1.0 Introduction

Actinobacteria are filamentous, branching bacteria that are widely distributed in nature. They are mainly found in soil, where they play a major role in the decomposition of organic matter. Actinobacteria may form a substrate mycelium only, or both aerial and substrate mycelia, or an aerial mycelium only. The hyphae are mostly about one micron or less in diameter. In some species, the cells are acid-fast. Although some species are holocarpic, eucarpic members may show highly complex mycelial structures with conidia and sporangia. Motility, when observed, is due to flagella. Conidia and sporangiospores may be variously arranged or variously structured with surface hairs, spines or ridges (Lechevalier and Pine, 1989; Locci, 1989).

Actinobacteria is a diverse group of filamentous Gram positive bacteria. They are prokaryotes having high G+C content (more than 55%) in their DNA, with extremely various metabolic possibilities. The metabolic diversity of the actinobacteria family is due to their extremely large genome, which has hundreds of transcription factors that control gene expression, allowing them to respond to specific needs (Goshi *et al*., 2002).

The majority of the actinobacteria are free living, saprophytic bacteria found widely distributed in soil, water and colonizing plants. Their population forms an important component of the soil microflora. Around 70-90% of the actinobacteria in virgin and cultivated soils are *Streptomyces* species (Alexander, 1961).

The demand for new antibiotics continues to grow due to the rapid spread of antibiotic-resistant pathogens causing life-threatening infections. Although

considerable progress is being made within the fields of chemical synthesis and engineered biosynthesis of antifungal compounds, tropical nature still remains the richest and the most versatile source for new antibiotics (Bredholt *et al*., 2008).

Actinobacteria are well known for their production of an extensive array of chemically diverse and medically important secondary metabolites to inhibit material degradation and in the protection of animal and plant health (Jimenez-Esquilin and Roane, 2005). They have been extensively used as sources of pharmaceuticals and agrochemicals because of their importance as producers of vitamins, enzymes, antitumour agents, immunomodifying agents, and mainly antibiotic compounds (Badji *et al*., 2007). The search for novel antimicrobial compounds to fight the emergence of pathogens has lead to wide-spread screening of actinobacteria from novel, unexplored habitats, both in academic and industrial laboratories (Basil *et al*., 2004).

It was suggested that, tropical nature should be used as a source of actinobacteria with properties antagonistic to disease producing organisms. The interest in nature as a source of antibiotic-producing actinobacteria resulted in an intensive competition by pharmaceutical industries to find such products. Actinobacteria produce a large and diverse array of bioactive compounds that inhibit the development of pathogens in the tropical nature. From the mid-1940s to mid-1980s many pharmaceutical companies supported intensive isolation, fermentation, and characterization studies on actinobacteria in a search for antifungal products (Gil *et al*., 2009).

Since actinobacteria has been a focus in the exploitation of excellent biocontrol agents against phytopathogenic fungi, this study was undertaken with the objective of

screening actinobacterial strains with antifungal activity. The determination of the antagonistic activity of the 52 strains of actinobacteria that have been previously isolated from tropical soil samples, earthworm gut sample and sediment sample against four species of yeasts (*Candida albicans* ATCC 14053, *Candida parapsilosis* ATCC 22019, *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*) as well as two species of filamentous fungi (*Ganoderma boninense* Pat. and *Fusarium oxysporum* f. sp. *cubense*) were carried out. Antagonistic properties of the actinobacteria strains were evaluated using agar plug-diffusion method.

Repetitive DNA sequences are dispersed throughout the genomes of diverse bacterial species including actinobacterial strains (Clark *et al*., 1998). In this study, the ten actinobacterial strains that had shown strong antifungal activity were dereplicated using enterobacterial repetitive intergenic consensus (ERIC)-PCR method. This technique was chosen because it could unambiguously detect the genomic differences among actinobacterial strains that were indistinguishable by other methods such as morphological observation on cultured media. This method also might give a brief picture on developing a foundation for future genetic studies of the actinobacterial strains.

Although polyene compounds have limited clinical use, which is to a large part due to their high toxicity and side effects, the superior antifungal activities of polyene compounds are still being considered in the development of improved antifungal drugs. Since the polyene CYP-specific PCR-guided genome screening approach is an efficient method for isolating potentially valuable polyene-producing actinobacterial strains, the actinobacterial strains used in this study that had shown strong antifungal activity were

screened for the presence of expected size of the CYP-specific DNA fragment in the actinobacterial chromosome (Hwang *et al*., 2007).

The actinobacterial strains that had shown strong antifungal activity were also identified by sequence comparison of their partial 16S rRNA gene sequence.

1.1 Objectives of Research

- To screen the actinobacterial strains with antifungal activity against *Candida albicans*, *Candida parapsilosis*, *Schizosaccharomyces pombe*, *Saccharomyces cerevisiae*, *Ganoderma boninense* and *Fusarium oxysporum*;
- To differentiate the actinobacterial strains that had shown strong antifungal activity by using ERIC-PCR method;
- To screen the presence of CYP-specific DNA fragment on actinobacterial strains with strong antifungal activity; and
- To identify the actinobacterial strains that had shown strong antifungal activity based on partial 16S rRNA gene sequence.

2.0 Literature Review

2.1 General Characteristics of Actinobacteria

Actinobacteria comprise a group of branching unicellular microorganisms. They form a mycelium which may be of a single kind designated as substrate and aerial. They reproduce by fission or by producing spores or conidia. Sporulation of actinobacteria has commonly been distinguished as fragmentation and segmentation or conidia formation. Most of the actinobacteria are aerobic. Very few are obligate anaerobes although some are microaerophilic (Hollick, 1995).

Colonies of actinobacteria are compact, often leathery and giving a conical appearance with a dry surface. They are frequently covered with aerial mycelium. If the colony is well developed and the aerial mycelium abundant, the surface spores can easily be picked with a sterile needle (Starr, 1981).

Actinobacteria produce a substrate mycelium that varies in size, shape and thickness. Its colour ranges from white or virtually colourless to yellowish, brownish, red, pink, orange, green or black. The aerial mycelium is usually thicker than the substrate mycelium. Under fixed condition of culture, the aerial mycelium shows sufficient differentiation that a miscellaneous assortment of isolates can be segregated into a number of groups having similar morphological characteristics. This is one of the most important criteria for the classification of the genus *Streptomyces* into species, comprising structure (cottony, velvety or powdery), formation of rings or concentric zones and pigmentation (Waksman, 1967; Batzing, 2002).

The actinobacteria spores are reproductive bodies. The spores germinate with the formation of one or more germ tubes. These grow into long branching hyphae, culminating in a complex mycelium. Some of the hyphae are straight and reach a length of more than 600μm. Others are only 50 to 100μm in length and are much branched and curved. The surface of the spores may be rough or smooth. The rough-surfaced spores are divided into those that possess spiny, hairy or warty surfaces (Atlas, 1984).

Actinobacterial strains may form water-soluble and water-insoluble pigments, depending on the nature of the organism and the composition of the medium. Pigments play various roles in the growth and survival of actinobacteria. One such role is for defence against foreign cells, they being referred to as antibiotic pigments (Parungao *et al*., 2007). The formation of deep brown to black pigments on organic media containing proteins and protein derivative, notably the amino acid tyrosine, is an important characteristic. Certain species may form only faint brown soluble pigments on organic or on synthetic media. On continued cultivation on artificial media, some cultures will show great variation in pigment production. Yellow, red, blue, green and other soluble pigments are produced by some species on synthetic media. There is considerable variation in the intensity of these pigments depending on the strain of organism (Waksman, 1967; Starr, 1981).

Presence of diaminopimelic acid (DAP) isomers is one of the most important cell wall properties of actinobacteria. The 2, 6- diaminopimelic acid (DAP) is widely distributed as a key amino acid and it has optical isomers. The systematic significance lies mostly in the key amino acid with two amino bases, and determination of the key amino acid is usually sufficient for characterisation. If DAP is present, bacteria

generally contain one of the isomers, the LL-form or the *meso*-form, mostly located in the peptidoglican (Lechevalier and Lechevalier, 1970).

The sugar composition often provides valuable information on the classification and identification of actinobacteria. Actinobacteria cells contain some kinds of sugars, in addition to the glucosamine and muramic acid of peptidoglican. The sugar pattern plays a key role in the identification of sporulating actinobacteria which have *meso*-DAP in their cell walls. However, the actinobacteria which have LL-DAP along with glycine have no characteristic pattern of sugars (Lechevalier and Lechevalier, 1970). On the basis of cell wall analysis actinobacteria family has been divided into eight types as in Table 2.1.

Table 2.1 Cell wall chemotypes with representative families (Lechevalier and Lechevalier, 1970).

Chemotype	**I**	**II**	**III**	**IV**	**V**	**VI**	**VII**	**VIII**
LL-DAP	+							
***Meso*-DAP**		+	+	+				
Diaminobutyric acid							+	
Aspartic Acid						V		
Glycine	+	+					+	
Lysine					+		V	
Ornithine					+			+
vArabinose				+				
Galactose				+		V		
	1	2	3	4	5	6	7	8

Note :
1 - *Streptomycetaceae*, *Nocardiaceae*
2 - *Micromonosporaceae*
3 - *Dermatophilaceae, Mycobacteriaceae, Thermomonosporaceae, Streptosporangiaceae, Frankiaceae*
4 - *Nocardiaceae, Mycobacteriaceae, Corynebacteriaceae, Pseudonocardiaceae*
5 - *Actinomycetacea*
6 - *Cellulomonadaceae*, *Micrococcaceae*, *Microbacteriaceae*, *Actinomycetaceae*
7 - *Microbacteriaceae*
8 - *Cellulomodaceae, Microbacteriaceae*
V - Variable

However, cell wall analysis is more troublesome to taxonomists and requires much work in comparing large numbers of organisms. Molecular techniques nowadays have been used extensively in order to establish a genus or family for new isolates as well as recognizing phylogenetic relationships among actinobacteria. These relationships often give decisive evidence that a genus or a family is exclusively independent from or inclusively related to otherwise different organisms (Yamaguchi, 1965).

Actinobacteria have been grouped into many genera based on their characteristics. The most common genera of actinobacteria are *Actinomyces*, *Nocardia*, *Streptomyces*, and *Actinoplanes*. By far the most successful genus in actinobacteria group is *Streptomyces* with over 500 species (El-Tarabily and Sivavithamparam, 2006).

Streptomyces species or streptomycetes are the most well known genus of the actinobacteria. It differs conspicuously from most other bacteria in their growth and developmental biology. They have been found to grow in marshes, soil and marine habitats. Streptomycetes that inhabit soil are responsible for giving the soil that earthy smell by secreting chemicals known as geosmins. They can break down organic matter, including sugars, alcohols, amino acids, organic acids and aromatic compounds, and can also metabolise other compounds. They do this by producing and releasing extracellular hydrolytic enzymes. There is considerable interest in these organisms as agents for bioremediation (El-Tarabily and Sivavithamparam, 2006).

In the conventional taxonomy of *Streptomyces* spp., its systematics is, in practice, based on characteristics of mycelial morphology, pigment production, and certain physiological properties. In 1964, the International *Streptomyces* Project (ISP),

a collaborative study group on *Streptomyces* spp. taxonomy, proposed an elaborate description of criteria for some of the authentic and extant type strains of *Streptomyces* and their related taxa, which is dependent on a limited number of taxonomic characteristics selected from those accumulated in conventional taxonomic test (Shirling and Gottlieb, 1969, 1972).

Streptomyces is the most studied genus of actinobacteria. The distinctive morphology of the sporing structures has been widely used in species delimitation. Spore chain morphology and spore surface ornamentation determined by microscopy have figured prominently in many classifications. One of the most recent and extensive taxonomic studies of this genus was carried out in the ISP. Over 400 type strains were examined independently by standardized procedures in three different laboratories where morphological criteria were figured out in the resulting species description (Shirling and Gottlieb, 1968a, 1968b, 1969, 1972).

Morphological differentiation in *Streptomyces* spp. involves the formation of vegetative mycelium and then the production of aerial hyphae as the nutrient becomes limiting. These aerial hyphae stand up in the air and differentiate into a chain of spores (Flardh, 2003). This action is unique among Gram-positive bacteria and requires a specialized coordination among Gram-positive bacteria (Flardh, 2003). As demonstrated by using pulse-labelling or lectin binding, the streptomycete cell wall is polymerised at the hyphal apex. Although some turnover may occur in the lateral walls, hyphae are in principle tubes of inert murein that grow only at the extreme ends. When disconnected from the apex by septation, a subapical cell appears unable to grow until it has created a new tip by lateral branching. Most models for *Streptomyces* spp. tip extension assume that new material is incorporated at the apex in a flexible form, which

becomes more rigid further back from the tip, and that stretching of the tip by turgor pressure is a major driving force for growth (Flardh, 2003).

In July 2001, the full genome sequence for *Streptomyces coelicolor* A3 was completed. It consists of 8.66 million base pairs, which is large for a bacterium. Of the 7,825 genes, an unprecedented proportion carries out regulatory functions in the cell. More than 12% of the genome is involved in facilitating biological processes, such as the bacterium's response to environmental stimuli and stress. This strain of *Streptomyces* was studied intensively as it has a complex life cycle. Approximately, one in 20 of the *Streptomyces coelicolor* genome consists of genes that encode enzyme to make secondary metabolites. Such secondary metabolites are antibiotics and bioactive compounds for human and veterinary medicine (El-Tarabily and Sivavithamparam, 2006).

Streptomyces spp. produce many pigments. Since colour is one of the easiest characteristics to observe, it has always been used in descriptions and has often been the prime distinguishing characteristic. It even forms the basis of separation of large groups of species in some classifications. There has been a tendency to describe colours of *Streptomyces* spp. in very narrow terms, examples are dull, yellowish brown, greyish, and greenish blue. Culture colour, however, can vary because of slight differences in media, environment, handling and age of culture. Furthermore, sensitivity to colour differs widely. Eventhough the standards are used, the descriptive terms used vary from individual to individual (Okanishi *et al*., 1972).

Over 50 different antibiotics have been isolated from streptomycetes, including streptomycin, neomycin, chloramphenicol and tetracyclines. The structure of these

secondary metabolites can also contain antibacterial, antifungal, antiviral and antitumor activities. Some secondary metabolites also have immunosuppresent, antihypersensitive and antihypercholesterolemic properties. The discovery of new antibiotics produced by streptomycetes still continues, for example, mediomycins A, B and clethramycin that were isolated from *Streptomyces mediocidicus* ATCC23936 (Flardh, 2003).

Brevibacterium is a genus of actinobacteria of the order Actinomycetales. It is the sole genus in the family *Brevibacteriaceae* that exhibit rod-coccus cycle, possess *meso*-diaminopimelic acid in the peptidoglycan of the cell wall, catalase-positive, non-spore-forming, non-motile, and aerobic Gram-positive actinobacteria (Brazzola *et al*., 2000). The genus is a heterogeneous mixture of coryneform organisms that have particular application to industrial production of vitamins, amino acids for fine chemical production, and are commonly used in cheese production (Seefeldt and Weimer, 2000). This genus contains nine species from diverse habitats, such as soil, poultry, fish, human skin, and food. They are found as normal human skin flora, in raw milk and on the surface of cheeses. Some species that are recovered from patients with septicemia and from the peritoneum of patients undergoing peritoneal dialysis appear to be opportunistic human pathogens (Brazzola *et al*., 2000).

One example of species under the genus *Brevibacterium* is *Brevibacterium linens* ATCC 9174. It is non-motile, non-spore forming, non-acid fast, Gram-positive coryneform that tolerates high salt concentrations (8-20%) and is capable of growing in a broad pH range (5.5-9.5), with an optimum of pH 7.0. They also survive carbohydrate starvation and drying for extended periods (Boyaval *et al*., 1985). *Brevibacterium linens* is unusual as they produce alkali as they grow, raising the pH to around 9.5 within 24-36 hours. Recent interest in *B. linens* has focused around their ability to

produce self-processing extracellular proteases, their ability to produce high levels of volatile sulfur compounds, bacteriocin production, cell membrane associated carotenoid pigment production and aromatic amino acid metabolism (Rattray and Fox, 1999).

2.2 Habitats of Actinobacteria

Actinobacteria are found in virtually every natural substrate such as soils and composts, fresh water basins, marine water, marine plants, marine sediment, foodstuffs and the atmosphere (You *et al*., 2005). Alkaline and neutral soils are more favourable habitats than acid soils. Some groups favour one habitat over another. For example, *Streptomyces* species are found most commonly in soils and in composts, while *Micromonospora* species are abundant in lake bottoms (El-Tarabily and Sivavithamparam, 2006).

Soil harbours various kinds of microorganisms. Actinobacteria are one of the dominant groups of soil populations. Numerous papers have been published on the number of actinobacteria, particularly streptomycetes in soil. Most of these studies are related to the antibiotic activity of the soil microflora and of the soil as habitat. They are found in both virgin and cultivated soils, in fertile and in unfertile soils, in various regions throughout the world. They are particularly abundant in alkaline soils and in soils rich in organic material. When the soil is rich with organic materials, such as proteins and lignins, the growth of actinobacteria is greatly stimulated. It has even been suggested that their major function in the soils consists in the decomposition of plant and animal residues (Arai, 1976).

Actinobacteria occur in the soil in the spore stage as well as in the mycelial stage. Mycelium develop most abundantly at 28°C to 37°C. At lower and higher temperatures, growth was slower but eventually reach the same density. The growth of vegetative mycelium of actinobacteria is very scanty at 5°C to 28°C, whereas at temperatures exceeding 37°C, there is no further stimulation of vegetative growth. At the higher temperatures, the mycelium undergo increasing fragmentation giving rise to abundant formation of spores. Treatments of soils, especially the use of fertilizers which results in changes in soil reaction, and the use of organic manures and cover crops, would also influence the abundance of actinobacteria in soil. For example, the addition of calcium carbonate ($CaCO_3$) to an acid soil results in a marked increase in actinobacteria population (Waksman, 1967; Starr, 1981).

Soil is a very complex substrate in which numerous factors influence the number of actinobacteria as well as the qualitative composition of its microflora. The important factors controlling the abundance of actinobacteria in the soil are nature and abundance of organic matter, reaction, relative moisture content, temperature, aeration of soil or oxygen supply, and soil vegetation. Actinobacteria are less favoured by higher moisture content than the bacteria. They are able to grow well at relatively low moisture, even at 15 to 20 percent of the moisture-holding capacity of the soil (El-Tarabily and Sivavithamparam, 2006).

There is a marked difference in the nature of actinobacteria types found in the different soils. Some of the species are very abundant and are found in several soils, whereas others are only of limited occurrence and are detected only seldom in one or two soils. At different depths of soil, there is a different proportion of actinobacteria, as compared to the total soil microbiological population (Starr, 1981). Based on a study of

two soil samples taken from different depths in a closed landfill in Southern Finland, the actinobacterial indicator was higher in the upper 2 metres of soil profile and was only found in low amounts below 2.5 metres (Bjorklof *et al*., 2009).

Actinobacteria, including streptomycetes and certain nocardia, occur abundantly in and around the root systems of higher plants. Some forms produce yellowish, orange or black pigments in organic media. Some are spiral producing and others form straight aerial mycelium (Waksman, 1967; Starr, 1981). Mangrove, unique woody and plant communities of intertidal coasts in tropical and subtropical coastal regions, are highly productive ecosystems though surprisingly little is known about the microbial communities living therein, although there is evidence that mangrove sediments contain high populations of micromonosporae and novel actinobacteria. Studies done by Hong *et al*. (2009) proved that *Micromonospora* and *Streptomyces* strains were present in dominant numbers among the samples isolated from mangrove forests in Fujian, Guangdong, Guangxi and Hainan Provinces in China. Eccleston *et al*. (2008) also reported that micromonosporae isolates could decompose complex organic compounds, such as cellulose, lignin and chitin that are not readily decomposed by the majority of aerobic bacteria and thus tend to accumulate in sediments. The isolation procedures undertaken from leaf specimens subjected to decomposition yielded greater densities of actinobacteria than those specimens without signs of decomposition. Accordingly, micromonosporae which are found to be predominantly associated with mangrove mud sediments in the study can be seen as important components of benthic microbial communities within mangrove habitats, and are active in the degradation of complex organic materials.

In the earthworm digestive tract, actinobacteria play an important role as a kind of useful bacteria which not only help in digestion but also produce a large range of useful chemicals. It has been confirmed by many studies that environments created by earthworms contain antifungal compounds like streptomycin, erythromycin and terramycin produced by actinobacteria living in the body of earthworms. These antifungal compounds are responsible for suppressing many kinds of fungal pathogens attacking plants, such as *Fusarium oxysporum* (Jayasinge and Parkinson, 2009)

A study done by Kristufek *et al*. (1993) has identified *Streptomyces diastochromogenes* subsp. *Variabilicolor* n. subsp., which is characteristic of soil within the digestive tracts of the earthworm. Meanwhile, a study done by Polyanskaya *et al*. (1996) has found that the actinobacteria community present in the intestine of earthworms, mainly *Streptomyces caeruleus* NBRC 13344, develops better in the intestines in comparison to soil and helps the earthworms to metabolize organic matter and decomposition of substances from plant origin. These studies indicate that the earthworm gut is promising and can be an important source of adopted habitat possessing antifungal activity. They should receive higher attention in research for biological controls worldwide.

Studies on the microbial diversity by 16S rRNA gene analysis showed that a group of high-GC actinobacteria are dominant in marine sediments. Within the marine sediments, taxonomically diverse bacterial groups 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 microorganisms (Eccleston *et al*., 2008). Actinobacteria showed many interesting activities in water, such as degradation of starch and casein, and

production of antimicrobial agents. With these bioactivities, actinobacteria play an important role in the webs of the marine environment. Studies done by You *et al*. (2005) showed that streptomycetes normally predominate at shallow depths, and actinoplanes (belonging to the genus *Micromonospora*) increase with increasing depth.

Micromonosporae are frequent inhabitants of aquatic habitats worldwide. They have been isolated from water samples from streams, rivers and lakes, from lake mud, river sediments, beach sands, littoral sediments and deep marine sediments. Micromonosporae have been found to be the dominant actinobacteria group in a range of aquatic environments, particularly in the deeper mud layers as well as in deep sea sediments (Eccleston *et al*., 2008) However, actinobacteria occur in seawater to only a limited extent, their occasional presence being due largely to land contamination or to algal material floating on the surface of the sea. When enriched with petroleum hydrocarbons, seawater permits the development of certain types of actinobacteria, notably *Nocardiae* and *Micromonosporas* (Waksman, 1967; Starr, 1981).

The large numbers of actinobacteria are also living in composts of stable manures and plant residues especially in high-temperature composts that frequently attain temperatures of 50 to 65°C and may even reach 80°C due to the activities of the microorganisms bringing about the decomposition. This habitat comprised almost entirely of certain specific genera related to *Streptomyces* and *Micromonospora* (Waksman, 1967; Starr, 1981).

Actinobacteria are also found in the air, both as mycelium and as spores. Because of the ability of actinobacteria to withstand desiccation, their presence is believed to be a result of the breaking-up of fine soil particles suspended in the

atmosphere and the drying of water drops. Wind currents are largely responsible for their wide distribution (Lima *et al*., 2006).

Actinobacteria are found extensively on and in various food products that are often causing considerable spoilage and bringing about some odour. This odour, characteristic of many species, is often referred to as 'earthy' since it is similar to the odour of well-aerated soil. Actinobacteria are also responsible for some of the undesirable odours and flavours in fresh milk. Their occurrence has been demonstrated in the cow's udder. They also occur in cacao beans and in a variety of other foodstuffs to which they cause undesirable flavours (Waksman, 1967; Starr, 1981).

2.3 Fungal Infections

2.3.1 Fungal Infections of Humans

Fungal infections that are caused by eukaryotic organisms generally present more difficult therapeutic problems than do bacterial prokaryotic infections. Ongoing demographic trends would tend to strongly suggest that the number of fungal infections will continue to increase due to the aging of the population in developed countries. Fungal diseases in humans can be classified into allergic reactions to fungal proteins, toxic reactions to toxins present in certain fungi and infections (mycoses) (Barret, 2002).

Candida spp. are the fourth most common cause of bloodstream infections in hospitalized patients. Up to 40% of patients with *Candida* strains isolated from

intravenous catheters have underlying fungemia and the mortality rate of patients with catheter-related candidemia approaches 40% (Kuhn *et al*., 2002).

Candida albicans normally live in the human mouth and gastrointestinal tract. Under common circumstances, *C. albicans* lives in 80% of the human population with no harmful effects, although overgrowth results in candidiasis. The syndrome was recognized sixty years ago as a result of the interactions of *C. albicans* with body tissues and fluids resulting in vaginal, mouth, throat and gastrointestinal infections. Candidiasis may also occur in the blood and in the genital tract (Kambizi and Afolayan, 2008).

Systemic fungal infections of *C. albicans* have emerged as important causes of morbidity and mortality in immunocompromised patients (for examples, AIDS, cancer chemotherapy, organ or bone marrow transplantation). Infections are difficult to treat and can be very serious, where 30 to 40% of systemic infections result in death. Increasing resistance in *Candida albicans* in patients with AIDS has been reported, where the typical rates are 10 to 15%. In addition, hospital-related infections in patients not previously considered at risk (for example, patients in an intensive care unit) have become a major health concern (Dieterich *et al*., 2002).

The *Candida* yeast colony normally lives as a saprophyte, which is by consuming dead tissue rather than living cells. However, the colony can become a pathogen when it is allowed to grow beyond its current food supply or when an event impacts the growth of the bacterial colonies that limit the *Candida* colony's expansion. Such events include the introduction of antibiotics, cortisone, birth control pills, or artificial hormones. The *Candida* colony is not directly affected by these drugs, and

when the competing bacteria are killed, the *Candida* expands rapidly. The colony overgrows its normal food supply and easily makes the transition from saprophyte to pathogen and continues to thrive on living tissue. The *Candida* colony is therefore 'opportunistic' as they will overgrow whenever the body's resistance is lowered by nutritional deficiency, infection or a debilitating agent or drug. The colony will increase its area of tissue involvement after conversion to the pathogenic form. This growing tissue involvement will ultimately result in death from blood poisoning known as *Candida* septicemia. The role of *Candida* in blood poisoning and death has recently become more evident as physicians treat AIDS patients whose immune systems are ineffective against the pathogenic effects of *Candida* overgrowth. The *Candida* colony that exceeds the environmental food supply will readily transform from its rounded yeast form to a puncturing mycelia form and secrete numerous toxins (Kambizi and Afolayan, 2008).

Septicemia is the most common severe infection encountered in the neonatal intensive care unit (NICU). Depending on the institution, *Candida* species rank second to forth as the most frequent cause of late-onset sepsis in vey low birth weight (VLBW) infants. Occurring 72 hours after birth, late-onset sepsis is referred to as nosocomial sepsis to emphasize the possibility of transmission in the NICU. Besides *C. albicans*, *Candida parapsilosis* has also emerged as the predominant fungal pathogen causing bloodstream infections (BSIs) among VLBW infants in some NICUs (Reiss *et al*., 2008).

Crude mortality caused by *Candida* species infections in the NICU was estimated at 25 to 30%. *Candida parapsilosis* sepsis of the newborn can have a high case-to-fatality ratio, which in one study resulted in death in 42% of 19 infants with *C.*

parapsilosis fungemia. Premature infants are susceptible to *Candida* infections because of an immature immune system and immature skin which is not an efficient barrier to *Candida*. Other risk factors are indwelling catheters of all types, broad spectrum antibiotics and steroid therapy. Transmission of *Candida* species to infants in the NICU may be via maternal contact, postnatally from healthcare workers (HCW) or from contaminated solutions and fomites (Reiss *et al*., 2008).

Although *C. parapsilosis* is described as a harmless commensal of the normal microflora residing on the skin surface, it is also an important pathogen commonly isolated from pathological lesions of the nails and skin. Furthermore, this species has recently emerged as an important nosocomial pathogen. Clinical manifestations include fungemia, endocarditis, endophtalmitis, septic arthritis and peritonitis. The shift from a non-pathogenic inhabitant into a pathogen is triggered by predisposing host factors or by iatrogenic factor such as antibiotics treatment (Ga'cser *et al*., 2005).

The presence of immunosuppressed individuals and the increasing use of diagnostic and therapeutic procedures requiring equipment and instruments which get contaminated and are difficult to sterilize has added to the risk of acquiring infection in modern hospitals. Very little published data is available on the incidence of hospital acquired infection in Malaysia. It is likely that such infections occur here regularly as they do in hospitals the world over. Prolonged hospital stay and the use of antifungal compounds alters the normal flora, both in the type of organisms and in their susceptibility towards antifungal compunds (Farida, 1981).

Over the past decade, the incidence of *C. parapsilosis* has dramatically increased. In fact, reports indicate that *C. parapsilosis* is often the second most

commonly isolated *Candida* species from blood cultures. Although it is often considered less virulent than *C. albicans*, it is the *Candida* species with the largest increased incidence since 1990 and even outranks *C. albicans* in some Asian hospitals (Trofa *et al*., 2008).

Amphotericin B is a polyene antibiotic that is the most successful substance currently available for the treatment of *Candida* spp. infections. The sensitivity of fungal cells to the drug depends on the characteristics of the sterol. Sensitivity to polyene antibiotics is a consistent feature of wild-type fungal strains. Experience with *Candida* species has suggested that the exposure of *Candida* species to sublethal concentrations of polyenes might lead to the development of resistant strains. Induced resistance to polyene antibiotics has been observed in *Candida* species including *C. albicans* and *C. parapsilosis* (Molzahn and Woods, 1972).

Amphotericin B has activity against a number of pathogenic fungi, and it is the treatment of choice for severe fungal infections (Rogers *et al*., 2003). Acquired resistance of isolates *C. albicans* on amphotericin B has been reported, but the frequency is very low. The problem has been seen more frequently on *Candida* species including *C. parapsilosis*. Most of the resistant yeasts have been recovered from immunocompromised patients who had received amphotericin B for prolonged periods. In a recent study, it has been found that there was a strong association between the *in vitro* decreased susceptibility to amphotericin B of *Candida* species isolated from severely immunocompromised patients with fungemia and subsequent poor clinical outcome. *Candida* species isolates resistant to 0.8μg of amphotericin B per millilitre were associated with a fatal outcome in many cases (Brajtburg *et al*., 1990).

With the increasing number of immunocompromised patients and the greater use of broad-spectrum antifungal agents, disseminated candidiasis has become a significant cause of mortality and morbidity. Treatment of disseminated candidiasis remains unsatisfactory because of a lack of non-toxic effective antifungal agents with favourable pharmacokinetic properties. There is an increasing need for safe and effective agents with high levels of tissue penetration and fungicidal activity against *Candida* species (Khardori *et al*., 1993)

2.3.2 Fungal Infections of Plants

Fusarium is a large genus of filamentous fungi widely distributed in soil and in association with plants. Most species are harmless saprobes and are relatively abundant members of the soil microbial community. Some species produce mycotoxins in cereal crops that can affect human and animal health if they enter the food chain.

Fusarium oxysporum is an asexual fungus that produces three types of spores: microconidia, macroconidia and chlamydospores. Microconidia are one or two celled, that is produced by *F. oxysporum* under all conditions, and produced the most within the infected plants. Macroconidia are three to five celled and are commonly found on the surface of plants that have been killed by *Fusarium* wilt. Chlamydospores can remain dormant in soil and infect other hosts for as long as 30 years, and all of these spores can spread through running water, on farm implements and machinery (Gutleb *et al*., 2002).

Fusarium oxysporum spores have also been proven to live on non-host plants in the absence of a susceptible host. This provides a means of survival for the fungus,

which remains virulent until a host plant appears. When non-host plants become infected, they show few, if any symptoms, and become a carrier of the pathogen. Underground rhizomes are often another means of spreading the disease. Bananas raised for human consumption do not produce seeds. So, rhizomes are used as 'planting material' to establish new banana plantations. When a banana plant is infected with *Fusarium* wilt, the rhizome usually doesn't show any symptoms, but if the infected rhizome is used to grow new bananas, the crop will always die of *Fusarium* wilt before the fruit matures (Gutleb *et al*., 2002).

A virulent strain of Panama wilt that can attack Cavendish (banana) was found in Asia in the 1990's. A virulent form of the disease, known as 'Tropical Race 4' (TR4), has destroyed banana plantations in Malaysia in the early 1990's and it continues to spread throughout Southeast Asia. The eminent extinction of bananas have shown serious implications for many farmers, who may soon be forced to consider producing other crops. Scientists are currently racing to develop new and genetically modified strains that are resistant to the fungus, yet this could potentially take decades to achieve. Even if a new variety was developed, it remains questionable as to whether it would be an acceptable alternative for a discerning general public.

Fusarium species are notoriously resistant to antimicrobial agents *in vivo* and *in vitro*. This organism is resistant *in vitro* to most antibiotics available and is resistant as judged by the poor results *in vivo* to either the antibiotic used or the poor virtual concentration obtained (Rowsey *et al*., 1979).

The oil palm, *Elaeis guineensis*, is the highest yielding among the oil-producing crops. The tree is a leading source of edible vegetable oil production in the world, with

production figures of more than 32 million tonnes of oil in 2003. In Malaysia, the hierarchy of palm oil as a leading cash crop has exceeded that of natural rubber and its importance has been further boosted by the recent introduction of bio-diesel.

Like any other crop, the oil palm also faces a lot of pest and disease (P&D) tribulations. From seed germination right up to field planting, the crop is exposed to several P&D problems, some of which are caused by fungi. Some of the P&D problems faced by oil palm industry are the basal stem rot, brown germ, upper stem rot, *Rhinoceros* beetles and bagworm. Among these, the most serious disease is Basal Stem Rot (BSR) caused by *Ganoderma boninense* (Shamala *et al*., 2006).

Ganoderma is a white rot fungus. It has been identified in Malaysia as the major pathogen on oil palm. It is considered to be from root to root contact, the usual perception, with spore spread from wind also possible. On the other hand, some researchers suggest that root to root contact may not be the manner of spread and that basidiospore dispersal or contact with residual inoculum is, although both, could be involved if true. Symptoms of BSR appear long after planting (Paterson, 2007).

For the past 50 years or more, BSR had been causing serious damage to the oil palm plantation in Malaysia and it is currently the most important disease of economic importance causing large amount of losses in revenue. This fatal disease is considered as the most serious disease of oil palm in Malaysia where losses can reach up to 80% after repeated planting cycles and causes severe economic losses during the past 10 to 20 years and continues to do so (Mazliham *et al*., 2007). In coastal estates where the disease has reached epidemic proportions, the pathogen can kill up to 85% of the original stands by the time palms are 25 years old (Shamala *et al*., 2006). Under severe

infestation situations, more than 50% of oil palm stand can be lost to the malady. The FELDA plantations, for example, recorded high incidence of the disease in the Peninsular Malaysia. It is estimated that if only 2.5% of the total acreage of the oil palm plantation in Malaysia is affected by BSR, the industry would lose about RM80 million each year (Markom *et al*., 2008).

Chemical treatments are considered as the immediate short-term control measures for BSR infection. The use of systemic fungicides, together with a correct technique of applications helps reduce the progress of the disease on the palm. Attempts to control BSR in the laboratory and field by the use of systemic fungicide, for example triadimefon, carboxin, carbendazim and methfuroxam, have been made by various workers and the results from the studies were all conclusive. However, current chemical control has not been effective and long lasting, even though *in vitro* screening has identified several chemicals that are effective against *G. boninense*. The effective use of chemical control for treatment of the infected palms is limited by the fact that both visibly infected and subclinical palms may harbour established infections by the time treatment is applied (Shamala *et al*., 2006).

2.4 Antifungal Activities of Actinobacteria

The emergence microbial pathogens and the increased threat of biological warfare have increased the demand for novel and efficacious antimicrobial agents. The need for less toxic, more potent antibiotics and the evolving resistance of major and opportunistic pathogens to existing antibiotics is among the medical problems posing challenges to the development of effective therapeutic agents (Basil *et al*., 2004). Although considerable progress is being made within the fields of chemical synthesis

and engineered biosynthesis of antifungal compounds, nature still remains the richest and the most versatile source for new antibiotics (Bredholt *et al*., 2008).

In the long list of currently available antibiotics in the market, antifungal antibiotics are very few, but they form a significant group of drugs having an important role in the control of life-threatening fungal infections (Thakur *et al*., 2009). New fungicides are needed in agriculture, food protection and medicine due to the increase in resistant pathogens, appearance of new infectious diseases, and the toxicity of currently used compounds. There are some strategies that have been employed to find new antifungal drugs including the use of formulations which can decrease the toxicity of known antifungals and intensified search of new bioactive drugs from natural sources (Badji *et al*., 2007).

The screening of microbial natural products continues to represent an important route to the discovery of novel chemicals and for development of new therapeutic agents. Microorganisms produce some of the most important medicines ever developed. They are the source of live saving treatments for bacterial and fungal infections. In spite of the success of the past secondary metabolite research, the number of terrestrial antibiotics seems currently being tremendously studied and enforced the search for metabolites in so far untouched habitats (Thakur *et al*., 2007). Antifungal agents are used in several fields including human and animal therapy, agriculture for protection of plant and crops, food industries and treatment of wood (Ouhdouch *et al*., 2001).

Chemical fungicides are extensively used in current agriculture practices. However, excessive use of chemical fungicides in agriculture has led to deteriorating human health, environmental pollution and development of pathogen resistance to

fungicide. Because of the worsening problems in fungal disease control, a serious search is needed to identify alternative methods for plant protection, which are less dependent on chemicals and are more environmental friendly. Microbial antagonists have been used for the biocontrol of fungal plant diseases. Many species of actinobacteria, particularly those belonging to the genus *Streptomyces*, are well known as antifungal biocontrol agents that inhibit several plant pathogenic fungi. The antagonistic activity of *Streptomyces* spp. to fungal pathogens is usually related to the production of antifungal compounds and extracellular hydrolytic enzymes, for example chitinase (Prapagdee *et al*., 2008). In this regard, actinobacteria deserve to be studied, as they are known to produce potent antifungal agents.

It has been estimated that approximately two-third of the thousands of naturally occurring antibiotics have been isolated from actinobacteria in nature. For example, the genus *Streptomyces* might produce at least 100,000 new compounds of biological interest (Thakur *et al*., 2007). In pure culture, some of the actinobacteria produce antibiotics, like cycloheximide, that possess antifungal properties (Waksman, 1967; Kominek, 1975). The rise of antibiotic-resistant pathogenic strains dictates an increasing need for the survey of unexplored and underexplored niche habitats for novel antibiotic-producing actinobacterial strains. Intense screening of actinobacteria especially rare actinobacteria is taking place all over the world (Ningthoujam *et al*., 2009).

There have been numerous studies done by scientists in order to discover the actinobacterial strains that have the potential to exhibit antifungal activity against pathogens. A study done by Fguira *et al*. (2005) found out that an actinobacterial strain, isolated from Tunisian oasis soil, has showed antifungal activity against *Fusarium* sp.

The cultural characteristic studies had strongly suggested that this strain belonged to the genus *Streptomyces*. The nucleotide sequence of the 16S rRNA gene exhibited 98% similarity with the 16S rRNA gene of *Streptomyces roseoflavus*. Meanwhile, based on a study done by Badji *et al*. (2007), one of the actinobacterial strains that have been isolated from Saharan soil has showed antifungal activity against numerous organism including *F. oxysporum*, *C. albicans* and *S. cerevisiae*. This strain was then identified as a novel *Nonomuraea* sp.

Based on a study done by Peela *et al*. (2005), 88 actinobacterial strains have been isolated from 26 marine sediment samples collected near nine islands of the Andaman Coast of the Bay of Bengal and out of this 88 strains, 17% of them showed antifungal activity against *C. albicans*. Most of these strains were identified as belonging to the genus *Streptomyces*. Meanwhile, a study done by Jimenez-Esquilin and Roane (2005) has found that among 122 actinobacterial strains isolated from the rhizosphere of low-altitude sagebrush root system, nine strains that have been identified as *Streptomyces hawaiiensis* showed antagonistic affect against *C. albicans* and *F. Oxysporum*, and five strains identified as *Streptomyces coeleorubidus* showed antagonistic affect against *F. oxysporum*.

Some actinobacteria species produce more than one antibiotic. For example, *Streptomyces fradiae* forms neomycin and fradicin. Meanwhile, *Streptomyces rimosus* produces oxytetracycline and rimocidin. Different strains of a single antibiotic-producing organism may form several antibiotics not even related chemically. For example, *Streptomyces griseus* strains may yield streptomycin, streptocin, cycloheximide, grisein and candicidin. A single strain may form several chemically-related antibiotic substances. This is true of the production of mannosidostreptomycin

and streptomycin by *S. griseus*. Meanwhile, neomycin B and C could be produced by *S. fradiae*. The same antibiotic may be formed by different species often found in different parts of the world. For example, cycloheximide can be produced by *Streptomyces noursei* and *S. griseus* (Dolezilova *et al*., 1965; Kominek, 1975).

Among all the genera of actinobacteria, the genus *Streptomyces* is represented in nature by the largest number of species and varieties. They differ greatly in their morphology, physiology and biochemical activities. The fact that the majority of antibiotic-producing actinobacteria are found among these species led to growing economic importance of this group of organisms (Badji *et al*., 2007). Over 200 gene clusters governing antibiotic biosynthesis in *Streptomyces* spp. have been isolated and analyzed, giving rise to a wealth of information regarding the enzymology of antibiotic formation (Volokhan *et al*., 2006).

Brevibacterium linens has long been recognized as an important dairy microorganism because of its presence on the surface of smear surface-ripened cheeses, such as Limburger. *Brevibacterium linens* has potential to be used as a biological control agent in dairy products and other foods to prevent mould growth and the production of mycotoxins. Antimicrobial biopreservatives have the potential to constitute suitable food preservatives that are safe, effective and acceptable to consumers, regulatory agencies and food industries if properly investigated and developed (Osman, 2004).

There is strong need for cheese surface flora exhibiting inhibitory properties against moulds, as mould growth makes the surface of the cheese unacceptable and may produce mycotoxins and aflatoxin (Rattray and Fox, 1999). The antifungal

compound produced by *B. linens* is not sensitive to several proteases, demonstrating that it may not be a proteinaceous substance. Therefore, the antifungal compound produced by this strain could probably survive the degradative effect of proteolytic enzymes (native milk enzymes, rennet and microbial enzymes) during cheese ripening when used to prevent mould growth and aflatoxin production (Osman, 2004).

The antifungal antibiotics of actinobacteria can be classified into polyenes and non-polyenic types. The polyene antibiotics, a category that includes nystatin, piramicin, amphotericin and candicidin, comprise a family of very promising antifungal polyketide compounds and are typically produced by soil actinobacteria. The biosynthetic gene clusters for this polyene have been previously investigated, revealing the presence of highly similar cytochrome P450 hydroxylase (CYP) genes (Lee *et al*., 2006). In this study, polyene CYP-specific PCR screening was done in order to determine the presence of unique polyene-specific CYP gene on selected actinobacteria isolates. The polyene CYP-specific screening approach may constitute an efficient method for the isolation of potentially valuable cryptic polyene biosynthetic gene clusters from various actinobacterial strains (Lee *et al*., 2006).

The polyene antifungal antibiotics comprise a family of type I polyketide macrolide ring compounds with 20 to 40 carbon backbones, containing three to eight conjugated double bonds. The primary antifungal mechanism by which these polyene antibiotics exert their effects is believed to involve specific binding to the ergosterol present in the fungal membrane and the formation of channels which allow for the leakage of cellular potassium ion (K^+) and magnesium ion (Mg^{2+}), eventually culminating in the death of the fungal cell. Although polyene compounds are limited with regard to their clinical use, due largely to their high toxicity and side effects, the

superior antifungal activities of polyene compounds are still being considered in the further development of improved antifungal drugs (Fjaervik and Zotchev, 2005).

Nystatin, produced by *Streptomyces noursei*, was the first polyene macrolide antifungal antibiotic to be discovered (Borgos *et al*., 2006). It is used in human therapy for treatment of certain types of mycoses. Its antifungal activity is facilitated through its binding to sterols in the cell membrane, which leads to leakage of intracellular compounds (Nedal *et al*., 2007). Nystatin has no affect against bacteria (Jonsbu *et al*., 2001). Nowadays, nystatin is mainly being used for tropical treatment of *Candida* infections and the world market for nystatin is estimated to have a yearly turnover in the range or 250 to 300 million US dollars (Jonsbu *et al*., 2001).

Nystatin has a lactone ring of 37 carbon atoms that contains a characteristic diene-tetrane chromophore. The synthesis of the lactone ring starts with acetyl-CoA and proceeds with elongation of 15 melonyl-CoA and three methylmalonyl-CoA. One mycosamine is linked to the lactone ring, probably during secretion of the antibiotic. It has been suggested that biosynthesis of the precursors takes place by non-specific methylmalonyl carboxyltransferase from oxaloacetate to acetyl-Coa/propionyl-CoA in cooperation with phosphoenol-pyruvate carboxylase for regeneration of oxaloacetate. An alternative pathway for the synthesis of methylmalonyl-CoA is through rearrangement of succinyl-CoA by methylmalonyl-CoA mutase, which was proved for the synthesis of erythromycin by *S. erythreus*. The polyketide chains of polyene macrolide antibiotics are synthesised by a large, type I, multifunctional polyketide synthase, where each active site carries out one reaction in the assembly of precursors and modification of the growing carbon chain (Jonsbu *et al*., 2001).

Besides nystatin, candicidin is also an aromatic polyene antibiotic produced by *Streptomyces griseus*. It was first described on 1953 and named antibiotic C135, although it was renamed candicidin because of its strong activity against species of *Candida* (Campelo and Gil, 2002). Candicidin is a strong antifungal agent and has shown promising results in the treatment of candidiasis infections. In fact, it is believed to be the most potent of the new group of antifungal compounds. To date, use of candicidin vaginal ointment appears to be the most effective therapy for candidiasis vaginitis.

Streptomycin is an antibiotic derived from soil bacteria, S*treptomyces griseus*. It is used to protect crops from spoilage by controlling fungi and algae growth. It is known as a General Use Pesticide (GUP) whish is registered for use on commercial fruit and vegetables and also on non-food crops such as tobacco. Streptomycin acts by interfering with a microorganism's ability to synthesize certain vital proteins and damaging cell membranes of susceptible microorganisms (Sutton *et al*., 1978).

Cycloheximide is an inhibitor of protein biosynthesis in eukaryotic organisms, produced by *Streptomyces griseus*. Cycloheximide exerts its effect by interfering with the translocation step in protein synthesis thus blocking translational elongation. Cycloheximide is widely used in biomedical research to inhibit protein synthesis in eukaryotic cells studied *in vitro*. It is inexpensive and works rapidly. Its effects are rapidly reversed by simply removing it from the culture medium (Sutton *et al*., 1978). Due to significant toxic side effects, including DNA damage, teragonesis, and other reproductive effects, including birth defects and toxicity to sperm, cycloheximide is generally used only in *in vitro* research applications, and is not suitable for human use as a therapeutic antibiotic compound. Although it has been used as a fungicide in

agricultural applications, this application is now decreasing as the health risks have become better understood (Sutton *et al*., 1978).

2.5 Molecular Characterisation of Actinobacteria

The successful isolation and enumeration of actinobacteria from the environment is usually achieved by dilution plate techniques with a medium containing selective nutrients and certain antibiotics. However, differentiation of the genera by physiological and biochemical methods is often time-consuming and laborious. The biochemical tests used rely on the expression of phenotypes that may not always allow differentiation between species or strains within a species (Shuhaimi *et al*., 2001).

Molecular biologist and microbiologists have been quick to recognise how molecular techniques could be used to answer major questions limiting the progress in strain differentiation. The rapid characterization and identification of microbiological isolates are prerequisite for an effective industrial screening program. Detection of duplicates is necessary to reduce redundancy in screening assays and help in the discovery of novel compounds (Zhao *et al*., 2004).

Rapid and accurate identification of aerobic actinobacteria is becoming an increasingly important objective for microbiology laboratories. Characterization of microorganisms is a crucial step in the screening for natural products of biocatalytical activities of industrial interest. In this context, the ability to exclude previously isolated organisms and to recognize microbial colonies on primary isolation plates that have developed from identical environmental propagules (dereplication), greatly assist the

choice of biological material for large commercial screening operations (Colquhoun *et al*., 2000).

In both clinical practice and screening, it is important to discriminate between organisms at the intraspecific level, i.e. to examine the genetic diversity within a defined species. Alternative methods of identification, including high-performance liquid chromatography (HPLC) and molecular technique have been applied. However, HPLC is limited by the inability to determine species-level identification. DNA amplification followed by PCR analysis has proven to be a more effective method of rapid identification (Patel *et al*., 2004).

Sequence-based identification is an alternative method of identifying clinical isolates that are either slowly growing or difficult to identify by biochemical profiling. Several reports have described the use of sequencing a portion of the 16S rRNA gene for identifying isolates of actinobacteria (Patel *et al*., 2004). 16S rRNA sequence data have proven invaluable in *Streptomyces* systematic, in which they have been used to identify several newly isolated *Streptomyces* spp. (Fguira *et al*., 2005).

However, some widely used molecular techniques such as small subunit rRNA gene sequencing lack the power to distinguish below the species level or between recently diverged species (Zhou *et al*., 2004). The ideal procedure for microbial characterization should be capable of operating on small, easily prepared samples, provide rapid and highly reproducible analysis and be capable of automation and handling high throughputs (Colquhoun *et al*., 2000).

In this study, selected actinobacterial strains are dereplicated by using enterobacterial repetitive intergenic consensus (ERIC)-PCR. The primers

corresponding to ERIC sequence coupled with the PCR technique (ERIC-PCR) is an approach to fingerprint the genomes of a variety of bacterial strains. ERIC was originally identified in the genome of *Escherichia coli* and *Salmonella typhimurium* (Wieser and Busse, 2000). The relative positions of the repeated sequences in the genome of a particular bacterial strain appear to be conserved in closely related strains and are distinct in diverse species and genera. Detected fingerprinting polymorphism between closely related samples is caused either by different localization of a repetitive sequence in the genome or by changes in the region between repetitive sequences (Fousek and Mraz, 2003). However, not much study has been done to prove the presence of this ERIC element in Gram-positive bacteria, including actinobacteria species (Sadowsky *et al*., 1996).

ERIC-PCR is a simple, rapid and inexpensive tool with sufficient discriminatory power and is applicable for characterization and genotyping of diverse types of DNA for many Gram-negative, and also some Gram-positive bacteria (Giovanni *et al*., 1999). It is also reproducible and advantageous over tedious and time consuming methods like Pulse Field Gel Electrophoresis (PFGE) and serotyping (Bishi *et al*., 2008).

ERIC elements are 126bp in size and are distributed throughout extragenic regions of the genomes of many bacterial genera (Mouwen *et al*., 2005). ERIC-PCR generated multiple distinct multiplication products of size ranging from approximately 50 to 3000bp. Discrimination among strains is achieved by analyzing individual electrophoretic profiles obtained from PCR products (Mouwen *et al*., 2005). The unique location of ERIC elements in bacterial genomes allows discrimination at genus, species, and strain levels based on the eletrophoretic pattern of amplification products.

The sequences contain highly conserved central inverted repeats and can be divided into two classes that do not share significant homology. Class I consists of the repetitive extragenic polindromic elements (REPs) and class II consists of the enterobacterial repetitive intergenic consensus (ERIC) sequences. Difference in band sizes represents polymorphisms in the distances between the repetitive elements of different strains.

3.0 Materials and Methods

3.1 Sources and Storage of the Strains

Fifty two actinobacterial strains were selected from the collection of Microbial Resources Laboratory, Institute of Biological Sciences, University of Malaya. Four yeasts (*Candida albicans, Candida parapsilosis*, *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*) and two filamentous fungi (*Ganoderma boninense* and *Fusarium oxysporum*) were obtained from Prof. Dr. S. Vikineswary, Mycology Lab, University of Malaya.

For the purpose of storage and preservation, the actinobacterial strains were prepared onto the surface of the Yeast Extract Malt Extract (ISP2) slant and incubated at 28°C for 7 days and kept at room temperature. Stocks of actinobacterial strains were also prepared in 20% (v/v) glycerol and kept at -20°C.

3.2 Characterization and KOH String Test

Yeast Extract Malt Extract (ISP2) plates were inoculated with actinobacterial strains, incubated at 28°C until good growth was observed. Actinobacteria colonies were initially characterized morphologically. Colours of aerial spore and substrate mycelium were visually recorded by using a colour chart (http://swiss.csail.mit.edu/~jaffer/Color/nbs-iscc.pdf).

The actinobacterial strains were also characterized morphologically by light microscopy by using Gram stain technique. By using a loop, a small sample of the actinobacterial colony was transferred to a drop of distilled water on a slide. The film was than allowed to dry. The dried film was fixed by passing it briefly through the Bunsen flame without exposing the dried film directly to the flame. The slide was then flooded with methyl violet and $NaHCO_3$ solution for up to one minute and washed off briefly with distilled water before draining. The slide was flooded with Gram's Iodine solution and allowed to act for about one minute before washing off with distilled water and drained. After removing the excess water from the slide, the slide was then flooded with aceton and washed off with distilled water and drained. The slide was flooded with safranin solution and allowed to counterstain for 30 seconds before washing off with distilled water and drained. The slide was examined at 100X magnification with a light microscope.

The strains were also observed for spore chain. This was done by using the cover slip method in which sterile square cover slips were inserted at an angle of 45° in ISP2 medium in petri dishes that has been individually cultured with the strains. The cover slips were removed after seven days of incubation, air-dried and observed at 400X magnification (Locci, 1989).

For KOH string test, a loopful of growth from an actinobacteria colony was emulsified on the surface of a glass slide in a drop of 3% KOH. The suspension was stirred continuously for 60s after which the strain would be considered Gram positive if no stringing occurred within the first 30s of mixing the strain in KOH solution. *Escherichia coli* was used as a negative control (Arthi *et al*., 2003).

3.3 Antifungal Assay

Antifungal activity was determined against *C. albicans*, *C. parapsilosis*, *S. pombe*, *S. cerevisiae*, *G. boninense* and *F. oxysporum* by the agar plug-diffusion method. The Sabouraud's Dextrose Agar (SDA) medium was lawned with three-day-old colony of yeast. A plug was cut from ten-day-old actinobacteria culture by using a sterile straw and transferred aseptically to the SDA medium. Each preparation was done in triplicate. Plates were examined for evidence of antifungal activities represented by an inhibition zone of the corresponding tested yeasts after 48 hours of incubation at 28°C. Nystatin and cycloheximide were used as the positive control (Barry and Thornsberry, 1985). The zones of inhibition of the test microorganisms were measured in millimetres.

For filamentous fungus, each actinobacterial strain was streaked onto one half of a Potato Dextrose Agar (PDA) plate and incubated at 25°C for ten days. An agar plug with actively growing fungal mycelia (two-week-old) was then placed near the front (20mm) of the actinobacteria grown on the plate. Each preparation was done in triplicate. Following 48h incubation, antagonism was determined by the distance between actinobacterial growth and fungal growth. The nearer the distance, the lesser antagonistic activity was presented. Nystatin and cycloheximide were used as positive controls (Crawford *et al*., 1993).

3.4 Molecular Studies

3.4.1 DNA Extraction

The actinobacterial strains that showed strong antifungal activity were streaked on ISP2 plates and grown for four days at 28°C. A loopful of the colony was transferred to 150µl Tris-EDTA buffer (pH8) containing Sigma glass beads, 2.5µl lysozyme (50mg/ml) and 5µl Proteinase K (20mg/ml) (Hopwood *et al*., 1985). The suspension was then vortexed to mix it and subsequently incubated at 37°C for two hours before being centrifuged at 13,400rpm for ten minutes. The supernatant was transferred to a new tube, incubated at 75°C for 15 minutes and kept at -20°C until required for use (Sambrook *et al*., 1989)

The quality of the crude DNA preparations was checked by agarose gel (0.8%, w/v) electrophoresis (30mins, at 100V, 0.5X TBE Buffer) with ethidium bromide (0.5µg/ml). The DNA preparation (4µl) was mixed with 3.0µl 1X DNA loading dye (Fermentas) and loaded onto the gel. The gel images were then captured using a computerized UV transilluminator (Cleaver Scientific Ltd.). 100bp DNA ladder (New England BioLabs) was used as a reference to estimate the size of bands (Sambrook *et al*., 1989).

3.4.2 Polyene CYP-Specific Fragment PCR Amplification

PCR amplification for selected strains was performed in a 50µl reaction mixture as table below:

Table 3.1 Reaction micture for Polyene CYP-specific Fragment PCR Amplification

Components	Volume of single reaction	Final concentration
Sterile distilled water	27.8μl	
10X PCR buffer	5.0μl	1X
3U *Taq* DNA polymerase	0.25μl	0.015U
0.5mM dNTP mix	1.0μl	0.01mM
10μM PEH1f primer	2.0μl	0.4μM
10μM PEH2r primer	2.0μl	0.4μM
25mM $MgCl_2$	7.0μl	3.5mM
Template DNA	5.0μl	
Total	50μl	

The PCR amplification profile was according to the following amplification profile:

Initial denaturation : 95°C for 1 minute

Denaturation : 94°C for 35 seconds
Annealing : 69°C for 40 seconds
Extension : 72°C for 2 minutes
} 30 cycles

Final extension : 72°C for 8 minutes

The amplified DNA fragments were subjected to 0.8% (w/v) agarose gel electrophoresis. Gels were submerged in 0.5X TBE buffer. The PCR product (4μl) was mixed with 3.0μl 1X DNA loading dye (Fermentas) and loaded onto the gel. The gel

was electrophoresed at 100V for 30min. 100bp DNA ladder (New England BioLabs) was used as a reference to estimate the size of bands and concentration of DNA.

3.4.3 ERIC-PCR

PCR amplification for selected strains was performed in a 25µl reaction mixture as table below:

Table 3.2 Reaction miture for ERIC-PCR

Components	Volume of single reaction	Final concentration
Sterile distilled water	4.32µl	
10X PCR buffer	2.5µl	1X
3U *Taq* DNA polymerase	0.83µl	1.13U
10mM dNTP mix	3.1µl	1.24mM
10µM ERIC 1R primer	5.0µl	2µM
10µM ERIC 2 primer	5.0µl	2µM
50mM $MgCl_2$	3.0µl	6mM
10% (v/v) DMSO	0.25µl	0.1%
Template DNA	1.0µl	
Total	**25µl**	

The PCR amplification profile was based on Versalovic *et al*. (1991) with slight modification as follows:

Initial denaturation : 95°C for 5 minutes

Denaturation : 95°C for 1 minute

Annealing : 40°C for 1 minute } 30 cycles

Extension : 65°C for 8 minutes

Final extansion : 65°C for 16 minutes

The amplified DNA fragments were subjected to 0.8% (w/v) agarose gel electrophoresis. Gels were submerged in 0.5X TBE buffer. 4µl of the PCR product was mixed with 3.0µl 1X DNA loading dye (Fermentas) and loaded onto the gel. The gel was electrophoresed at 100V for 30min. 100bp DNA ladder (New England BioLabs) was used as a reference to estimate the size of bands (Hopwood *et al*., 1985).

3.4.4 Partial 16S rRNA Gene Analysis

PCR amplification for selected strains was performed in a 50µl reaction mixture as table below:

Table 3.3 Reaction mixture for Partial 16S rRNA Gene Analysis

Components	Volume of single reaction	Final concentration
Sterile distilled water	33.8µl	
10X PCR buffer	5.0µl	1X
3U *Taq* DNA polymerase	0.25µl	1.5U
10mM dNTP mix	1.0µl	0.2mM
10µM 27f primer	2.5µl	0.5µM
10µM 1492r primer	2.5µl	0.5µM
25mM $MgCl_2$	3.0µl	1.5mM
Template DNA	2.0µl	
Total	50µl	

The PCR amplification profile was based on Savic and Vasiljevic (2006) with slight modification as follows:

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 minutes 30 seconds

Final extension : 72°C for 10 minutes

The amplified DNA fragments were subjected to 0.8% (w/v) agarose gel electrophoresis. Gels were submerged in 0.5X TBE buffer. 4µl of the PCR product was mixed with 3.0µl 1X DNA loading dye (Fermentas) and loaded onto the gel. The gel was electrophoresed at 100V for 30min. 100bp DNA ladder (New England BioLabs) was used as a reference to estimate the size of bands (Hopwood *et al*., 1985).

3.4.5 Purification and Partial Sequencing of 16S rRNA Gene

The 16S rRNA gene products were purified by using QIAGEN (QIAquick® PCR purification kit, USA) and sent for sequencing to 1st Base (Shah Alam, Malaysia), which used Applied Biosystems 3730X1 DNA Analyzer.

3.4.6 Sequence Analysis

The sequence results were analysed by using BLAST search (Altsctiul *et al*., 1990) in the NCBI database (http://www.ncbi.nlm.nih.gov/blast). 16S rRNA gene sequences were then aligned. Neighbour-joining tree was generated using ClustalX software and performed by using Treecon software.

4.0 Results and Discussion

4.1 Morphology and Cultural Characteristics of Actinobacteria

Characterisation of isolates on an agar media plate was very important for practical purposes. Until recently, bacterial systematic was still based on the morphological and behavioural properties of microorganisms, as well as molecular approaches. Morphological study is important in the characterization of actinobacteria but it depends on the nature of the organisms, the composition of the medium and conditions of growth. Most of the colonies that formed on ISP2 media were dry, chalky, compact, raised with white aerial mycelium and adhered to the surface of the agar.

Based on morphological observation after incubation for 10 days at 28°C, all 52 actinobacterial strains used in this study can be divided into 20 colour groups, as shown in Table 4.1. The morphology of mycelium produced was varied among the actinobacterial strains studied. They were categorized into different groups according to the colour of aerial and substrate mycelium produced. Majority of the strains showed similar morphology with one another and they were categorized into the same group, Group I to Group XIII. Meanwhile, there were seven strains that were categorized into single-member group because they were not sharing similar morphological characteristics with the other strains studied. In addition, almost half of the total strains produced diffusible pigments, with the brown colour series being the most abundant. The diffusible pigment production by each of the strains was examined on ISP2 medium.

Table 4.1 Characterization of strains based on aerial and substrate mycelia colour and diffusible pigment on ISP2 medium.

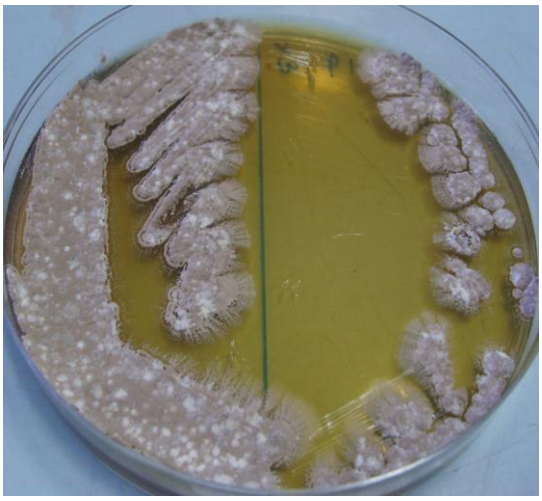
Group	Strain	Colony morphology		
		Aerial mycelium colour	**Substrate mycelium colour**	**Diffusible pigment**
I	CM1, CM2, CM4, CM17, MA04014, RA1, RA6	None	Dark yellow	None
II	CM15, MA04007, MA04013, OPCB, MA04020	White	Light Brown	None
III	CM8, CM14, MA04117, OPCC, RA2	Black	Dark brown	Dark brown
IV	CM6, CM11, OPSe, OPB1, RA5	Dark yellow	Brown	None
V	4, RHRS, OPSb, RHOP	Grey	Brown	None
VI	HT7, RA10, RAB1, RC1,	Pale yellow	Yellow	None
VII	CM3, CM10, A12	None	Dark yellow	None
VIII	CM5, RAPC	White	Light yellow	None
IX	CM9, MA04119	Grey	Light brown	Light brown
X	CM7, H23	Grey	Grey	None
XI	A21, RB2	Dark brown	Dark brown	Dark brown
XII	MA04120, RA9	Dark olive	Light brown	Dark brown
XIII	CM16, RC3	Light grey	Light brown	Pale yellow
XIV	9	White	Brown	Pale yellow
XV	CM13	None	Dark brown	None
XVI	MA04111	Black	Light orange	Light brown
XVII	A15	Reddish black	Black	Dark brown
XVIII	OPSa	Light grey	Brown	None
XIX	PDA4	Grey	Brown	Light brown
XX	MA04118	Brownish orange	Dark red	Dark brown

The actinobacterial strains showed the production of aerial mycelia ranging from white to black (Table 4.1). However, strains in Groups I, VII and XV were not showing any production of aerial mycelia. The variety of aerial and substrate mycelia produced have shown that there were a variety of actinobacterial strains abundant in

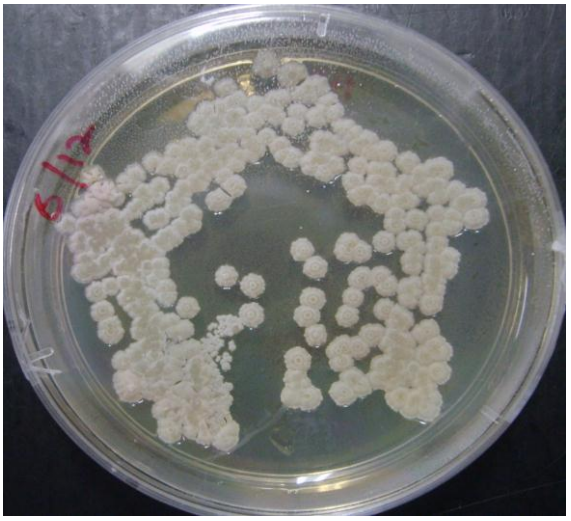
nature. In this study, the actinobacterial strains used were previously isolated from different samples taken from different habitats. For example, the strains 4, 9, A21, A15, OPSa and H23 were previously isolated from the earthworm gut samples, the strains MA04007, MA04013, MA04017, MA04119 and MA04020 were from various soil samples, the strains MA04111, MA04117, MA04118, PDA4 and MA04120 were from mangrove soil samples, the strains CM1 to CM17 were from the sediments of the Straits of Malacca, meanwhile the remaining strains were from soil samples taken from the palm oil and rubber plantations.

Colony morphology has also shown that the actinobacterial strains have margins ranging from circular and entire to irregular and undulate edges. For example, strains 9 and CM6 had circular colony edge, while strain H23 showed irregular colony margin (Figure 4.1). Under light microscopy observation, all of the strains produced branched filaments, as shown in Figure 4.2, except for the strains A21, MA04111, PDA4 and RC1, which produced non-branching filaments with straight to spiral structures. Gram stained slides of the isolated actinobacteria were visualized as purple or dark-blue coloured mycelia against a lighter background. In some strains, the branching patterns of the spore-bearing mycelia were also visible from the Gram stained glass slides. However, this was not applicable to most of the strains as the mycelium was always damaged most of the time during the preparation of the bacterial smear. To counter this problem, further visualization of the mycelium branching patterns were done using the coverslip method where there was minimal damage to the mycelia.

Strain MA04020

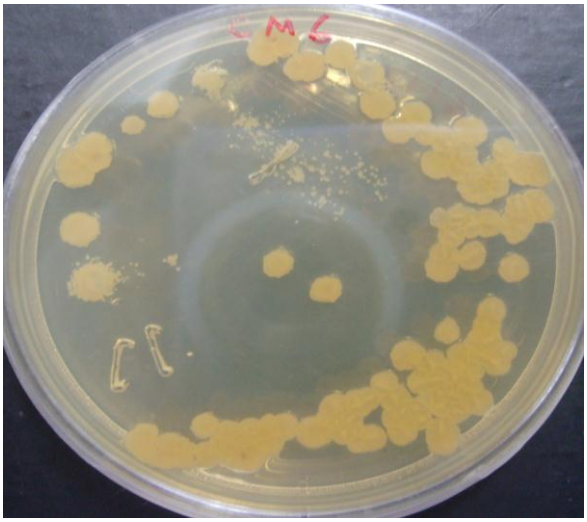


Strain PDA4



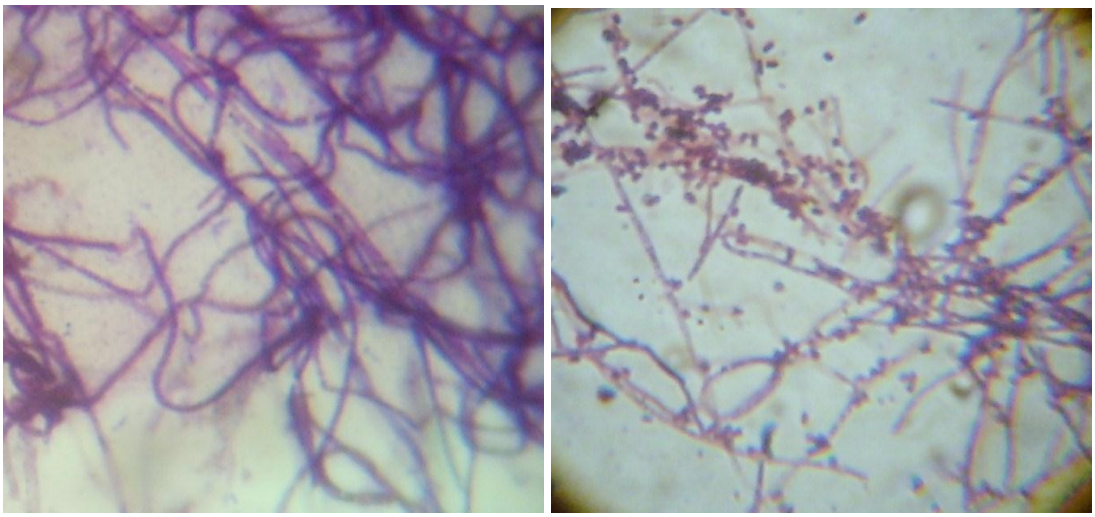
Strain H23

Strain 9

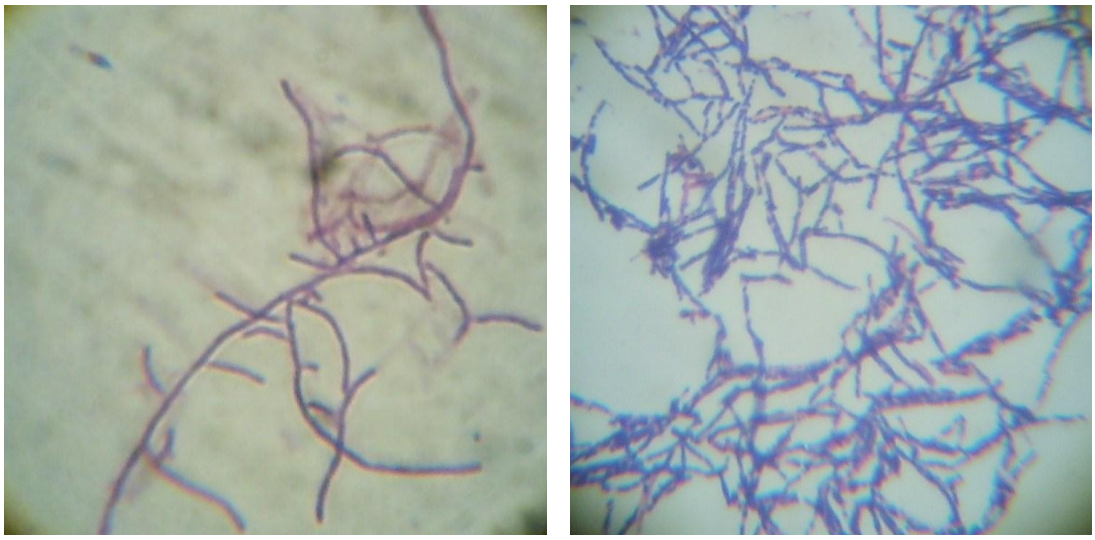


Strain CM6

Figure 4.1 Colony morphology of selected actinobacterial strains grown on ISP2 at 28°C for ten days.



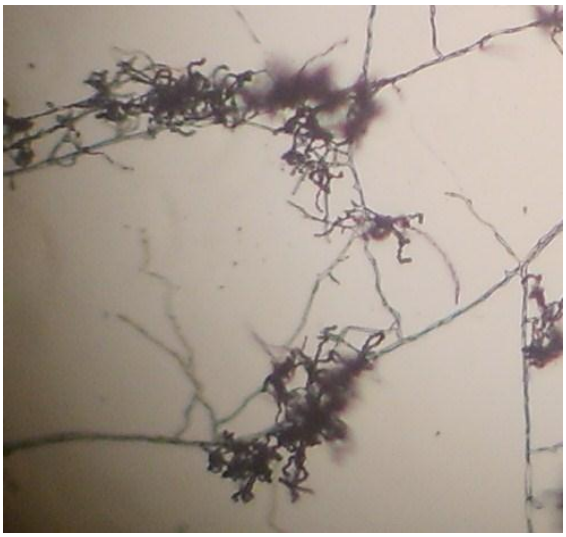
Strain CM3 Strain MA04020



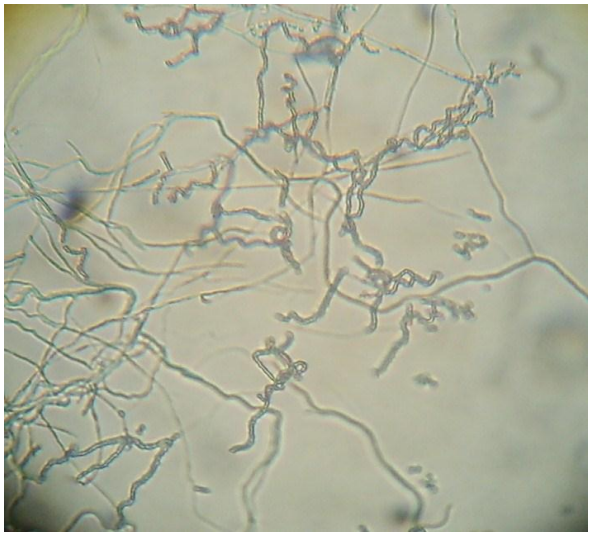
Strain H23 Strain OPSa

Figure 4.2 Gram stain of selected actinobacteria showing strains CM3, MA04020, H23 and OPSa. All strains were observed under 100X magnification with a light microscope and showing purple or dark-blue coloured mycelia against a lighter background (proved to be Gram-positive bacteria).

Strain 9



Strain A21



Strain H23

Figure 4.3 Spore chain morphology of actinobacterial strains on coverslip. All strains were observed under 100X magnification with a light microscope.

Besides that, differences in KOH solubility have been used successfully to categorise bacteria including actinobacteria which display variable Gram staining reactions. In this study, all 52 actinobacterial strains tested showed negative results for KOH string test. Negative results indicate that the strains are Gram-positive bacteria. Like the Gram stain reaction, the KOH test is based on the differences in the chemistry of the bacterial cell wall. The cell wall of Gram positive bacteria is not easily being disrupted when exposed to dilute alkali solutions (Arthi *et al*., 2003).

According to the shape of the spore chains observed under the light microscope (100X magnification), the strains were grouped as Rectus-Flexibiles (RF), Spira (S) and Retinaculiaperti (RA). The variety in spore chains observed among the actinobacterial strains in this study suggest that they are different from each other. The variety of spore chains depends on the species, showing straight or flexuous forms, hook, open loops and coils, which are used among other features to establish differences between them (Thakur *et al*., 2007). For example, the strain A21 was showing spiral spore chain, while the strain H23 was producing RA type of spore chain (Figure 4.3).

Group I contained the most number of actinobacterial strains with strains CM1, CM2, CM4, CM17, MA04014 and RA6 (Table 4.1). All of them were not producing any aerial mycelium or diffusible pigment on ISP2 medium used. The substrate mycelium produced was dark yellow in colour. However, based on their colony morphology, only strains CM1, CM2 and CM4 were producing irregular and undulate colony margin, whereas the remaining strains were producing circular and entire margin. All of the strains were non-sporing type of actinobacteria. Under the light microscopy, all of them were showing the presence of branched filament.

There were seven actinobacterial strains (9, A15, OPSa, CM13, MA04111, MA04118 and PDA4) categorized in a single-member group. The colour of the aerial and substrate mycelium produced were varied from white to reddish black aerial mycelia and from light orange to black colour of substrate mycelia. Strain 9 was the only strain that produced white aerial mycelia and brown substrate mycelia. Based on morphological characteristics, this strain showed the typical morphology of *Brevibacterium* spp. With branched, well-developed and fragmented aerial mycelia

(Figure 4.3a), producing colonies that were round with entire margins (Figure 4.1e), and showing smooth surface with pale yellow pigment. The strain did not form spores.

Almost all of the actinobacterial strains in this study showed morphology typical of *Streptomyces* spp. since the colonies were slow growing, aerobic, glabrous or chalky (as shown in Figure 4.1 for the strains MA04020, PDA4 and A21) and with aerial and substrate mycelia of different colours (Table 4.1). In addition, the colonies possessed an earthy odour. Most of the actinobacterial strains used were previously isolated from soil samples. For example, the strains MA04111, MA04117, MA04118, PDA4 and MA04120 were previously isolated from mangrove soil sample, the strain MA04020 from the soil sample taken from the bushes beside the Microbiology block of the Genetics and Microbiology Department, University of Malaya, while the strains RA1, RA2, RA5, RA6, RA9, RA10, RB2, RC1, RC3, RHOP and A12 were from soil samples taken from the palm oil and rubber plantation. This is consistent with the previous study where the genus *Streptomyces* forms the majority of the isolates from various soil samples (Thakur *et al*., 2007). However, more study into the physiological characteristics of the actinobacterial strains are recommended to be carried out in future including cell wall analysis where the presence of *LL*-DAP isomer on the cell wall will confirm the classification of the strains into the genus *Streptomyces*. However, due to time constraint, this has not done in the present study.

According to Shirling and Gottlieb (1966), the standard culture media for morphological studies, especially for colour determination for all cultures are ISP2, oatmeal agar, inorganic salts-starch agar and glycerol-asparagine agar. Because of this reason, the ISP2 medium was used in this study. The crosshatch plates that were prepared for morphological studies were also used for identification purpose.

Some of the actinobacterial strains in the study were isolated from earthworm gut samples and they showed similar characteristics to some of the actinobacterial strains isolated from soil samples. For example, in Group XI the strain 4 that was isolated from the earthworm gut sample showed similar morphological characteristics with the strains RHRS and OPSb which were isolated from soil samples taken from palm oil plantation. This might be due to the feeding habit of the earthworms that are also consuming material from the soil. In many cases, there is an increase in microbial population and activity during passage through the earthworm gut. Parle (1963) has showed that the number of actinobacteria increased 1,000 fold during passage through the gut and oxygen consumption remained higher in earthworm casts than in soil for 50 days, indicating an increased microbial activity. The gut of the earthworms act as 'bioreactors', where under ideal conditions of temperature, moisture and pH, desired strains of aerobic antinobacteria get multiplied.

The same observation was also noted between the actinobacterial strains isolated from sediment sample and soil sample. For example, in Group III the strains CM8 and CM14 isolated from the sediment sample taken from the Straits of Malacca showed similar morphological characteristics with the strains MA04117 isolated from mangrove soil sample and the strains RA1 and OPCC from soil sample taken from palm oil plantation. The dissolved and particulate materials in the sediments might have originated from the terrestrial material when the run-off occurred that brought them from the terrestrial to the sediments. The presence of actinobacterial strains in sediment might be due largely to land contamination or to algal material floating on the surface of the sea (Bredholt *et al*., 2008).

4.2 Antifungal Assays

4.2.1 Antiyeast Assays

The antifungal activities of the different actinobacterial strains on the tested yeasts are shown in Tables 4.2 and 4.3. Out of the 52 actinobacterial strains, eight showed strong antifungal activities (zones of inhibition of more than 15mm in diameter). Two strains (OPSa and RHRS) were highly active against *C. albicans*, one strain (MA04020) against *C. parapsilosis*, four strains (9, A21, OPSa and RC3) against *S. cerevisiae* and two strains (MA04119 and RHRS) against *S. pombe*. The broad (more than 15mm in diameter) clear zone appeared in the test lawn within 48 hours. None of the strains showed antagonism against all of the test yeasts.

Table 4.2 Antifungal activity of the actinobacterial strains, grown on ISP2 media after ten days, against *S. pombe*, *S. cerevisiae*, *C. albicans* and *C. parapsilosis*

Strain	**Antifungal activity**			
	C. albicans	***C. parapsilosis***	***S. cerevisiae***	***S. pombe***
9	-	-	++++	+
A21	++	+	++++	-
A15	+	-	-	++
OPSa	+++	++	+++	+
H23	-	+	-	+
MA04119	-	-	-	++++
CM13	-	-	-	++
RHRS	+++	-	+	++++
OPSe	+	+	++	++
MA04020	-	++++	-	+
RA5	+	-	+	++
RA6	-	-	-	+
RA9	+	-	-	+
RB2	+	-	+	+
RC3	++	-	++++	+
RHOP	+	-	+	+
Nystatin	+++	+++	+++	+++
Cycloheximide	+++	++++	++++	+++

- : Not showing any inhibition

+ : Showing zones of inhibition of 6-10mm diameter

++ : Showing zones of inhibition of 11-15mm diameter

+++ : Showing zones of inhibition of 16-20mm diameter

++++ : Showing zones of inhibition of more than 20mm diameter

The method used for screening of antifungal activity of 52 isolated actinobacteria was an agar plug diffusion method on the Sabouraud's Dextrose Agar (SDA) medium. Variable inhibitory patterns were evident. From the total number of actinobacterial strains, a spectrum of antifungal activity against yeasts tested was observed in seven of the total strains studied and appeared promising.

Figure 4.4 (a) shows antifungal activity of strain OPSa against *C. albicans*. The zone of inhibition produced was between 16-20mm in diameter. Strain MA04020 had shown a potential for producing antifungal agent when it showed strong inhibition activitiy (more than 20mm diameter of inhibition zone) against *C. parapsilosis*, as shown in Figure 4.4(b). Strains OPSa and MA04119 showed antagonistic activity against *S. cerevisiae* and *S. pombe* respectively (Figures 4.4c and d).

4.2.2 Antagonistic Activities against Filamentous Fungi

The assay on filamentous fungus was done qualitatively. The antifungal activities of the different actinobacterial strains on filamentous fungi are presented in Table 4.3. In the agar diffusion assay, strain H23 has shown strong antifungal activity with the wide clearing zone against *F. oxysporum*, as shown in Figure 4.4(e). This showed that the strain might have a potential in producing potent antifungal agent.

Meanwhile, strains OPSa, RHRS and A12 only showed a slight inhibition zone against *F. oxysporum* growth.

For the screening against *G. boninense*, strains 9 and PDA4 were found to have a significant degree of inhibition against *G. boninense* growth on the SDA plates. The inhibition activity of strain PDA4 is shown in Figure 4.4(f). Meanwhile, the activity of strain A15 against *G. boninense* was not very significant and only a very slight clearing zone was observed while the hyphal growth of the *G. boninense* was still present.

Table 4.3 Actinobacterial strains showing antifungal activity against *F. oxysporum* and *G. boninense*

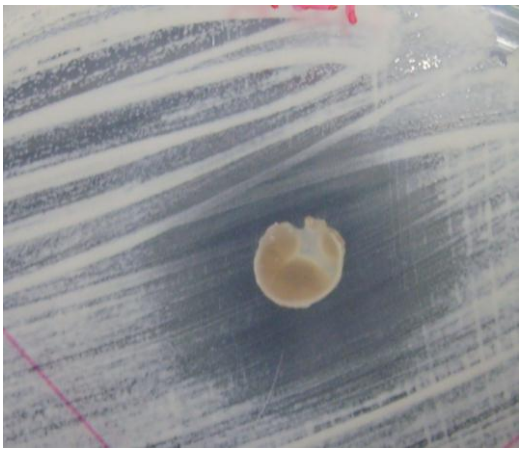
Strain	Antifungal activity	
	F. oxysporum	*G. boninense*
9	-	+++
A15	-	+
OPSa	++	-
H23	+++	-
PDA4	+	+++
MA04120	+	-
RHRS	++	-
RA1	+	-
RA2	+	-
A12	++	-
Nystatin	+++	++
Cycloheximide	+++	++

- : No antifungal activity

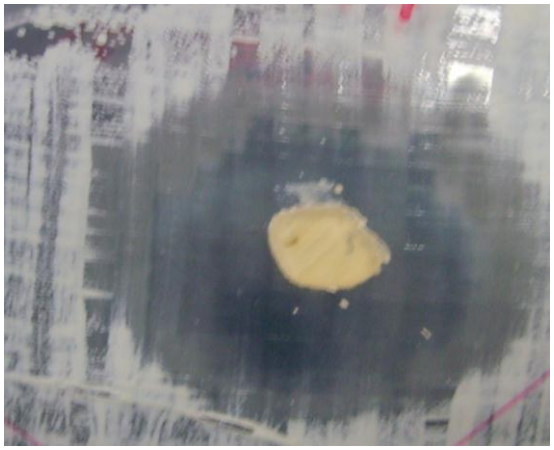
\+ : Very slight antifungal activity (less than 10mm)

++ : Slight antifungal activity (between 10 to 15mm)

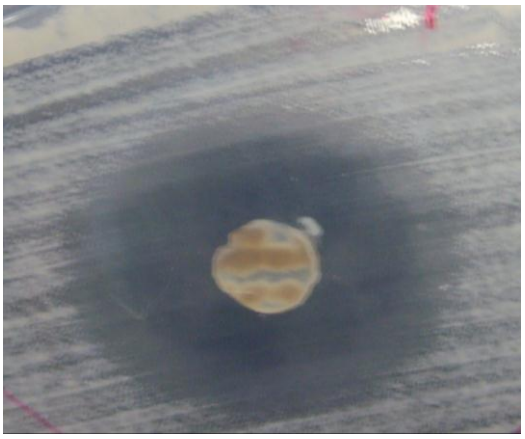
+++ : Strong antifungal activity (more than 15mm)



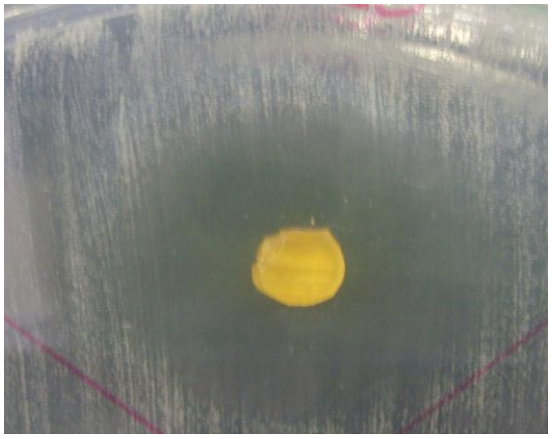
a) Strain OPSa against *C. albicans*



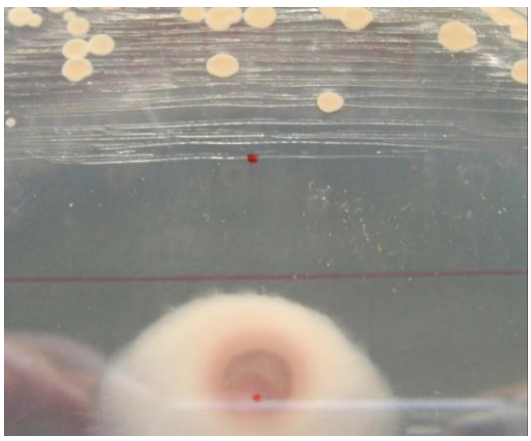
b) Strain MA04020 against *C. Parapsilosis*



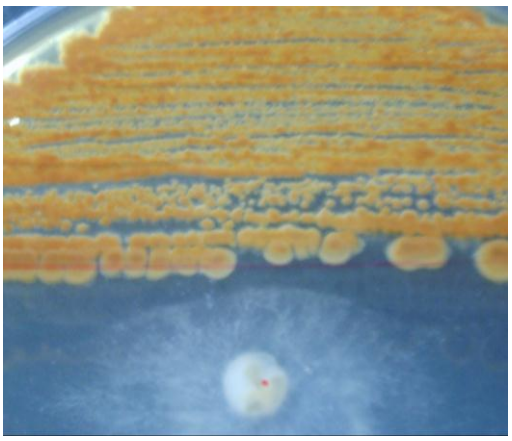
c) Strain OPSa against *S. cerevisiae*



d) Strain MA04119 against *S. Pombe*



e) Strain H23 against *F. oxysporium*



f) Strain PDA4 against *G. boninense*

Figure 4.4 Inhibition of yeast (a-d) and filamentous fungi (e and f) by actinobacterial strains.

Schizosaccharomyces pombe and *S. cerevisiae* are non-pathogenic model of fungal pathogens. They have been used in many studies as models in order to understand the mechanisms of action of certain metabolites and identify the targets of metabolite and explore their unique action in mammals with specific activities. This shows that *S. pombe* and *S. cerevisiae* are the better model for mammalian cell biology (Cardenas *et al*., 1999). Because of this reason, both *S. pombe* and *S. cerevisiae* were used as reference spesies for antifungal test in this study.

It is now possible to use *S. cerevisiae* and *S. pombe*, the most extensively studied eukaryotic microorganisms, to directly examine conserved pathways for their role in survival *in vivo*, which will improve the understanding of fungal pathogenesis (Goldstein and McCusker, 2001). They are established model organisms for investigation of the modes of action of antifungal and therapeutic compounds. They have also been used to study the biological properties of defensins, another class of molecule that play a role in plant defence against fungal attack (Simons *et al*., 2006).

In some cases, an antifungal agent is a broad spectrum agent. However, an antifungal agent or activity can instead be specific to one or more particular fungi. Both *Saccharomyces* spp. and mammalian cells are eukaryotic cells. They might be performing the same basis of targets for fungal-specific drug interactions. For example, the strain OPSa had shown strong antagonistic activity against both *C. albicans* and *S. cerevisiae*. This showed that the antifungal compound produced by this strain might be affective against the infection caused by *C. albicans* but at the same time having toxicity against human cells. Meanwhile, the strain MA04020 showed antagonistic activity against *C. parapsilosis* but did not show any antifungal activity against either *S. cerevisiae* or *S. pombe*. This shows that the strain MA04020 has the potential to

produce the antifungal compound which is effective against the *C. parapsilosis* infection without any toxic affect on human cells.

Antifungal agents that specifically target fungal cell wall synthesis, fungal DNA synthesis or enzymes in key fungal biology pathways are extremely useful in destroying or eradicating an infecting fungus, while having minimal toxicity, if any, to the affected subject. It is important to identify antifungal compounds that might be less toxic to many species than current fungicides. The ability to identify antifungal agents which are less toxic to the host organism specifically and the environment in general, is extremely advantageous both in the clinical and agricultural setting. Development of new antifungal agents is difficult because there are relatively few fungal genes and proteins that are not present in the human genome. Therefore a need exists to identify fungal genes that are sufficiently distinct from any human counterpart that they can form the basis of fungal-specific drug interactions, and specifically be used to identify drugs targeted against fungi.

There have been reports by many investigators on actinobacteria as sources of novel antibiotics with new modes of action. The frequency of the resultant antifungal activities was considered as an indicator of the capability of the different actinobacterial strains to produce bioactive metabolites. The biological activities of the actinobacterial strains can affect the growth of other microorganisms either through affecting the nutrient supply or the *in situ* production of secondary metabolites which stimulate or depress vegetative development (Taechowisan *et al.*, 2003). However, the characterization of metabolites involved in antifungal activities is not an easy task, and there is a need to have a better knowledge of antifungal activities, corresponding to a

species or genotype before understanding the chemical identification of compounds responsible for such activities (Basil *et al*., 2004).

Antifungal compounds are produced by some actinobacteria in order to overcome competition with other microorganisms in the same habitat by killing or destroying them. The samples from which the actinobacterial strains were isolated were having a rich fungal population which compete with them. As a means of adaptation, they produce antifungal compounds which are harmful to these fungi (Parungao *et al*., 2007). The ability to produce bioactive compound that play an important role in antifungal activity is dependent on the strain, environment (pH and temperature), substrate used and the diffusion rate of the bioactive compound produced by actinobacterial strain (Thakur *et al*., 2009).

In this study, the antifungal test was done by using the actinobacterial strains that has been incubated for ten days at 28°C since the best temperature range suitable for the production of antifungal compound by mesophilic actinobacteria was 27 to 30°C. Study done by Osman (2004) found that the formation of antifungal compounds always start from the logarithmic phase of growth, which vary among actinobacteria (normally at day one to day two of growth) and increase during stationary and death phases of growth (normally at day three to day ten of growth).

Out of 52 actinobacterial strains studied, only 10 (strains 9, A21, OPSa, MA04119, PDA4, A12, RHRS, MA04020, RC3 and H23) showed strong antifungal activity especially on *C. albicans*, *C. parapsilosis*, *F. oxysporum* and *G. boninense*. The remaining strains only showed slight or no inhibition activity at all. This shows that the other 42 strains do not produce any desired antifungal compound, or the environment or

media used in this study were not suitable to produce the antifungal compound. In this study, the media used was not set at specific pH. Based on study done by Valdes-Stauber and Schrer (1994), the linocin M18, produced by *B. linens* M18, was stable between pH 3.0 and 12.0, while linenscin OC2, produced by *B. linens* OC2, was stable between pH 2.0 and 11.0. These studies showed that the pH is one of the important factors for the actinobacteria strains to produce antifungal compound in order to show the inhibition activity against tested fungus.

A study made by Thakur *et al*. (2009) showed that being a mesophilic organism, *Streptomyces* spp. showed a narrow range of incubation temperature for relatively good growth and antibiotic production. Maximum mycelial growth (70mg dry weight/25ml medium) was recorded at 35°C, while production of bioactive metabolite was highest at 30°C. The increase of the incubation temperature from 25 to 35°C enhanced the growth of the cells. However, a higher incubation temperature (more than 35°C) had an adverse effect on growth and antibiotic production. The study also recorded that *Streptomyces* spp. could not grow when the initial reaction of the medium was adjusted to pH 5-6. The strain was able to grow in pH ranging from 7-8. However, biosynthesis of antifungal agent as well as growth was maximum at pH 7.5.

The study done by Suetsuna and Osajima (1990) showed that 28°C was the optimum temperature for griseohordin production by *S. californicus* JCM 6910. Meanwhile, study done by James *et al*. (1991) reported that granaticin, an antibiotic produced by *S. thermoviolaceus* required a pH of 6.5 to 7.0. Similarly, actinorhidin, a blue-pigment antibiotic, was produced extracellularly in *S. coelicolor* cultures at pH values around 7 (Bystrykh *et al*., 1996). A study done by Raytapadar and Paul (2001) proved that the maximum production of antifungal antibiotic IDA-28 by *S.*

aburavirensis depended on the optimum culture conditions. They have found that supplementation of medium with casein hydrolysate improved both growth and antibiotic titre where the yeast extract exhibited marked inhibition.

The ability of actinobacteria cultures to form antibiotics is not a fixed property but can be greatly increased or completely lost under different conditions of nutrition and cultivation. Therefore, the medium constitution together with the metabolic capacity of the producing organism greatly influence antibiotic biosynthesis. Changes in the nature and type of carbon, nitrogen or phosphate sources and trace elements have been reported to influence antibiotic biosynthesis in actinobacteria. In addition, antibiotic productivity has a tendency to decrease when metal ion deficient media is used and the strains are incubated for long periods at high temperatures. To achieve maximum production of antibiotic by any producer strain, it is necessary to optimize nutrient and environmental conditions (Thakur *et al*., 2009).

Shake flask condition is an essential parameter for optimum antibiotic yield as reported by many investigators. Thakur *et al*. (2009) has reported that the size of inoculum influenced the ability of *Streptomyces* spp. studied to produce the antibiotic in the tested cultures. It was found that the maximum growth and yield of antibiotic by the *Streptomyces* spp. were at an inoculum size of 2×10^9 spores per ml. These results showed that the growth and yield of antibiotic was influenced by the size of inoculum.

All the studies mention above had strongly proved that the production of any antifungal compounds are markedly influence by many factors including environment (pH and temperature) and components of the substrate used during the antifungal assay, growth phase of the actinobacterial strains and the size of inoculum. Further

investigation is needed to find out the best condition in order to optimize the production of antifungal activity by the actinobacterial strains.

In this study, only one method of antifungal screening was used. There are two techniques in screening the antifungal properties that should have be tried, secondary screening using liquid culture and secondary screening using shaken liquid culture. The secondary screening is a way to verify the consistent activity of the actinobacterial strains on the same test microorganism (Parungao *et al*., 2007). However, because of time constraint, both techniques could not be carried out. Besides that, further investigation is also needed in order to determine the active compounds that take part in the antifungal activity, as well as the structure of the compounds.

Some studies had indicated that the competitive effects of some organisms were largely due to specific antibiotic action (Stevenson, 1956). Some antifungal compounds, inhibit only closely related fungi. For example, candicidin was highly active against yeast, such as *Candida* species, but not against filamentous fungi (Waksman *et al*., 1965). However, there are also some antifungal compounds that could inhibit various fungal genera, including the filamentous fungi. For example, cycloheximide could exhibit an extraordinarily wide activity spectrum which includes strains of more than one genus, such as *Aspergillus fumigatus* and *Pseudallescheria boydii* (Salkin and Hurd, 1972; Berger *et al*., 1973).

The search for antifungal compounds requires a large number of isolates (over thousands) in order to discover an actinobacteria population with compounds of pharmaceutical interest. Because of this, the research will be more promising if diverse and more actinobacteria are screened. Such attempts need to be continued both in the

same area as well as from the adjoining places during various climatic conditions as to screen more isolates especially for novel therapeutics.

4.3 Molecular Characterization

4.3.1 DNA Extraction

The electrophoresis gel run for the DNA extractions in Figure 4.5 shows the presence of bands which indicate the presence of the genomic DNA. Ten bands with estimated molecular masses of more than 2000bp were observed. The band for strains H23, A21, MA04119, A12, and RC3 were very bright, indicating that the concentration of the extracted DNA was very high (more than 500mg/ml).

Two critical factors that have been taken into account in the DNA extraction protocols are the DNA yield and the DNA quality. The usual contaminant during DNA extraction is protein (Nguyen *et al*., 2009). In order to get good quality of DNA samples, the DNA extraction method has to be appropriate to the sample used. Therefore, it is recommended to do some modification in DNA extraction by adding 1% CTAB to the extraction buffer and a final purification step using mini-columns from DNA purification kit. Genomic DNA purification kit provides an easy and rapid way to purify high quality genome DNA from a variety of samples, including actinobacterial samples. The purified DNA obtained is suitable for PCR amplification procedures. Purification is based on the usage of the strong chaotropic agent which provides effective lysis of the cells, denaturation of proteins and release of genomic DNA (Siun and Beow, 2009).

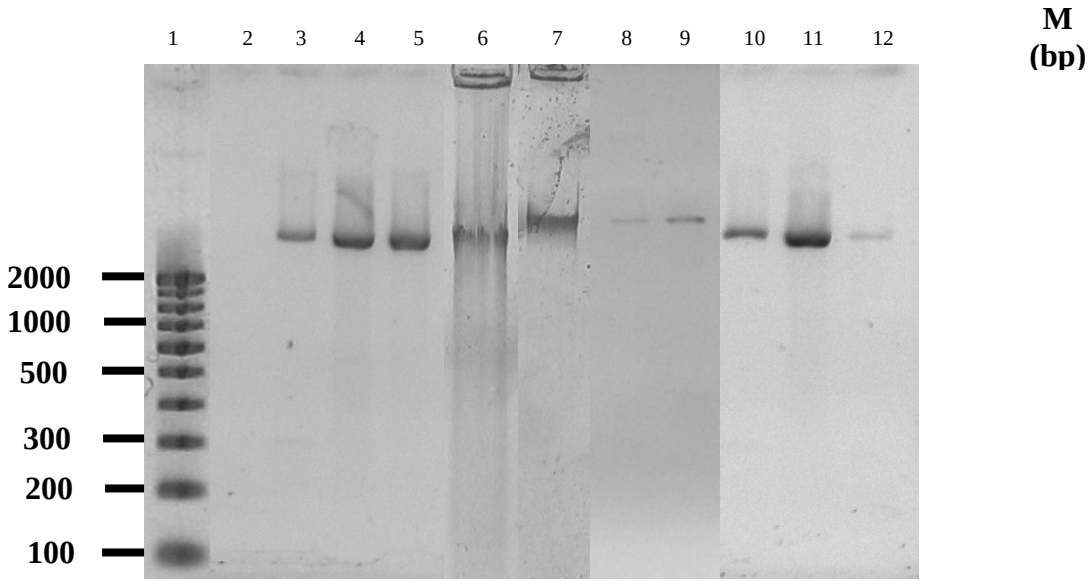


Figure 4.5 DNA isolated from selected actinobacterial strains; lane 1, 100bp ladder; lane 2, negative control; lane 3, strain H23; lane 4, strain A21; lane 5, strain MA04119; lane 6, strain 9; lane 7, strain MA04020; lane 8, strain RHRS; lane 9, strain PDA4; lane 10, strain A12; lane 11, strain RC3; lane 12, strain OPSa.

4.3.2 Polyene CYP-specific Fragment PCR amplification

In order to detect the presence of potential antifungal biosynthetic genes among the actinobacterial strains (as discussed in 4.2), polyene CYP-specific fragment PCR amplification was carried out. The gene polyene CYP-specific fragment is normally present in organisms that could inhibit the growth of fungus. The polyene antifungal agents form complexes with ergosterol and disrupt the fungal plasma membrane, resulting in increased membrane permeability, leakage of the cytoplasmic contents and death of the fungal cell. Thus, the polyenes are fungicidal and have the broadest spectrum of antifungal activity of any of the clinically available agents (Andriole, 1999).

Figure 4.6 shows the gel electrophoresis image of representative polyene CYP specific fragment PCR products of genomic actinobacterial strains tested. The ten actinobacterial strains which show antifungal activities were screened for the presence of polyene CYP-specific fragment genes in the genomes. Only four of them (strains H23, RC3, MA04020, and RHRS) produced the expected 350bp DNA fragment of the polyene CYP-specific internal region. This result suggests that these four strains might have the potential to produce valuable cryptic polyene biosynthetic gene, and antifungal compound is probably a polyene.

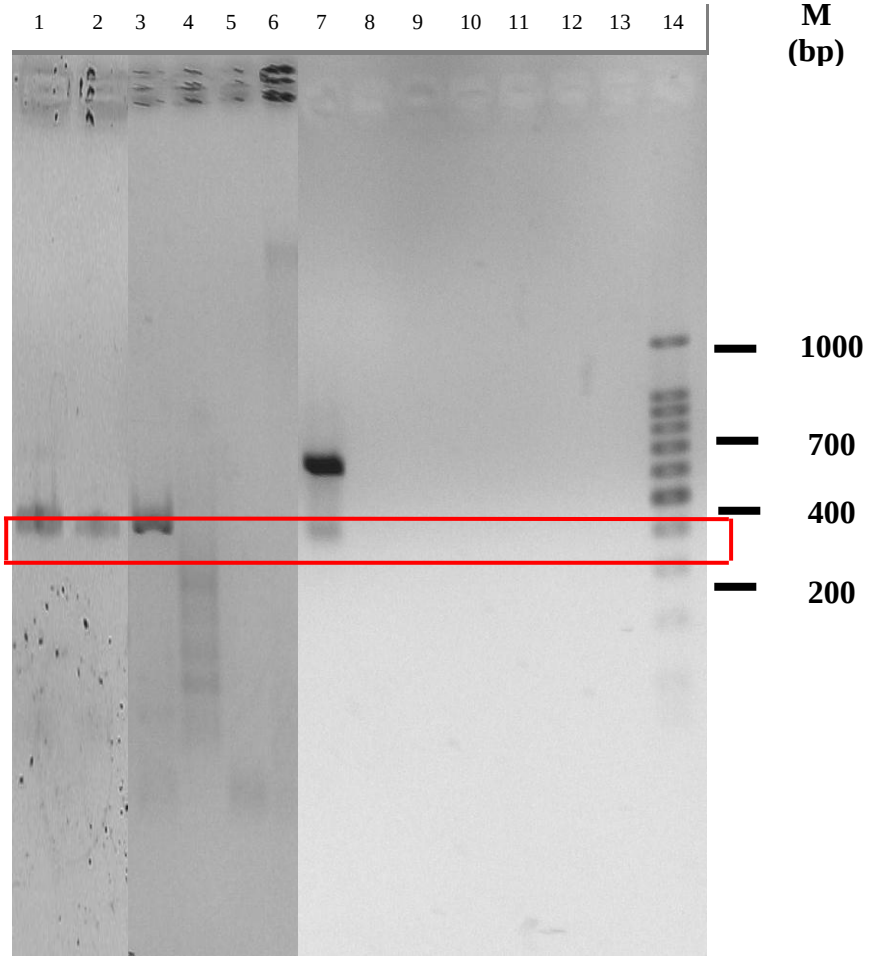


Figure 4.6 Polyene CYP specific fragment amplified from selected actinobacterial strains; lane 1, strain H23; lane 2, strain RC3; lane 3, strain MA04020; lane 4, strain MA04119; lane 5, strain 9; lane 6, strain 9; lane 7, strain RHRS; lane 8, strain A21; lane 9, strain OPSa; lane 10, strain OPSa; lane 11, strain A12; lane 12, strain A12; lane 13, strain PDA4; lane 14, 100bp DNA ladder.

Although actinobacterial strains had shown the antifungal activity against tested fungi, six of them did not show the presence of polyene CYP-specific fragment gene. This might be due to several factors. Since a PCR-based screening strategy using low stringency cycling conditions might influence the yield of PCR products, the possibility of presence of more polyene positive strains could not be completely ruled out (Hwang *et al*., 2007).

It is not currently known whether the detection of high amplification frequencies in a given actinobacteria population reflects a potential to produce secondary metabolites with biological activity. If so, combining the data derived from traditional molecular fingerprinting approaches based on the metabolic potential to produce secondary metabolites would enable screening efforts to focus on the most talented groups, increasing the chances of finding secondary metabolites with interesting biological activities (Ayuso *et al*., 2005).

Clear relationships can be established between the occurrence of biosynthetic gene sequences and the production of antifungal activities. It should be considered that not all of the sequences detected with the primers used are necessarily involved in the synthesis of industrially relevant bioactive metabolites and that the amplification products can reflect the presence of other genes involved in the biosynthesis of other types of metabolites, such as pigments or structural components of the microbial cell. It is also important to note that biological activities of interest are governed by the screen to which the strain is subjected (Ayuso *et al*., 2005).

The presence of glucose could affect the polyene production. Glucose is required for the biosynthesis of polyenes, but high levels of glucose have a negative

effect on the production of polyene antibiotics. Slow feeding of glucose to the fermentation have been shown to give rise to a considerable increase in the yields of nystatin and other polyene macrolides. Recent studies done by Fjaervik and Zotchev (2005) in batch cultures with *S. noursei* wild type strain demonstrated that the specific yield of nystatin (per biomass) was low under high glucose concentrations, or in the presence of a rapidly metabolised nitrogen source such as ammonium. The data indicated that growth rate is important in determining the specific rate of nystatin biosynthesis. This probably explains why complex nitrogen sources (for example, soybean meal) are traditionally chosen for the production of polyene antibiotics. In addition to the production of antibiotics, the antagonistic activity of fungi could also be caused by the production of other secondary metabolites, such as biologically active enzymes which are able to directly degrade the fungal cell wall.

The bands produced by strains H23, RC3, MA04020 and RHRS were faint (Figure 4.6). Meanwhile, no band was produced by the other strains (MA04119, 9, A21, OPSa, A12 and PDA4). It might be due to the low concentration of DNA template that caused the low concentration on PCR product. The availability of high-quality genomic DNA is a crucial prerequisite for molecular genetic analysis. The degrading or shearing of DNA samples could prevent the PCR amplification process to take place. During DNA extraction process, it is important to be aware of the concentration of $MgCl_2$ used. Too much $MgCl_2$ will result in smearing or extraneous bands. It may cause multiple bands to appear, as shown by strain RHRS (Lane 7, Figure 4.6). Moreover, the presence of extraneous bands might also be due to the non-specific band amplification. This non-specific band could be from the contamination of template DNA with foreign DNA (Rao, 1994).

If the target template DNA is present in very few copies or if there are inhibitory substances in the template DNA solution, it would normally not obtain a distinct single PCR product. A commonly used strategy to improve the yield in such cases has been to perform two successive PCR amplifications, with the products from the first amplification taking the role as template for the second amplification. Nested PCR is a very reliable way to get better PCR product when results are marginal for a number of reasons. This method could be used to eliminate extra bands and produce robust bands where the first PCR was messy, weak or even invisible. This method could also save genomic DNA. It needs very little of the primary product because it is of very low complexity (Wolff *et al*.,1995). However, because of time constraint, this method was not used.

During DNA extraction technique, Tris-EDTA buffer was used. EDTA is a powerful chelating agent. The remaining Tris-EDTA buffer in DNA preparation during PCR amplification might possibly bind with Mg used and thus inhibit the PCR. DNA preparations that use a minimum of Tris-EDTA buffer or any other chemicals, or techniques that can prevent inhibition of PCR such as DNA extraction kit, are optimal for creating PCR templates (Siun and Beow, 2009).

The non-producing band in strains MA04119, 9, A21, OPSa, A12 and PDA4 might also be due to the presence of other antifungal compounds or genes in the strain other than polyene CYP-specific gene. One example of compound that could also give the inhibition activity is chitinase. Chitinase is a glycosyl hydrolase which catalyzes the degradation of chitin, an important structural component for microorganisms including *C. albicans*, *C. parapsilosis* and *F. oxysporum* (Chapman *et al*., 1992; Madrid *et al*., 2003). A study done by Watanabe *et al*. (1999) has proved that ChiC (a gene that codes

for an extracellular chitinolytic enzyme) has the ability to inhibit hyphal extension of *Trichoderma reesei*. However, because of time constraint, the analysis to confirm the presence of this enzyme was not done.

Hwang *et al*. (2007) has stated that the toxicity of polyenes limits their usefulness as antifungal drugs. Therefore, the study on the presence of non-polyene antifungal agents is recommended to further study especially the agents isolated from the strains that had shown positive results in the antifungal test.

4.3.3 ERIC PCR

The ERIC-PCR study was carried out on ten selected actinobacterial strains (strains 9, A21, OPSa, MA04119, PDA4, A12, RHRS, MA04020, RC3 and H23) that showed the best antifungal activity against tested yeasts and filamentous fungi, especially *C. albicans*, *C. parapsilosis*, *F. oxysporum* and *G. boninense*. The banding pattern shows four to nine major bands with varying molecular sizes ranging from 1000bp to 300bp (Figure 4.7). 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 products varied, which showed the genetic diversity among different strains.

Lane 11 in Figure 4.7 was a negative control, where template DNA was not added to the ERIC-PCR reaction. The absence of an amplified DNA band in this lane indicated that these DNA fingerprints were free of any primer artefacts. In this study, the result was evaluated on the basis of visualization of banding patterns. There are several bands of equal mobility with a number of minor variations. This is consistent

with the concept that selection for a specialized niche affects genome organization which corresponds to a unique distribution of repetitive sequences in the bacterial genome (Koh-Luar *et al*., 1998).

Strains RHRS and OPSa has shown similar pattern of band (Figure 4.7). Strain RHRS was isolated from soil sample taken from oil palm plantations. Meanwhile, strain OPSa was from earthworm gut sample. Although they were isolated from different habitats, but because of the feeding habit of earthworms that also consume materials from the soil, this might give an indication that both of them are closely related. Besides that, strainMA04020 and RC3 s might also be related with each other. Both of them were isolated from soil samples. This is consistent with the concept that the strains isolated from the same sample might show similar distribution of repetitive sequences in the bacterial genome.

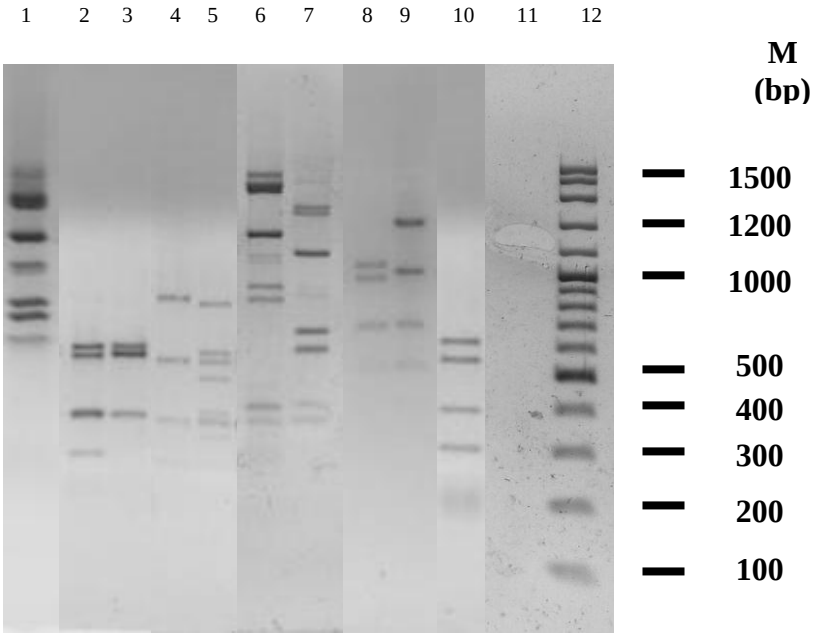


Figure 4.7 ERIC-PCR fingerprinting patterns of selected actinobacterial strains; lane 1, strain RHRS; lane 2, strain MA04020; lane 3, strain RC3; lane 4, strain PDA4; lane 5, strain A12; lane 6, strain OPSa; lane 7, strain H23; lane 8, strain MA04119; lane 9, strain 9; lane 10, strain A21; lane 11, negative control; lane 12, 100bp DNA ladder.

However, the other strains show variation in banding pattern is with three to nine major bands with varying molecular sizes mostly ranging from 1000bp to 300bp. From the banding pattern produced, the strains could be categorized into three groups, where strains RHRS and OPSa belong to one group, strains MA04020, RC3, PDA4, A12 and A21 into one group, while the another group comprises strains H23, MA04119 and 9.

The ERIC-PCR has become a powerful tool for the molecular genetic analysis of bacteria because it allows the fingerprinting of individual genera, species and strains (Yong *et al*., 2008). The ERIC sequences are highly conserved at the nucleotide sequence level but their chromosomal locations and numbers differ among species (Yong *et al*., 2008). So the ERIC-PCR method can serve as a valuable and sensitive tool for genetic differentiation of isolates from different sites. This study was conducted to determine whether ERIC-like sequences are present in the genomes of actinobacteria studied. The ERIC-PCR bands were compared based on the presence or absence of fragments at a specific position. The bands were present in tested strains, but there were differences in the intensity of some amplified fragments as well as in the occurrence of several polymorphic bands.

Discrimination among strains is achieved by analyzing individual electrophoretic profiles obtained from ERIC-PCR products. The unique location of ERIC elements in bacterial genomes allows discrimination at genus, species, and strain levels based on the eletrophoretic pattern of amplification products. Difference in band sizes represents polymorphisms in the distances between the repetitive elements of different strains (Shuhaimi *et al*., 2001).

ERIC-PCR has been proven to hybridize the genomic DNA from Enterobacteriaceae, however not much study has been done to prove the presence of this element in Gram-positive bacteria, including actinobacteria species. However, it is believed that the ERIC-PCR technique may be broadly applicable to fingerprinting all bacteria, especially actinobacteria species that possess such repetitive elements (Towner and Cockayne, 1993).

A recent study established that ERIC-PCR is not invariably directed towards ERIC elements and that under certain PCR conditions ERIC-PCR may generate fingerprints for almost any DNA sample (Giovanni *et al*., 1999). ERIC-PCR does not necessarily amplify bands directly from genuine ERIC sequences. ERIC primers may act as arbitrary or random primers as in Randomly Amplified Polymorphic DNA (RAPD). The use of larger primers (more than 22 nucleotides) and higher annealing temperature renders ERIC-PCR less sensitive to changes in reaction conditions. Although the banding patterns were reproducible and were able to type the unrelated strains, the real basis of discrimination was not clear. Therefore, the sequences that act as targets for ERIC primer within the genome in the isolates studied cannot be stated absolutely (Bishi *et al*., 2008).

The greatest advantages of genotyping by ERIC-PCR lie in its accessibility, speed, relative ease of use and general stability. Unfortunately, ERIC-does not always offer a complete picture of genetic relatedness. Identical bands are based on their size and not necessarily their genetic make-up. It is possible that two genetically diverse segments between two ERIC segments can be counted as equal, provided that the ERIC targets are the same distance apart on the DNA. The presence of ERIC sequences throughout the bacterial genome is only one of many molecular traits that can be used

to compare bacteria. Further detail could be obtained by combining ERIC-PCR data with additional rep-PCRs, such as REP elements, or the presence or absence of other genetic markers or virulence factors (Dyer, 2005).

4.3.4 Amplification of 16S rRNA Gene

Figure 4.8 shows the gel electrophoresis image of 16S rRNA PCR amplified gene product of actinobacterial strains tested. All the bands obtained from the PCR amplification of the strains, 9, H23, RHRS, MA04119, MA04020, OPSa, RC3, PDA4, A12 and A21, were visible. All the bands obtained from 16S rRNA amplified gene of actinobacterial strains were determined to be at the length of 1500bp.

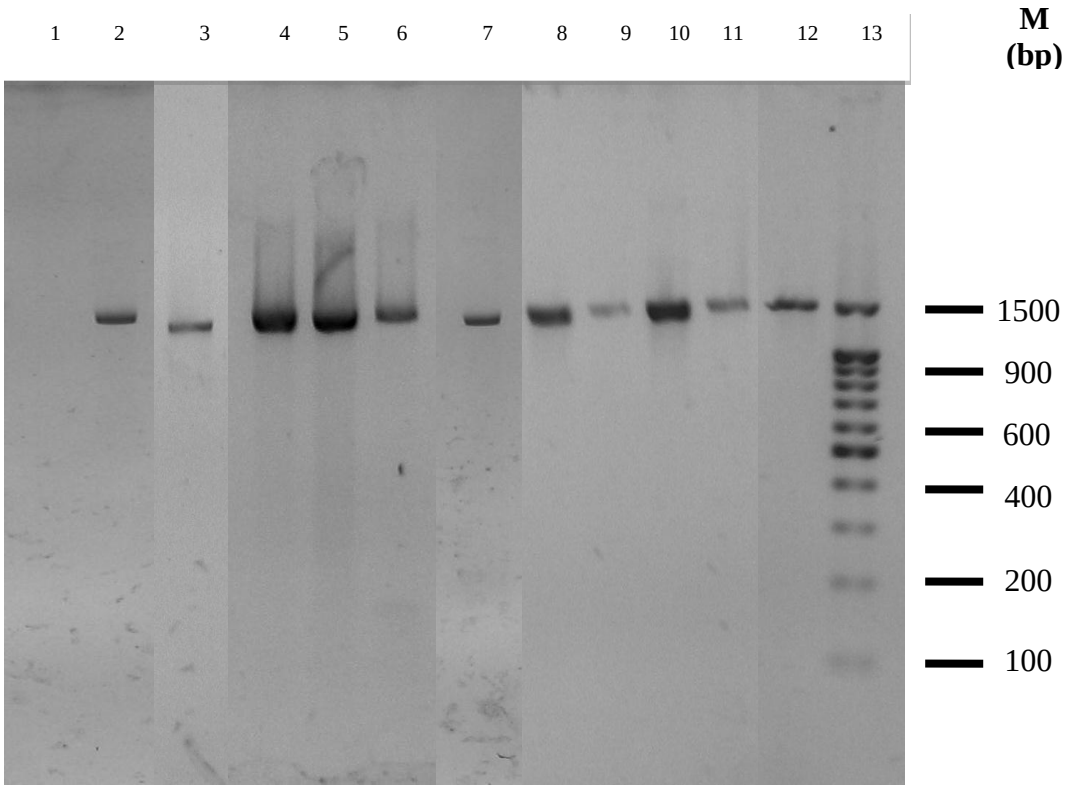


Figure 4.8 Partial 16S rRNA gene fragment amplified from selected actinobacterial; lane 1, negative control; lane 2, strain 9; lane 3, strain H23; lane 4, strain RHRS; lane 5, strain MA04119; lane 6, strain MA04020; lane 7, strain OPSa; lane 8, strain RC3; lane 9, strain PDA4; lane 10, strain A12; lane 11, strain A21; lane 12, 100bp DNA ladder.

The production of faint bands, for example strain PDA4 at lane 9 in Figure 4.8 showed a low quality of DNA sample. This might be due to the technique used during the preparation of DNA samples. It is essential to make sure the concentration of DNA used are measured correctly. If the DNA is too concentrated, the sequencing reaction could drop off quickly and greatly diminish the length. Conversely, an overly weak DNA concentration will produce excess background noise and introduce errors during the sequence analysis later.

Nevertheless, studies on actinobacteria diversity by 16S rRNA gene amplification and sequencing have some limitations. The biases in genomic DNA extraction, PCR amplification and cellular rDNA copy number are well known limitations for all molecular approaches relying on PCR amplification of rRNA genes from genomic DNA (Sun *et al*., 2008). The universal PCR allowed for the identification to the level of genus, but at the level of the species there is an ambiguity that can be explained by the fact that the primers used in the work is not specific to actinobacteria (Yong *et al*., 2008).

4.3.5 Partial 16S rRNA Gene Analysis

Sequencing is one of many methods that can use amplification and analysis of 16S rRNA genes to compare the composition, richness and structure of actinobacterial communities (Dunbar *et al*., 1999). The 16S rRNA gene from ten of the samples (strains 9, A21, OPSa, MA04119, PDA4, A12, RHRS, MA04020, RC3 and H23) were sent for sequencing. The amplified region was sequenced and compared against known sequences in the GenBank database to find the identity of the actinobacteria. Results obtained after sequencing are presented in Table 4.4.

Table 4.4 Closest matches from the GenBank using BLAST

Strain	Closest match	Accession number	% similarity
9	*Brevibacterium linens* VKM Ac-2119	AY243345	97.25
A21	*Streptomyces rubrogriseus* DSM 41477	AF503501	96.08
OPSa	*Streptomyces coeruleofuscus* ISP 5144	AJ399473	100.00
A12	*Streptomyces aureofaciens* NRRL 2209	Y15504	99.97
H23	*Streptomyces libani* subsp. *libani* IFO 13452	AB184414	99.00
MA04119	*Streptomyces carpinensis* ACC 27116	AB122728	100.00
PDA4	*Streptomyces parvulus* IFO 13193	AB184326	96.00
RHRS	*Streptomyces galilaeus* ISP 5481	AB045878	95.92
MA04020	*Streptomyces spiralis* ACC 25664	AB122727	97.18
RC3	*Streptomyces aburaviensis* IFO 12830	AB122746	98.16

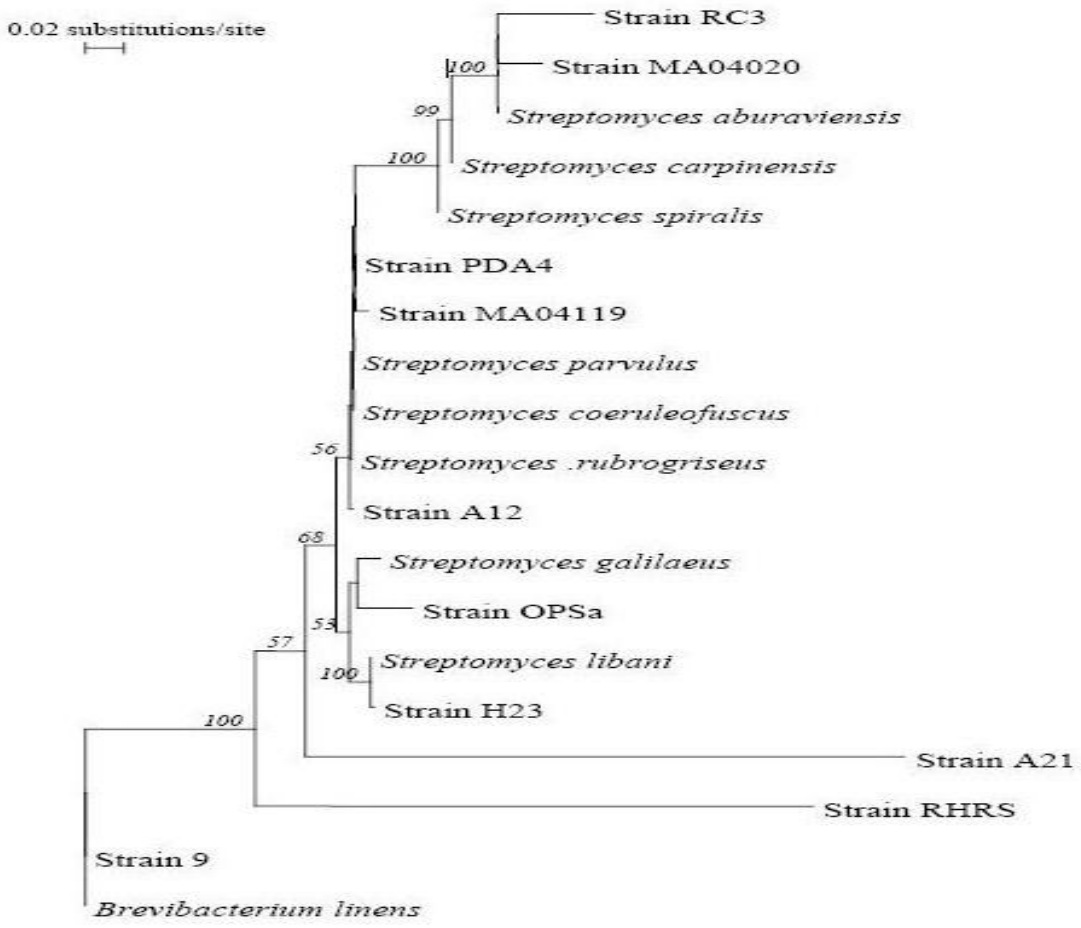


Figure 4.9 Neighbour-joining phylogenetic tree showing relationship between the tested actinobacterial strains and closely related validly described *Streptomyces* spp. and *Brevibacterium linens*.

The analysis using partial 16S rRNA sequence confirmed that the strain 9 belonged to the genus *Brevibacterium*. This was also proven by the neighbour-joining analysis, as shown in Figure 4.9. Although the similarity level between the strain and *Brevibacterium linens* VKM Ac-2119 (Genbank accession number AY243345) was only 97.25%, but they are sharing the same lineage. Strain 9 was not closely related to the other tested strains. This shows that it has the different genetic information (nucleotide sequence) compared to the other strains. However, because of low percentage similarity with *Brevibacterium linens* VKM Ac-2119, the result suggests that this strain is a potentially new species. However, further studies have to be done to confirm the identity of this strain at the species level. *Brevibacterium linens* VKM Ac-2119 has been listed in Approved Lists of Bacterial Names in IJSEM (Skerman *et al*., 1980). The strain had been validly described and was first found by Wolff in 1910, isolated from soil sample taken from rice field in Krasnodar, Russian (Skerman *et al*., 1980).

Strain 9 was isolated from earthworm gut sample. It forms an extensively fragmented substrate mycelium and aerial hyphae. This species is an aerobic and non-acid-fast actinomycete that produces a brown substrate mycelium and a white aerial spore-mass. It also produced diffusible pigment that was pale yellow in colour and did not form spores. The strain had circular type of colony edge. It was categorized in the single-member group because it did not have similar morphological characteristics with the other strains studied.

Strain 9 showed an antifungal activity against *S. cerevisiae* and *G. boninense*. However, it did not show the presence of polyene CYP-specific fragment gene. This suggests that the strain might be using other mechanism or producing other antifungal

compound that could also give the inhibition activity on the tested fungi. Osman (2004) has proven that *B. linens* was producing aflatoxins in order to inhibit the growth of *Aspergilus flavus*. This result gives a basis to recommend the strain for use as a biological control agent.

The analysis using partial 16S rRNA sequence confirmed that the strain MA04119 belonged to the genus *Streptomyces*. The similarity level was 100.00% with *Streptomyces parvulus* IFO 13193 (Genbank accession number AB184326). *Streptomyces parvulus* IFO 13193 has been listed in Bergey's Manual Trust Taxonomy Online (Ludwig *et al*., 2009). The strain was identified to be related to strain PDA4. Only a small portion of their sequences might be different. This is based on Figure 4.9 that shows the close relationship between them. It shows that both of the strains share the same lineage. In addition, they were also isolated from the same soil sample. This may indicate that they might have originated from the same ancestor.

Strain MA04119 showed an antifungal activity against *S. Pombe.*. However, it did not show the presence of polyene CYP-specific fragment gene. This suggests that the strain might be using other mechanism or produce other antifungal compound that could also give the inhibition activity on the fungi tested. However, there are not many reports that *S. parvulus* has a potential as an antifungal compound producer.

Strain PDA4 showed an antagonistic activity against *G. boninense*. However, it did not show the presence of polyene CYP-specific fragment gene. This suggests that the strain might be using other mechanism or producing other antifungal compound that could also give the inhibition activity on the tested fungi. In many studies done by other researchers, this strain has been shown to be promising in the agricultural industry. El-

Tarabily *et al*. (2010) reported that *S. spiralis* has the potential as a biological control agent against Pythium disease of cucumber, a disease cause by *Phythium aphanidermatum*, and could be used in place of the chemical fungicide which is currently recommended for the management of the disease in United Arab Emirates.

The analysis using partial 16S rRNA sequence confirmed that the strain A21 belonged to the genus *Streptomyces*. The similarity level was 96.08% with *Streptomyces coeruleofuscus* ISP 5144 (Genbank accession number AJ399473), the most closely related species. *Streptomyces coeruleofuscus* ISP 5144 has been listed in Approved Lists of Bacterial Names in IJSEM (Skerman *et al*., 1980). The strain had been validly described and was first found by Pridham *et al*. in 1958 and was isolated from soil sample taken in Daghestan and cultivated at 28°C in Peptone Yeast Extract Agar (Skerman *et al*., 1980). Phylogenetic tree in Figure 4.9 also shows that the strain is not closely related to the other strains tested. In addition, because of low similarity between the strain and *Streptomyces coeruleofuscus* ISP 5144, this may indicate that the strain is a potentially new species. However, further studies have to be done to confirm the identity of this strain at the species level.

Strain A21 was isolated from earthworm gut sample. It was producing branched filaments with straight to spiral structures. The mature spore chains were spiral and long, may be more than 50 spores per chain. According to Shirling and Gottlieb (1968), *Streptomyces coeruleofuscus* ISP 5144 also produce similar morphology on oatmeal agar, salts-starch agar and glycerol-asparagine agar. The spore surface was spiny**.** It produced aerial mass and dark brown substrate mycelia on ISP2 medium. It also produced dark brown diffusible pigment, and had circular colony edge.

Strain A21 showed an antifungal activity against *S. cerevisiae*. However, it did not show the presence of polyene CYP-specific fragment gene. This suggests that the strain might be using other mechanism or producing other antifungal compound that could also give the inhibition activity on the fungi tested. However, there are not many reports that *S. coeruleofuscus* has a potential as an antifungal compound producer.

The analysis using partial 16S rRNA sequence confirmed that the strain OPSa belonged to the genus *Streptomyces*. The similarity level was 100.00% with *Streptomyces galilaeus* ISP 5481 (Genbank accession number AB045878). *Streptomyces galilaeus* ISP 5481 has been listed in Bergey's Manual Trust Taxonomy Online (Ludwig *et al*., 2009).

Strain OPSa was also isolated from earthworm gut sample. It produced branched filaments and the mature spore chains were spiral with moderately long chains of 10 to 50 or more spores. The spore surface was rough**.** The aerial mass produced was light grey in colour, while the substrate mycelium was brown on ISP2 medium. No diffusible pigments were produced. According to Shirling and Gottlieb (1972), *Streptomyces galilaeus* ISP 5481 also produce similar morphology on oatmeal agar, salts-starch agar and glycerol-asparagine agar. The strain had irregular colony margin. In this study, strain OPSa was categorized in the single-member group because it did not share similar morphological characteristics with the other strains studied.

Strain OPSa showed an antifungal activity against *C. albicans, S. cerevisiae* and *F. oxysporum*. However, it did not show the presence of polyene CYP-specific fragment gene. This suggests that the strain might be using other mechanism or producing other antifungal compounds that could also give the inhibition activity on the

fungi tested. However, there are not many reports that *S. galilaeus* has a potential as an antifungal compound producer. Most of the studies done reported that it has a potential to be an antitumor agent. For example, study done by Chung *et al*. (2002) has shown that *S. galilaeus* could produce aclacinomycins, an anthracycline antibiotic with a potent antitumor activity. It has been clinically used in France, Japan and other Asian countries for the treatment of carcinoma of the stomach, pulmonary carcinoma, oophoroma, malignant lymphadnoma and acute leukemia.

The analysis using partial 16S rRNA sequence confirmed that the strain A12 belonged to the genus *Streptomyces*. The similarity level was 99.97% with *Streptomyces rubrogriseus* DSM 41477 (Genbank accession number AF503501), the most closely related species. *Streptomyces rubrogriseus* DSM 41477 was first found in 1986 (Garrity *et al*., 2002).

Strain A12 was isolated from soil sample taken from a rubber plantation. It was produced branched filaments. Spore-chain morphology was spirals. Tight terminal spirals were usually seen on moderately short chains of 10 or more spores. No aerial mass color was observed on ISP2 medium, while the reverse side of colony was dark yellow. Diffusible pigments were not formed. The strain had circular colony edge.

Strain A12 showed an antifungal activity against *F. oxysporum*. However, it did not show the presence of polyene CYP-specific fragment gene. This suggests that the strain might be using other mechanism or producing other antifungal compound that could also give the inhibition activity on the fungi tested. Many studies have shown that *S. rubrogriseus* is an antifungal compound producer. For example, study done by Doolotkeldieva and Totubaeva (2009) has shown that the preparation of *S. rubrogriseus*

was effective in protection of sprouts from *Fusarium* sp. cultures. It provided protection of sprouts up to 80% on average.

The 16S rRNA partial sequence analysis showed that the strain H23 was closely related to the genus *Streptomyces*, with a 100.00% sequence similarity to *Streptomyces libani* subsp. *libani* IFO 13452 (Genbank accession number AB184414). *Streptomyces libani* subsp. *libani* IFO 13452 has been listed in Bergey's Manual Trust Taxonomy Online (Ludwig *et al*., 2009).

Strain H23 was isolated from earthworm gut sample. It produced branched filaments. Tight terminal spirals were usually seen on moderately short chains of 10 or more spores. The aerial mass and substrate mycelium were grey in color on ISP2 medium. Diffusible pigments were not formed. The strain had irregular colony margin. The spore surface was rough. According to Shirling and Gottlieb (1972), *Streptomyces libani* subsp. *libani* IFO 13452 also produce similar morphology on oatmeal agar, salts-starch agar and glycerol-asparagine agar.

Strain H23 showed an antifungal activity against *F. oxysporum*. It also showed the presence of polyene CYP-specific fragment gene. This suggests that the strain might be producing the polyene CYP-specific antifungal compound that gives the inhibition activity on the fungi tested. In many studies, *S. libani* has been known to be the producer of many antifungal compounds. For example, it was proved by Kekuda *et al*. (2010) showed tha *S. libani* produce a macrolide antibiotic, Oligomycin A, that used in inhibiting pathogenic fungi.

The 16S rRNA partial sequence analysis of the strain RHRS showed a low percentage of similarity to the genus *Streptomyces*, with a 95.22% sequence similarity to *Streptomyces aureofaciens* NRRL 2209 (Genbank accession number Y15504). *Streptomyces aureofaciens* NRRL 2209 was first found in 1948 (Skerman *et al*., 1980). Because of the low similarity between the strain and *Streptomyces aureofaciens* NRRL 2209, this might indicate that the strain is a potentially new species. Phylogenetic tree in Figure 4.9 also shows that the strain is not closely related to the other strains tested. However, further studies have to be done to confirm the identity of this strain at the species level.

In this study, strain RHRS was isolated from the soil sample taken in a palm oil plantation. This strain was an aerobic and non-acid-fast actinobacteria that produced a brown substrate mycelium and grey aerial spore-mass. It was not producing diffusible pigments. The strain had circular colony margin. It formed an extensively branched substrate mycelium and aerial hyphae that differentiated into chains of spores. The spore chain was in Retinaculiaperti (RA) structure where the matured spore chains were generally 10 to 50 spores per chain. The spore surface was smooth. According to Shirling and Gottlieb (1968b), *Streptomyces aureofaciens* NRRL 2209 also produce similar morphology on oatmeal agar, salts-starch agar and glycerol-asparagine agar.

Strain RHRS appeared to be an antagonist of *C. albicans*, *S. pombe* and *F. oxysporum*. It also showed the presence of polyene CYP-specific fragment gene. A study done by Taechowisan *et al*. (2005) had also found that the strain was producing potential antifungal compound that inhibit *F. oxysporum*. The evidence was demonstrated in their *in vitro* study by the zone of fungal-growth inhibition. Microscopic observation showed the thick and bulbous structures at the edges of the

inhibited fungal hyphae. The major active ingredients from the culture filtrate of the strain was purified and identified to be 5,7 – dimethoxy-4-ρ-methoxylphenylcoumarin and 5,7 – dimethoxy-4-phenylcoumarin. Bioassay studies showed that these compounds had antifungal activities against the fungi tested. Besides that, Taechowisan and Lumyong (2003) had found that *S. aureofaciens* had an effective antifungal activity against *Candida albicans*. The extracts of its fermentation broth had activity against fungi tested at a concentration of 10mg/ml.

The analysis using partial 16S rRNA sequence confirmed that strain MA04020 belonged to the genus *Streptomyces*. The similarity level was only 97.18% with *Streptomyces carpinensis* ACC 27116 (Genbank accession number AB122728), the most closely related species. Because of the low percentage similarity, the result might suggest that this strain is a potentially new species. However, further studies have to be done to confirm the identity of this strain at the species level. *Streptomyces carpinensis* ACC 27116 was first found by Morais *et al*. in 1971. It was isolated from soil sample taken in Carpina, Brazil and cultivated at 28°C in Glucose Yeast Extract Agar (GYEA) (Garrity *et al*., 2002).

Strain MA04020 showed an antifungal activity against *C. parapsilosis*. It also showed the presence of polyene CYP-specific fragment gene. This suggests that the strain might be producing the polyene CYP-specific antifungal compound that gives the inhibition activity on the tested fungal. However, there are not many reports that *S. carpinensis* has the potential as an antifungal compound producer. The ability of this strain to inhibit *C. parapsilosis* and the presence of polyene CYP-specific fragment gene indicate that it has the potential to be developed as a source of antifungal compound. However, further studies have to be done on it.

Based on the phylogenetic tree in Figure 4.9, strain MA04020 shares the same lineage with the strain RC3. The analysis using partial 16S rRNA sequence confirmed that the strain RC3 belonged to the genus *Streptomyces*. The similarity level was 98.16% with *Streptomyces aburaviensis* IFO 12830 (Genbank accession number AB122746). Strains MA04020 and RC3 were isolated from soil sample. Although strain RC3 could only inhibit *S. cerevisiae* while strain MA04020 was only inhibiting *C. parapsilosis*, the ability of producing polyene CYP-specific gene indicates that both of them are closely related and might share the same origin.

5.0 Conclusion

From the antifungal assays of the 52 actinobacterial strains, ten of them showed strong antifungal activities against the fungi tested. Three strains have shown antifungal activity against both yeasts and filamentous fungi. Strain 9 was producing 21.0mm of inhibition zone when tested on *S. cerevisiae* and showed strong antifungal activity against *G. boninense,* producing more than 15mm of inhibition zone. Strain OPSa was showing antifungal activity against *C. albicans* and *S. cerevisiae* with zones of inhibition in the range of 16 to 20mm. It also showed slight antifungal activity against *F. oxysporum*. Strain RHRS was highly active against *S. pombe* with 20.0mm inhibition zone produced. It also showed antifungal activity against *C. albicans* and *F. oxysporum*.

Four strains showed zones of inhibition of more than 15mm in diameter against tested yeasts (*Candida albicans, Candida parapsilosis*, *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*). Strain MA04020 was highly active against *C. parapsilosis* with inhibition zone of 22.0mm. Strains A21 and RC3 were highly active against *S. cerevisiae,* while strain MA04119 was showing an inhibition activity against *S. pombe*.

For antifungal assays against filamentous fungi, strains A12 and H23 have shown strong antifungal activity with wide clearing zones (more than 15mm) against *F. Oxysporum*, while, strain PDA4 showed an inhibition zone of more than 15mm against *G. boninense*.

These results demonstrate that some of actinobacteria have the potential for inhibiting the growth of pathogenic fungi tested.

By using ERIC-PCR amplification technique, ten of the actinobacterial strains that had shown strong antifungal activity are different from one another. The bands produced by each of the strains were compared based on the presence or absence of fragments at a specific position. The bands were present in tested strains, but there were differences in the patterns of some amplified fragments as well as in the occurrence of several polymorphic bands. In addition, this technique could unambiguously detect genomic differences among strains. However, strains RHRS and OPSa have shown similar pattern of bands. The same band patterns were also observed between strains MA04020 and RC3. From the variation in banding patterns produced by all the ten strains tested, three groups were formed for categorizing the strains. strains RHRS and OPSa belong to one group, strains MA04020, RC3, PDA4, A12 and A21 into one group, while the another group comprises strains H23, MA04119 and 9.

The polyene CYP-specific PCR-based genome screening approach is an efficient and powerful means of identifying the putative polyene producing actinobacterial strains. However, only four (strains H23, RC3, RHRS and MA04020) out of ten strains studied show the presence of the expected 350bp DNA fragment of the polyene CYP-specific internal region. This shows that the strains could produce polyene CYP-specific in order to show the antifungal activity against the fungi tested. The remaining strains might be using other mechanism of inhibition or producing other antifungal conpounds to inhibit the fungi. However, more detailed investigation is required to demonstrate the potential of the strains in producing the biocontrol agents against plant and human pathogens.

From the 16S rRNA gene sequence analysis of the ten actinobacterial strains that exhibited antifungal activity, nine of them (strains A21, OPSa, A12, MA04119, PDA4, H23, RHRS, RC3 and MA04020) were identified as belonging to the genus *Streptomyces*, family *Streptomycetaceae*, and one of them (strain 9) as *Brevibacterium*, family *Brevibacteriaceae*.

The approaches discussed in this study help to identify strains with a potential application. A rapid method to investigate fingerprint also has been done using PCR products as an alternative characterization tool to be applied in the study of distinct actinobacterial strains. In the need for new antifungal compounds, further investigation of actinobacteria habitats will prove a fruitful enterprise. New classes of antibiotics are necessary to combat the increased incidence of multiple resistances among pathogenic microorganisms to the available drugs that are currently in clinical use. It is anticipated that the current effort for the isolation, characterization and the study on actinobacteria can be a milestone for the discovery of novel antibiotics effective against pathogens.

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7.0 Appendix

7.1 Media Preparation

7.1.1 Yeast Extract Malt Extract Agar (ISP2)

Ingredients for preparing 1 litre ISP2 medium:

4g Bacto™ yeast extract

10g Bacto™ malt extract

4g dextrose

15g Bacto™ agar

The ingredients were dissolved into 1,000ml distilled water and autoclaved at 121°C 15 p.s.i. for 20 minutes before poured into sterile disposable petri dishes (plates) and allow to solidify.

7.1.2 Sabouraud's Dextrose Agar (SDA)

65g Oxoid Sabouraud's Dextrose Agar powder was dissolved into 1,000ml distilled water and autoclaved at 121°C 15 p.s.i. for 20 minutes before poured into into sterile disposable petri dishes (plates) and allow to solidify.

7.1.3 Potato Dextrose Agar (PDA)

35g Bacto™ Potato Dextrose Agar powder was dissolved into 1,000ml distilled water and autoclaved at 121°C 15 p.s.i. for 20 minutes before poured into into sterile disposable petri dishes (plates) and allow to solidify.

7.2 Source of Isolation

7.2.1 Source of Actinobacteria Strains Isolation

No	Strain	Source of isolation
1	CM1, CM2, CM3, CM4, CM5, CM6, CM7, CM8, CM9, CM10, CM11, CM13, CM17, CM14, CM15, CM16	Sediments of the Straits of Malacca
2	4, 9, A12, A15, A21, H23	Earthworm gut sample
3	MA04007, MA04013, MA04014, MA04020, MA04111, MA04117, MA04118, MA04119, MA04120, PDA4	Mangrove soil sample
4	RA1, RA2, RA5, RA6, RA9, RA10, RB2, RC3, OPCB, OPCC, OPB1, OPSa, OPSb , OPSe, RAB1, RC1 RHOP, HT7	Soil sample from palm oil plantation
5	RHRS, RAPC	Soil sample from rubber plantation

7.3 Antifungal Activity

7.3.1 Raw data of antifungal activity of the actinobacterial strains, grown in ISP2 media after ten days, against *S. pombe, S. cerevisiae, C. albicans* and *C. parapsilosis*

Strain	Inhibition zone (mm)															
	C. albicans				*C. parapsilosis*				*S. pombe*				*S. cerevisiae*			
	R1	**R2**	**R3**	**Average**	**R1**	**R2**	**R3**	**Average**	**R1**	**R2**	**R3**	**Average**	**R1**	**R2**	**R3**	**Average**
9	-	-	-	-	-	-	-	-	8.0	9.0	8.0	8.33±0.6	21.0	21.0	21.0	21.00±0.0
A21	11.0	12.0	11.0	11.33±0.6	10.0	12.0	11.0	11.00±1.0	-	-	-	-	21.0	21.0	20.0	20.67±0.6
A15	6.0	6.0	5.0	5.67±0.6	-	-	-	-	12.0	15.0	15.0	14.00±1.7	-	-	-	-
OPSa	20.0	17.0	17.0	18.00±1.7	15.0	12.0	15.0	14.00±1.7	18.0	16.0	15.0	16.33±1.5	20.0	21.0	18.0	19.67±1.5
H23	-	-	-	-	8.0	7.0	8.0	7.67±0.6	7.0	7.0	7.0	7.00±0.0	-	-	-	-
MA04119	-	-	-	-	-	-	-	-	22.0	20.0	22.0	21.33±1.2	-	-	-	
CM13	-	-	-	-	-	-	-	-	15.0	15.0	15.0	15.00±0.0	-	-	-	-
RHRS	18.0	18.0	18.0	18.00±0.0	-	-	-	-	20.0	20.0	20.0	20.00±0.0	9.0	9.0	9.0	9.00±0.0
OPSe	9.0	8.0	9.0	6.67±0.6	8.0	8.0	9.0	8.33±0.6	18.0	15.0	18.0	17.00±1.7	12.0	15.0	12.0	13.00±1.7
MA04020	-	-	-	-	23.0	20.0	23.0	22.00±1.7	10.0	9.0	8.0	9.00±1.0	-	-	-	-
RA5	7.0	5.0	7.0	6.37±1.2	-	-	-	-	10.0	8.0	11.0	9.67±1.5	8.0	11.0	11.0	10.00±1.7
RA6	-	-	-	-	-	-	-	-	9.0	9.0	9.0	9.00±0.0	-	-	-	-
RA9	7.0	7.0	7.0	7.00±0.0	-	-	-	-	9.0	9.0	9.0	9.00±0.0	-	-	-	-
RB2	6.0	6.0	9.0	7.00±1.7	-	-	-	-	9.0	8.0	8.0	8.33±0.6	9.0	9.0	9.0	9.00±0.0
RC3	15.0	15.0	13.0	14.33±1.2	-	-	-	-	8.0	8.0	8.0	8.00±0.0	26.0	24.0	27.0	25.67±1.5
RHOP	8.0	9.0	9.0	8.33±0.6	-	-	-	-	7.0	8.0	8.0	7.67±0.6	9.0	7.0	7.0	7.67±1.6
Nystatin	27.0	26.0	25.0	26.00±1.0	25.0	25.0	28.0	26.00±1.7	28.0	29.0	28.0	28.33±0.6	29.0	28.0	26.0	27.67±1.5
Cycloheximide	27.0	27.0	27.0	27.00±0.0	27.0	28.0	28.0	27.67±0.6	22.0	25.0	25.0	24.00±1.7	28.0	28.0	26.0	27.33±1.2

7.4 Strains Sequence

7.4.1 Strain 9 Sequence

```
  1 tcgatcgggg atgggctcgc ggcctatcag cttgttggtg gggtaatggg cctaccaagg
 61 gcgacgacgg gtagccggcc tgagagggcg accggccaca ctgggactga gacacggccc
121 agactcctac gggaggcagc agtggggaat attgcacaat gggggaaacc ctgatgcagc
181 gacgcagcgt gcgggatgac ggccttcggg ttgtaaaccg ctttcagcag ggaagaagcg
241 gacgcagcgt gcgggatgac ggccttcggg ttgtaaaccg ctttcagcag ggaagaagcg
301 aaagtgacgg tacctgcaga agaagtaccg gctaactacg tgccagcagc cgcggtaata
361 cgtagggtac gagcgttgtc cggaattatt gggcgtaaag agctcgtagg tggttggtca
421 cgtctgctgt ggaaacgcaa cgcttaacgt tgcgcgtgca gtgggtacgg gctgactaga
481 gtgcagtagg ggagtctgga attcctggtg tagcggtgaa atgcgcagat atcaggagga
541 acaccggtgg cgaaggcggg actctgggct gtaactgaca ctgaggagcg aaagcatggg
601 gagcgaacag gattagatac cctggtagtc catgccgtaa acgttgggca ctaggtgtgg
661 gggacattcc acgttctccg cgccgtagct aacgcattaa gtgccccgcc tggggagtac
721 ggtcgcaagg ctaaaactca aaggaattga cgggggcccg cacaagcggc ggagcatgcg
781 gattaattcg atgcaacgcg aagaacctta ccaaggcttg acatacactg gaccgttctg
841 gaaacag
```

7.4.2 Strain A12 Sequence

```
  1 atcagcttgt tggtgaggta atggctcacc aaggcgacga cgggtagccg gcctgagagg
 61 gcgaccggcc acactgggac tgagacacgg cccagactgg tacgggaggc agcagtgggg
121 aatattgcac aatgggcgaa agcctgatgc agcgacgccg cgtgagggat gacggccttc
181 gggttgtaaa cctctttcag cagggaagaa gcgaaagtga cggtacctga agaagaagcg
241 ccggctaact acgtgccagc agccgcggta atacgtaggg cgcaagcgtt gtccggaatt
301 attgggcgta aagagctcgt aggcggcttg tcgcgtcggt tgtgaaagcc cggggcttaa
361 ccccgggtct gcagtcgata cgggcaggct agagttcggt aggggagatc ggaattcctg
421 gtgtagcggt gaaatgcgca gatatcagga ggaacaccgg tggcgaaggc ggatctctgg
481 gccgatactg acgctgagga gcgaaagcgt ggggagcgaa caggattaga taccctggta
541 gtccacgccg taaacggtgg gcactaggtg tgggcaacat tccacgttgt ccgtgccgca
601 gctaacgcat taagtgcccc gcctggggag tacggccgca aggctaaaac tcaaaggaat
661 tgacgggggc ccgcacaagc ggcggagcat gtggcttaat tcgacgcaac gcgaagaacc
721 ttaccaaggc ttgacataca ccggaaacac tcagagatgg gtgccccctt gtggtcggtg
781 tacag
```

7.4.3 Strain A21 Sequence

```
  1 cgatactgac gctgaggagc gaaagcgtgg ggagcgaaca ggattagata ccctggtagt
 61 ctcgtaggcg gcttgtcacg tcggttgtga aataccgggg cttaaccccg ggtctgcagt
121 cgatacgggc aggctagagt tcggtcgggg agatcggaat tcctggtgta gcggtgaaat
181 gcgcagatat caggaggaac accggtggcg aaggcggatc tctgggccga tactgacgct
241 gaggagcgaa agcgtgggga gcgaacagga ttagataccc tggtagtctc gtaggcggct
301 ggtcacgtcg gttgtgaaat accggggctt aaccccgggt ctgcagtcga tacgggcagg
361 gaggaacacc ggtggcgaag gcggatctct gggccgctac tgacgctgag gagcgaaagc
421 gtggggagcg aacaggatta gataccctgg tagtctcgta ggcggcttgt cacgtcggtt
481 gtgaaatacc ggggcttaac cccgggtctg cagtcgatac gggcaggcta gagttcggta
541 ggggagatcg gcattcctgg tgtagcggtg aaatgcgcag atatcaggag gaacaccggt
601 ggcgaaggcg gatctctggg ccgatactga cgctgaggag cgaaagcgtg gggagcgaac
661 aggattagat accctggtag tctcgtaggc ggcttgtcac gtcggtggtg aaataccggg
721 gctta
```

7.4.4 Strain RHRS Sequence

```
  1 gggggatcgc aagacctctc actattggag cggccgatat cggattagct agttggtggg
 61 gtaaaggctc accaaggcaa cgatccgtag ctggtttgag aggacgacca gccacactgg
121 gactgagaca cggcccagac tcctacggga ggcagcagtg gggaattttg gacaatgggg
181 gaaaccctga tccagccatc ccgcgtgtat gatgaaggcc ttcgggttgt aaagtacttt
241 tggcagagaa gaaaaggtat cccctaatac gggatactgc tgacggtatc tgcagaataa
301 gcaccggcta actacgtgcc agcagccgcg gtaatacgta gggtgcaagc gttaatcgga
361 attactgggc gtaaagcgtg tgtaggcggt tcggaaagaa agatgtgaaa tcccagggct
421 caaccttgga actgcatttt taactgccga gctagagtat gtcagagggg ggtagaattc
481 cacgtgtagc agtgaaatgc gtagatatgt ggaggaatac cgatggcgaa ggcagccccc
541 tgggataata ctgacgctca gacacgaaag cgtggggagc aaacaggatt agataccctg
601 gtagtccacg ccctaaacga tgtcaactag ctgttggggc cgtaaggcct tagtagcgca
661 gctaacgcgt gaagttgacc gcctggggag tacggtcgca agattaaaac tcaaaggaat
721 tgacggggac ccgcacaagc ggtggatgat gtggattaat tcgatgcaac gcgaaaaacc
```

7.4.5 Strain H23 Sequence

```
  1 aaagctccgg cggtgaagga tgagcccgcg gcctatcagc ttgttggtgg ggtgatggcc
 61 taccaaggcg acgacgggta gccggcctga gagggcgacc ggccacactg ggactgagac
121 acggcccaga ctcctacggg aggcagcagt ggggaatctt gcacaatggg cgaaagcctg
181 atgcagcgac gccgcgtgag ggatgacggc cttcgggttg taaacctctt tcagcaggga
241 agaagcgaga gtgacggtac ctgcagaaga agcgccggct aactacgtga cagcagccgc
301 ggtaatacgt agggcgcaag cgttgtccgg aattattggg cgtaaagagc tcgtaggcgg
361 cttgtcacgt cggatgtgaa agcccggggc ttaaccccgg gtctgcattc gatacgggca
421 ggctagagtt cggtagggga gatcggaatt cctggtgtag cggtgaaatg cgcagatatc
481 aggaggaaca ccggtggcga aggcggatct ctgggccgat actgacgctg aggagcgaaa
541 gcgtggggag cgaacaggat tagataccct ggtagtccac gccgtaaacg ttgggaacta
601 ggtgtgggcg acattccacg tcgtccgtgc cgcagctaac gcattccgtt ccccgcctgg
661 ggagtacggc cgcaaggcta aaactcaaag gaattgacgg gggcccgcac aagcagcgga
721 gcatgtggct taattcgacg caacgcgaag aaccttacca aggcttgaca tacaccggaa
```

7.4.6 Strain MA04020 Sequence

```
  1 tggctcacca aggcgacgac gggtagccgg cctgagaggg cgaccggcca cactgggact
 61 gagacacggc ccagactcct acgggaggca gcagtgggga atattgcaca atgggcgaaa
121 gcctgatgca gcgacgccgc gtgagggagg acggccttcg ggttgtaaac ctctttcagc
181 agggaagaag cgaaagtgac ggtacctgca gaagaagcgc cggctaacta cgtgccagca
241 gccgcggtaa tacgtagggc gcaagcgttg tccggaatta ttgggcgtaa agagctcgta
301 ggcggcgtgt cacgtcggtt gtgaaagccc ggggcttaac cccgggtctg cagtcgatac
361 gggcaggcta gagttcggta ggggagatcg gaattcctta gtggcgaacg ggtgagtaac
421 acgtgggcaa tctgccctgc actctgggac aagccctgga accggggtct aataccggat
481 actgaccttc acgggcatct gtgaaggtcg aaagctccgg cggtgcagga tcagcccgcg
541 gcctatcagc ttgttggtga ggtaatggct caccaaggcg acgacgggta gccggcctga
601 gagggcgacc ggccacccctg ggactgagac acggcccaga ctcctacggg aggcagcagt
661 ggggaatatt gcacaatggg cgaaagcctg atgcagcgac gccgcgtgag ggatgacggc
721 cttcgggttg taaacctctt tcagcaggga agaagcgaaa gtgacggtac cggcagaaga
```

7.4.7 Strain MA04119 Sequence

```
  1 cggcggtgca ggatgagccc gcggcctatc agcttgttgg tgaggtaacg gctcaccaag
 61 gcgacgacgg gtagccggcc tgagagggcg accggccaca ctgggactga gacacggccc
121 agactcctac gggaggcagc agtggggaat attgcacaat gggcgaaagc ctgatgcagc
181 gacgccgcgt gagggatgac ggccttcggg ttgtaaacct ctttcagcag ggaagaagcg
241 agagtgacgg tacctgcaga agaagcgccg gctaactacg tgccagcagc cgcggtaata
301 cgtagggcgc aagcgttgtc cggaattatt gggcgtaaag agctcgtagg cggcttgtcg
361 cgtcggttgt gaaagcccgg ggcttaaccc cgggtctgca gtcgatacgg gcaggctaga
421 gttcggtagg ggagatcgga attcctggtg tagcggtgaa atgcgcagat atcaggagga
481 acaccggtgg cgaaggcgga tctctgggcc gatactgacg ctgaggagcg aaagcgtggg
541 gagcgaacag gattagatac cctggtagtc cacgccgtaa acggtgggca ctaggtgtgg
601 gcgacattcc acgtcgtccg tgccgcagct aacgcattaa gtgccccgcc tggggagtac
661 ggccgcaagg ctaaaactca aaggaattga cgggggcccg cacaagcggc ggagcatgtg
721 gcttaattcg acgcaacgcg aagaaacctt accaaggctt gacatacacc ggaaacgtcc
```

7.4.8 Strain OPSA Sequence

```
  1 tggtggaaag ctccggcggt gcaggatgag cccgcggcct atcagcttgt tggtggggta
 61 atggcctgcc aaggcgacga cgggtagccg gcctgagagg gcgaccggcc acactgggac
121 tgagacacgg cccagactcc tacgggaggc agcagtgggg aatattgcac aatgggcgaa
181 agcctgatgc agcgacgccg cgtgagggat gacggccttc gggttgtgaa cctctttcag
241 cagggaagaa gcgcaagtga cggtacctgc agaagaagca ccggctaact acgtgccagc
301 agccgcggta atacgtaggg tgcgagcgtt gtccggaatt attgggcgta aagagctcgt
361 aggcggcctg tcgcgtcgga tgcgaaagcc cggggcttaa ccccgggtct gcattcgata
421 cgggcaggct agagtgtggt aggggagatc ggaattcctg gtgtagcggt gaaatgcgca
481 gatatcagga ggaacaccgg tggcgaaggc ggatctctgg gccattactg acgctgaggg
541 gcgaaagcgt ggggagcgaa caggattaga taccctggta gtccacgccg taaacgttgg
601 gaactaggtg ttggcgacat tccacgtcgt cggtgccgca gctaacgcat taagttcccc
661 gcctggggag tacggccgca aggctaaaac tcaaaggaat tgacgggggc ccgcacaagc
721 agcggagcat gtggcttaat tcgacgcaac gcgaagaacc ttaccaaggc ttgacatatg
```

7.4.9 Strain PDA4 Sequence

```
  1 taaagctccg gcggtgcagg atgagcccgc ggcctatcag cttgttggtg aggtaatggc
 61 gacgacgggt agccggcctg agagggcgac cggccacact gggactgaga cacggcccag
121 actcctacgg gaggcagcag tggggaatat tgcacaatgg gcgaaagcct gatgcagcga
181 cgccgcgtga gggatgacgg ccttcgggtt gtaaacctct tcagcaggg aagaagcgaa
241 agtgacggta cctgcagaag aagcgccggc taactacgtg ccagcagccg cggtaatacg
301 tagggcgcaa gcgttgtccg gaattattgg gcgtaaagag ctcgtaggcg gcttgtcgcg
361 tcggttgtga aagcccgggg cttaaccccg ggtctgcagt cgatacgggc aggctagagt
421 tcggtagggg agatcggaat tcctggtgta gcggtgaaat gcgcagatat caggaggaac
481 accggtggcg aaggcggatc tctgggccga tactgacgct gaggagcgaa agcgtgggga
541 gcgaacagga ttagataccc tggtagtcca cgccgtaaac ggtgggcact aggtgtgggc
601 aacattccac gttgtccgtg ccgcagctaa cgcattaagt gccccgcctg gggagtacgg
661 ccgcaaggct aaaactcaaa ggaattgacg ggggcccgca caagcggcgg agcatgtggc
721 ttaattcgac gcaacgcgaa gaaccttacc aaggcttgac atacaccgga aacgt
```

7.4.10 Strain RC3 Sequence

```
 61 ggcctaccaa ggcgacgacg ggtagccggc ctgagagggc gaccggccac actgggactg
 61 agacacggcc cagactccta cgggaggcag cagtggggaa tattgcacaa tgggcgaaag
121 cctgatgcag cgacgccgcg tgagggctga cggccttcgg gttgtaaacc tctttcagca
181 gggaagaagc gcaagtgacg gtacctgcag aagaagcacc ggctaactac gtgccagcag
241 ccgcggtaat acgtagggtg cgagcgttgt ccggaattat tgggcgtaaa gcgctcgtag
301 gggccggtcg cgtcggatgt gaaagcccgg ggcttaaccc cgggtctgca ttcgatacgg
361 gcaggctaga gtgtggtagg ggagatcgga attcctcagt ggcgaacggg tgagtaacac
421 gtgggcaatc tgccctgcac tctgggacaa gccctggaaa cgggggctaa taccggatat
481 gaccttcctc cgcatggggg ttggtggaaa gctccggcgg tgcaggatgc gcccgcggcc
541 tatcagcttg ttggtggggt aatggcctac caaggcgacg acgggtagcc ggcctgagag
601 ggcgaccggc cacactggga cggagacacg gcccagactc ctacgggagg cagcagtggg
661 gaatattgca caatgggcga aagcctgatg cagcgacgcc gcgtgaggga tgacggcctt
721 cgggttgtaa acctctttca gcagggaaga agcgcaagtg acggtacctg cagaagcagc
```