + | + | + | + | - | + | + | - | - | - |
| | TY029-008 | NS | Pasoh | 0.2 | - | - | + | + | + | + | + | + | ++ | + | - | - | + | - | - | - |
| | TY029-014 | NS | Pasoh | 0.3 | - | - | + | + | - | + | + | + | ++ | + | - | - | + | - | - | - |
| Mercury Specific | TY048-047 | S | Bidor | 0.1 | - | ++ | + | ++ | + | + | + | + | + | - | - | + | + | + | + | + |
| | TY046-017 | S | Bidor | 0.3 | - | - | - | ++ | + | + | ++ | ++ | ++ | - | + | + | + | + | - | + |
| Cadmium Specific | TY049-057 | S | Bidor | 0.3 | - | - | - | + | + | ± | + | + | ++ | + | - | - | + | - | + | + |
| | TY046-037 | NS | Bidor | 0.2 | - | ± | - | + | + | + | ++ | + | ++ | + | + | - | + | - | + | + |
| | TY047-020 | NS | Bidor | 0.2 | - | - | + | ++ | + | + | + | + | + | + | + | + | + | - | + | + |
| | TY048-042 | NS | Bidor | 0.2 | - | - | + | ++ | + | + | + | + | + | + | + | + | + | - | + | + |
| | TY029-029 | NS | Pasoh | 0.3 | - | - | - | ++ | + | + | ++ | ++ | ++ | + | + | + | + | - | + | - |

"Table 4.5, continued"

| | Isolate | Type | Site | MAR | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | TY030-054 | S | Pasoh | 0.2 | - | - | + | + | + | + | + | + | + | s | - | + | + | - | + | - |
| | TY028-047 | S | Pasoh | 0.1 | - | + | + | + | + | ++ | + | + | + | + | - | + | + | - | + | + |
| Cuprum | TY046-070 | S | Bidor | 0.3 | - | - | - | ± | + | + | + | ++ | ++ | + | - | + | - | + | - | + |
| Specific | TY047-014 | NS | Bidor | 0.2 | - | - | + | ++ | + | + | + | + | ++ | + | - | - | - | + | + | + |
| | TY047-023 | S | Bidor | 0.1 | - | ± | + | + | + | + | + | + | + | + | + | - | - | + | + | + |
| | TY047-025 | S | Bidor | 0 | ± | ± | + | + | + | + | + | + | + | + | + | + | - | + | - | + |
| | TY047-024 | S | Bidor | 0 | + | + | + | + | + | + | + | + | + | + | + | + | - | + | - | + |
| | TY028-043 | S | Pasoh | 0 | ± | + | + | + | + | ++ | + | + | + | - | + | - | - | + | + | + |
| | TY028-019 | S | Pasoh | 0.1 | - | + | + | + | + | ± | + | + | + | + | + | + | - | + | + | + |
| | TY047-027 | S | Bidor | 0.1 | - | ± | + | + | + | + | + | + | + | + | + | - | - | + | + | + |

1) *"*S*"* - *Streptomyces-like* & "NS" - non-*Streptomyces*

2) Antibiotic resistance assay: resistant (-) = no inhibition zone, weakly susceptible (±) = DIZ < 1 cm, susceptible (+) = 1 cm ≤ DIZ < 3cm, extremely susceptible (++) = DIZ ≥ 3cm

3) Heavy metal tolerance screening: tolerance (-) = no inhibition zone present, sensitive (+) = DIZ > 0.6cm

4) DIZ = Diameter of inhibition zone

5) Am = Ampicilin, Na = Nalidixic asid, E = Erythromycin, Gm = Gentamycin, Ch = Chloramphenicol, An = Ampicilin, K = Kanamycin, Va = Vancomycin, P = Penicillin

6) MAR index = a/b where a = number of isolates resistant to antibiotics, b = total number of antibiotic tested
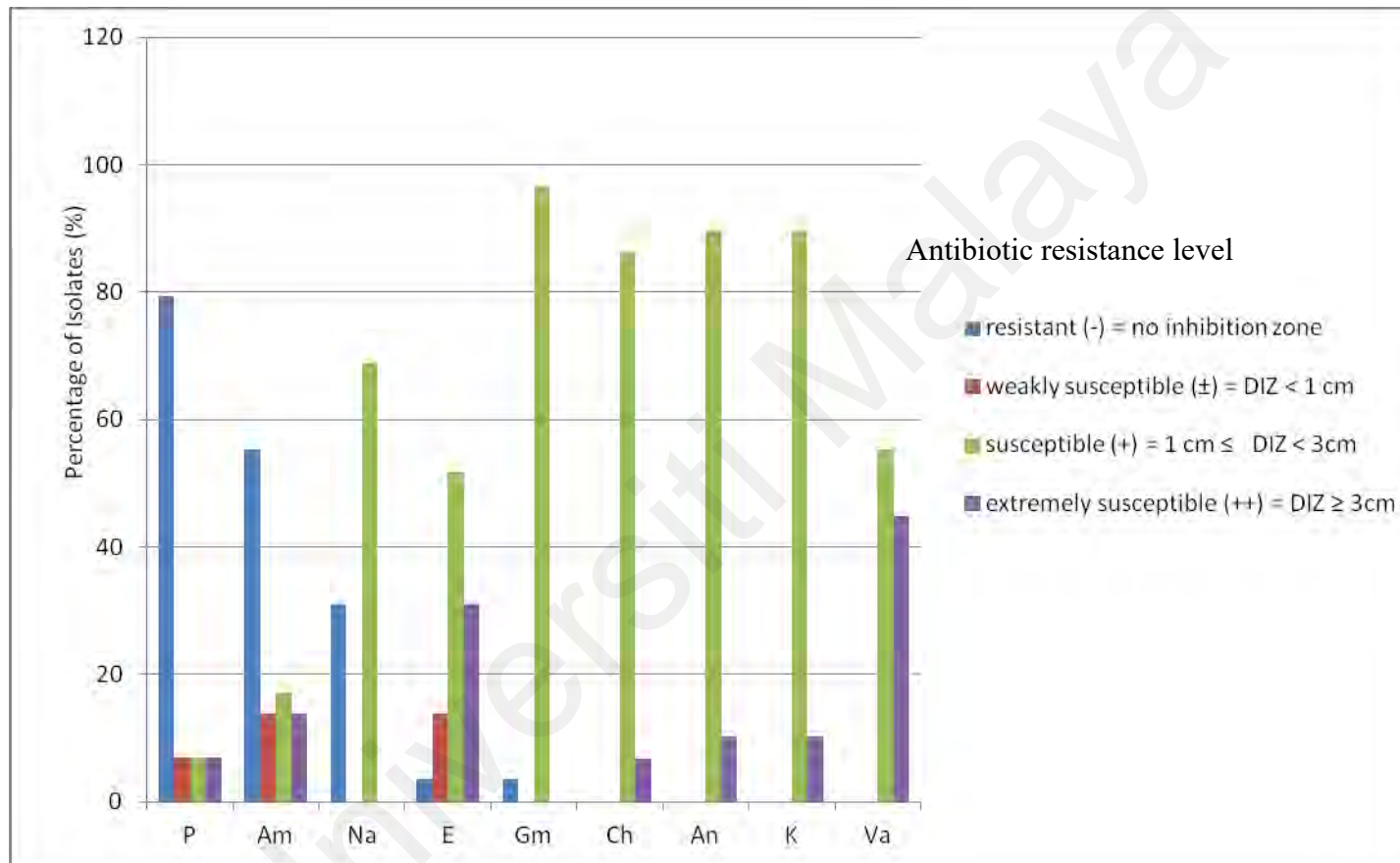
**Figure 4.4** Total percentage of isolates showing resistance to different antibiotics.

Out of 29 tested isolates, none was found resistant to all antibiotics and a total of six isolates were susceptible to all antibiotics. Only isolate TY046-018 from Bidor showed resistance to four antibiotics (Table 4.5). Figure 4.4 showed that more than 50% of the selected metal tolerant isolates were resistant to penicillin and ampicillin. Ampicillin is the semi synthetic class of antibiotics similar to penicillin with the same mode of action which inhibits cell wall synthesis (Table 4.6). A total of 31% isolates showed resistance to nalidixic acid belongs to the qiunolone class. Eight out of the nine isolates were from Bidor and this is followed by erythromycin with one isolate showing resistance and four weakly susceptible. All isolates were from Bidor. More than 90% of the metal tolerant isolates showed susceptibility to chloramphenicol and to the aminoglycosides such as gentamycin, amikacin, and kanamycin (Table 4.5). All the metal tolerant isolates showed either susceptible or extremely susceptible towards glycopeptide vancomycin (55.2%) compared to the rest of the tested antibiotics even though glycopeptides and beta lactams are inhibitors of cell wall synthesis.

Similar patterns of antibiotic resistance were observed between the three different groups of metal (multi metal, toxic metal and trace metal). There are at least one isolate from the three different groups which able to tolerate more than two antibiotics. Further, three isolates (TY047-025, TY047-024 and TY028-043) from the trace metal group showed susceptibility to all the tested antibiotics and the same profile was observed for another three isolates (TY046-021, TY046-016 and TY047-062) from the multi metal tolerant group (Table 4.5).

A number of studies have indicated correlation between multiple antibiotic resistance with heavy metal tolerance (Jain *et al*., 2012; Bahig *et al*., 2008; Spain, 2003). In this study, Pearson Correlation analysis showed that a positive correlation (r = 0.359) exist between multiple antibiotic resistance and heavy metal tolerance in the

**Table 4.6**     Chemical classes, biological sources and mode of action of selected antibiotics

| Antibiotics | Chemical Classes* | Biological Sources** | Mode of Action^ |
|---|---|---|---|
| Penicillin G | Beta lactams | *Penicillium notatum* | Inhibition of cell wall synthesis |
| Ampcilin | | Semi synthetic | Inhibition of cell wall synthesis |
| Vancomycin | Glycopeptides | *Amycolatopsis orientalis* (formerly designated *Norcardia orientalis* | Inhibition of cell wall synthesis |
| Nalidixic acid | Qiunolones | Synthetic | Inhibition of nucleic acid synthesis |
| Erythromycin | Macrolides | *Streptomyces erythreus* | Inhibition of protein synthesis (Action on 50S ribosomal subunit) |
| Chloramphenicol | Chloramphenicol | *Streptomyces venezuelae* | Inhibition on protein synthesis (Action on 50S ribosomal subunit) |
| Gentamycin | Aminoglycosides | *Micromonospora* species | Inhibition of protein synthesis (Action on 30S ribosomal subunit) |
| Kanamycin | Aminoglycosides | *Streptomyces kanamyceticus* | Inhibition of protein synthesis (Action on 30S ribosomal subunit) |
| Amikacin | Aminoglycosides | Semi synthetic | Inhibition of protein synthesis (Action on 30S ribosomal subunit) |

*Source adapted from Levinson, 2006 & Todar, 2009; ** Source adapted from Todar, 2009; ^Source adapted from Levinson, 2006

actinomycetes isolated from Bidor while no correlation was observed among the Pasoh isolates (r = - 0.192) (Table 4.7/Appendix B-4).

Penicillin and ampicillin resistance were widespread among the metal tolerant isolates, and not targeted to a particular metal. High number of actinomycetes showing resistance to penicillin, ampicilin and nalidixic acid may be quite common because most bacteria have developed partial or complete resistance to these antibiotics (Chaudhuri *et al.*, 1997). A similar pattern of antibiotic resistance among the three different groups of metal showed that antibiotic resistance was not influenced by a particular group of metal. However, the positive correlation observed between multiple antibiotic resistance and heavy metal tolerance in the actinomycetes isolated from Bidor and negative correlation for Pasoh clearly explains selective pressure had taken place among actinomycetes isolated from Bidor and not Pasoh.

**Table 4.7**       Correlation between multiple antibiotic resistance and heavy metal tolerance of actinomycete isolates from Bidor and Pasoh using Pearson Correlation

| Group | Pearson Correlation (r value) | p value* |
|-------|-------------------------------|----------|
| Bidor | 0.359 | 0.228 |
| Pasoh | -0.192 | 0.808 |

*p value less than the 5% level of significance (Pearson Correlation Test)

Microorganisms have acquired a variety of mechanisms for adaptation to the presence of toxic heavy metals in the environment. Among the various adaptation mechanisms, enzymatic oxidation or reduction to a less toxic form, metal uptake and accumulation and metal efflux systems have been reported (Silver and Phung, 2005;

Nageswaran *et al*., 2012). Thus, multiple tolerance is common phenomena among heavy metal resistant bacteria due to their similarities in toxicity mechanisms (Samanta *et al*., 2012). It is ecologically favorable for bacteria to acquire antibiotic resistance in the presence or absence of antibiotics when excessive amount of heavy metals are found in the environment (Calomiris *et al*., 1984). This happens because of the likelihood that resistance genes to both (antibiotics and heavy metals) may be located closely together on the same plasmid in bacteria (Sobecky, 1999; Dalsgarrd and Guardbassi, 2002). Therefore, both the selective pressures (heavy metals or antibiotics) indirectly select both sets of resistance in the microorganism.

A further study was conducted to determine the minimum tolerable concentration levels of heavy metals in broth cultures of the selected metal tolerant isolates. A semi-quantitative metal toxicity assay was carried out.

## 4.4 Determination of Minimum Tolerable Concentration (MTC) Levels of Heavy Metals in Actinomycetes

Metal toxicity assays of selected actinomycetes are shown in Figure 4.5. Out of the 29 isolates tested, a total of 13 isolates showed the ability to tolerate high concentrations of at least one metal in the metal toxicity assay. Growth was observed only in Bidor isolates at concentration of 43.2 mmol/L for $Pb^{2+}$ (three isolates) and 138.9 mmol/L for $Ni^{2+}$ (five isolates). For metals such as $As^{3+}$ and $Cd^{2+}$, growth was observed in Bidor and Pasoh isolates at the highest concentration of 182 mmol/L and 21.8 mmol/L, respectively. Only one isolates each from Bidor and Pasoh could tolerate the high test concentration of 21.8 mmol/L for $Cd^{2+}$. Three isolates from Bidor and two from Pasoh were tolerant to $As^{3+}$ at 182 mmol/L (Table 4.8/Appendix B-5).

A) Plumbum

a) Control without actinomycetes
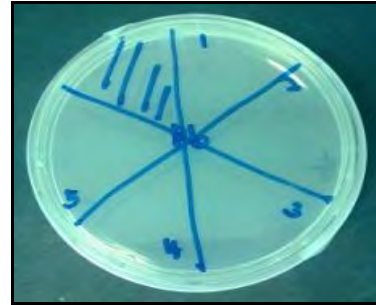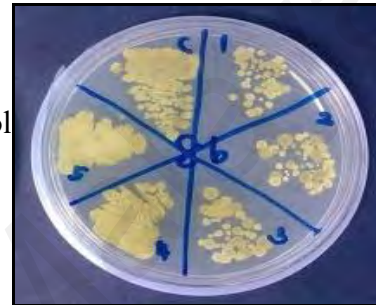


b) TY046-021



c) TY046-016
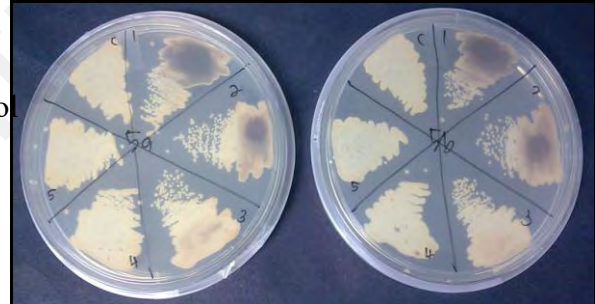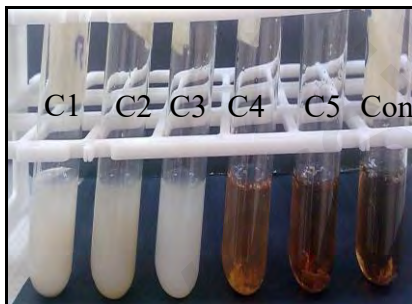


d) TY046-078
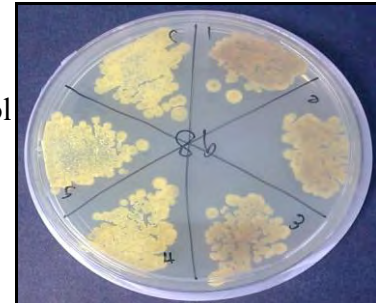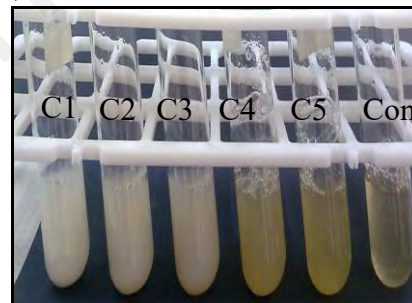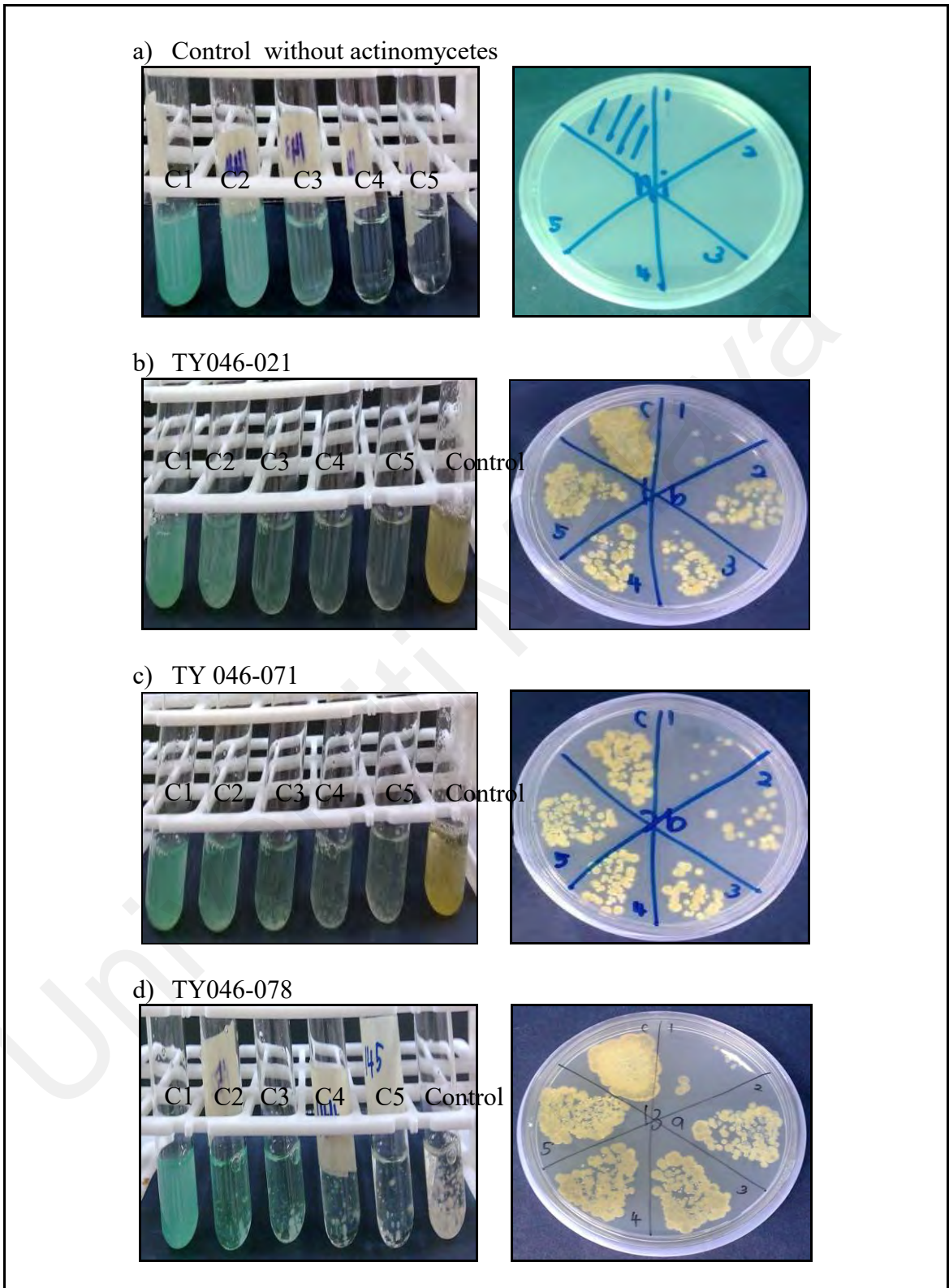
B) Nickel

a) Control without actinomycetes



b) TY046-021



c) TY 046-071



d) TY046-078

C) Arsenic



a) Control without actinomycetes

b) TY046-078

c) TY046-021

D) Cadmium

a) Control without actinomycetes



b) TY 028-047



c) TY 049-057

E) Mercury



a) Control without actinomycetes

a) TY046-017
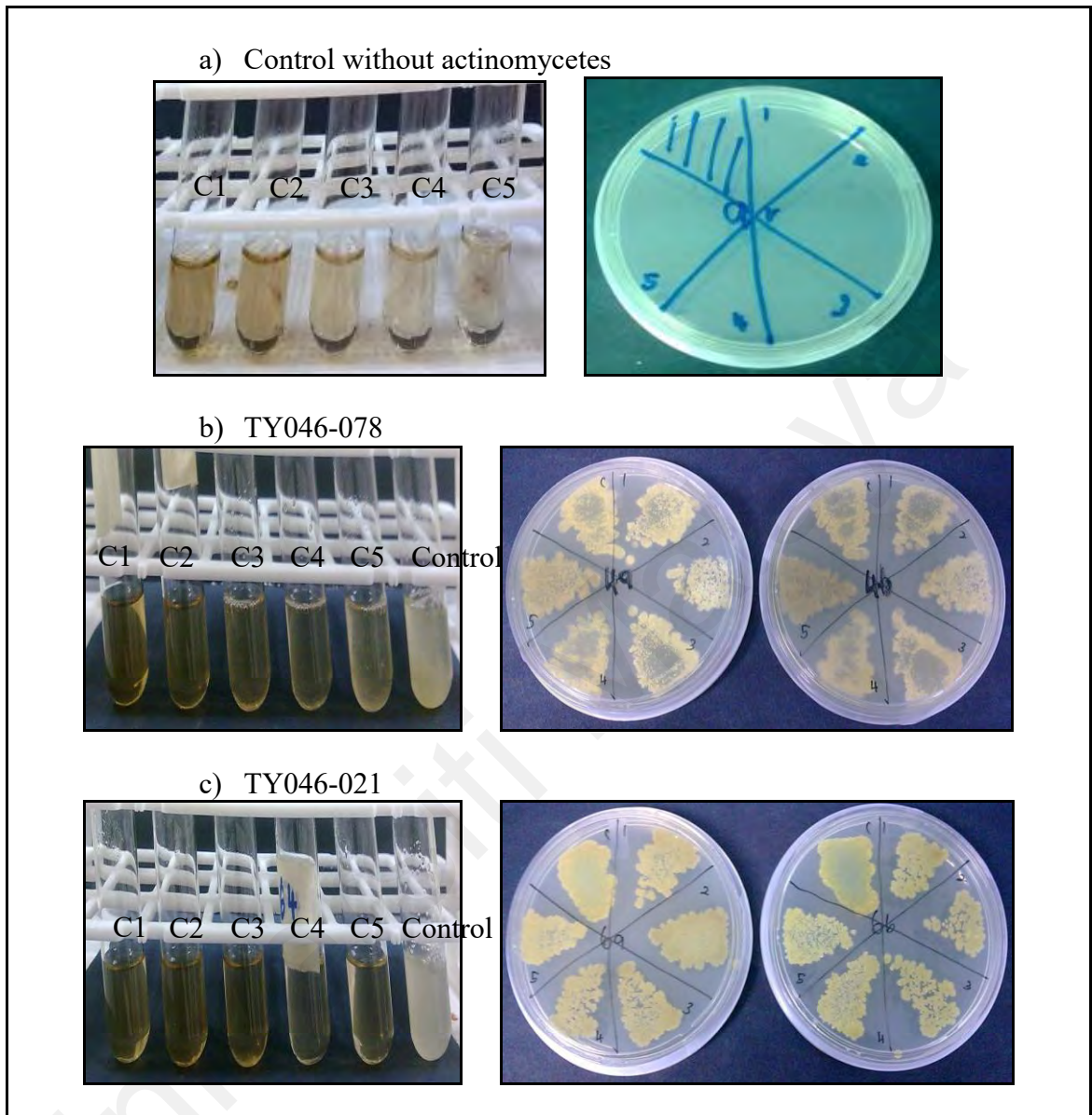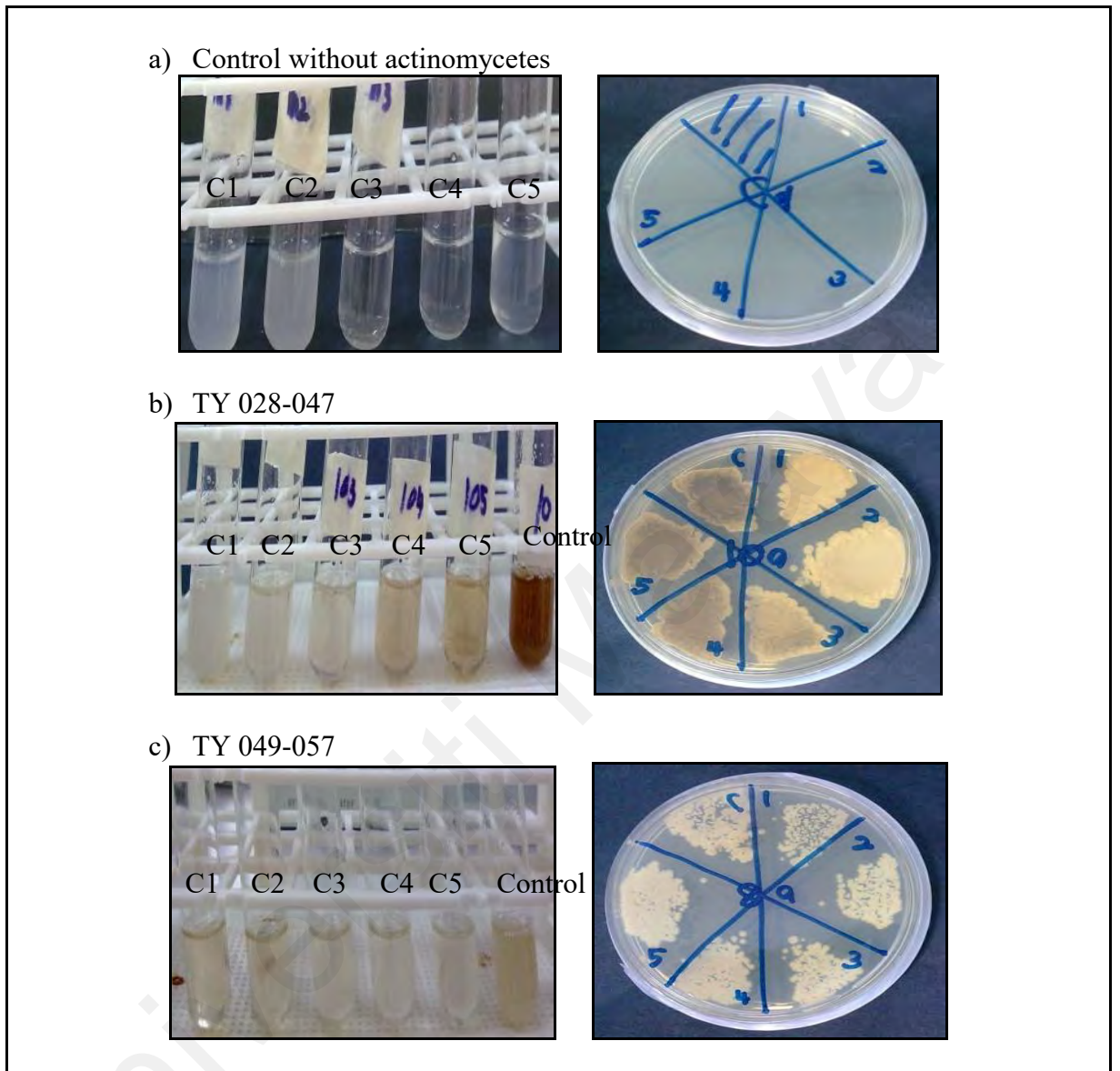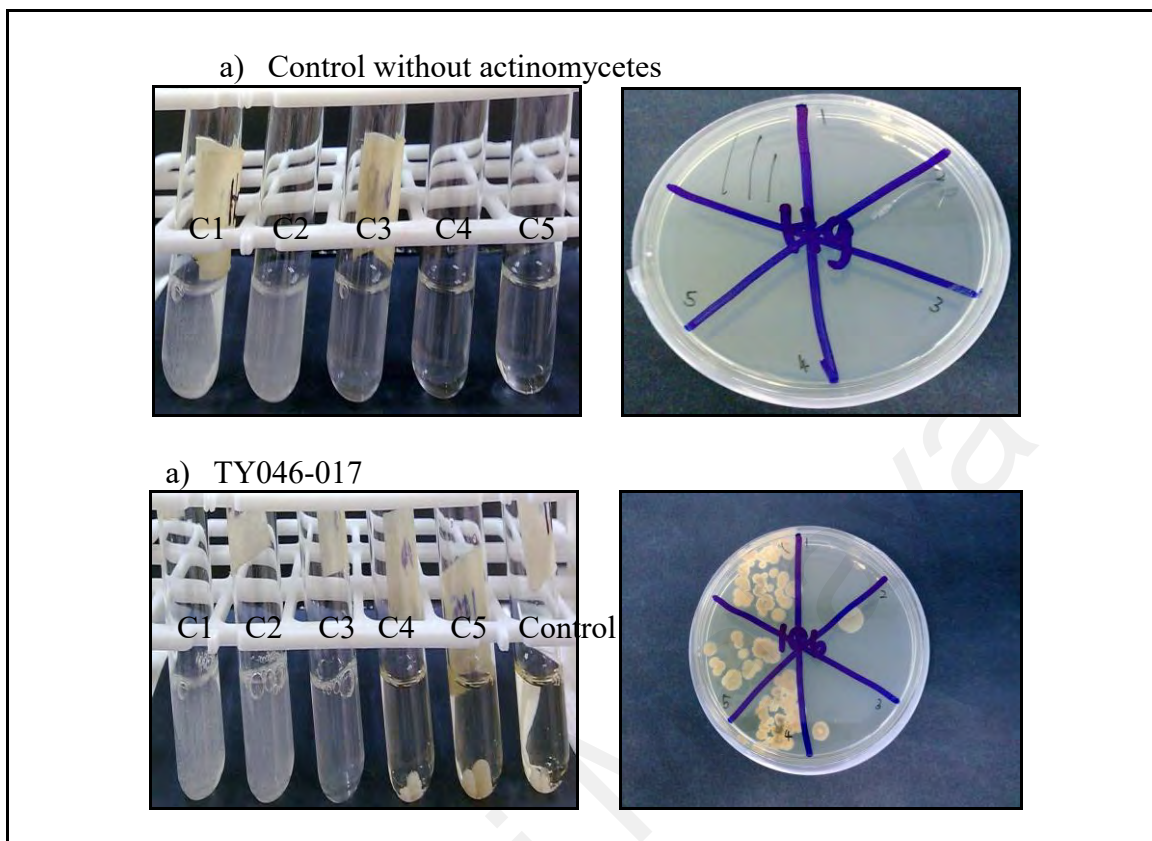
**Figure 4.5** Metal toxicity assays of potential actinomycete isolates which showed

tolerance to (A) Plumbum, (B) Nickel, (C) Arsenic, (D) Cadmium and (E) Mercury.

Concentration = C1 (highest test concentration) - C5 (lowest test concentration)
Control = no metal supplement

**Table 4.8**      Metal MTC values of highly tolerant isolates from Bidor and Pasoh

| Sampling Site | Metal Groups | Isolate Codes | MAR Index | Metal MTC values for different heavy metals obtained in Metal Toxicity Assay (mmol/L) | | | | | | Tolerance level obtained in Metal Tolerance Screening (mmol/L) # |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | $Pb^{2+}$ | $Ni^{2+}$ | $As^{3+}$ | $Cd^{2+}$ | $Cu^{2+}$ | $Hg^{2+}$ | |
| Bidor | Multi | TY046-078 | 0.3 | 43.2* | 138.9* | 182* | nt | nt | nt | $Pb^{2+}$(18); $Ni^{2+}$(77); $As^{3+}$(51) |
| Bidor | metal | TY046-021 | 0 | 43.2* | 138.9* | 182* | 10.9 | nt | nt | $Pb^{2+}$(18); $Ni^{2+}$(77); $As^{3+}$(51); $Cd^{2+}$(6) |
| Bidor | | TY046-071 | 0.3 | 5.4 | 138.9* | 45.5 | 2.7 | nt | nt | $Pb^{2+}$(18); $Ni^{2+}$(77); $As^{3+}$(51); $Cd^{2+}$(6) |
| Bidor | | TY047-019 | 0.2 | 5.4 | 138.9* | 11.4 | nt | nt | nt | $Pb^{2+}$(18); $Ni^{2+}$(77); $As^{3+}$(51) |
| Bidor | | TY046-027 | 0.2 | 5.4 | 138.9* | 11.4 | nt | nt | nt | $Pb^{2+}$(18); $Ni^{2+}$(77); $As^{3+}$(51) |
| Bidor | | TY046-073 | 0.3 | 2.7 | 17.4 | 182* | nt | nt | nt | $Pb^{2+}$(18); $Ni^{2+}$(77); $As^{3+}$(51) |
| Pasoh | | TY029-008 | 0.2 | 2.7 | nt | 182* | 2.7 | nt | nt | $Pb^{2+}$(18); $As^{3+}$(51); $Cd^{2+}$(6) |
| Pasoh | | TY029-014 | 0.3 | 5.4 | nt | 182* | 1.4 | nt | nt | $Pb^{2+}$(18); $As^{3+}$(51); $Cd^{2+}$(6) |
| Bidor | | TY046-016 | 0 | 43.2* | 69.45 | 11.4 | nt | nt | nt | $Pb^{2+}$(18); $Ni^{2+}$(77); $As^{3+}$(51) |
| Bidor | | TY046-018 | 0.4 | 5.4 | 17.4 | 91 | nt | nt | nt | $Pb^{2+}$(18); $Ni^{2+}$(77); $As^{3+}$(51) |
| Bidor | | TY046-004 | 0.1 | 5.4 | 34.7 | - | nt | nt | nt | $Pb^{2+}$(18); $Ni^{2+}$(77); $As^{3+}$(51) |
| Bidor | | TY047-062 | 0 | nt | 69.45 | 22.8 | 10.9 | nt | nt | $Ni^{2+}$(77); $As^{3+}$(51); $Cd^{2+}$(6) |
| Bidor | Mercury | TY046-017 | 0.3 | nt | 69.45 | nt | nt | nt | 0.8* | $Ni^{2+}$(77); $Hg^{2+}$ (4) |
| Bidor | Specific | TY048-047 | 0.1 | nt | nt | 11.4 | nt | nt | - | $As^{3+}$(51); $Hg^{2+}$ (4) |
| Bidor | Cadmium | TY049-057 | 0.3 | 5.4 | nt | 11.4 | 21.8* | nt | nt | $Pb^{2+}$(18); $As^{3+}$(51); $Cd^{2+}$(6) |
| Pasoh | Specific | TY028-047 | 0.1 | nt | nt | 91 | 21.8* | nt | nt | $As^{3+}$(51); $Cd^{2+}$(6) |
| Pasoh | | TY030-054 | 0.2 | nt | nt | 91 | 10.9 | nt | nt | $As^{3+}$(51); $Cd^{2+}$(6) |
| Bidor | | TY048-042 | 0.2 | nt | nt | nt | 5.45 | nt | nt | $Cd^{2+}$(6) |
| Bidor | | TY047-020 | 0.2 | nt | nt | nt | 2.7 | nt | nt | $Cd^{2+}$(6) |
| Pasoh | | TY029-029 | 0.3 | nt | nt | nt | 1.4 | nt | nt | $Cd^{2+}$(6) |
| Bidor | | TY046-037 | 0.2 | 5.4 | nt | nt | 1.4 | nt | nt | $Pb^{2+}$(18); $Cd^{2+}$(6) |
| Bidor | Cuprum | TY046-070 | 0.3 | nt | 34.7 | 22.8 | nt | - | nt | $Ni^{2+}$(77); $As^{3+}$(51); $Cu^{2+}$(63) |
| Bidor | Specific | TY047-024 | 0 | nt | 17.4 | nt | nt | - | nt | $As^{3+}$(51); $Cu^{2+}$(63) |

| Pasoh | TY028-043 | 0 | 5.4 | nt | nt | nt | 14.1** | ng | $Pb^{2+}$(18); $Cu^{2+}$(63); $Hg^{2+}$(4) |
|---|---|---|---|---|---|---|---|---|---|
| Pasoh | TY028-019 | 0.1 | nt | nt | nt | nt | - | nt | $Cu^{2+}$(63) |
| Bidor | TY047-025 | 0 | nt | 34.7 | nt | nt | - | nt | $Ni^{2+}$(77); $Cu^{2+}$(63) |
| Bidor | TY047-014 | 0.2 | 5.4 | nt | 11.4 | nt | - | nt | $Pb^{2+}$(18); $As^{3+}$(51); $Cu^{2+}$(63) |
| Bidor | TY047-023 | 0.1 | 5.4 | nt | nt | nt | - | nt | $Pb^{2+}$(18); $Cu^{2+}$(63) |
| Bidor | TY047-027 | 0.1 | 5.4 | nt | nt | nt | - | nt | $Pb^{2+}$(18); $Cu^{2+}$(63) |

"Table 4.8, continued"

\#   Test concentration showing bacterial tolerance (Figure 4.2.1)

\*   Metal MTC values supporting actinobacteria growth (good growth as control)

\*\* Metal MTC values supporting actinobacteria growth (moderate growth compared to control)

-   Bacterial growth not observed

nt  not tested

ng no growth

Final concentrations of $Pb^{2+}$, $Ni^{2+}$, $As^{3+}$ and $Cd^{2+}$ that isolates could tolerate in the metal toxicity assay were higher than concentrations showing bacterial tolerance in the rapid metal tolerance screening. The concentrations of metal supporting bacterial growth in metal toxicity assay were higher than those from previous studies; 60-90mmol/L for $As^{3+}$ and 4-10mmol/L for $Pb^{2+}$ (Kermanshahi *et al.*, 2007), 10mmol/L for $Cd^{2+}$ (Amoroso *et al.*, 1998), and 42.6mmol/L for $Ni^{2+}$ (Van Nostrand *et al.*, 2007). Only one isolate from Bidor could tolerate $Hg^{2+}$ at 0.8 mmol/L and one isolate from Pasoh tolerated $Cu^{2+}$ at 14 mmol/L.

Not all of the isolates that showed tolerance to more than one metal in the metal tolerance screening were able to tolerate all the metals at high concentrations in the metal toxicity assay. Isolates that showed tolerance to $Cu^{2+}$ and $Hg^{2+}$ in the metal tolerance screening were able to tolerate higher metal concentrations compared with results obtained in the metal toxicity assay (Table 4.8). However, the concentrations that supported the actinomycetes growth in metal toxicity assay were still much higher than the results reported by Albarracin *et al.* (2005) which was 6mmol/L for $Cu^{2+}$ and Prithviraj *et al.* (2012) which was 0.1 mmol/L for $Hg^{2+}$.

Various studies have been conducted for heavy metal removal and all the available commercial remediation methods are becoming uneconomical and unfavorable to remove heavy metals and still failing to provide the needed requirements for a safe, cheap, effective and environmental friendly metal remediation technique (Kumar *et al.*, 2011). Thus, the metal tolerant isolates obtained from the current study could be further explored for use in heavy metal removal to fulfill the needed requirements such as safe, eco-friendly, cheap and effective methods of bioremediation.

Compared with metal tolerance screening, the metal toxicity assay clearly showed that metal tolerant isolates obtained from Bidor able to tolerate $Pb^{2+}$, $Ni^{2+}$, $As^{3+}$,

$Cd^{2+}$ and $Hg^{2+}$ at a very high concentration. However, this was not observed in isolates obtained from Pasoh (Table 4.8). Thus, the distribution of metal tolerance in actinomycetes to specific metal ions is heavily influenced by environmental conditions from where the isolates are isolated.

In the metal toxicity assay, liquid medium was used as the test media for bacterial growth whereas in the metal tolerance screening, a solid agar medium was used. Thus, the conditions of diffusion, complexation and availability of metals are different in both methods. Hassen *et al.* (1998) who tested the levels of tolerance in *Pseudomonas aeruginosa* to different concentrations of divalent metal ions in nutrient broth showed that the test in liquid media was sensitive at concentrations 10 to 1000 times lower than those obtained in solid media. This may be the reason why isolates tolerant to $Hg^{2+}$ and $Cu^{2+}$ in rapid metal tolerance screening became sensitive to metal ions in the metal toxicity assay.

A total of eight isolates showing tolerance to at least one metal ion at the highest test concentration, three isolates tolerant to $Ni^{2+}$ at the second highest concentration and another two isolates showing moderate tolerance to $Cu^{2+}$ and $Hg^{2+}$, respectively (Table 4.8) were listed as potential heavy metal tolerant isolates for metal uptake study. However, the objective of this study was to compare metal accumulation ability of potential tolerant isolates obtained from two different study sites for one metal. Only $As^{3+}$ and $Cd^{2+}$ tolerant isolates represented the different sites. In the metal toxicity assay, two isolates from Bidor and Pasoh, respectively were able to tolerate $Cd^{2+}$. While, three isolates from Bidor and two from Pasoh were able to tolerate $As^{3+}$ at the highest test concentration (Table 4.8). Isolates TY049-057 and TY028-047 were selected for further study to determine their ability in $Cd^{2+}$ uptake. Both of these isolates are from the *Streptomyces*-like group. In order to de-replicate the selected isolates, all

13 potential heavy metal tolerant isolates were clustered based on their DNA fingerprint similarity using the repetitive element-based BOX-PCR fingerprinting technique. This de-replication technique is useful for a rapid identification percentage of similarity between the two $Cd^{2+}$ tolerant isolates.

## 4.5 Actinomycetes De-replication using BOX-PCR Method

### 4.5.1 Band Analysis using Gel Compar II Software Analysis

All 13 potential heavy metal tolerant isolates showed optimal ratio of DNA purity around 1.8 and DNA concentration ranging from 21.3 to 121.6µg/µl (Table 4.9). Good quality DNA is required for BOX-PCR method. According to Santella, 2006, to determine DNA quality the ratio absorbance at 260 and 280 nm (A260 nm/A280 nm) are used. Absorbance ratio around 1.8 indicates good-quality DNA whereas lower values indicate protein contamination while higher values indicate ribonucleic acid (RNA) contamination. Thus, all 13 extracted DNA was eligible for BOX-PCR method.

**Table 4.9**    DNA concentration of the potential heavy metal tolerant isolates

| Sample | DNA Concentration (µg/µl) | A260/280 |
|--------|---------------------------|----------|
| TY046-016 | 26.6 | 1.92 |
| TY046-017 | 55.7 | 1.88 |
| TY046-021 | 21.3 | 1.95 |
| TY046-027 | 69.4 | 1.89 |
| TY046-071 | 36.5 | 2.00 |
| TY046-073 | 100.1 | 1.82 |
| TY046-078 | 39 | 1.84 |
| TY049-057 | 118.7 | 1.89 |
| TY028-047 | 27.7 | 1.83 |
| TY029-008 | 22.2 | 1.94 |
| TY029-014 | 79.5 | 1.89 |
| TY047-019 | 55.6 | 1.88 |
| TY028-043 | 121.6 | 1.97 |

In this study, the use of the repetitive PCR DNA fingerprinting technique is described by carrying out BOX-PCR using BOXA1R primer to differentiate 13 potential heavy metal tolerant actinomycete isolates from tin tailings and natural forest soil.

DNA fingerprint patterns were compared by measurement of band positions of PCR products. The banding pattern shows at least four major bands with varying molecular sizes ranging from 1000bp to 300bp for all the tested isolates (Figure 4.6). The lane 14 in Figure 4.6 was a negative control, where template DNA was not added to the BOX-PCR reaction. The absence of an amplified DNA band in this lane indicates that the DNA fingerprints were free from contamination. The PCRs were repeated at least two times and the patterns of PCR products were consistently reproducible.

The position and intensity of the amplified PCR product varied between some strains, which showed the genetic diversity among different isolates (Figure 4.6). However, there are also isolates with exactly or nearly same position and intensity bands such as TY046-078, TY047-019 and TY046-071, TY046-021 and TY046-027 with TY049-057 and TY046-073. Thus, for more precise results a computer-assisted cluster analyses were performed with the biostatistical analysis program Gel Compar Software II (Applied Maths, Inc., Belgium) to derive the similarity groups using the measured qualitative data (Figure 4.7).

A dendrogram with three major clusters was produced (Figure 4.7). The first cluster was consisting of two minor clusters (Cluster IA and IB). A total of five isolates were obtained in cluster IA. Three out of five isolates (TY046-078, TY047-019 and TY046-071) has 100% similarity as expected. All 3 isolates posses the same DNA fingerprint (Figure 4.6) and they are also macro morphologically same (Figure 4.8). While, the other two isolates (TY029-014 and TY046-017) were approximately 27.3%

**Figure 4.6**    Agarose electrophoresis of BOX-PCR gels. Lanes labeled M was run with the DNA size standard (100bp ladder; Fermentas Corp.), sizes of fragments (bp) are indicated on the left. Lane 1, isolate TY046-021; lane 2, TY028-047; lane 3, TY046-016; lane 4, TY046-071; lane 5, TY046-078; lane 6, TY046-017; lane 7, TY046-027; lane 8, TY046-073; lane 9, TY049-057; lane 10, TY029-014; lane 11, TY047-019; lane 12, TY028-043; lane 13, TY029-008; lane 14, negative control.

**Figure 4.7** Dendrogram showing BOX-PCR fingerprints and percentage similiraty of 13 potential heavy metal tolerant actinomycetes.

(i) Grey Series

a) TY046-073



b) TY049-057



c) TY046-021
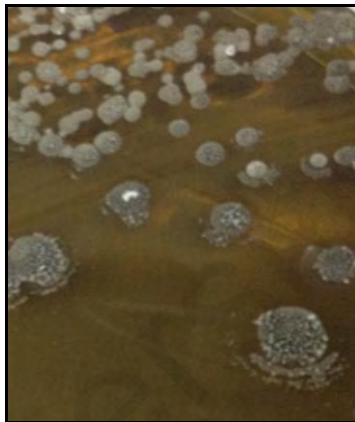


d) TY046-027
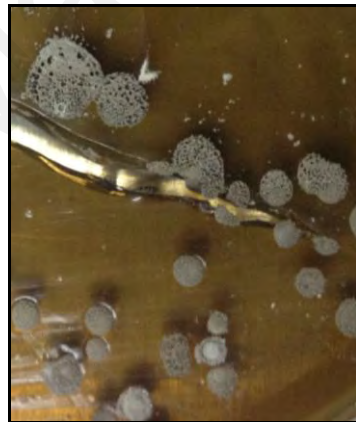


e) TY028-047

(ii) White Series

a) TY046-016

b) TY028-043
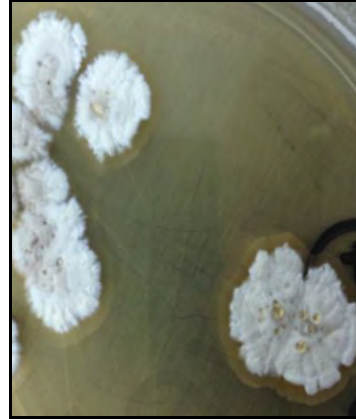


c) TY046-078

d) TY047-019



e) TY046-071

"Figure 4.8, continued"

f ) TY029-014          g) TY046-017



(B)

a)  TY029-008
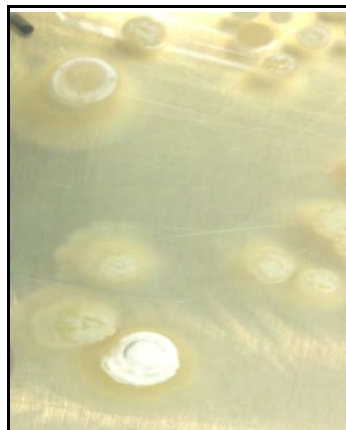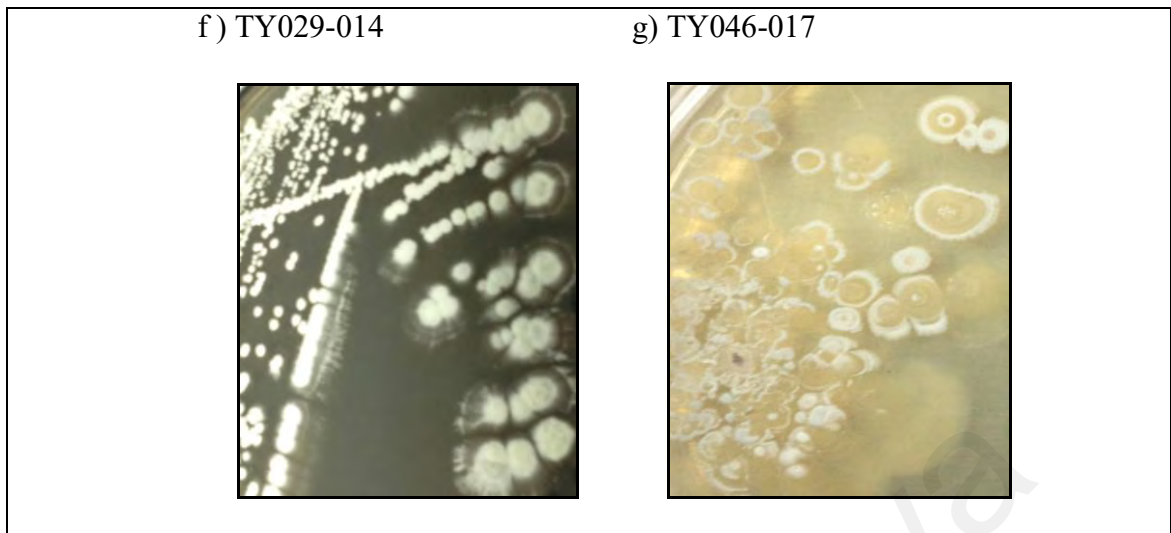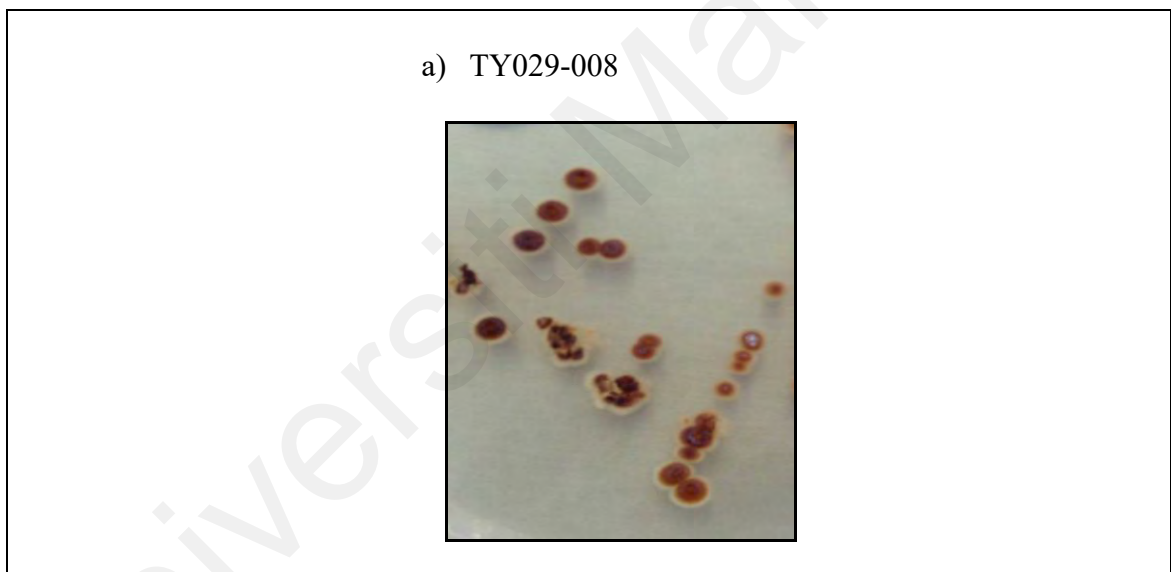


**Figure 4.8**   Colour grouping of 13 potential heavy metal tolerant actinomycete isolates

A: *Streptomyces*-like, B: non-*Streptomyces*.

and 14.3% similar with the other three isolates, respectively. However, all 5 isolates from cluster IA belongs to the white series even though the two isolates (TY029-014 and TY046-017) are with large different of similarity. On the other hand, another five isolates were obtained from cluster IB. This cluster can be divided into two groups of TY046-016 and TY028-043 from white series and TY028-047, TY046-021, and TY046-027 from grey series (Figure 4.8). Both clusters had different of 5%. Further, they also possess different of 5% and 10%, respectively with the isolates from cluster IA. Thus, the white series sitting in cluster IB is actually siting inter between of white series and grey series even though they are separated into two different minor clusters. Both white series in cluster IB were 27.3% similar. Further, in grey series, isolate TY046-021 and TY046-027 has 100% similarity as expected and both isolate were 30.8% similar with TY028-047. Both TY046-021 and TY046-027, were macro morphologically same (Figure 4.8) and they do have the same DNA fingerprint pattern (Figure 4.6).

Meanwhile, a total of two isolates were obtained in cluster II and one isolate in cluster III. Both isolate from cluster II were 100% similar as expected. They were macro morphologically same (Figure 4.8) and they has the same DNA fingerprint band pattern (Figure 4.6). This isolates were only 2% different from the isolates in cluster I but the single isolate in cluster III were 7% different from isolates in cluster I. This happens because the isolates in cluster II are from *Streptomyces*-like group same as the isolates from cluster I meanwhile the single isolate in cluster III is from non-*Streptomyces* group (Figure 4.8). Thus, a great difference is observed between cluster III and cluster I compared to cluster II and cluster I. Therefore, it can be concluded that a fast and precise grouping of actinomycete isolates can be done based on DNA fingerprint band patterns.

In this study, BOX-PCR was performed for the selected actinomycetes as it appears highly conserved among diverse bacteria and it was the first repetitive sequences identified in a Gram-positive organism (*Streptococcus pneumoniae*) (Elke and Christoph, 2003). Result showed the cluster analysis of similarity coefficients using BOX-PCR data can be used for grouping of unidentified isolates after doing comparison between the DNA fingerprint, similarity value and the macroporphological characteristic. Even in previous studies, the repetitive-PCR fingerprinting method have managed to distinguish pathogenic and non-pathogenic strains of *Streptomyces scabies* and other streptomycetes used for biocontrol of potato scab (Spooner *et al*., 1995). It also detected genomic differences among strains and pathovars of different strains of *Pseudomonas* and *Xanthomonas* that were indistinguishable by other methods used in classification (Louws *et al*., 1992).

However, the cluster analysis or known as dendogram was not useful in grouping the potential heavy metal tolerant isolates based on their metal tolerant ability. One glaring example was the two potential cadmium tolerant isolates. TY028-047 from Pasoh was sitting in cluster I meanwhile TY049-057 from Bidor was sitting in cluster II. Both isolates were only having 15.4% similarity even though both isolates able to tolerate cadmium at the highest test concentration. Further, TY049-057 from Bidor has 100% similarity with TY046-073 but both isolates had different ability in tolerating metal. TY046-073 able to tolerate multi metal with arsenic as the best tolerable metal while TY049-057 does not tolerate multi metal but able to tolerate only cadmium at the highest test concentration (Table 4.8).

Based on the DNA fingerprinting study, it is distinguished that both isolates TY049-057 from Bidor and TY028-047 from Pasoh are widely different (15.4% similarity) even though they fall under the same *Streptomyces*-like group. Thus, further

characterization was done on these isolates in order to identify them in species level. A further study to evaluate the effect of cadmium supplement on metal uptake and growth were then carried out on the identified isolates.

## 4.6 Identification of Isolate TY028-047 and TY049-057

### 4.6.1 Cultural, Micromorphological and Physiological Characterization

Morphological observations are best made on a variety of standard cultivation media (Labeda, 1987). Thus, the culture characteristics of both TY028-047 and TY049-057 were observed after 14 days of incubation on ISP2, ISP3 and ISP4 media. Isolate TY028-047 exhibited good growth on all three different types of media. Grey greenish aerial mycelium is observed on ISP2 and ISP3 media. While, the aerial mycelium is white in colour on ISP4 agar media. The substrate mycelium is dark brown on ISP2, brown on ISP3 and peach on ISP4 media with diffusible pigment ranging from light brown (ISP2) to brown (ISP3 & ISP4). Isolate TY049-057 also exhibited good growth with grey whitish aerial mycelium on all three different types of agar media. The substrate mycelium is brown on ISP2 and cream on both ISP3 and ISP4 media. No diffusible pigments were observed on all tested media (Table 4.10). In conclusion, both isolates grown on the three different media showed filamentous growth with abundant of aerial and vegetative hyphae. The colonies of both TY028-047 and TY049-057 were well grown into the medium with their threads, and have convex form (Figure 4.9).

In this study, observation of spore chain arrangements under the light microscope at 150X and 600X showed that sporophores of both isolate were spirals. However, sporophores of isolate TY028-047 were close spirals while for TY049-057 were open spirals (Figure 4.10). According to Shirling and Gottlieb (1966), the spore morphology of genus *Streptomyces* can be grouped into three different categories

**Table 4.10**    Morphology and Physiology Characteristics of Isolate TY028-047 and

TY049-057

| Characteristics | Name of Medium | TY 028-047 | TY049-057 |
|---|---|---|---|
| Growth | Yeast extract malt | Good | Good |
| Aerial mycelium | extract dextrose agar | Gray | Gray |
| Substrate mycelium | (ISP2) | Dark brown | Brown |
| Pigmentation | | Light brown | None |
| | | | |
| Growth | Oat-meal agar (ISP3) | Good | Good |
| Aerial mycelium | | Gray | Gray |
| Substrate mycelium | | Brown | Cream |
| Pigmentation | | Brown | None |
| | | | |
| Growth | Inorganic salts–starch | Good | Good |
| Aerial mycelium | agar (ISP4) | White | Gray |
| Substrate mycelium | | Peach | Cream |
| Pigmentation | | Brown | None |
| | | | |
| Spore chain morphology | | Closed spirals | Open spirals |
| | | | |
| Growth at: | Inorganic salts–starch | | |
| 45°C * | agar (ISP4) | - | - |
| 28°C * | | + | + |
| 10°C ** | | - | - |
| 4°C ** | | - | - |
| pH 4 | | - | ± |
| pH 5-10 | | + | + |
| | | | |
| Growth in the presence of | Modified Benett's Agar (MBA) | | |
| (% w/v) | | | |
| NaCl (0-2) | | + | + |
| NaCl (3-5) | | ± | ± |
| NaCl (6-10) | | - | - |

 *Observed after 14 days of incubation. **Observed after 6 weeks of incubation
 - No growth + Good growth ± Weak growth

**Figure 4.9**    Colony morphology of TY028-047 (i) and TY049-057 (ii) grown on ISP2 for 14 days at 28$^{o}$C.
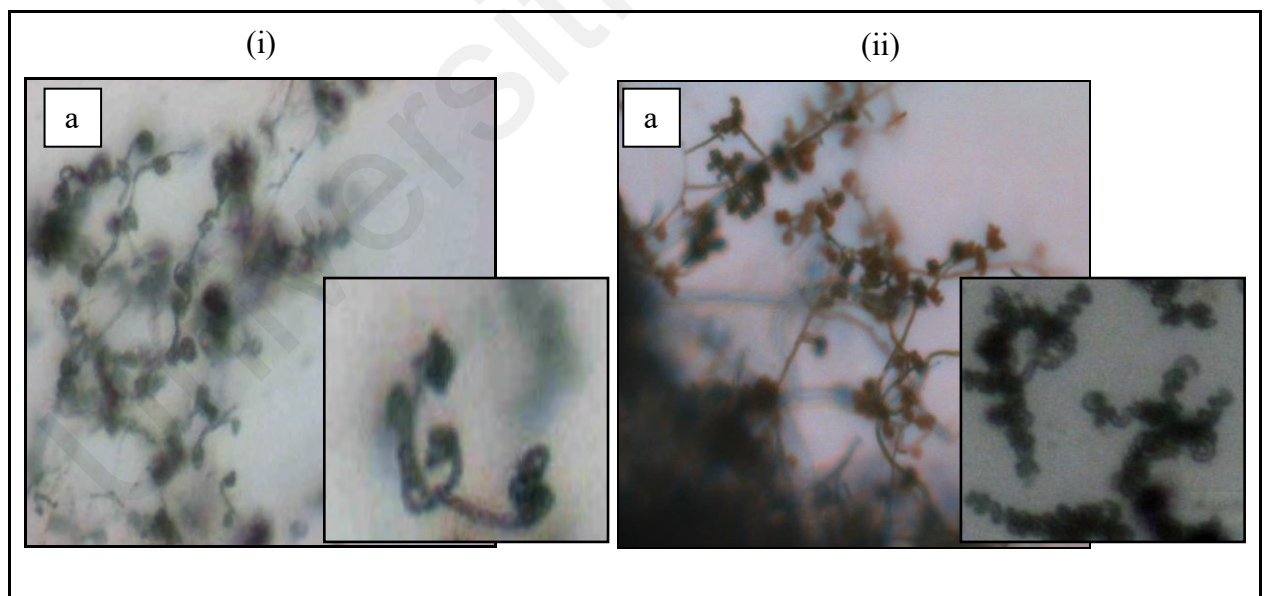


**Figure 4.10**    Spore chain morphology of TY028-047 (i) and TY049-057 (ii) on coverslip. Isolate were observed at a. 150X and b. 600X magnification (cs=close spirals, op=open spirals) under a light microscope.

namely rectiflexibiles (RF), retinaculiaperti (RA) and spirals (S). This indicates that, both TY028-047 and TY049-057 closely relates to the genus *Streptomyces*. Further, thephysiological characteristic of both isolate were summarized in Table 4.10.

Good growth was observed at pH 5-10 with optimum temperature of $28^0C$ after 8 days of incubation for both isolates. Tendler (1959) has shown that ability to grow at different pH and temperatures is a function of nutritional requirements rather than a specific character. Both isolates also showed good growth at 0-2% (w/v) NaCl and the growth was weak at 3-5% NaCl. No growth was observed at higher concentration of NaCl (6-10%). Hence, the isolates can be placed in an intermediate salt tolerance group (Tresner *et al.* 1968).

Morphology has always been an important characteristic used to identify actinomycete isolates. In section 4.1, macromorphology of isolates was observed to distinguish between *Streptomyces*-like and non-*Streptomyces* groups. However, for a further detailed characterization of a particular actinomycetes isolate, data generated from morphological and physiological studies can be used as markers for strain identification (Siva Kumar, 2001).

### 4.6.2  Phylogenetic Analysis of Isolate TY028-047 and TY049-057

### 4.6.2.1 Amplification and Sequencing of 16S rRNA gene

The 16S ribosomal ribonucleic acid (rRNA) gene for isolates TY028-047 and TY049-057 were amplified using PCR. Figure 4.11 shows the gel electrophoresis image of 16S rRNA PCR amplified gene product of two potential heavy metal tolerant isolates. All the bands obtained were visible and they were estimated to be at the length of 1500bp.
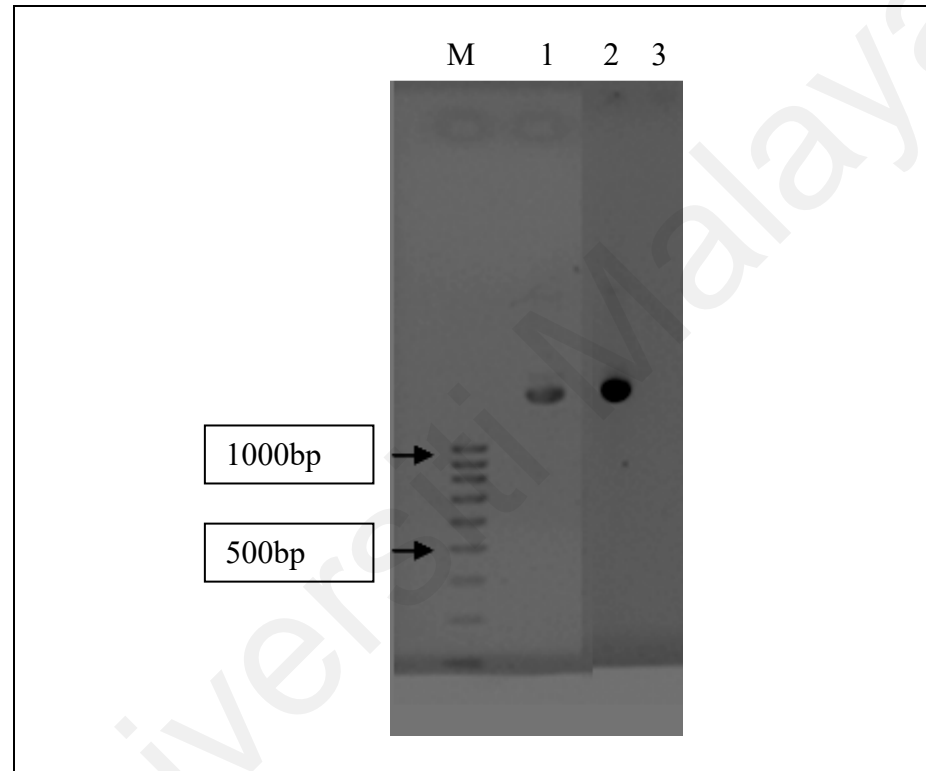
**Figure 4.11** Partial 16S rRNA gene fragment amplified from selected actinomycetes; Lanes labeled M was run with the DNA size standard (100bp ladder; Fermentas Corp.), sizes of fragments (bp) are indicated on the left. Lane 1, isolate TY028-047; lane 2, TY049-057; lane 3, negative control isolate.

It is important to measure correctly the concentration of DNA that is being used for PCR amplification because if the DNA is too concentrated, the sequencing reaction could drop off quickly and greatly diminish the read length. While, an overly weak DNA concentration will produce excess background noise and introduce errors during the sequence analysis. Purified PCR product was then send for sequencing to First Base Sdn. Bhd.

Partial 16S rRNA gene sequence of the isolates TY028-047 and TY049-057 with length of 881bp and 1049bp (Figure 4.12), respectively, were obtained. The partial sequences were aligned and compared with 16S rRNA gene sequences available from the GenBank database by using BLAST comparison tool available in the website of National Centre for Biotechnology Information (Table 4.11). A 100% 16S rRNA sequence similarity value was obtained for both *Streptomyces lannensis* (TY028-047) and *Streptomyces malaysiensis* (TY049-057) which highly indicate that they may belong to these species. DNA-DNA hybridization can be done in order to confirm this result.

**4.6.2.2 Phylogenetic Analysis**

16S rRNA gene sequences from twenty closest matches were aligned using the CLUSTAL W programme from the MEGA4 software. Sequence comparison of the isolates TY028-047 and TY049-057 with the corresponding sequences of the close representative strains of *Streptomyces* from the GenBank database showed that the strains formed a same distinct phyletic line with *Streptomyces lannensis* and *Streptomyces malaysiensis*, respectively (Figure 4.13). Both *Streptomyces lannensis* and *Streptomyces malaysiensis* have been listed in LPSN (List of prokaryotic names with standing in nomenclature) bacterio net (Euzrby, 2013). Therefore, both isolates are not novel species but has been validly described and found by Promnuan *et al*. (2013) and

i) TY028-047

GGCGAACGGGTGAGTAACACGTGGGCAATCTGCCCTGCACTCTGGGACAAGCCCTGGAAA
CGGGGTCTAATACCGGATACGAGCCTCCCGGGCATCTGGGAGGTTGGAAAGCTCCGGCGG
TGCAGGATGAGCCCGCGGCCTATCAGCTTGTTGGTGAGGTAACGGCTCACCAAGGCGACG
ACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAGACACGGCCCAGACTC
CTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCC
GCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAAGCGAAAGTG
ACGGTACCTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGG
GCGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGCTTGTCGCGTCGA
TTGTGAAAGCCCGGGGCTTAACTCCGGGTCTGCAGTCGATACGGGCAGGCTAGAGTGTGG
TAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCG
GTGGCGAAGGCGGATCTCTGGGCCATTACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGA
ACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGGTGGGCACTAGGTGTTGGCGACA
TTCCACGTCGTCGGTGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGC
AAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGTGGCTTAA
TTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACA

ii) TY049-057

CCGGTTTCGGCCGGGGATTAGTGGCGAACGGGTGAGTAACACGTGGGCAATCTGCCCTGC
ACTCTGGGACAAGCCCTGGAAACGGGGTCTAATACCGGATATGACGCGTTCCCGCATGGG
ATACGTGTGGAAAGCTCCGGCGGTGCAGGATGAGCCCGCGGCCTATCAGCTTGTTGGTGG
GGTGATGGCCTACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACACTG
GGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGG
CGCAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTT
TCAGCAGGGAAGAAGCGTGAGTGACGGTACCTGCAGAAGAAGCGCCGGCTAACTACGTGC
CAGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGAGC
TCGTAGGCGGCTTGTCGCGTCGGATGTGAAAGCCCGGGGCTTAACTCCGGGTCTGCATTC
GATACGGGCAGGCTAGAGTTCGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATG
CGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGATCTCTGGGCCGATACTGACGCTG
AGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACG
TTGGGAACTAGGTGTGGGCGACATTCCACGTTGTCCGTGCCGCAGCTAACGCATTAAGTT
CCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCAC
AAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACA
TACACCGGAAACATCCAGAGATGGGTGCCCCCTTGTGGTCGGTGTACAGGTGGTGCATGG
CTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGT
CCTGTGTTGCCAGCGGGTTATGCCGGGGA

**Figure 4.12**    Partial 16S rRNA sequence data of TY028-047 and TY049-057.

**Table 4.11**     Closest matches from GenBank using BLAST

| Strains | Closest matches | Accession number | % similarity |
|---------|-----------------|------------------|--------------|
| TY049-057 | *Streptomyces malaysiensis* strain MJM10645 | GU350494 | 100% |
| | *Streptomyces yatensis* strain *NBRC 101000* | NR041427 | 99% |
| | *Streptomyces violaceusniger Tu 4113 strain Tu 4113* | NR074570 | 99% |
| | *Streptomyces asiaticus* strain *NBRC 100774* | NR041418 | 99% |
| | *Streptomyces rhizosphaericus* strain *NBRC 100778* | NR041415 | 99% |
| | *Streptomyces sporoclivatus* strain *NBRC 100767* | NR041413 | 99% |
| | *Streptomyces antimycoticus* strain *NBRC 12839* | NR041080 | 99% |
| | *Streptomyces indonesiensis* strain *DSM 41759; A4R2* | NR043724 | 99% |
| | *Streptomyces geldanamycininus* strain *NRRL 3602* | NR043722 | 99% |
| | *Streptomyces griseiniger* strain *NRRL B-1865* | NR042099 | 98% |
| | *Streptomyces castelarensis* strain *BJ-608* | NR029114 | 98% |
| | *Streptomyces cangkringensis* strain *D13P3* | NR028957 | 98% |
| | *Streptomyces hygroscopicus* strain *NRRL 1346* | NR044200 | 98% |
| | *Streptomyces melanosporofaciens* strain *NRRL B-12234* | NR028917 | 98% |
| | *Streptomyces sporocinereus* strain *NBRC 100766* | NR041412 | 98% |
| | *Streptomyces endus* strain *NRRL 2339* | NR043379 | 98% |
| | *Streptomyces demainii* strain *NRRL B-1478* | NR043723 | 98% |
| | *Streptomyces celluloflavus* strain *NBRC 13780* | NR041150 | 98% |
| | *Streptomyces yogyakartensis* strain *NBRC 100779* | NR041416 | 98% |
| | *Streptomyces kasugaensis* strain *M338-M1* | NR024724 | 98% |
| | | | |
| TY028-047 | *Streptomyces lannensis* strain JCM 16578 | AB562508 | 100% |
| | *Streptomyces durhamensis* strain NRRL B-3309 | NR043352 | 99% |
| | *Streptomyces filipinensis* strain NBRC 12860 | NR041083 | 99% |
| | *Streptomyces djakartensis* strain NBRC 15409 | NR041178 | 98% |
| | *Streptomyces minutiscleroticus* strain NRRL B-12202 | NR044149 | 98% |
| | *Streptomyces geysiriensis* strain NRRL B-12102 | NR043818 | 98% |
| | *Streptomyces glomeratus* strain NBRC 15898 1 | NR041409 | 98% |
| | *Streptomyces flavoviridis* strain NBRC 12772 | NR041218 | 98% |
| | *Streptomyces cinerochromogenes* strain NBRC 13822 | NR041153 | 98% |
| | *Streptomyces pilosus* strain NBRC 12807 | NR041073 | 98% |
| | *Streptomyces ghanaensis* strain KCTC 9882 | NR043366 | 98% |
| | *Streptomyces eurythermus* strain ATCC 14975 | NR025869 | 98% |
| | *Streptomyces rubrogriseus* strain NBRC 15455 | NR041188 | 98% |
| | *Streptomyces anandii* strain NBRC 13438 | NR041135 | 98% |
| | *Streptomyces malachitofuscus* strain NBRC 13059 | NR041105 | 98% |
| | *Streptomyces rochei* strain NBRC 12908 | NR041091 | 98% |
| | *Streptomyces chromofuscus* strain NBRC 12851 | NR041082 | 98% |
| | *Streptomyces vinaceusdrappus* strain NRRL 2363 | NR043383 | 98% |
| | *Streptomyces speibonae* strain PK-Blue | NR025212 | 98% |
| | *Streptomyces showdoensis* strain NBRC 13417 | NR041129 | 98% |
| | *Streptomyces roseolilacinus* strain NBRC 12815 | NR041075 | 98% |
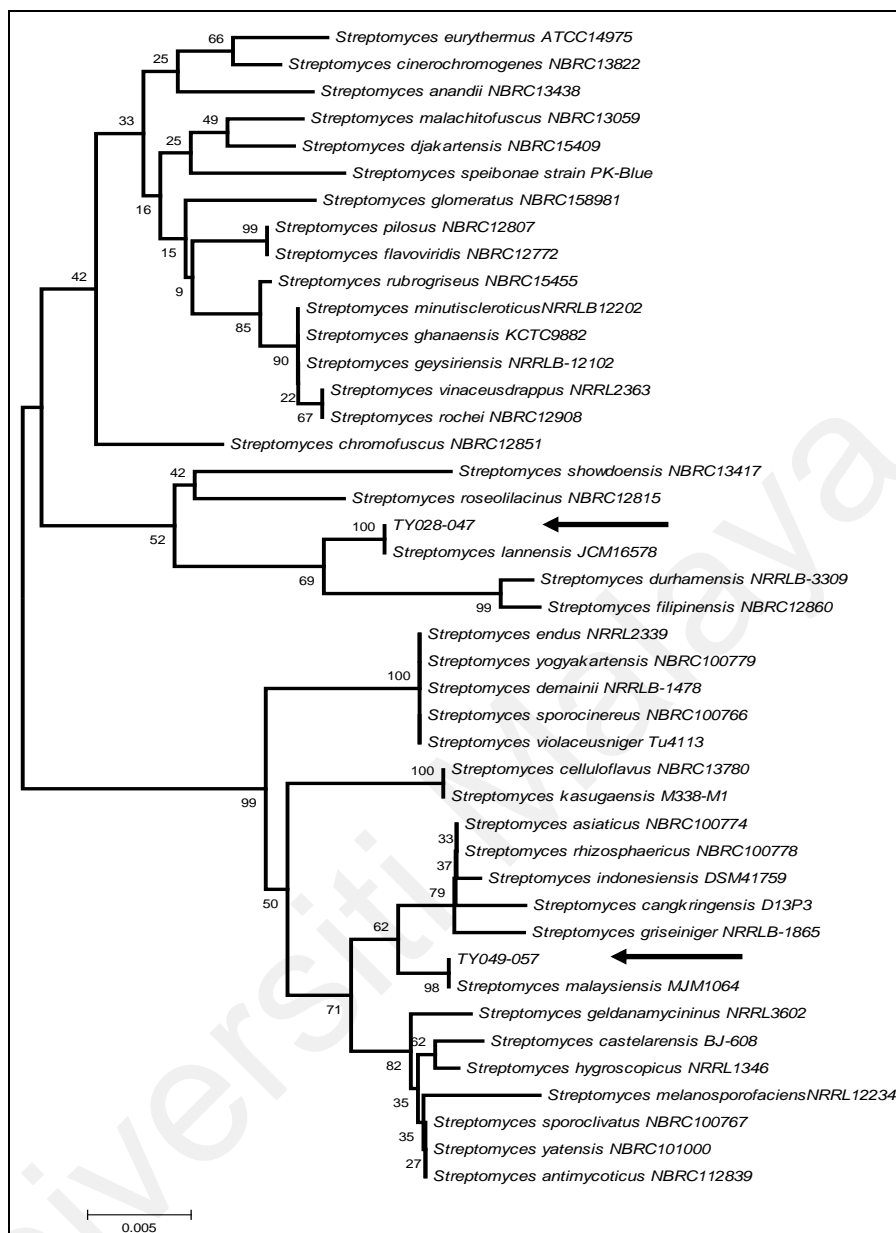
**Figure 4.13** Phylogenetic tree (the neighbor-joining tree) based on partial 16S rRNA gene sequence showing relationship between *Streptomyces* isolate TY028-047 and TY049-057 with related members of the *Streptomyces* species. The numbers at the nodes indicate the level of bootstrap support based on Kimura 2-parameter analysis of 1000 resampled datasets; the arrow indicates the root position of the tree for TY028-047 and TY049-057 the scale bar indicates 5 substitutions per 1000 nucleotide position.

Al-Tai *et al*. (1999), respectively. The *Streptomyces lannensis* strain identified by Promnuan *et al*. (2013) was isolated from a South-East Asian stingless bee compared to isolate TY028-047 which originates from soil. While, the *Streptomyces malaysiensis* strain identified by Al-Tai *et al*. (1999) was a soil isolate found from Malaysian soil, just as the current isolate TY049-057. Therefore, it indicates that the recently found novel species *Streptomyces lannensis* by Promnuan *et al*. (2013) can also be found in Malaysian soil. While, *Streptomyces malaysiensis* obtained in this study and by Al-Tai *et al*. (1999) indicates that Malaysian soil is rich with this species.

## 4.7 The Effect of Cadmium Supplement on Metal Uptake and Growth of Selected Tolerant Isolates

Cadmium uptake ability of the two selected isolates from Pasoh and Bidor and one control (metal sensitive) isolate was assessed to determine their potential uses in bioremediation processes. The time course of $Cd^{2+}$ uptake in the selected isolates when grown in the presence of 1mM of $CdCl_2$ in individual 500 ml flasks is shown in Figure 4.14/Appendix B-6.

At day 0, a very low amount of $Cd^{2+}$ uptake was observed for all three isolates (Figure 4.14). A slight increase in $Cd^{2+}$ uptake was observed between day 0 to 2 for Bidor isolate (TY049-057) while a slight drop was observed in the Pasoh isolate (TY028-047). After an increasing $Cd^{2+}$ uptake from day 2 to 4, there was a slight drop between day 4 to 6 and once again showed a significant increase between day 6 to 8 (Figure 4.14). While, a significant decrease was observed between day 8 to 10 in the Pasoh isolate and a slight decrease in the Bidor isolate (Figure 4.14). The maximum uptake of $Cd^{2+}$ in isolate TY049-057 was 3.17 umol $Cd^{2+}$/mg dry cell mass while 4.66 umol $Cd^{2+}$/mg dry cell mass was recorded in isolate TY028-047 at day 8 which was significantly higher than the metal sensitive isolate (TY049-044). Cadmium uptake was
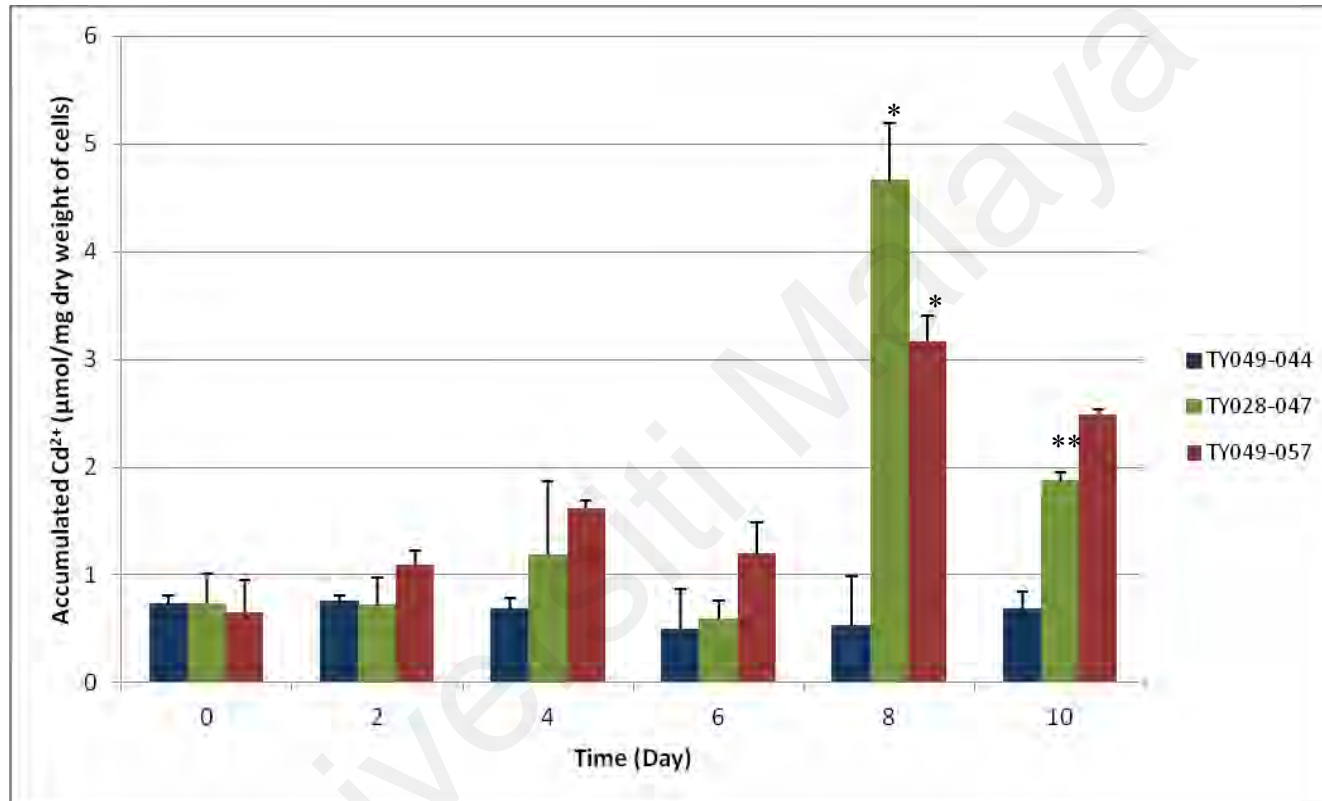
**Figure 4.14**   Time-dependent profile of $Cd^{2+}$ uptake (μmol/mg of cells) in TY049-044 (metal sensitive isolate), TY028-047 (Pasoh) and TY049-057 (Bidor) grown in media containing 1mM of $CdCl_2$.

Positive Significant,*: $p < 0.05$; Negative Significant, **: $p < 0.05$ (Scheffe Test)

not observed in all controls of the test isolates. Overall, both test isolates showed significant different in $Cd^{2+}$ uptake compared to the metal sensitive isolate (Table 4.12/Appendix B-7).

In parallel to the above study, growth viability of these metal sensitive and tolerant isolates in liquid media with and without 1mM $CdCl_2$ was studied by determining number of viable cells in a time-dependent study (Figure 4.15/Appendix B-8). A maximum increase in viable cells was observed in the control cultures of Bidor at day 2 while day 4 for control cultures of metal sensitive and Pasoh isolate (Figure 4.15). The maximum increase of $5.3 \times 10^6$ CFUs/ml viable cells at day 4 shows that the Pasoh isolate has higher growth rate compared to the metal sensitive ($6.8 \times 10^5$ CFUs/ml viable cells) and Bidor isolate ($7.9 \times 10^5$ CFUs/ml viable cells). Further, a clear decrease in number of viable cells from day 6 to 8 and followed by a slight decrease at day 10 were observed in all the controls (Figure 4.15/Appendix B-8a).

**Table 4.12**   Analysis of variance (ANOVA) on the $Cd^{2+}$uptake of day 0, 2, 4, 6, 8, 10 for TY049-044 (metal sensitive isolate), TY028-047 (Pasoh isolate) and TY049-057 (Bidor isolate)

| Isolate Code | F ratio | p-Value |
|---|---|---|
| TY049-044 | 0.352 | 0.864 |
| TY028-047 | 94.505 | 0.000* |
| TY049-057 | 42.761 | 0.000* |

*Significant, $p < 0.05$
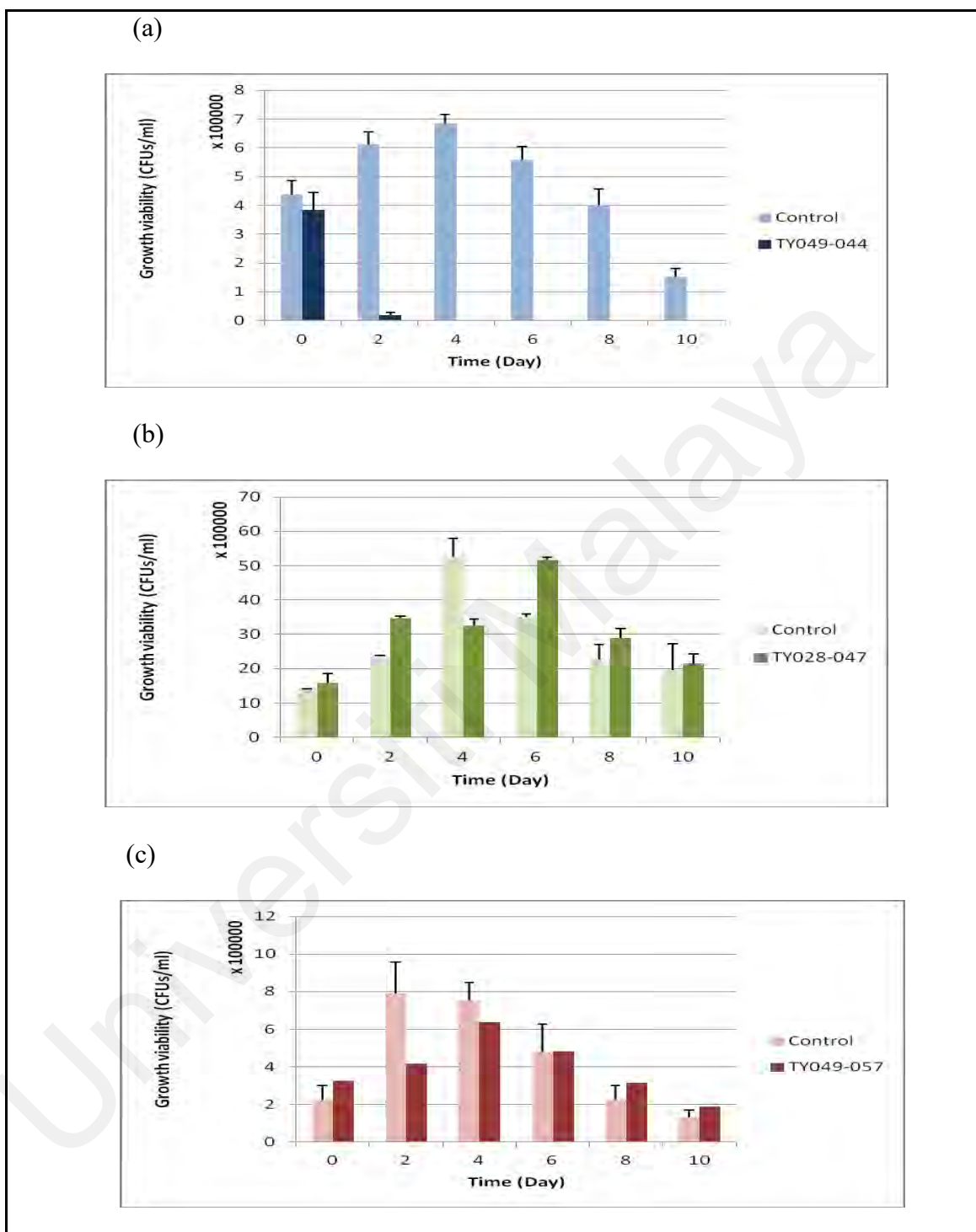
**Figure 4.15** Growth viability of a) TY049-044 (metal sensitive isolate) b) TY028-047 (Pasoh isolate) and c) TY049-057(Bidor isolate) at Day 0, 2, 4, 6, 8, and 10 with and without 1mM initial concentration of $CdCl_2$.

*Control- Actinomycete cultures without $CdCl_2$ supplement

On the other hand, all three test isolates showed different profile of growth viability when grown in 1mM $CdCl_2$. The metal sensitive isolate could not survive from the start of the experiment but both Bidor and Pasoh cadmium tolerant isolates showed an increase in viable cells at day 4 and day 6, respectively (Figure 4.15/Appendix B-8b). This results were similar to the ANOVA analysis of $Cd^{2+}$ uptake where both test isolates showed a significant $Cd^{2+}$ uptake compared to the metal sensitive isolate. Based on both studies, it clearly explains that only metal tolerant actinomycetes able to uptake metal and survive in the metal contaminated environment.

Among the heavy metals, cadmium is a highly toxic, carcinogenic (Mohamed Fahmy Gad El-Rab *et al*., 2006) and responsible for serious decreases in biological activity (Bruins *et al*., 2000) It is at number seven in Agency for Toxic Substances and Disease Registry (ATSDR) 2011 hazardous substance priority list. This might due to the wide usage of cadmium in various industries such as electroplating, tanneries, textiles, fertilizers, metallurgical processing, mineral processing and battery manufacturing (Lodeiro *et al*., 2004; Amoroso *et al*., 1998). Cadmium is found to be most toxic in its free (ionic) form. Thus, it may easily dissolve and move in wastewaters from these industries and threatens the groundwater supplies and soil (Anu *et al*., 2010). Further, cadmium enriched soil enhance food crops to accumulate cadmium and eventually causes a long term effect in living organism (Rani *et al*., 2008; Tripathi *et al*., 2005). Its exposure to human may result in adverse effects especially to the kidney which is a critical organ of intoxication after the exposure to cadmium (Krishna *et al*., 2012; Doshi *et al*., 2007). Thus, cadmium contamination in the soil and industrial effluents are serious and growing concern which requires immediate remediation measures.

The maximum increase in $Cd^{2+}$ uptake at day 8 by both Pasoh and Bidor cadmium tolerant isolates (Figure 4.14) showed that both isolates have the ability to accumulate $Cd^{2+}$ compared to the metal sensitive isolate. This maximum $Cd^{2+}$ uptake was observed during the stationary growth phase of both tolerant isolates. According to Daughney (2001), tolerant isolates should yield higher metal tolerance ability during exponential phase due to abundance of nutrients which allow them to develop cell walls with high concentration of suitable binding sites compared to stationary phase. However, a higher yield during stationary phase can also be expected due to partial deficiency of nutrient during this stage which increases negative charge of the cell membrane and hence leads to a higher metal binding capacity. Thus, nutrient in the media and membrane alterations determines which phase attains the higher metal capacity in bacteria (Anagnostopoulos *et al*., 2010). Low level of $Cd^{2+}$ uptake detected in all three test isolates at day 0 (Figure 4.14) may have caused by the residual $Cd^{2+}$ in culture broth even after rinsing the cell pellets with sterilized distilled $H_2O$.

Based on the growth viability result, a maximum increase in growth viability were observed at the end of exponential phase for both $Cd^{2+}$ supplemented isolates compared to their controls (Figure 4.15). El-Hellow *et al* (2000) have reported on *B. thuringiensis* strain where it able to accumulate $Cd^{2+}$ and has relatively a long lag phase in the presence of $Cd^{2+}$ while Shapiro and Keasling (1996) and Sinha and Mukherjee (2009) have reported that cadmium causes a longer lag phase compared to the normal bacteria growth phase. According to Matthew *et al* (2012), bacteria cells can sense the changes in their surroundings quickly and initiate transcription of genes during early lag phase to prepare for exponential growth. Thus, the effect shows an adaptive phenomenon involving the development of protective mechanisms in the Cd tolerant isolates. A reduction was observed in both $Cd^{2+}$ uptake (Figure 4.14) and growth viability (Figure 4.15) experiments at day 10. This explains, the reduction in $Cd^{2+}$

uptake occurs when viable cells which plays an important role in metal uptake reduces (Hamzah *et al*., 2008).

The control and cadmium supplemented cultures of Pasoh showed higher growth rate compared to the control and cadmium supplemented cultures of Bidor and metal sensitive isolates (Figure 4.15), even though the inoculum size used in each experiment were the same for all three test isolates. This might be the reason for cadmium tolerant isolate from Pasoh to be able to uptake a higher concentration of $Cd^{2+}$ compared to cadmium tolerant isolate from Bidor. This may also be explained by the metal tolerance screening and metal toxicity assay results where strains isolated from Pasoh showed higher ability to tolerate cadmium compared to Bidor (Table 4.2). Thus, not all isolates from heavy metal contaminated environment have potential in tolerating heavy metal because its effect of growth phase seems to be metal- and species– specific (Anagnostopoulos *et al.*, 2010). Further, many other aspects influence the level of cadmium uptake such as nutrients, metal concentration in the media and growth viability rate of bacteria. Thus, cadmium tolerant isolate from Bidor is interesting for further studies as they can uptake a high amount of $Cd^{2+}$ even with a low growth rate.

Most of the previous studies listed in Table 4.13 looked at $Cd^{2+}$ uptake at a very low initial concentration of 1mM $CdCl_2$ or more. Further, these were the very few studies uses viable cells to determine $Cd^{2+}$ uptake. Toxic metals even at a low concentration can disrupt the microorganism's metabolism and cause damage to the cells (Zouboulis *et al*., 2010). Hence, they lost their binding abilities and perform a remarkably lower uptake capacity. (Kadukova and Vircikova, 2005). Viable cells are rarely used in studies to determine uptake of metal because their metabolic resistance mechanisms are more complex. However, Chang *et al* (1997) showed that resting viable cells were able to uptake higher amount of Pb than inactivated dead cells of

**Table 4.13** Comparison between different literatures on actinomycetes maximum uptake for cadmium

| Biosorbents | Initial $Cd^{2+}$ Concentration mg/L | Maximum $Cd^{2+}$ uptake mg/g biomass | Time taken for maximum uptake | References |
|---|---|---|---|---|
| Actinomycetes | 183.32 (1mM) | 1831.34 mg/g (9.99 umol/mg) | 48 hours | Amoroso *et al*., 1998 |
| *Streptomyces* spp. | 8 | 41.7 | 7 days | Sineriz *et al*., 2009 |
| Actinomycetes | 8 | 37.3 | 7 days | Amoroso *et al*., 2012 |
| *Streptomyces zinciresistens* | 366.64 (2mM) | 85.0 | 8 days | Lin *et al*., 2012 |
| *Streptomyces lannensis* (TY028-047) | 183.32 (1mM) | 524 (4.66 umol/mg) | 8 days | Present study |
| *Streptomyces malaysiensis* (TY049-057) | 183.32 (1mM) | 356.3 (3.17 umol/mg) | 8 days | Present study |

*Pseudomonas aeruginosa*. Therefore, viable cells may be potential in uptake of heavy metals since absorbtion may add to the total accumulation capacity (Mullen *et al.*, 1989) which may results in higher yield in absorption (uptake using viable cells) than adsorption (biosorption using dead cell) into cell surface alone. Thus, low initial concentration of 1mM $CdCl_2$ and viable cells were used in current study.

Based on the previous work (Table 4.13), a high initial $Cd^{2+}$ concentration of 2mM used by Lin (2012) showed much lower $Cd^{2+}$ uptake compared to Amoroso (1998) and the present study. Thus, the actinomycetes isolated by Amoroso (1998) showed the highest level of $Cd^{2+}$ in a short period of time. However, the cadmium tolerant isolate from Pasoh is still interesting to be studied further as it showed excellent ability to uptake $Cd^{2+}$ in viable cell form, even though the isolate was obtained from a non-contaminated environment compared to Amoroso (1998) who obtained the isolate from a heavy metal polluted river. Further studies on mechanisms of the uptake system and kinetic studies are needed in order to use the isolate from the current study in future as a bioremediation agent to treat the cadmium contaminated wastewater and soil.

# CHAPTER 5

## CONCLUSION AND RECOMMENDATION

The aim of the research study to select and identify potential heavy metal tolerant actinomycetes from heavy metal contaminated and forest soil as an early exploration into their bioremediation capabilities were achieved and the following are the conclusion and summarize of the finding of this study.

The conclusion of the study:

i.   A total of 29 potential heavy metal tolerant actinomycetes were successfully selected using direct agar diffusion assay and further studied in liquid medium of two different assays (metal toxicity assay and metal uptake assay).

ii.  Based on statistical analysis (Pearson Correlation), a positive correlation were successfully observed between multiple antibiotic resistance and heavy metal tolerance in the actinomycetes isolated from Bidor while no correlation were determined among the Pasoh isolates.

iii. A total of 13 potential heavy metal tolerant actinomycetes selected based on the metal toxicity assay were successfully differentiated using BOX-PCR method into three major clusters.

iv.  Two selected isolates for cadmium uptake study were successfully identified based on morphological, physiological, and genotypic characteristics as *Streptomyces lannensis* and *Streptomyces malaysiensis*.

A total of 421 actinomycete isolates obtained from FRIM Actinobacteria Culture Collection (FACC) were successfully revived and culturally characterized after 7-14 days of incubation on International *Streptomyces* Project (ISP) 2. The isolates were then

grouped into *Streptomyces*-like (Bd: 124; Pa: 107) and non-*Streptomyces* (Bd: 114; Pa: 76) group by observing their presence or absence of aerial mycelia in order to evaluate and compare the heavy metal tolerance ability of both groups in two different study sites (Bidor and Pasoh).

Heavy metal tolerance test was conducted using control cultures and seven different metal salts with three different concentrations ($HgCl_2$: 0.04, 0.4, 4.0 mmol/L, $As_2O_3$: 5.0, 25.0, 51.0 mmol/L, $PbCl_2$: 0.4, 4.0, 18.0 mmol/L, $Cu_2SO_4$: 6, 31, 63 mmol/L, $CdCl_2$: 0.06, 0.6, 6.0 mmol/L, $NiCl_2$: 8.0, 36.0, 77.0 mmol/L and $ZnCl_2$: 7, 37, 73 mmol/L). Among the tested metal, a significantly higher number of Bidor isolates showed tolerance to $Ni^{2+}$ compared to Pasoh. A total of 40% and 33% of $Ni^{2+}$ tolerant isolates obtained from Bidor and Pasoh, respectively showed multi metal tolerant (tolerant to four and more metals). Thus, metal phenotype of co-tolerance was much easily seen among the $Ni^{2+}$ tolerance isolates from Bidor compared to Pasoh. In Bidor, Ni was reported to be found in high concentration in both the sand tailings (0.29-8.78 $mg/g^{-1}$ Ni at 0-20cm depth of tin tailings) and slime tailings (3.64-29.31 $mg/g^{-1}$ Ni at 0-20cm depth of tin tailings) (Ang and Ang, 1997). Thus, high Ni contamination in Bidor may have created a selective pressure for the soil microbe and this could be widespread among actinomycetes growing in the tin tailings. On the other hand, Mann Whitney test showed a significantly higher percentage of *Streptomyces*-like isolates from Bidor tolerant to $As^{3+}$, $Pb^{2+}$, $Ni^{2+}$ and $Zn^{2+}$. Meanwhile, the non-*Streptomyces* isolates from Pasoh showed a significantly higher percentage of tolerance towards $Cd^{2+}$. Therefore, metal tolerance in actinomycetes may not directly relate to a particular genera or group of actinomycete but is widely distributed in different genera. A total of 44 metal tolerant isolates were selected based on the selection criteria for heavy metal tolerant actinomycetes. However, out of 44 isolates a total of 15 isolates from both Bidor and Pasoh showed poor growth after a few times of re-culture on working plate even though

they showed a good growth when it was taken from cryovials. Out of 15, 12 isolates belonged to the non-*Streptomyces* group. This proves that the non-*Streptomyces* groups are very difficult to cultivate and maintain. All 15 isolates including the three *Streptomyces*-like isolates were dropped as they did not show a good growth on working plate. A total of 29 (Bd: 22; Pa: 7) potential heavy metal tolerant actinomycetes were successfully selected to evaluate and compare their antibiotic resistance pattern.

Antibiotic resistance test were conducted using nine different antibiotic discs [BD BBL™ Sensi-Disc™ - kanamycin (K-30 µg), ampicilin (Am-10 µg), nalidixic asid (Na-30 µg), chloramphenicol (Ch-30 µg), amikacin (An-30 µg), penicillin (P-10 µg), gentamycin (Gn-10 µg), vancomycin (Va-30 µg), erythromycin (E-15 µg)]. Three different groups of metal tolerant isolates which are from multi metal, toxic metal and trace metal were compared in this study. A similar pattern of antibiotic resistance among the three different groups of metal showed that antibiotic resistance was not influenced by a particular group of metal. Further, statistical analysis (Pearson Correlation) showed a positive correlation (r = 0.359) exist between multiple antibiotic resistance and heavy metal tolerance in the actinomycetes isolated from Bidor while no correlation was observed among the Pasoh isolates (r = - 0.192). This explains that selective pressure had taken place among actinomycetes isolated from Bidor and not Pasoh.

In metal toxicity assay, six different types of metal salt at five different concentrations labeled as C1 (highest) - C5 (lowest) with two sets of control were studied ($NiCl_2$:17.4, 34.7, 69.45, 138.9, 277.8 mmol/L, $PbCl_2$: 2.7, 5.4, 10.8, 21.6, 43.2 mmol/L, $CdCl_2$: 1.4, 2.7, 5.45, 10.9, 21.8 mmol/L, $CuSO_4$: 14.1, 28.2, 56.4, 112.8, 225.6 mmol/L, $HgCl_2$: 0.4, 0.8, 1.5, 3, 6 mmol/L, and $As_2O_3$: 11.4, 22.8, 45.5, 91, 182 mmol/L). Compared to heavy metal tolerance screening, metal toxicity assay gave a

clearer view by showing Bidor isolates ability to with stand a higher concentration of the selected heavy metals ($Pb^{2+}$, $Ni^{2+}$, $As^{3+}$ and $Cd^{2+}$) compared to Pasoh isolates ($As^{3+}$ and $Cd^{2+}$). Thus, the distribution of metal tolerance in actinomycetes to specific metal ions is heavily influenced by environmental conditions from where the isolates are isolated. Further, the tolerance level of the isolates to different metal ions was lower in liquid medium compared to solid medium. This might due to the conditions of diffusion, complexation and availability of metals which are different in both methods. Thus, isolates become more sensitive in metal toxicity assay compared to heavy metal tolerance test. A total of eight isolates showing tolerance to at least one metal ion at the highest test concentration, three isolates tolerant to $Ni^{2+}$ at the second highest concentration and another two isolates showing moderate tolerance to $Cu^{2+}$ and $Hg^{2+}$, respectively were listed as potential heavy metal tolerant isolates for metal uptake study.

All 13 potential heavy metal tolerant isolates were first clustered based on their deoxyribonucleic acid (DNA) fingerprint similarity using the repetitive element-based BOX-PCR fingerprinting technique in order to identify the percentage of similarity between the isolates. The obtained cluster analysis of similarity coefficients using BOX-PCR data were compared with their macromorphology results. Results showed that, isolates with same DNA fingerprinting (100% similarity) has the same macromorphology profile. Thus, such de-replication technique is much precise, faster and a better tool to group unidentified isolates compared to morphology characterization grouping. Based on the DNA fingerprinting study, both TY049-057 and TY028-047 with 15.4% similarity were selected to represent Bidor and Pasoh, respectively for further studies to determine the cadmium supplement effect on metal uptake and growth ability.

Both selected isolates grown on the ISP2, ISP3 and ISP4 media showed filamentous growth with abundant of aerial and vegetative hyphae. The colonies of both TY028-047 and TY049-057 were well grown into the medium with their threads, and have convex form While, observation of spore chain arrangements under the light microscope at 150X and 600X showed that sporophores of both TY028-047 and TY049-057 were close spirals and open spirals, respectively. Good growths were observed at pH range 5-10 with optimum temperature of 28$^{\circ}$C after 8 days of incubation for both potential isolates. Both isolates also showed good growth at 0-2% NaCl concentration and the growth was weak at 3-5% NaCl. No growths were observed at higher concentration of NaCl (6-10%). Based on 16S ribosomal ribonucleic acid (rRNA) sequence available in the GenBank database both strains were identified as belonging to the genus *Streptomyces*. However, both isolates showed 100% similarity to two different species which was *Streptomyces lannensis* for Pasoh isolate and *Streptomyces malaysiensis* for Bidor isolate.

Finally, TY049-057, TY028-047 and a control isolate (metal sensitive isolate) were grown in 1mM $Cd^{2+}$ in order to determine the $Cd^{2+}$ supplements effect on metal uptake and growth viability. Both isolates showed maximum $Cd^{2+}$ uptake at day 8 when grown in MM liquid media containing an initial concentration of 1mM $Cd^{2+}$. The cadmium tolerant isolate from Pasoh able to uptake a higher concentration of $Cd^{2+}$ compared to cadmium tolerant isolate from Bidor. Strain TY049-057 was able to accumulate 3.17 μmol $Cd^{2+}$/mg dry cell mass while TY028-047 was able to accumulate 4.66 μmol$Cd^{2+}$/mg. This might due to the higher growth ability of control and cadmium tolerant cultures from Pasoh compared to the control and cadmium tolerant cultures of Bidor and metal sensitive isolate, even though the inoculum size used in each experiment were the same for all three test isolates. Thus, cadmium tolerant isolate from Bidor is an interesting isolate for further studies as they can uptake a high amount of

$Cd^{2+}$ even with a low growth viability rate. This may also be explained by the metal tolerance screening and metal toxicity assay results where strains isolated from Pasoh showed higher ability to tolerate cadmium compared to Bidor. Thus, not all isolates from heavy metal contaminated environment have potential in tolerating heavy metal because its effect of growth phase seems to be metal- and species– specific. Further, many other aspects influence the level of cadmium uptake such as nutrients, metal concentration in the media and growth viability rate of bacteria.

The approaches discussed in this study are very helpful to identify strains with heavy metal tolerant ability especially among the actinomycetes strains. Further, a rapid method to investigate fingerprint have also been done by using BOX-PCR as an alternative characterization tool for the study of distinct actinomycetes strains. In the need for an appropriate bioremediation treatment for removal of heavy metal, further investigation of actinomycetes on metal uptake ability will bring to a fruitful enterprise as microbes are known as an eco-friendly, effective and low cost material compared to other conventional adsorbents and physic-chemical methods. It is anticipated that the current effort of screening, characterization and the study on heavy metal tolerant actinomycetes can be a milestone for the discovery of novel actinomycetes with metal tolerant ability for bioremediation purpose.

**Recommendation and Future Studies to be Carried Out**

- A total of 44 actinomycetes have showed metal tolerant ability in the primary screening. However, only 29 isolates were further studied as the rest of 15 isolates were difficult to cultivate and maintain. It is interesting to look at this isolates as most of it are belonging to the rare or non-*Streptomyces* group. Thus, a suitable ISP growth media established by Shirling and Gottlieb (1966) should be used for these isolates and further studied for metal toxicity.

- A total of 13 potential heavy metal tolerant actinomycetes were identified in metal toxicity assay and all 13 were differentiated using BOX-PCR. The BOX-PCR data were then compared with macromorphology characterization in order to validate the data. However, in a previous study by Marques *et al*. (2008), he identified the species of each strain in order to validate the BOX-PCR data and he manages to observe that BOX-PCR able to identify bacterial strains at species level. Thus, for a better validation, 16S rRNA gene amplification must be done for all 13 isolates.

- Pasoh isolates showed better ability in cadmium tolerant in both heavy metal tolerance and metal toxicity assay compared to the rest of the test metals. As a natural forest soil, it should not have shown such profile. Thus, it will be good to go back to the forest and determine the concentration of cadmium in the soil in order to validate this result.

- Among the 13 potential heavy metal tolerant isolates only two were studied against one metal in order to compare two different environments. Thus, it will be interesting to look at all the 13 isolates against their respective tolerable metal in order to find a good candidate for bioremediation.

- The control culture of Pasoh was doing much better than the control culture of Bidor and metal sensitive isolate. Thus, in order to synchronize the experiment, growth profile of the selected isolates must be determined first before comparing isolates for metal uptake and growth viability.

- In future, more studies such as mechanisms of metal uptake system, kinetic studies (Lin *et al*., 2012) and gene basis studies (Rajendran *et al*., 2003; Silver and Phung, 2005) should carried out using the potential metal tolerant isolates obtained from this study in order to use it as a bioremediation agent.

- Based on 16S rRNA gene sequences available in the GenBank database both Pasoh and Bidor isolates showed 100% similarity to two different species which was *Streptomyces lannensis* and *Streptomyces malaysiensis*, respectively. However, due to the partial

sequence obtained, further full sequence analysis may be carried out to ascertain the identity of the isolates.

# CHAPTER 6

# REFERENCES

Adegboye, M.F., & Babalola, O.O. (2012). Taxonomy and ecology of antibiotic producing actinomycetes. *Journal of Agricultural Research, 7*, 2255-2261. doi:10.5897/AJARX11.071

Agamuthu, P., & Fauziah, S.H. (2010). Heavy metal pollution in landfill environment: A Malaysian case study. *4th International Conference of Bioinformatics and Biomedical Engineering (iCBBE)*, (pp. 1 - 4).

Alan, C.W., & Nagamani, B. (2006). Diversity and biogeography of marine actinobacteria. *Current Opinion in Microbiolog, 9*, 279-286.

Albarracin, V.H., Amoroso, M.J., & Abate, C.M. (2005). Isolation and characterization of indigenous copper-resistant actinomycete strains. *Chemi der Erde-Geochemistry, 65*, 6145-6156.

Alexander, S.S., Natalia, V.P., Naumova, I.B., Evtushenko, L.I., & Widmalm, G. (1999). Cell wall teichoic acids of *Actinomadura viridis* VKM Ac-1315T. *European Journal of Biochemistry, 262*, 688-695.

Al-tai, A., Kim, B., Kim, S.B., Manfio, G.P., & Goodfellow, M. (1999). *Streptomyces malaysiensis* sp. nov., a new streptomycete species with rugose ornamented spores. *International Journal of Systematic and Evolutionary Microbiology, 49*, 1395-1402.

Altschul, S.F., Gish,W., Miller, W., Myers, E.W., & Lipman, D.J. (1990). Basic local alignment search tool. *Journal of Molecular Biology, 215*, 403-410.

Alvarez, V.M., Marques, J.M., Korenblum, E., & Seldin, L. (2011). Comparative bioremediation of crude oil-amended tropical soil microcosms by natural attenuation, bioaugmentation or bioenrichment. *Applied and Environmental Soil Science, Article ID 156320.* doi:10.1155/2011/156320

Amoroso, M.J., & Abate, C.M. (2012). Bioremediation of copper, chromium and cadmium by actinomycetes from contaminated soil. *Soil Biology, 31* , 349-364.

Amoroso, M.J., Castro, G., Carlino, Federico, J., Romero, N.C., Hill, R., & Oliver, G.

(1998). Screening of heavy metal tolerant actinomycetes isolates from the Sali River. *Journal of General Applied Microbiology, 44*, 29-32.

Anagnostopoulos, V.A., Symeopoulos, B.D., & Soupioni, M.J. (2010). Effect of growth conditions on biosorption of cadmium and copper by yeast cells. *Global Nest, 12*, 288-295.

Andre, S., Gotz, H., Astrid, S., Manuel S., Dirk, M., Felicia G., George B., & Erika, K. (2005). Heavy metal resistance mechanisms in actinobacteria for survival in AMD contaminated soils. *Chemi der Erde, 65*, 131-144.

Anu, Upadhyaya, S.K., & Avinash, B. (2010). Seasonal Analysis of soil sediment of Shahpura Lake of Bhopal (M.P.). *International Journal of Environmental Science and Development, 1*, 333 - 335.

Albarracin, V.H., Amoroso, M.J., & Abate, C.M. (2005). Isolation and characterization of indigenous copper-resistant actinomycete strains. *Chemi der Erde-Geochemistry, 65*, 6145-6156.

Alvarez, V.M., Marques, J.M., Korenblum, E., & Seldin, L. (2011). Comparative bioremediation of crude oil-amended tropical soil microcosms by natural attenuation, bioaugmentation or bioenrichment. *Applied and Environmental Soil Science, Article ID 156320.* doi:10.1155/2011/156320

Ang, L.H., & Ang, T.B. (1997). Greening the tin tailings-are we ready? In K.C Khoo, & S. Apanah (Ed.), *Confrence of Forestry and Forest Product Research.* Kuala Lumpur, Malaysia.

Ang, L.H., & Ng, L.T. (2000). Trace element concentration in mango (*Mangifera indica L.*), seedless guava (*Psidium guajava L.*) and papaya (*Carica papaya L.*) grown on agricultural and ex-mining lands of Bidor, Perak. *Pertanika Journal of Tropical Agriculture Science, 23*, 15-22.

Ang, L.H., Ho, W.M., Ramli, M.O., Maimon, A., Chung, P.Y. & Ng, L.T. (2000). The update of potentially toxic elements in some economically important plants and fish produced from ex-mining sites in Bidor, Perak. *Proceedings of the Malaysian Science Technology Convention 2000*, (pp. 296-303). Kota Kinabalu, Sabah.

Ang, L.H., Tang, L.K., Ho, W.M., Hui, T.F. & Theseira, G.W. (2010). Pyhotoremediation of Cd and Pb by four tropical timber species grown on an ex-tin mine in peninsular Malaysia. *World Academy of Science, Engineering and Technology, 62*, 459-463.

Anne, S. (2003). Implication of microbial heavy metal tolerance in the environment. *Reviews in Undergraduate Research, 2*, 1-6.

Anton, A., Grobe, C., Reimann, J., Pribyl, T., & Nies, D.H. (1999). CzcD is a heavy metal transporter involved in regulation of heavy metal resistance in *Ralstonia* sp. strain CH34. *Journal of Bacteriology, 181*, 6876-6881.

Arifuzzaman, M., Khatun, M.R., & Rahman, H. (2010). Isolation and screening of actinomycetes from Sundarbans soil for antibacterial activity. *African Journal of Biotechnology , 9*, 4615-4619.

Arsenic. (2013). *Arsenic*. Retrieved January 5, 2013, from Wikipedia: http://en.wikipedia.org/wiki/Arsenic

ATSDR. (2011). *The ATSDR 2011 substance priority list*. Retrieved May 27, 2013, from Agency for Toxic Substances and Disease Registry (ATSDR): http://www.atsdr.cdc.gov/SPL/index.html

Babalola, O.O., Kirby, B.M., Le Roes-Hill, M., Cook, A.E., Cary, S.C., Burton, S.G., & Cowan, D.A. (2009). Phylogenetic analysis of actinobacterial populations associated with Antarctic dry valley mineral soils. *Environmental Microbiology, 11*, 566-576.

Bahig, A.E., Aly, E.A., Khaled, A.A., & Amel, K.A. (2008). Isolation, characterization and application of bacterial population from agricultural soil at Sohag Province, Egypt. *Malaysian Journal of Microbiology, 4*, 42-50.

Baker-Austin, C., Wright, M. S., Stepanauskas, R., & McArthur, J.V. (2006). Co-selection of antibiotic and metal resistance. *Trends Microbiology, 14*, 176–182.

Barabas, G.Y., Vargha, G., Szabo, I.M., Penyige, A., Damjanovich, S., Szollosi, J., Matk,J., Hirano, T., & Atyus, A.M. (2001). n-Alkane uptake and utilization by *Streptomyces* strains. *Antonie Van Leeuwenhoek, 79*, 269–276.

Bauer, A.W., Kirby, W.M., Sherris, J.C., & Turch, M. (1966). Antibiotic susceptibility testing by a standardized single disk method. *American Journal of Clinical Pathology, 45*, 493-496.

Benson, A.A., & Summons, R.E. (1981). Arsenic accumulation in Great Barrier Reef invertebrates. *Science, 211*, 482-483.

Benson, H. J. (2002). *Microbiological Applications: Laboratory Manual in General Microbiology, Short Version* (8 ed.). Bonston, MA: McGraw Hill.

Berdy, J. (2005). Bioactive microbial metabolites: A personal view. *Journal of Antibiotics, 58*, 1-26. doi:10.1038/ja.2005.1

Bouizgarne, B., Lanoot, B., Loqman, S., Sproer, C., Klenk, H. P., Swings, J., & Ouhdouch, Y. (2009). *Streptomyces marokkonensis* sp. nov., isolated from rhizosphere soil of Argania spinosa L. *International Journal of Systematic and Evolutionary Microbiology, 59*, 2857-2863.

Bruins, M.R., Kapil, S., & Oehme, F.W. (2000). Microbial resistance to metals in the environment. *Ecotoxicology and Environmental Safety, 45*, 198–207.

Buchanan, R.E., & Gibbons, N.E. (1974). *Bergey's manual of determinative* (8 ed.). Baltimore: The Williams and Wilkins Co.

Bull, A.T., Goodfellow, M., & Slater, J.H. ( 1992). Annual Review of Microbiology. *Biodiversity as a source of innovation in biotechnology, 46*, 219-252.

Cadmium. (2013). *Cadmium*. Retrieved January 15, 2013, from Wikepedia: http://en.wikipedia.org/wiki/Cadmium

Calomiris, J.J., Armstrong, J.L., & Seidler, R.J. (1984). Association of metal tolerance with multiple antibiotic resistance of bacteria isolated from drinking water. *Applied Environmental Microbiology, 47*, 1238-1242.

Canadian Drinking Water Regulation. (2013). *Drinking water quality standards*. Retrieved January 25, 2013, from Wikepedia: http://en.wikipedia.org/wiki/Drinking_water_quality_standards

Carlsohn, M.R., Groth, I., Haus-Peter, S., Schumann, P., & Stackebrandt, E. (2008). *Fodinicola feengrottensis* gen. nov., sp. Nov., an actinomycete isolated from a medieval mine. *International Journal of Systematic and Evolutionary Microbiology, 58*, 1529-1536.

Catara, V., Sutra, L., Morineau, A., Achouak, W., Christen, R., & Gardan, L. (2002). Phenotypic and genomic evidence for the revision of *Pseudomonas corrugata* and proposal of *Pseudomonas mediterranea* sp. *International Journal of Systematic and Evolutionary Microbiology, 52*, 1749-1758.

Catherine, V., Hawkyard., & Roland, J.K. (2007). The use of erythromycin as gastrointestinal prokinetic agent in adult critical care: Benefits versus risks. *Journal of Antimicrobial Chemotherapy, 59*, 347-58. doi:10.1093/jac/dkl537

Cavicchioli, R., & Thomas, T. (2002). *Extremophiles: Encyclopedia of Microbiology* (2 ed., Vol. 2). San Diego: Academic Press.

Chan, Y.K. (15–16 October 1990). The mining land – an overview of the current situation in Peninsular Malaysia. *Ex-mining land and BRIS soils: Prospects and Profit*, (p. 17). Kuala Lumpur.

Chandy, J.P. (1998). Heavy metal tolerance chromogenic and non- chromogenic marine bacteria from Arabian gulf. *Environmental Monitoring and Assessment, 59*, 321-330.

Chang, J.S., Law, R. & Chang, C.C. (1997). Biosoprtion of lead, copper and cadmium by Biomass of *Pseudomonas aeruginosa* PU21. *Water Research, 31*, 1651-1658.

Chaudhuri, B.N., Maiti, P.K., & Sil, J. (1997). Antibiotic sensitivity patterns of actinomycetes isolated from patients of actinomycetoma. *Indian Journal of Medical Research, 105*, 162-166.

Cherif, A., Brusetti, L., Borin, S., Rizzi, A., Boudabous, A., Khyami-Horani, H., & Daffonchio, D. (2003). Genetic relationship in the "*Bacillus cereus* group" by rep-PCR fingerprinting and sequencing of a *Bacillus anthracis*-specific rep-PCR fragment. *Journal of Applied Microbiology, 94*, 1108-1119.

Chmielewski, R., Wieliczko, A., Kuczkowski, M., Mazurkiewicz, M., & Ugorski, M. (2002). Comparison of ITS profiling, REP- and ERIC-PCR of *Salmonella enteritidis* isolates from Poland. *Journal of Veterinary Internal Medicine , 49*, 163-168.

Chronopoulos, J., Haidouti, C., Chronopoulou, A., & Massas, I . (1997). Variations in plant and soil lead and cadmium content in urban parks in Athens, Greece. *Science Total Environment, 196*, 91–98.

Clark, C.A., Chen, N.C., Rainey, W., & Pettis, G.S. (1998). Diversity within *Streptomyces ipomoeae* based on inhibitory interactions, rep-PCR, and plasmid profiles. *Bacteriology, 88*, 1179-1186.

Cold Spring Harb Protocols. (2006). *Phosphate-buffered saline (PBS) Recipe*. doi:10.1101/pdb.rec8247

Collin, P.H. (2001). *Dictionary of Ecology and the Environment* (4 ed.). London: Peter Collin Publishing.

Compant, S., Clement, C., & Sessitsch, A. (2010). Plant growth-promoting bacteria in the rhizo- and endosphere of plants: Their role, colonization, mechanisms involved and prospects for utilization. *Soil Biology and Biochemistry, 42*, 669-678.

Congeevaram S., Dhanarani, S., Park, J., Dexilin, M., Thamaraiselvi, K. (2007). Biosorption of chromium and nickel by heavy metal resistant fungal and bacterial isolates. *Journal of Hazardous Materials , 146*, 270–277.

Copper. (2013). *Copper*. Retrieved January 15, 2013, from Wikepedia: http://en.wikipedia.org/wiki/Copper

Cross, T., & William, S.T. (1971). *Method in microbiology: Actinomycetes* (4 ed.). London: Academic press.

Dalsgarrd, A., & Guardbassi, L. (2002). *Occurrence and fate of antibiotics resistant bacteria in sewage.* Danish: EPA Environment Project No. 722.

Dastjerdi, M.V., Attar, H.M., & Bina, B. (2013). Accumulation of heavy metals in agricultural products irrigated with treated municipal wastewater. *International Journal of Environmental Health Engineering, 2*, 9.

Daughney, C.J., Fowle, D.A., & Fortin, D. (2001). The effect of growth phase on proton and metal adsorption by Bacillus subtilis. *Geochimica et Cosmochimica Acta, 65*, 1025-1035.

De Schrijver, A., De Mot, R., & Source, F.A. (1999). Degradation of pesticides by actinomycetes. *Critical Reviews in Microbiology, 25*, 85-119.

Department of Environment (DOE). (1997). *Guidelines for environmental impact assessment of ground water and /or surface water supply project* . Kuala Lumpur: DOE Press.

Dhanasekaran, D., Rajakumar, G., Sivamani, P., Selvamani, S., Paneerselvam, A., & Thajuddin, N. (2005). Screening of Salt Pans Actinomycetes for Antibacterial Agents. *The Internet Journal of Microbiology, 1*, 6-12.

Dominic, R.M., Shenoy, S., & Baliga, S. (2007). Candida biofilms in medical devices:

evolving trends. *Kathmandu University Medical Journal , 5*, 431-436.

Doshi, H., Ray, A., & Kothari, I. (2007). Biosorption of cadmium by live and dead Spirulina: IR spectroscopic, kinetics, and SEM studies. *Current Microbiology, 54*, 213–218.

Eitinger, T., & Mandrand-Berthelot, M.A. (2000). Nickel transport systems in microorganisms. *Archives of Microbiology , 173*, 1-9.

El Tassa, S.O.M., Moraes, M.G., & Duarte, V. (1999). Identificação de *Pseudomonas syringae* pv. coronafaciens através de ERIC- e BOX-PCR (Abstract in english). *Fito Bras, 24*, 503-508.

El-Helow, E.R., Sabry, S.A., & Amer, R.M. (2000). Cadmium biosorption by cadmium resisting strain of *Bacillus thuringiensis*: regulation and optimization of cell surface affinity for metal cations. *Biometals, 13*, 273-280.

Elke, G., & Christoph, O. (2003). The use of repetitive element PCR fingerprinting (rep-PCR) for genetic subtyping of German field isolates of *Paenibacillus larvae* subsp. larvae. *Apidologie, 34*, 195–206. doi:10.1051/apido:2003025

El-Tarabily, K. A., & Sivasithamparam, K. (2006). Non-streptomycete actinobacteria as biocontrol agents of soil-borne fungal plant pathogens and as plant growth promoters. *Soil Biology & Biochemistry, 38*, 1505–1520.

European Union, EPA. (2013). *Drinking water quality standards*. Retrieved January 25, 2013, from Wikipedia: http://en.wikipedia.org/wiki/Drinking_water_quality_standards

Euzeby, J. (2013). *List of prokaryotic names with standing in nomenclature (LPSN) bacterioa net*. Retrieved July 10, 2013, from http://www.bacterio.net/s/streptomycesb.html

Felsenstein, J. (1985). Confidence limits on phylogenies: An approach using the bootstrap. *Evolution, 39*, 783-791.

Fenical, W., & Jensen, P. R. (2006). Developing a new resource for drug discovery: Marine actinomycete bacteria. *Nature Chemical Biology, 2*, 666–673.

Franco-Correa, M., Quintana, A., Duque, C., Suarez, C., Rodriguez, M.X., & Barea, J.M. (2010). Evaluation of actinomycete strains for key traits related with plant

growth promotion and mycorrhiza helping activities. *Applied Soil Ecology , 45*, 209-217.

Funk, S. B., Roberts, D.J., Crawford, D.L, & Crawford, R.L. (1993). Initial-phase optimization for the bioremediation of munition compound-contaminated soils. *Applied and Environmental Microbiology, 59*, 2171-2177.

Galal, A.A., Yei, K., el Daoudi, Y.H., Shihata, Z.A., & Ouf, M.F. (2003). A comparative study on the identification of races and biovars of some Egyptian isolates of *Ralstonia solanacearum*. *Egyptian Journal of Phytopathology, 31*, 103-117.

Gao, B., & Gupta, R.S. (2005). Conserved indels in protein sequences that are characteristic of the phylum Actinobacteria. *International Journal of Systematic Bacteriology, 55*, 2401-2412.

Getha, K., Mohd Ilham, A., Lee, S.S., Chang, Y.S., Nimura, S., & Hatsu, M . (2008). Exploratory studies of actinomycetes biodiversity of Frim forests in aid of drug discovery. *Highlights of Frim's MOSTI Projects 2007 in Frim, Kepong*, (pp. 105-109). Kepong, Selangor.

Getha, K. & Vikineswary, S. (2002). Antagonistic effects of *Streptomyces violaceusniger* strain G10 on *Fusarium oxysporum f.* sp. cubense race 4: Indirect evidence for the role of antibiosis in the antagonistic process. *Journal of Industrial Microbiology and Biotechnology, 28*, 303-310.

Getha, K., Vikineswary, S., Wong, W.H., Seki, T., Ward, A., & Goodfellow, M. (2004). Characterization of selected isolates of indigenous *Streptomyces* species and evaluation of their antifungal activity against selected plant pathogenic fungi. *Malaysian Journal of Science, 23*, 37-47.

Goodfellow, M., & O'Donnell, A.G. (1989). *Search and discovery of industrially significant actinomycetes. In: Baumberg, S., Haunter, I.S., Rhodes, P.M. (Eds.), Microbial Products: New Approaches.* Cambridge: University Press.

Goodfellow, M., & Wiliams, S.T. (1983). Ecology of actinomycetes. *Annual Review of Microbiology, 37 ,* 189-216.

Goodfellow, M., Williams, S.T., & Mordarski, M. (1988). *Actinomycetes in biotechnology.* London, United Kingdom: Academic Press Ltd.

Greco, S., Bella, P., Tessitori, M., & Catara . (2004). Indagini  sulla  disseminazione  in

vivaio di Xanthomonas hortorum pv. Pelargonii (Abstract in english). *Colture Protette, 33*, 65-68.

Gupta, S.K., Herren, T., Wenger, K., Krebs, R., & Hari, T. (2000). *In situ gentle remediation measures for heavy metal-polluted soils: Phytoremediation of Contaminated Soil and Water.* (N. Banuelos, & G. Terry, Eds.) Boca Raton,Florida, USA: Lewis Publishers.

Gurung, T.D., Sherpa, C., Agrawal, V.P., & Nepal, B.L. (2009). Isolation and characterization of antibacterial actinomycetes from soil samples of Kalapatthar,Mount Everest Region. *Journal of Science and Technology, 10*, 173-182.

Gust, B., Challis, G. L., Fowler, K., Kieser, T., & Chater, K. F. (2003). PCR-targeted Streptomyces gene replacement identifies a protein domain needed for biosynthesis of thesesquiterpene soil odor geosmin. *Proceedings of the National Academy of Sciences of the USA, 100*, pp. 1541-1546.

Hagedorn, C. (1996). *Waste Disposal Technology in Soils*. Retrieved May 27, 2013, fromhttp://www.webapps.cee.vt.edu/ewr/environmental/teach/gwprimer/group09/trace1.htm

Hamzah, M., Al-Qadiri, Nivin, I., Al-Alami, Lin, M., Al-Holy, M., Cavinato, A.G., & Rasco, B.A. (2008). Studying of the bacterial growth phases using fourier transform infrared spectroscopy and multivariate analysis. *Journal of Rapid Methods and Automation in Microbiology, 16*, 73-89.

Hasegawa, S., Meguro, A., Shimizu, M., Nishimura, T., & Kunoh, H. (2006). Endophytic actinomycetes and their interactions with host plants. *Actinomycetologica, 20*, 72-81.

Hassen, A., Saidi, N., Cherif, M., & Boudabous, A. (1998). Resistance of environmental bacteria to heavy metals. *Biosource Technology, 64*, 7-15.

Hayakawa, H. (2008). Studies on the isolation and distribution of rare actinomycetes in soil. *Actinomycetologica, 22*, 12–19. doi:10.3209/saj.SAJ220103

Hiroki, M. (1992). Effects of heavy metal contamination on soil microbial population. *Soil Science Plant Nutritional, 38*, 141–147.

Holt, J.G., Krieg, H.R., Sneath, P.H.A., Stacey, J.T., & Williams, S.T. (1994). *Beygey's*

*manual of determinative bacteriology* (9 ed.). Baltimore: The Williams and Wilkins Co.

Hugh C., Archambault M., & Prescott, J.F. (2003). 16S ribosomal RNA sequence–based identification of veterinary clinical bacteria. *Journal of Veterinary Diiagnostic, 15*, 465–469.

Imada, C., Koseki, N., Kamata, M., Kobayashi, T., & Hamada-Sato, N. (2007). Isolation and characterization of antibacterial substances produced by marine actinomycetes in the presence of seawater. *Actinomycetologica, 21*, 27-31.

Jain, P., Amatullah, A., Rajib, S.A., & Reza, H.M. (2012). Antibiotic resistance and chromium reduction pattern among actinomycetes. *American Journal of Biochemistry and Biotechnology, 8*, 111-117.

Jimi, E., Aoki, K., Saito, H., D'Acquisto, F., May, M.J. & Nakamura, I. (2004). Selective inhibition of NF-kappa B blocks osteoclasto-genesis and prevents inflammatory bone destruction in vivo. *Nature Medicine, 10*, 617–624.

Johnsen, A.R., Winding, A., Karlson, U. & Roslev, P. (2002). Linking of microorganisms to phenanthrene metabolism in soil by analysis of 13C-labeled cell lipids. *Applied and Environmental Microbiology, 68*, 6106-6113.

Jose P.A., & Jebakumar S.R.D. (2012). Phylogenetic diversity of actinomycetes cultured from coastal multipond solar saltern in Tuticorin, India. *Aquatic Biosystems, 8*, 23. doi:10.1186/2046-9063-8-23.

Kadukova, J., & Vircıkova, E. (2005). Comparison of differences between copper bioaccumulation and biosorption. *Environment International, 31*, 227– 232.

Kang, H.P., & Dunne, W.M . (2003). Stability of repetitive-sequence PCR patterns with respect to culture age and subculture frequency. *Journal of Clinical Microbiology, 41*, 2694-2696.

Kanopka, A., & Zakharova, T. (1999). Quantification of bacterial lead resistance via activity assays. *Journal of Microbiological Methods, 37*, 17-22.

Kaplan, D. L., & Kaplan, A.M. (1982). Thermophilic biotransformations of 2,4,6-trinitrotoluene under simulated composting conditions. *Applied and Environmental Microbiology, 44*, 757-760.

Kaye, K.S., Fraimow, H.S., & Abrutyn, E. (2000). Pathogens resistant to antimicrobial agents: Epidemiology molecular mechanisms, and clinical management. *Infectious Disease Clinics of North America , 14*, 293-319.

Kekuda, T.R.P., Shobha, K.S., & Onkarappa, R. (2010). Fascinating diversity and potent biological activities of actinomycetes metabolites. *Journal of Pharmacy Research, 3*, 250-256.

Kermanshahi, K.R., Ghazifard, A., & Tavakoli, A. (2007). Identification of bacteria to heavy metals in the soils of Isfahan Province. *Iranian Journal of Science and Technology, 31*, A1.

Khanna, M., Solankil, R., & Lal, R. (2011). Selective isolation of rare actinomycetes producing novel antimicrobial compounds. *International Journal of Advanced Biotechnology and Research, 2*, 357-375.

Khucharoenphaisan, K., Sripairoj, N., & Sinma, K. (2012). Isolation and Identification of Actinomycetes from Termite's Gut against Human Pathogen. *Asian Journal of Animal and Veterinary Advances, 7*, 68-73.

Kieser, T., Bibb, M.J., Buttner, M.J., Clater, K.F., & Hopwood, D.A . (2000). *Practical Streptomyces Genetics.* Crowes, England: John Innes Foundation.

Kim, B. S., Sahin, N., Minnikin, D. E., Screwinska, J. Z., Mordarski, M., & Goodfellow, M. (1999). Classification of thermophilic streptomycetes including the description of *Streptomyces thermoalcalitolerance* sp. nov. *International Journal of Systematic and Evolutionary Microbiology, 49*, 7-17.

Kim, E.J., Chung, H.J., Suh, H.J., Hah, Y.C., & Roe, J.H. (1998). Transcriptional and post-transcriptional regulation by nickel of sodN gene encoding nickel containing superoxide dismutase from *Streptomyces coelicolor* Muller. *Molecular Microbiology, 27*, 187-195.

Kim, J.S., Kang, S.O., & Lee, K.J. (2003). The protein complex composed of nickel-binding SrnQ and DNA binding motif-bearing SrnR of *Streptomyces griseus* represses sodF transcription in the presence of nickel. *The Journal of Biological Chemistry, 278*, 18455-18463.

Kimura, M. (1980). A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution, 16*, 111-120.

Klausmeier, R. E., Osmon, J.L., & Walls, D.R. (1973). The effect of trinitrotoluene on microorganisms. *Developments in Industrial Microbiology , 15*, 309-317.

Kluyver, A.J., & Van Niel, C.B. (1936). Prospects for a natural system of classification of bacteria. *Zentrbl. Bakteriol. Parasitenkd. Infektkrankh. Hyg., Abt. II, 94*, 369-403.

Krasilnikov, N. A. (1961). *The soil as environment for microorganisms, part II. In Soil microorganisms and higher plants.* (N. A. Krasilnikov, Ed.) Jerusalem, Israel: S. Monson.

Krasilnikov, N.A . (1949). *Guide to the bacteria and actinomycetes.* SSSR, Moscow: Academiya Nauk.

Krishna, M.P., Varghese, R., Babu, A.V., & Mohamed Hatha, A.A. (2012). Bioaccumulation of cadmium by *Pseudomonas* Sp. isolated from metal polluted industrial region. *Environmental Research, Engineering and Management, 3*, 58-64.

Krumpermann, P.H. (1983). Multiple antibiotic resistance indexing of *Escherichia coli* to identify high risks sources of faecal contamination of foods. *Applied Environmental Microbiology, 46*, 165-170.

Kumar, A., Bisht, B.S. & Joshi, V.D. (2010.). Biosorption of heavy metals by four acclimated microbial species, *Baciluss* spp., *Pseudomonas* spp., *Staphylococcus* spp. and *Aspergillus* niger. *Journal of Biology and Environmental Science, 4*, 97-108.

Kumar, A., Bisht, B.S., Joshi, V.D., & Dhewa, T. (2011). Review on bioremediation of polluted environment: A management tool. *International Journal of Environmental Sciences, 1*, 1079-1093.

Kumar, A., Bisht, B.S., & Joshi, V.D . (2011a). Bioremediation potential of three acclimated bacteria with reference to heavy metal removal from waste . *International Journal of Environmental Sciences, 2*, 896-908.

Labeda, D.P. (1987). Actinomycete taxonomy: generic characterization. *Journal of Industrial Microbiology, 28*, 115-121.

Lam, K.S. (2006). Discovery of novel metabolites from marine actinomycetes. *Current Opinion in Microbiology, 9*, 245-251.

Lawrence, J.G. (2000). Clustering of antibiotic resistance genes: beyond the selfish operon. *ASM News, 66*, 281-286.

Laxman, S.R., & More, S. (2002). Reduction of hexavalent chromium by *Streptomyces griseus*. *Minerals Engineering, 15*, 831-837.

Lazzarini, A., Cavaletti, L., Toppo, G., & Marinelli, F. (2000). Rare genera of actinomycetes as potential producers of new antibiotics. *Antonie Van Leeuwenhoek , 78*, 399-405.

Lead. (2013). *Lead*. Retrieved January 15, 2013, from Wikepedia: http://en.wikipedia.org/wiki/Lead

Lechevalier, M. P., & Lechevalier, H. A. (1970). Chemical composition as a criterion in the classification of aerobic actinomycetes. *International Journal of Systematic and Evolutionary, 20*, 435–443.

Lee, A., & Wong, E. (2009). Optimization and robustness of BOX A1R PCR for DNA fingerprinting using Trout Lake *E.coli* isolates. *Journal of Microbiology, Immunology and Infection, 13*, 104-113.

Levinson, W. (2006). *Review of Medical Microbiology and Immunology* (9 ed.). USA: Mc Graw Hill.

Levy, D.B., Barbarick, K.A., Siemer, E.G., & Sommers, L.E . (1992). Distribution andpartitioning of trace metals in contaminated soils near Leadville, Colorado. *Journal of Environmental Quality, 21*, 185-195.

Lide, D. (1992). *Selected values of chemical thermodynamic properties: CRC Handbook of Chemistry and Physics* (73 ed.). Boca Raton, Florida, USA: CRC Press.

Lin, Y.B., Wang, X.Y., Wang, B.P., Mohamad, O., & Wei, G. (2012). Bioaccumulation characterization of zinc and cadmium by *Streptomyces zinciresistens*, a novel actinomycete. *Ecotoxicology and Environmental Safety, 77*, 7-17.

Lo, C.W., Lai, N.S., Cheah, H.Y., Wong, N.K.I., & Ho, C.C. (2002). *Actinomycetes isolated from soil samples from the crocker range Sabah*. Retrieved January 15, 2013, from ASEAN Review of Biodiversity and Environmental Conservation (ARBEC): http::/www.arbec.com.my/pdf/art21julysep02.pdf

Lodeiro P., Cordero B., Grille Z., Herrero R., & Sastre de Vicente M.E. (2004). Physicochemical studies of cadmium(II) biosorption by the invasive alga in Europe, *Sargassum muticum*. *Biotechnology and Bioengineering, 88*, 237-247.

Lopez-Maury, L., Garcia-Dominguez, M., Florencio, F.J., & Reyes, J.C. (2002). A two component signal transduction system involved in nickel sensing in the cyanobacterium *Synechocystis* sp. PCC 6803. *Molecular Microbiology, 43*, 247-256.

Louws, F.J., Fulbright, D.W., Stephens, C.T., & King, R.R. (1992). Specific genomic fingerprinting of phytopathogenic *Xanthomonas* and *Pseudomonas* pathovars and strains generated with repetitive sequences and PCR. *Applied Environmental Microbiology, 60*, 2286-2295.

Luo, Y.M. (2009). Current Research and Development in Soil Remediation Technologies. *Progress in Chemistry, 21*, 558-565.

Macagnan, D., Romeiro, Rd.S., Pomella, A.W.V., & deSouza, J.T. (2008). Production of lytic enzymes and siderophores, and inhibition of germination of basidiospores of Moniliophthora (ex Crinipellis) perniciosa by phylloplane actinomycetes. *Biological Control, 3*, 309-314.

Madigan, M.T., & Martinko, J.M. (2006). *In Brock Biology of Microorganisms* (11 ed.). New Jersey: Pearson Education, Upper Saddle River.

Maheshwari, D.K., & Shimizu, M. (2011). *Bacteria in Agrobiology: Plant Growth Responses*. Springer Berlin Heidelberg.

Malaysian Environmental Quality Act. (1998). *Overview of environmental issues and environmental conservation practices in Malaysia*. Retrieved Febuary 19, 2013, from Chapter 1: http://www.env.go.jp/earth/coop/oemjc/malay/e/malaye1.pdf

Malik, A. (2004). Metal bioremediation through growing cells. *Environment International, 30*, 261-278.

Malle, K.G. (1992). Zink in the environment. *Acta Hydrochemica and Hydrobiological, 20*, 196-204.

Marco, V., Carlos, C., Andreas, T., Govind, C., Gerald, F.F., Keith, F.C., & Douwe, S. (2007). Genomics of Actinobacteria: Tracing the Evolutionary History of an Ancient Phylum . *American Society for Microbiology, 71*, 495-548.

Marques, A.S.A, Corbiere, R., Gardan, L., Tourte, C., Manceau, C., Taylor, J.D., & Samson, R. (2000). Multiphasic approach for the identification of the different classification levels of *Pseudomonas savastanoi* pv. phaseolicola. *European Journal of Plant Pathology, 106*, 715-734.

Marques, A.S.A., Marchaison, A., Gardan, L., & Samson, R. (2008). BOX-PCR- based identification of bacterial species belonging to *Pseudomonas syringae*-Pviridiflava group. *Genetics and Molecular Biology, 31*, 106-115.

Marsh, P., & Wellington, E.M.H. (2007). *Molecular Ecology of Rhizosphere Microorganisms.* Wiley-VCH Verlag GmbH.

Martin, T.A., & Ruby M.V. (2004). Review of in situ remediation technologies for lead. *Remediation Journal, Summer 2004*, 37-53.

Masanori. (2006). *Expert and citizen participation in the pollution control: The case of itai-itai disease in Japan.* Japan: The Japan Times (eds) Minamata's legacy after 50 years May2006.

Mason, M.G., Ball, A.S., Reeder, B.J., Silkstone, G., Nicholls, P., & Wilson, M.T. (2011). Extracellulear heme peroxidases in actinomycetes: a case of mistaken identity. *Applied Environmental Microbiology, 67*, 4512-4519.

Matthew, D., Rolfe, Christopher, J., Rice, Lucchini, S., Pin, C., Thompson, A., Andrew, D. S., Cameron, Alston, M., Michae,l F., Stringer, Roy, P., Betts, Baranyi, J., Michael, W., Peck, Jay, C. D., & Hinton. (2012). Lag phase is a distinct growth phase that prepares bacteria for exponential growth and involves transient metal accumulation. *Journal of Bacteriology, 194*, 686–701. doi: 10.1128/JB.06112-11

McGregor, J. (1954). Nuclear division and the life cycle in a *Streptomyces* sp. *Journal of General Microbiology, 11*, 52-62.

Mercury. (2013). *Mercury*. Retrieved January 15, 2013, from Wikepedia: http://en.wikipedia.org/wiki/Mercury_(element)

Mergeay, M., Monchy, A., Vallaeys, T., Auquier, V., Benotmane, A., Bertin, P., Taghavi, S., Dunn, J., Van der Lelie, D., & Wattiez, R. (2003). *Ralstonia metallidurans*, a bacterium specifically adapted to toxic metals towards a catalogue of metal-responsive genes. *FEMS Microbiology Reviews, 27*, 385-410.

Mighall, T.M., Grattan, J.P., Timberlake, S., Lees, J.A., & Forsyth, S. (2002). An atmospheric pollution history for lead-zinc mining from the Ystwyth Valley,

Dyfed, Mid-Wales, UK as recorded by an upland peat. *Environment, 2*, 175-184.

Milic, J.S., Beskosko, V.P., Ilic, M.L., Ali Sam, Gojgic-Cvijovic, G.D., & Vrvic, M.M . (2009). Bioremediation of soil heavily contaminated with crude oil and its products: Composition of the microbial consortium. *Journal of the Serbian Chemical Society, 71*, 455-460.

Mincer, T.J., Jensen. P.R., Kauffman, A.C., & Fenical, W . (2002). Widespread and persistent populations of major new marine actinomycete taxon in ocean sediments. *Applied and Environmental Microbiology, 68*, 5005-5001.

Mohamed Fahmy Gad El-Rab, S., Abdel-Fattah Shoreit, A., & Fukumori, Y. (2006). Effects of cadmium stress on growth, morphology and protein expression in *Rhodobacter capsulatus* B10. *Bioscience Biotechnology and Biochemistry, 70*, 2394–2402.

Mullen, M.D., Wolf, D.C., Ferris, F.G., Beveridge, T.J., Flemming, C.A., & Bailey, G.W. (1989). Bacterial sorption of heavy metals. *Applied and Environmental Microbiology, 55*, 3143-3149.

Mundie, D.A. (1995). *NBS/ISCC Color System*. Retrieved April 15, 2012, from NBS/ISCC Color System: http://www.tx4.us/nbs-iscc.htm

Nageswaran, N., Ramteke, P.W., Verma, O.P., & Pandey, A. (2012). Antibiotic susceptibility and heavy metal tolerance pattern of *Serratia Marcescens* isolated from soil and water. *Journal of Bioremediation and Biodegradiation, 3*, 158.

Nemeth, T., & Kadar, I. (2005). Leaching of microelement contaminants: A long-term field study. *Journal of Biosciences, 60*, 260-264.

Neu, H.C. (1989). Overview of mechanisms of bacterial resistance. *Diagnostic Microbiology and Infectious Disease , 12*, 109-116.

Nickel. (2013). *Nickel*. Retrieved January 15, 2013, from Wikepedia: http://en.wikipedia.org/wiki/Nickel

Niemann, S., Puhler, A., Ticky, H.V., Simon, R., & Selbitschka, W. (1997). Evaluation of the resolving power of three different DNA fingerprinting methods to discriminate among isolates of a natural *Rhizobium meliloti* population. *Journal of Applied Microbiology, 82*, 477-484.

Nies, D.H. (1999). Microbial heavy metal resistance. *Applied Microbiol Biotechnology, 51*, 730-750.

Nies, D.H., & Silver, S. (1995). Ion efflux systems involved in bacterial metal resistances. *Journal of Industrial Microbiology, 14*, 186-199.

Nimnoi, P., Pongsilp, N., & Lumyong, S. (2010). Genetic diversity and community of endophytic actinomycetes within the roots of *Aquilaria crassna* Pierre ex Lec assessed by Actinomycetes-specific PCR and PCR-DGGE of 16S rRNA gene. *Biochemical Systematics and Ecology , 38*, 595-601.

Nithya, B., Ponmurugan, P., & Fredimoses, M. (2012). 16S rRNA phylogenetic analysis of actinomycetes isolated from Eastern Ghats and marine mangrove associated with antibacterial and anticancerous activities. *African Journal of Biotechnology, 11*, 12379-12388. doi:10.5897/AJB12.772

Nogales, B., Moore, E.R.B., llobet-Brossa, E., Rosello-Mora, R., Amann, R., & Timmis, K.N. (2001). Combined use of 16S ribosomal DNAand 16S rRNA to study the bacterial community of polychlorinated biphenyl-polluted soil. *Applied and Environmental Microbiology, 67*, 1874-1884.

Nonomura, H. (1974). Key for classification and identification of 458 species of the *Streptomyces* included in ISP. *Journal of Fermentation Technology, 52*, 78-92.

Normark. B.H., & Normark, S. (2002). Evolution and spread of antibiotic resistance. *Journal of Internal Medicine, 91*, 91-106.

Numuta, K., & Nimura, S. (2003). Access to soil Actinomycetes in Malaysian tropical rain forests. *Actinomycetologica, 17*, 54-56.

Oda, Y., Star, B., Huisman, L.A., Gottschal, J.C., & Forney, L.J. (2003). Biogeography of the purple nonsulfur bacterium *Rhodopseudomonas palustris*. *Applied and Environmental Microbiology, 69*, 5186-5191.

Olafson, R.W., McCubbin, W.D., & Cyril, M.K. (1988). Primary- and secondary-structural analysis of a unique prokaryotic metallothionein from a *Synechococcus* sp. cyanobacterium. *Biochemical Journal, 251*, 691-699.

Olive, D.M., & Bean, P. (1999). Principles and applications of methods for DNA-based typing of microbial organisms. *Journal of Clinical Microbiology, 37*, 1661–1669.

Pace, B., & Campbell, L.L . (1971). Homology of the ribosomal ribonucleic acid of Desulfovibrio species with *Desulfovibrio vulgaris*. *Journal of Bacteriology, 106*, 717-719.

Pandey, B., Ghimire, P., & Agrawal, V.P. (2004). Studies on the antibacterial activity of actinomycetes. *International Conference on the Great Himalayas: Climate, Health, Ecology, Management andConservation,Kathmandu.* Canada: Kathmandu University and the Aquatic Ecosystem Health and Management Society.

Patel, J.B. (2001). 16S rRNA gene sequencing for bacterial pathogen identification in the clinical laboratory. *Molecular Diagnosis and Therapy, 6*, 313-21.

Pathom-aree, W., James, E. M.S., Alan, C.W., Horikoshi, K., Alan T. B., & Goodfellow, M. (2006). Diversity of actinomycetes isolated from challenger deep sediment (10,898 m) from Marina Trench. *Extremophiles, 10*, 181-189. doi:10.1007/s00792-005-0482-z

Pattanapipitpaisal, N.L., Brown, N.L., & Mecaskie, L.E. (2001). Chromate reduction by *Microbacterium liquefaciens* immobilised in polyvinyl alcohol. *Biotechnology Letters, 23*, 61-65.

Peela, S., Kurada, B., & Terli, R. (2005). Studies on antagonistic marine actinomycetes from the Bay of Bengal. *World Journal of Microbiology & Biotechnology, 21*, 583–585.

Polti, M.A., Amoroso, M.J., & Abate, C.M. (2007). Chromium (VI) resistance and removal by actinomycete strains isolated from sediments. *Chemosphere, 67*, 660-667.

Poopal, A.C., & Laxman R.S. (2009). Studies on biological reduction of chromate by Streptomyces griseus. *Journal of Hazardous Materials, 169*, 539-545.

Praseetha P. K., Sugunan, V. S., Shabi, R., & Jegadambikadevi, S. (2012). Stress response of actinomycetes to toxins in their bioremediation processes. *Journal of Microbiology and Biotechnology Research , 2*, 657-664.

Prescott, L.M., Harley, J.P., Klein, D.A. (2005). *Microbiology* (6 ed.). New York: McGraw Hill.

Pridham, T.G., & Gottlieb, D. (1948). The utilization of carbon compounds by some

actinomycetales as an aid for species determination. *Journal of Bacteriology, 56*, 107-114.

Prithviraj, D., Divya, P., Patel, K., Aursang, R., Neelu, N., Balasaheb, K., Madhukar, K., & Abul, M. (2012). Biosorption of heavy metals by actinomycetes for treatment of industrial effluents. *Proceedings of the UMT 11th International Annual Symposium on Sustainability Science and Management*, (pp. 389-393). Kuala Terengganu, Malaysia.

Promnuan, Y., Kudo, T., Ohkuma, M., & Chantawannakul, P. (2013). *Streptomyces chiangmaiensis* sp. nov. and *Streptomyces lannensis* sp. nov., isolated from the South-East Asian stingless bee (*Tetragonilla collina*). *International Journal of Systematic and Evolutionary Microbiology, 63*, 1896-1901.

Qin, S., Li, J., Hua-Hong, C., Guo-Zhen, Z., Wen-Yong, Z., Cheng-Lin, J., Li-Hua, X., & Wen-Jun, L. (2009). Isolation, diversity, and antimicrobial activity of rare actinobacteria from medicinal plants of tropical rain forests in Xishuangbanna, China. *Applied and Environmental Microbiology, 75*, 6176-6186.

Raab, A., & Fieldman, J. (2003). Micobial transformation of metals and metalloids. *Science Progress, 86*, 179-202.

Radajewski, S., Webster, G., Reay, D.S., Morris, S.A., Ineson, P., Nedwell, D.B., Prosser, J.I., & Murrell, J.C. (2002). Identification of active methylotroph populations in an acidic forest soil by stable isotope probing. *Microbiology, 148*, 2331–2342.

Radwan, S.S., Barabas, G.Y., Sorkhoh, N.A., Damjanovich, S., Szabo, I., Szllosi, J., Matko, J., Penyige, A., Hirano, T., & Szabo, I.M. (1998). Hydrocarbon uptake by *Streptomyces*. *FEMS Microbiology Letters , 169*, 87-94.

Rai, R.A., Roychoudhury, M., Singh, R.P., & Roychoudhury, K. (2014). Application of PCR fingerprinting for identification and comperative phylogenetic study of antagonistic *Streptomyces*. *India Journal of Applied Research, 4*, 440-443.

Rajendran, P., Muthukrishnan, J., & Gunasekaran, P. (2003). Microbes in heavy metal remediation. *Indian Journal of Experimental Biology, 41*, 935-944.

Rani, A., Shouche, Y., & Goel, R. (2008). Declination of copper toxicity in pigeon pea and soil system by growth promoting *Proteus vulgaris* KNP3 strain. *Current Microbiology, 57*, 78–82.

Rao, S.N.S. (1999). *Soil Microbiology: Soil Microorganisms and Plant Growth* (4th ed.). Science Publication Incorporation.

Rascio, N., La Rocca, N., Erik, J.S., & Brian, F. (2008). *Encyclopedia of Ecology.* Oxford: Academic Press.

Rastogi, G., & Sani, R,K. (2011). Molecular Techniques to Assess Microbial Community Structure, Function, and Dynamics in the Environment. In F. A. I. Ahmad. (Ed.), *Microbes and Microbial Technology Agricultural and Environmental Applications.* Springer.

Ravel, J., Amoroso, M.J., Colwell, R., & Hill, R.T. (1998). Mercury-resistant actinomycetes from Chesapeake Bay. *FEMS Microbiology Letters, 162*, 177–184.

Raymond A.W., and Felix E. O. (2011). Heavy metals in contaminated soils: A review of sources, chemistry, risks and best available strategies for remediation. *International Scholarly Research Network.* doi:10.5402/2011/402647

Reddy, N.G., Ramakrishna, D.P.N., & Gopal, R.S.V. (2011). A morphological, physiological and biochemical studies of marine Streptomyces rochei (MTCC 10109) showing antagonistic activity against selective human pathogenic microorganisms. *Asian Journal of Biological Sciences, 4*, 1-14.

Reyes, F.L., Ritter, W., & Raskin, L. (1997). Group-specific small-subunit rRNA hybridization probes to characterize filamentous foaming in activated sludge systems. *Applied and Enviromental Microbiology, 63*, 1107-1117.

Robinson, N.J., Whitehall, S.K.,& Cavet, J.S. (2001). Microbial metallothioneins. *Advances in Microbial Physiology , 44*, 183-213.

Ross, S. (1994). *Toxic metals in Soil-Plant Systems.* Chichester, UK: John Wiley & Sons.

Rossello-Mora, R., & Amann, R. (2002). The species concept for prokaryotes. *Microbiology Reviews, 25*, 39-67.

Routh, J., Saraswathy, A., & Collins, D. (2007). Arsenicicoccus bolidensis a novel arsenic reducing actinomycete in contaminated sediments near the Adan mine (northen Sweden): impact on water chemistry. *Science of the Total Environment, 379*, 216-225.

Sabry, S.A., Ghozlan, H. A., & Abou-zeid, D.M. (1997). Metal tolerance and antibiotic resistance patterns of a bacterial population isolates from sea water. *Journal of Applied Microbiology, 82*, 245-252.

Saitou, N., & Nei, M. (1987). The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution, 4*, 406-425.

Samanta, A., Bera, P., Khatun, M., Sinha, C., Pal, P., Lalee, A., & Mandal, A. (2012). An investigation on heavy metal tolerance and antibiotic resistance properties of bacterial strain *Bacillu*s sp. isolated from municipal waste. *Journal of Microbiology and Biotechnology Research, 2*, 178-189.

Sambrook, J., Fritsch, E.F., & Maniatis, T. (1989). *Molecular Cloning: a laboratory manual* (2 ed.). New York, US: Cold Spring Harbour Laboratory Press.

Santella, R.M. (2006). Amplification approaches to DNA/RNA extraction and whole genome. *Cancer Epidemiol Biomarkers Prev, 15*, 1585-1587.

Sathiyaseelan, K., & Stella, D. (2012). Isolation and screening of α-glucosidase enzyme inhibitor producing marine actinobacteria isolated from Pichavaram mangrove. *International Journal of Pharmaceutical & Biological Archives, 3*, 1142-1149.

Savic, M., & Vasiljevic, B. (2006). Targeting polyketide synthase gene pool within actinobacteria: new degenerate primers. *Journal of Industrial Microbiology and Biotechnology, 33*, 423-430.

Schmidt, A., Haferburg, G., Schmidt, A., Lischke, U., Merten, D., Ghergel, F., Buchel, G., & Kothe, E. (2009). Heavy metal resistance to the extreme: *Streptomyces* strains from a former uranium mining area. *Chemi der Erde-Geochemistry, 69*, 35-44.

Schmidt, A., Haferburg, G., Sineriz, M., Merten, D., Buchel, G., & Kothe, E. (2005). Heavy metal resistance in ctinobacteria for survival in AMD contaminated soils. *Chemi der Erde-Geochemistry, 65*, 131-144.

Selvameenal, L., Radhakrishnan, M., & Balagurunathan, R. (2009). Antibiotic pigment from desert soil actinomycetes; Biological activity, purification and chemical screening. *Indian Journal of Pharmaceutical Sciences, 71*, 499–504.

Selvin, J., Shanmugha, P.S., Seghal, K.G., Thangavelu, T., & Sapna Bai, N. (2007). Sponge- associated marine bacteria as indicators of heavy metal pollution. *Microbiological Research, 164*, 352-363.

Sen, S., & Charaya, M.U. (2010). Copper tolerant microfungi for bioremediation. *Progressive Agriculture, 10*, 68-71.

Seong, C.N., Choi, J.H., & Keun-Shik, B. (2001). An improved selective isolation of rare actinomycetes from forest soil. *The Journal of Microbiology, 39*, 17-23.

Shaaban, M.T, El-Sabbagh, S.M.M., & Alam, A. (2013). Studies on an Actinomycete producing a melanin pigment inhibiting aflatoxin B1 production by *Aspergillus flavus*. *Life Science Journal, 10*, 1437-1448.

Shapiro, N., & Keasling, J.D. (1996). The recA gene and cadmium toxicity in *Escherichia coli* K-12. *Microbios, 86*, 23-26.

Shimizu, M., Nakagawa, Y., Sato, Y., Furamai, T., Igarashi, Y., Onaka, H., Yoshida, R., & Kunch, H. (2000). Studies on endophytic actinomycetes *Streptomycetes* sp. isolated from *Rhododendron* and its antimicrobial activity. *Journal of General Plant Pathology, 66*, 360-366.

Shirling, E. B., & Gottlieb, D. (1966). Methods for characterization of Streptomyces species. *International Journal of Systematic Bacteriology, 16*, 313-340.

Shivika, B. (2011). *Heavy Metal Resistance of Microorganisms.* Retrieved from Biotech Article: Environmental Biotecnology: http://www.biotecharticles.com/EnvironmentalBiotechnologyArticle/Heavy-Metal-Resistance-of-Microorganisms-1099.html

Shparyk, Y.S., & Parpan, V.I. (2004). Heavy metal pollution and forest health in the Ukrainian Carpathians. *Environmental Pollution, 130*, 55-63.

Shuhaimi, M., Ali, A.M., Saleh, N.M., & Yazid, A.M. (2001). Utilisation of enterobacterial repetitive intergenic consensus (ERIC) sequemce-band PCR to fingerprint the genomes of *Bifidobacterium* isolates and other probiotic bacteria. *Biotechnology Letters, 23*, 731-736.

Silver, S. (1996). Bacterial resistances to toxic metal ions--a review. *Gene, 179*, 9-19.

Silver, S., & Phung, L.T. (2005). A bacterial view of the periodic table: genes and proteins for toxic inorganic ions. *Journal of Industrial Microbiology and Biotechnology, 32*, 587-605.

Sineriz, M.L., Kothe, E., & Abate, C.M. (2009). Cadmium biosorption by *Streptomyces*

sp.F4 isolated from former uranium mime. *Journal of Basic Microbiology, 1*, 55-62.

Singh, D.V., Matte, M.H., Matte, G.R., Jiang, S., Sabeena, F., Shukla, B.N., Sanyal, S.C., Huq, A., & Colwell, R.R. (2001). Molecular analysis of *Vibrio cholerae* O1, O139, non-O1, and non-O139 strains: clonal relationship between clinical and environmental isolates. *Applied and Environmental Microbiology, 67*, 910-921.

Singh, R., Gautam, N., Mishra, A., & Gupta, R. (2011). Heavy metals and living systems: An overview. *Indian Journal of Pharmacology, 43*, 246-253. doi:10.4103/0253-7613.81505

Sinha, S., & Mukherjee, S.K. (2009). *Pseudomonas aeruginosa* KUCD, A possible candidate for cadmium bioremediation. *Brazilian Journal of Microbiology, 40*, 55-662.

Sivakumar, K. (2001). *Actinomycetes of an Indian mangrove (Pitchavaram) environment; an inventory* . Ph.D. Thesis: Annamalai University, Tamil Nadu.

Sobecky, P.A. (1999). Plasmid ecology of marine sediment microbial communities. *Hydrobiologia, 401*, 9-18.

Spain, A. (2003). Implications of microbial heavy metal tolerance in the environment. *Review of Undergraduate Research, 2*, 1-6.

Spooner, F., Medina, C., & Fulbright, D. (1995). Fingerprinting genomes of potato scab-causing *Streptomyces species* withrepetitive extragenic sequences and the polymerase chain reaction technique (Abstract). *Phytopathology, 85*, 1162.

Subhajit, J.R. (2012). Producttion of biosurfactant using an isolated bacterial strain of *Bacillus* sp (m28). *Journal of Microbiology and Biotechnology Research, 2*, 402-415.

Syed Amir, M. (2011). *Actinomycetes and bioremediation*. Retrieved Febuary 17, 2013, from Biotech Article: Environmental Biotecnology: http://www.biotecharticles.com/Environmental-Biotechnology Article/Actinomycetes-and-Bioremediation-1091.html

Tacao, M., Alves, A., Saavedra, M.J., & Correia, A. (2005). BOX-PCR is an adequate tool for typing *Aeromonas* spp. *Antoine-van-Leeuwenhoek, 88*, 173-179.

Taechowisan, T., Peberdy, J. F., & Lumyong, S. (2003). Isolation of endophytic actinomycetes from selected plants and their antifungal activity. *World Journal of Microbiology and Biotechnology, 19*, 381-385.

Tamura, K., Dudley, J., Nei, M., & Kumar, S. (2007). MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Molecular Biology and Evolution, 24*, 1596-1599.

Tendler, D. (1959). Studies on thermophilic actinomycetes. *Torrey bot. Cl, 86*, 17.

Timoney, J.F., Jennifer, P., Janis, G., & Spanier, J. (1978). Heavy Metal and antibiotic resistance in bacterial flora of sediments of New York bight. *Applied and Enviromental Microbiology, 36*, 465-472.

Todar, K. (2009). *The microbial world: Antimicrobial Agents Used in the Treatment of Infectious Disease.* Retrieved July 27, 2012, from http://www.textbookofbacteriology.net/themicrobialworld/antimicrobial.html

Todar, K. (2012). *Bacterial resistance to antimicrobial agents*. Retrieved November 26, 2012, from Todar's online textbook of bacteriology: http://textbookofbacteriology.net/Todar's Online Textbook of Bacteriology

Toshihide, T., & Takashi, Y. (2011). The history of minamata disease and public health policy. *Epidemiology, 22*, S99.

Tresner, H.D., Hayes, J.A., & Backns, E.J. (1968). Differential tolerance of *Streptomycetes* to sodium chloride as a taxonomic aid. *Applied Microbiology, 6*, 1134-1136.

Tripathi, M., Munot, H.P., Souche, Y., Meyer, J.M., & Goel, R. (2005). Isolation and functional characterization of siderophore producing lead and cadmium resistant *Pseudomonas putida* KNP9. *Current Microbiology, 50*, 233–237.

Tsai, K.J. (2006). Bacterial heavy metal resistance. *Pakistan Research Repository, 11*, 101-110.

US Environmental Protection Agency, USEPA. (1997). Food IngestionFactors: Exposure Factors Handbook. Washington, DC, USA: Office of Research and Development.

Valls, M., & de Lorenzo, V. (2002). Exploiting the genetic and biochemical capacities

of bacteria for the remediation of heavy metal pollution. *FEMS microbiology Reviews, 26*, 327-338.

Van Hop, D., Sakiyama, Y., Binh, C.T.T., Otoguro, M., Hang, D.T., Miyadoh, S., Luong, D.T., & Ando, K. (2011). Taxonomic and ecological studies ofactinomycetes from Vietnam: isolation and genus-level diversity. *Journal of Antibiotics*. doi:org/10.1038/ja.2011.1040

Van Nostrand, J.D., Khijniak, T.V., Gentry, T.J., Novak, M.T., Sowdwr, A.G., Zhou, J.Z., Bertsch, P.M., & Morris, P.J. (2007). Isolation and characterization of four gram positive nickel tolerant microorganisms from contaminated sediments. *Microbial Ecology, 53*, 670-682.

Vargas Gil, S., Pastor, S., & March, G.J. (2009). Quantitative isolation of biocontrol agents *Trichoderma* spp., *Gliocladium* spp. and. *Microbiology Research, 164*, 196-205.

Vijayakumar, R., Muthukumar, C., Thajuddin, N., Panneerselvam, A., & Saravanamuthu, R. (2007). Studies on the diversity of actinomycetes in the Palk Strait region of Bay of Bengal, India. *Actinomycetologica, 21*, 59–65.

Vijayaraghavan, K., & Yun, Y.S. (2008). Bacterial biosorbents and biosorption. *Biotechnology Advances, 26*, 266-291.

Volesky, B., & Holan, Z.R. (1995). Biosorption of heavy metals. *Biotechnology Progress , 11*, 235-250.

Waisberg, M., Joseph, P., Hale, B. & Beyersmann, D. (2003). Molecular and cellular mechanisms of cadmium carcinogenesis. *Toxicology, 192*, 95-117.

Waksman, S.A. (1952). *Streptomycin: background, isolation, properties, and utilization.* Amsterdam: Nobel Lecture.

Waksman, S.A., & Henrici, A.T. (1943). The nomenclature and classification of the actinomycetes. *Journal of Bacteriology, 46*, 337-341.

Watve, M.G., Tickoo, R., Jog, M.M., & Bhole, B.D. (2001). How many antibiotics are produced by the genus Streptomyces. *Archives of Microbiology, 176*, 386-390.

Weast, R.C. (1984). *CRC handbook  of chemistry and  physics* (64 ed.). Boca Raton, Florida, USA: CRC Press, Inc.

Whitman, W.B., Goodfellow, M., Kampfer, P., Busse, H.J., Martha, E., Trujillo, Suzuki, K., & Ludwig, W. (2012). *Bergey's Manual of Systematic Bacteriology: Volume 5: The Actinobacteria.* Springer-Verlag.

Willey, J. S. (2010). *Prescott's Microbiology* (7 ed.). New York: McGraw-Hill.

Williams, S. L. (1984). *Ecology of actinomycetes.The biology of the actonomycetes.* London, United Kingdom: Academic Press.

Wilson, L. (2012). *Toxic metals and human health*. Retrieved May 27, 2013, from http://drlwilson.com/articles/TOXIC%20METALS.htm

Woese, C.R. (1987). Bacterial evolution. *Microbiology Reviews, 51*, 221-271.

Zajicek, G. (2013). *The antibiotic paradox plasmids and transposons*. Retrieved March 5, 2013, from http://www.what-is-cancer.com/papers/AntibioticParadox.html

Zhao, H.J., Parry, R.L, Ellis, D.I., Griffith, G.W., & Goodcare, R. (2005). The rapid differentiation of *Streptomyces* isolates using Fourier transform infrared spectroscopy. *Vibrational Spectroscopy, 40*, 213–218.

Zhou, H., Kassama, Y., Young, M., Kell, D.B., & Goodacre, R. (2004). Differentiation of *Micromonospora* isolates from a coastal sediment in Wales an the basis fourier transform infrared spectroscopy, 16S rRNA sequence analysis, and the amplified fragment length polymorphism technique. *Applied and Environmental Microbiology, 20*, 6619-6627.

Zinc. (2013). *Zinc*. Retrieved January 14, 2013, from Wikipedia: http://en.wikipedia.org/wiki/Zinc

Zouboulis, A.I., Lazaridis, N. K., Karapantsios, T.D., & Matis, K.A. (2010). Heavy metals removal from industrial wastewaters by biosorption. *International Journal of Environment and Pollution, 1*, 57-78.

Zouboulis, A.I., Loukidou, M.X., & Matis, K.A. (2004). Biosorption of toxic metals from aqueous solutions by bacteria strains isolated from metal polluted soils. *Process Biochemistry, 39*, 909-916.