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นางสาวพิมพา ขอวางกลาง

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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ISOLATION OF ANTIMICROBIAL-PRODUCING MICROORGANISMS FROM SOIL

Phimpha Khowangklang

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ISOLATION OF ANTIMICROBIAL-PRODUCING MICROORGANISMS FROM SOIL

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for a Master's Degree.

Thesis Examining Committree

(Asst. Prof. Dr. Rungrudee Srisawat)

Chairperson

(Asst. Prof. Dr. Nawarat Nantapong)

Member (Thesis Advisor)

(Dr. Pongrit Krubphachaya)

Member

(Dr. Mantana Jamklang)

Member

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Vice Rector for Academic Affairs and Internationalization

(Assoc. Prof. Dr. Worawat Meevasana)

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สายพันธุ์แอคติโนแบคทีเรีย 77 สายพันธุ์ และเชื้อรา 177 สายพันธุ์ ได้ถูกแยกจากดินในเขตมหาวิทยาลัยเทคโนโลยีสุรนารี จังหวัดนครราชสีมา เชื้อเหล่านี้ได้ถูกนำมาทดสอบความสามารถในการสร้างสารต้านจุลชีพก่อโรค ได้แก่ *Staphylococcus aureus* TISTR1466 methicillin-resistant *Staphylococcus aureus* DMST20654 (MRSA) *Bacillus subtilis* TISTR008 *Bacillus cereus* TISTR687 *Escherichia coli* TISTR780 *Enterobacter aerogenes* TISTR1540 *Salmonella typhi* TISTR292 *Proteus mirabilis* TISTR100 *Candida albicans* TISTR5779 *Candida tropicalis* TISTR5174 และ *Saccharomyces cerevisiae* TISTR5049 พบว่ามีเพียงเชื้อรา 11 สายพันธุ์ และเชื้อแอคติโนแบคทีเรีย 6 สายพันธุ์ที่สามารถต้านการเจริญของเชื้อจุลินทรีย์ทดสอบได้ โดยแอคติโนแบคทีเรียสายพันธุ์ PKA45 และ PKA51 มีความสามารถในการสร้างสารต้านจุลชีพทดสอบทั้งแบคทีเรียแกรมบวก แบคทีเรียแกรมลบ และยีสต์ได้ดีที่สุด จากผลการทดสอบความสามารถในการยับยั้งการเจริญดังกล่าวสามารถคัดเลือกเชื้อจุลินทรีย์จากดินทั้งหมดจำนวน 17 สายพันธุ์มาจัดจำแนกชนิดโดยการวิเคราะห์เชิงเปรียบเทียบลำดับนิวคลีโอไทด์ของ Internal transcribed spacer (ITS) region และยีนส์ 16S rRNA พบว่า เชื้อราทั้งหมด 11 สายพันธุ์ สามารถระบุชนิดได้ 4 จีนัส ที่พบเป็นกลุ่มใหญ่คือ *Aspergillus* (45.5%) *Penicillium* (27.3%) *Talaromyces* (18.2%) และ *Clonostachys* (9.1%) และ 6 สายพันธุ์ของเชื้อแอคติโนแบคทีเรีย พบว่า ทุกสายพันธุ์เป็นสมาชิกในกลุ่ม *Streptomyces* และจากการศึกษาแผนภูมิวิวัฒนาการของยีน 16S rRNA พบว่าแบคทีเรียสายพันธุ์ PKA45 มีสายวิวัฒนาการที่แยกออกไปจากเชื้อสายพันธุ์อ้างอิง คือเชื้อ *Streptomyces sioyaensis* ซึ่งมีความเป็นไปได้ว่าแบคที่เรียสายพันธุ์ PKA45 อาจจะเป็นสายพันธุ์ใหม่ของเชื้อ *Streptomyces sioyaensis* จากผลการทดสอบความสามารถในการต้านเชื้อจุลชีพก่อโรค เชื้อแอคติ-โนแบคทีเรียสายพันธุ์ PKA45 และ PKA51 ที่สามารถสร้างสารต้านจุลชีพที่มีขอบเขตการออกฤทธิ์กว้างได้ถูกคัดเลือกนำมาใช้ในการศึกษาผลของอาหารเลี้ยงเชื้อ ระยะเวลาในการบ่ม และชนิดของสารสกัดต่อการออกฤทธิ์ต้านเชื้อจุลินทรีย์ทดสอบ พบว่าอาหารที่ใช้เลี้ยงเชื้อต่างชนิดกัน และระยะเวลาการบ่มที่ต่างกันมีผลต่อการออกฤทธิ์ต้านเชื้อจุลชีพทดสอบ โดยเชื้อที่เลี้ยงในอาหารสูตร MHB และใช้ระยะเวลาในการบ่ม 3 วัน เชื้อจะผลิตสารต้านจุลชีพทดสอบได้ดีที่สุด นอกจากนี้ยังพบว่าสารสกัดหยาบที่สกัดด้วยตัวทำละลายเอทิล อะซิเตท ให้ผลการต้านจุลชีพทดสอบทั้งแบคทีเรียแกรมบวก แบคทีเรียแกรมลบ และยีสต์ได้ดีที่สุด ผลจากการศึกษาในครั้งนี้

พบว่าเชื้อแอคติโนแบคทีเรียสายพันธุ์ PKA45 และ PKA 51 สามารถสร้างสารต้านจุลชีพก่อโรคได้ดีที่สุด รวมทั้งเชื้อดื้อยาสายพันธุ์ methicillin-resistant *Staphylococcus aureus* DMST20654 (MRSA) ดังนั้นการศึกษาสารออกฤทธิ์และการทำให้สารออกฤทธิ์บริสุทธิ์อาจถูกนำมาศึกษาเพิ่มเติมเพื่อใช้ในการรักษาโรคติดเชื้อ MRSA นอกจากนี้ งานวิจัยนี้ยังเป็นการรายงานการคัดแยกเชื้อ *Streptomyces sioyaensis* จากดินในประเทศไทย ที่สามารถสร้างสารต้านจุลชีพก่อโรคได้เป็นครั้งแรก

สาขาวิชาปรีคลินิก ลายมือชื่อนักศึกษา_____

ปีการศึกษา 2560 ลายมือชื่ออาจารย์ที่ปรึกษา_____

PHIMPHA KHOWANGKLANG : ISOLATION OF ANTIMICROBIAL-PRODUCING MICROORGANISMS FROM SOIL. THESIS ADVISOR : ASST. PROF. NAWARAT NANTAPONG, Ph.D. 93 PP.

ACTINOBACTERIA/ METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS* *STREPTOMYCES SIOYAENSIS*/ ANTIMICROBIAL ACTIVITY

Total of 77 actinobacteria strains and 177 fungal strains were isolated from soil in Suranaree University of Technology, Nakhon Ratchasima, Thailand. They were used for the screening of their antimicrobial activity against selected bacterial pathogens. They were *Staphylococcus aureus* TISTR1466, methicillin-resistant *Staphylococcus aureus* DMST20654 (MRSA), *Bacillus subtilis* TISTR008, *Bacillus cereus* TISTR687, *Escherichia coli* TISTR780, *Enterobacter aerogenes* TISTR1540, *Salmonella typhi* TISTR292, *Proteus mirabilis* TISTR100, *Candida albicans* TISTR5779, *Candida tropicalis* TISTR5174 and *Saccharomyces cerevisiae* TISTR5049. Eleven strains of fungi and 6 of actinobacteria isolates showed antimicrobial activity against test pathogens. Among them, PKA45 and PKA51 showed highest antimicrobial activity against gram-positive bacteria, gram-negative bacteria and yeasts. A total of 17 antimicrobial-producing soil isolates were used for identification of antimicrobial-producing soil microorganisms by internal transcribed spacer (ITS) region and 16S rRNA gene sequences analysis. The result showed that 11 antimicrobial-producing fungi isolates were classified into four genera. The dominant genera found in this area were the genus *Aspergillus* (45.5%). Others were

Penicillium (27.3%), *Talaromyces* (18.2%) and *Clonostachys* (9.1%). Six strains of actinobacteria were assigned into the genus *Streptomyces*. The phylogenetic tree analysis of 16S rRNA gene sequence showed that PKA45 are not clustered with known reference strain, *Streptomyces sioyaensis*. Thus, PKA45 might be suggested a new strain of *Streptomyces sioyaensis*. Based on the results of preliminary screening of antimicrobial activity against test pathogens from antimicrobial-producing soil isolates, PKA45 and PKA51 were more active against all the test pathogens. Thus, they were selected to study the effects of culture media, incubation periods and extraction solvents on antimicrobial activity against test pathogens. The cultivation of actinobacteria with different culture media and different incubation time affected for the antimicrobial activity against test pathogens. PKA45 and PKA51 showed highest antimicrobial activity with MHB medium on 3rd day of incubation time. In addition, the crude ethyl acetate of PKA45 and PKA51 exhibited high activity against gram-positive bacteria, gram-negative bacteria and yeast. In conclusion, antimicrobial-producing actinobacteria, PKA45 and PKA51 exhibited highest antimicrobial activity against test pathogens including methicillin-resistant *Staphylococcus aureus* DMST20654 (MRSA) . This study of active compound and purification of active ingredient from these strains might be further used for the treatment of MRSA infection. To our best knowledge, this study constitutes the first antimicrobial properties of *Streptomyces sioyaensis* isolated from soil in Thailand.

School of Preclinic Student's Signature _____

Academic Year 2017 Advisor's Signature _____

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LIST OF ABBREVIATIONS

µg/ml	=	Microgram per milliliter
µl	=	Microliter
mg/ml	=	Milligram per milliliter
CFU	=	Colony forming unit
cm	=	Centimeter
MBC	=	Minimum bactericidal concentration
MFC	=	Minimum fungicidal concentration
MIC	=	Minimum inhibitory concentration
ml	=	Milliliter
mm	=	Millimeter
PCR	=	Polymerase chain reaction
rpm	=	Round per minute
rRNA	=	Ribosomal ribonucleic acid
ITS	=	Internal transcribed spacer
TBE	=	Tris-borate-EDTA
v/v	=	Volume by volume
sq.m.	=	Square meter

CHAPTER I

INTRODUCTION

1.1 Background/ Problem

Infectious diseases caused by pathogenic microorganisms have been the leading cause of illness and death in human, causing over 15 million deaths in the 2010 (Dye, 2014). The improper use of antibiotics in human and animal leads to the emergence of antibiotic-resistant bacteria (Khachatourians, 1998). In order to overcome this problem, a growing need to discover for a new and effective antibiotic is necessary. One potential source of novel antibiotics is microorganisms. Many previous studies have shown that antimicrobial-producing microorganisms are widely distributed in natural habitats, especially in soil (Lihan, Choon, Hua and Wasli, 2014; Rabah, Elshafei, Saker, Cheikh and Hocine, 2007; Singh and Rai, 2012). Soil microorganisms are the major source for isolation of several important products such as antimicrobial drugs, anticancer drugs, herbicides, insecticides and biological enzymes (Rondon, Goodman and Handelsman, 1999). The numbers of antibiotics drugs have been discovered from soil microorganisms are 10% of eubacteria, 20% of fungi and 70% of actinobacteria (Makut and Owolewa, 2011). It has been shown that fungi and actinobacteria are potential source for bioactive secondary metabolites including antimicrobial agents. Soil fungi play an important role as decomposers in the soil ecosystem (Aislabie, Deslippe and Dymond, 2013). They are eukaryotic cells that have rigid cell walls and lack chlorophyll. Fungi can produce hyphae are able to

extract nutrients and water within the soil matrix (Loynachan, 2008). Actinobacteria are filamentous gram-positive bacteria, mostly found in soil. They play a major role in decomposition and humus formation. Actinobacterial colony show powdery consistency and stick firmly to agar surface, producing hyphae and conidia/ sporangia-like fungi in culture media (Ranjani, Dharumadurai and Gopinath, 2016).

Although 5×10^{30} microbes live on Earth, only 1% of actinobacteria and 5% of known fungi have been tested for of bioactivity (Bérdy, 2012). This means that the vast majority of known species have never been evaluated for their antimicrobial action. In 2014, Shetty and co-worker have reported that only one fifth of the global soil has been screened for antibiotic-producing microorganisms (Shetty, Buddana, Tatipamula, Naga and Ahmad, 2014). Although, there are substantial amount of terrestrial soils left for the screening of the novel antibiotic-producing soil microbes, the attempt for the screening of these organisms has decreased (Watve, Tickoo, Jog and Bhole, 2001). Thus, in the past two decades, only a few new chemical structures were discovered (Bérdy, 2012).

As of 2015, the World Bank estimates Thai's forest coverage to be 32.1% of the country's area (World Bank Group, 2015). Types of forest in Thailand include deciduous forests and evergreen forests. In Thailand 70% of all forests are considered deciduous forests. Deciduous forests can be divided into three groups, they are mixed deciduous forest, dry deciduous and bamboo forest (Wildlife Thailand, 2010). In Thailand, the strains of antimicrobial-producing microorganisms were isolated from soil collected in mixed deciduous dipterocarp forest, dry dipterocarp forest and riparian forest. They were *Nonomuraea monospora* sp. nov., *Streptomyces* sp., *Talaromyces flavus*, *Neosartorya fischeri* and *Eurotium* sp. (Chanthasena and

Nantapong, 2016; Jantasorn, Mongon, Moungsrimuangdee and Oiuphisittraiwat, 2016; Nakaew, Sungthong, Yokota and Lumyong, 2012).

Several areas in Suranaree University of Technology (SUT) are occupied by dry dipterocarp forest. Approximately 8,131,718 sq.m. of SUT's total area are covered with dry dipterocarp forest (SUT, 2017). Dry dipterocarp forest is commonly found in dry area where the soil condition is acidic, infertile, shallow and sandy or gravelly lateritic soil. It has been reported that spores of streptomycetes survived for long periods in dry soil. Their tolerance of high moisture tensions was greater than that of their own vegetative hyphae or cells of non-sporing bacteria (Williams, Shameemullah, Watson and Mayfield, 1972). Thus, soil of Suranaree University of Technology could be an interesting source for the search of novel antimicrobial agents. This present study attempted to screening and isolates the antimicrobial-producing microorganisms from forest soil in Suranaree University of Technology.

1.2 Research objectives

1.2.1 To isolate the antimicrobial-producing microorganisms from forest soil at Suranaree University of Technology (SUT).

1.2.2 To evaluate an antimicrobial activity of crude extracts produced by soil microorganisms against bacterial pathogens.

1.2.3 To identify the antimicrobial-producing microorganisms isolates by morphological characteristics and molecular technique.

1.3 Research hypothesis

1.3.1 Forest soil in Suranaree University of Technology is served as a potential resource for antimicrobial-producing microorganisms.

1.3.2 The crude extracts produced from soil microorganisms shows an antimicrobial activity against opportunistic pathogens.

1.4 Scope and limitations of study

This work involves the isolation of potential antimicrobial-producing microorganisms from soil. The soil isolates strains were used for evaluation of antimicrobial activities against pathogenic bacteria, such as, *Staphylococcus aureus, Bacillus cereus, Escherichia coli, Pseudomonas aeruginosa* and *Candida albicans*. The strains that show high antimicrobial activities were used for extraction of their bioactive compounds. The crude extracts were used for determination of minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC).

CHAPTER II

LITERATURE REVIEWS

2.1 History of antibiotics

Antibiotics are a drug used to treat infections caused by bacteria that can cause illness and death to humans and animals. Function of antibiotic is to inhibit or destroy the bacteria and/or fungi cells that cause certain disease (Lihan et al., 2014). Penicillin is the oldest available antibiotic that was first discovered in 1928 by Sir Alexander Fleming. He found that growth of the bacteria *Staphylococcus aureus* was inhibited by blue green mold. This mold was later identified as *Penicillium notatum*. In 1940, penicillin was introduced into clinical use and revolutionized the treatment of infections caused by gram-positive bacteria, especially, *staphylococci* and *streptococci*. This discovery marked the beginning of the development of antibacterial compounds produced by living organisms (Lihan et al., 2014; Singh and Rai, 2012). Gramicidin, first discovered by Rene Dubos in 1939, was derived from soil bacteria *Bacillus brevis*, which inhibited growth of gram-positive bacteria. The finding of gramicidin initiated the isolation and screening of bacteria and fungi from soil (Van Epps, 2006).

During the years 1940-1970, many antibacterial agents were identified and developed (Figure 2.1). Most of the bioactive compounds are divided into several major structural classes such as amino glycosides, ansamycins, anthracyclines, β-lactam, macrolides and tetracycline (Singh and Rai, 2012). In 1940s, Benjamin Minge

Duggar was first discovered tetracycline producing *streptomyces aureofaciens*. It is a family of antibiotics that inhibit protein synthesis. Tetracycline is broad-spectrum agents, against a wide range of gram-positive and gram-negative bacteria (Chopra and Roberts, 2001). Another antibiotic, streptomycin was isolated in 1944 by Waksman. It was produced from a soil bacterium *streptomyces griseus*. Streptomycin provided the first effective antibiotic against tuberculosis (Tripathi, Tewari, Dwivedi and Tiwari, 2005). Cephalosporin was isolated in 1945 from *Cephalosporium acremonium* by Giuseppe Brotzu. It had powerful action on a wide spectrum activity against Gram-positive and also some gram-negative bacteria (Muñiz, Zelaya, Esquivel and Fernández, 2007). Vancomycin was isolated from *Streptomyces orientalis* in 1955. Vancomycin was effective against gram-positive bacteria, especially resistant *staphylococci* (Butler, 2005).

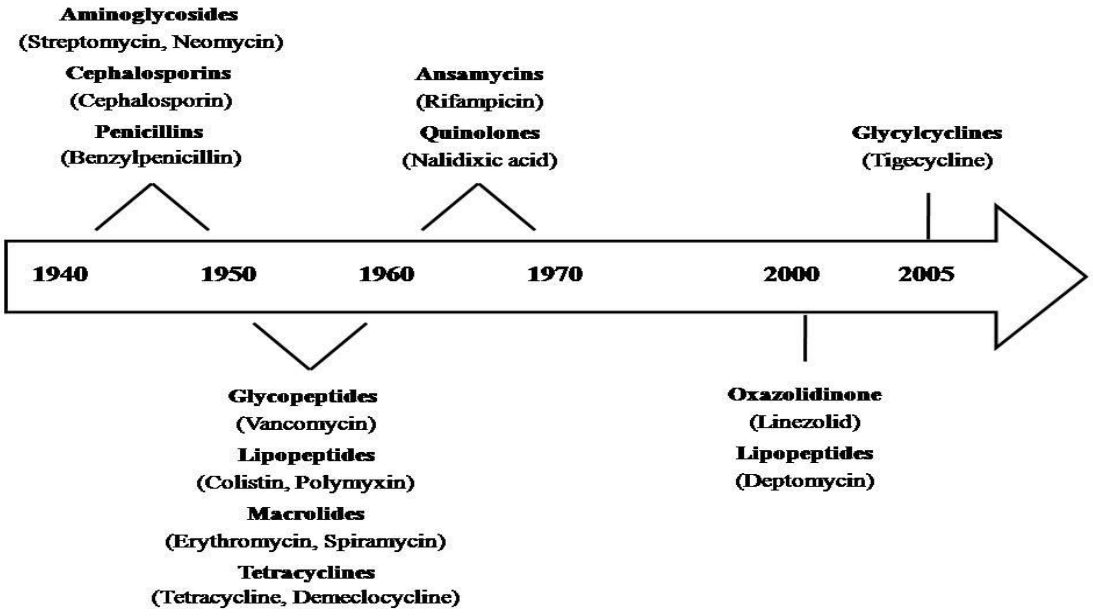


Figure 2.1 The timeline of antibiotic drug discovery during the years 1940-2005 (Wright, 2007).

2.2 Antimicrobial-producing microorganisms

It is a well known fact that natural products isolated from microorganisms have been the source of most of the antibiotics. The discovery of penicillin and its use in the clinic in 1940s was soon followed by the discovery of a large number of antibiotics from microbes, in particular, actinobacteria and fungi (Pelaez, 2006). Soil microorganisms are an excellent source for the discovery of new antibiotic drugs (Berdy, 2005). A number of antibiotic drugs have been discovered from soil-inhabiting microorganisms, which include fungi (20%), actinomycetes (70%) and eubacteria (10%) (Makut and Owolewa, 2011). They have continually been screened for their useful biological active metabolites, such as antibiotics since long ago (Lihan et al., 2014).

2.2.1 Antibiotic-producing actinobacteria

Actinobacteria are the major microbial population in soil that can produce active secondary metabolites, such as antimicrobial, antitumor and/or antiviral activities. They produce most of the medically useful natural antibiotics. Many of these secondary metabolites are of industrial and pharmaceutical interests, which they produce approximately 75% of commercial and medical antibiotics (Berdy, 2005; Lee et al., 2014). Many important drug used in hospitals around the world come from genus *Streptomyces* because they produce a variety of secondary metabolites. The history of antibiotics derived from *Streptomyces* has begun with the discovery of actinomycin and streptomycin during 1940s (de Lima Procópio, da Silva, Martins, de Azevedo and de Araújo, 2012). After the discovery of actinomycin and streptomycin, many scientists intensified the search for antibiotics within the genus *Streptomyces*.

In 1948, tetracycline was discovered by Benjamin Minge Duggar. This antibiotic was derived from *Streptomyces aureofaciens* isolated from soil in University of Missouri campus, USA. It is a wide spectrum of microbial pathogens and potential against inflammation-based mammalian cell diseases (Wermuth, 2007).

In 1949, erythromycin from soil bacterium, *Streptomyces erythrecus* by McGuire (Butler, 2008). Erythromycin has been in clinical use since 1952 to combat gram-positive and some gram-negative bacteria (Jelić and Antolović, 2016).

In 1957, kanamycin was isolated from *Streptomyces kanamyceticus* by Hamao Umezawa (Umezawa, 1958). It is an aminoglycoside antibiotic which was reserved for the treatment of tuberculosis (Yanai, Murakami and Bibb, 2006).

In 1985, Eli Lilly isolated a lipopeptide antibiotic, daptomycin from soil actinomycete, *Streptomyces roseosporus*. It has been in clinical use in the USA since 2003 for the treatment of infection caused by gram-positive bacteria (Rybak, 2006) by disrupting multiple aspects of bacterial cell membrane function.

Several antibiotics are derived from the genus *Streptomyces* which are shown in Table 2.1.

Table 2.1 List of antimicrobial drug produced by *Streptomyces* spp. and their mode of action.

Antibiotics	Source	Antibiotic classes	Mode of Action
Streptomycin	*S. griseus*	Aminoglycosides	Inhibits protein synthesis
Cephalosporin	*S. clavuligerus*	Cephalosporins	Inhibits cell wall synthesis
Chloramphenical	*S. venezuelae*	Chloramphenicol	Inhibits protein synthesis
Neomycin	*S. fradiae*	Aminoglycosides	Inhibits cell wall synthesis
Tetracycline	*S. aureofaciens*	Tetracyclines	Inhibits protein synthesis
Nystatin	*S. noursei*	Polyenes	Inhibits cell membrane function
Viomycin	*S. vinaceus* and *S. capreolus*	Hybrid peptides	Inhibits protein synthesis
Virginiamycin	*S. pristinaespiralis* and *S. virginiae*	Cyclic polypeptide	Inhibits protein synthesis
Erythromycin	*S. erythreus*	Macrolides	Inhibits protein synthesis
Lincomycin	*S. lincolnensis*	Lincosamides	Inhibits protein synthesis
Vancomycin	*S. orientalis*	Glycopeptides	Inhibits cell wall synthesis
Novobiocin	*S. niveus*	Aminocoumarin	Inhibits nucleic acid synthesis and inhibits metabolic processes

Table 2.1 List of antimicrobial drug produced by *Streptomyces* spp. and their mode of action (Continued).

Antibiotics	Source	Antibiotic classes	Mode of Action
Kanamycin	*S. kanamyceticus*	Aminoglycosides	Inhibits protein synthesis
Fosfomycin	*S. fradiae*	Epoxides	Inhibits cell wall synthesis
Ribostamycin	*S. ribosidificus*	Aminoglycosides	Inhibits protein synthesis
Daptomycin	*S. roseosporus*	Lipopeptides	Inhibits cell membrane function

Source: (de Lima Procópio et al., 2012).

Actinobacteria are aerobic, gram-positive filamentous bacteria. They are present in various ecological habitats such as soil, fresh water and marine. Actinobacteria play an important role in decomposition of organic materials. Most actinobacteria are free-living microorganisms and a few are pathogens (Dwivedi, Singh, Pandey and Kumar, 2011). They produce antibiotics to inhibit growth of other microorganisms and survive in nature.

Actinobacteria typically exhibit filamentous growth and produce spores. The filaments produced from actinobacteria are similar with fungal hyphae. A chain of sexual spore called conidia is produce on their hyphae. The colonies are powdery mass over the surface of culture medium, often these are pigment when the aerial spore are produced. Typically, a spore germinates under the favorable conditions of temperature, nutrients and moisture to generate a substrate mycelium. This consists of a net of branching hyphae that grows and penetrates into the substrate to reach

nutrients. When nutrients are scarce (or in response to other signals), some hyphae start growing away from the substrate into the air generating aerial mycelium. At the same time, the substrate mycelium suffers a process of programmed cell death and its content is reused by the growing aerial mycelium. Finally, on the distal parts of aerial hyphae, the partition process is complete and yields the chains of spores (Figure 2.2).

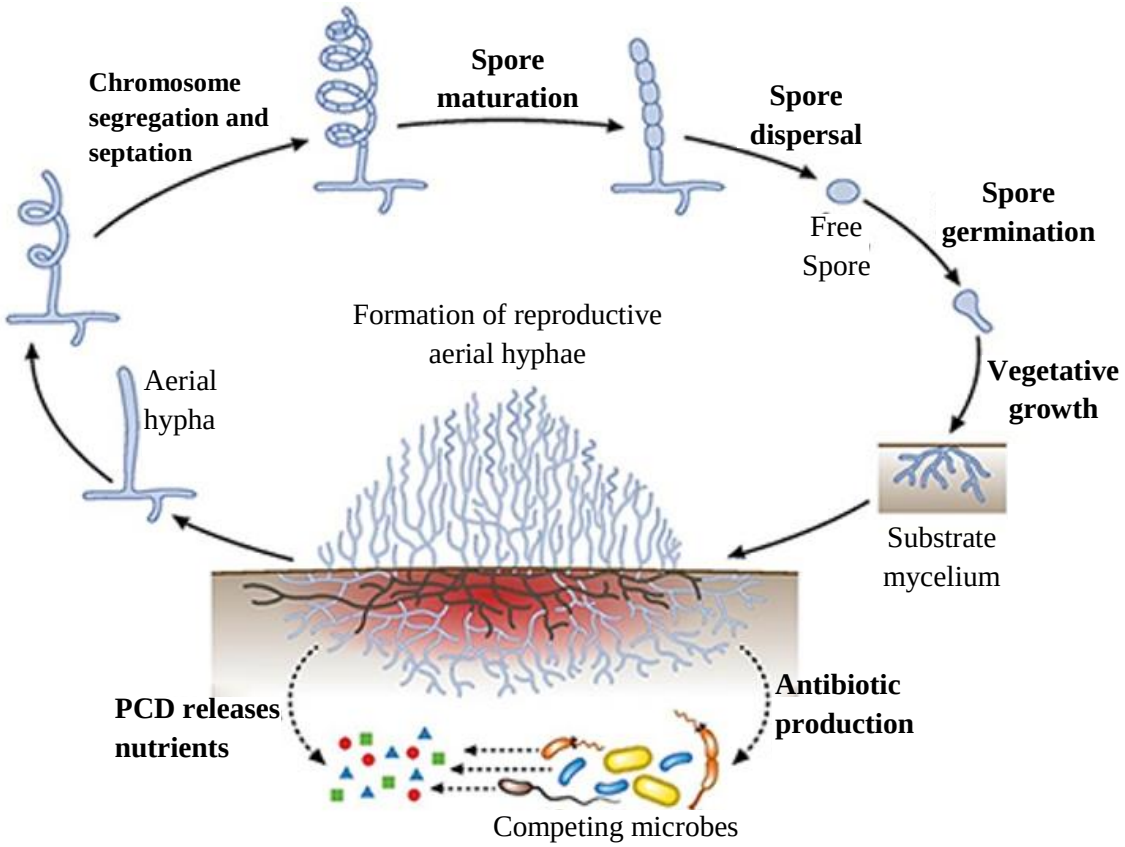


Figure 2.2 The life cycle of Actinobacteria.

There are many types of reproduction involving asexual sporulation. Spores may be formed on substrate and/or the aerial mycelium as single cells, in chains of various length or harbored in special vesicles (sporangia) and may be endowed with flagella (Kalakoutskii and Agre, 1976).

Morphological characters are widely used for the characterization of Actinomycetes, for example, the cellular morphology, the color of mycelium, the surface of colony and the presence or absence of spores on the mycelium (Anderson and Wellington, 2001). The spore morphologies are the important characteristics for identification of Actinomycetes. Several types of spores are produced by Actinomycetes species, many of which are involved in the dispersal of Actinomycetes. The colonies of Actinomycetes are grey-white which often turn to yellowish and pinkish colors due to the formation of pigmented spores. There is a typical velvety appearance to the colonies of most Actinomycetes which distinguishes them from other bacteria. The soil Actinomycetes produce a volatile compound called geosmin which literally translates to "earth smell" (Gerber and Lechevalier, 1965).

2.2.2 Antibiotic-producing fungi

Fungi are well known for producing antimicrobial agents, several of which have formed the basis for development of new clinical antimicrobial agents. Today, the largest group of microbial metabolites is produced by fungi (45%), which include basidiomycetes (11%) and microscopic filamentous fungi (33%). Filamentous fungi, such as *Penicillium*, *Aspergillus* and *Trichoderma* are important genus for production of antimicrobial agents. These strains represent almost 99% of all fungal metabolites (Bérdy, 2012). The total number of bioactive fungal products is approximately 86,000, representing 38% of all microbial products (Berdy, 2005). The history of antibiotics derived from fungi has begun with the discovery of penicillin in 1928 by Alexander Fleming, who showed its efficacy in laboratory cultures against many pathogenic bacteria. This discovery marked the beginning of the development of antibacterial compounds produced by living organisms (Makut and Owolewa, 2011).

Antimicrobial drugs produced by fungi include griseofulvin. It was discovered from *Penicillium griseofulvin* in 1939. It is used for the treatment of fungal infection of nails and skin (Blank et al., 1959). In 1948, cephalosporin was isolated from *Cephalosporium acremonium* by Giuseppe Brotzu. It is a broad spectrum of microbial pathogens and potential inhibit the growth of *Salmonella typhi* and *Staphylococcus aureus* (Buss and Hayes, 2000).

Several antibiotics are derived from fungi which are shown in Table 2.2.

Table 2.2 List of antimicrobial drug produced by fungi and their range of activity.

Antibiotics	**Source**	**Rang of Activity**
Penicillin	*Penicillium notatum*	Gram-positive and Gram-negative bacteria
Griseofulvin	*Penicillium griseofulvum*	Fungi
Cephalosporin	*Cephalosporium acremonium*	Gram-positive and Gram-negative bacteria
Citrinin	*Penicillium citrinum*	Fungi
Palutin	*Penicillium palutum*	Fungi and Bacteria
Fusidic acid	*Fusidium coccineum*	Bacteria
Viridin	*Trichoderma viridi*	Fungi
Clavacin	*Aspergillus clavatus*	Fungi

Source: (Kück, Bloemendal and Teichert, 2014).

Fungi are eukaryotic cells, consisting from unicellular yeasts to muticellular molds and mushrooms. They grow as long threadlike structure or hyphae that make a mass called mycelium. The mycelium produces special hyphae that create the reproductive spores. They are cosmopolitan in distribution and grow in diverse habitats, wherever moisture and organic materials are available. Most fungi are

saprophytic feeding on dead organic material. Many saprophytic fungi promote the suppression of plant disease, either by producing antibiotics inhibitory to disease-causing organisms or outcompeting pathogens for available resources (Reddy, 2001).

In general, Fungi begin their live as a spore, then germinate and develop into a mycelium. This process, the fungus is excreting digestive enzyme and absorb the nutrients. In addition, many fungi are secreting antibiotics and mycotoxin to discourage competition. Many fungi begin their life cycle producing asexual fruiting bodies called conidia. These are generated quickly and help the fungus occupy the area quickly. If fungus encounters another of its own species and condition are right. It may mate and begin the process of producing sexual fruiting bodies (Figure 2.3).

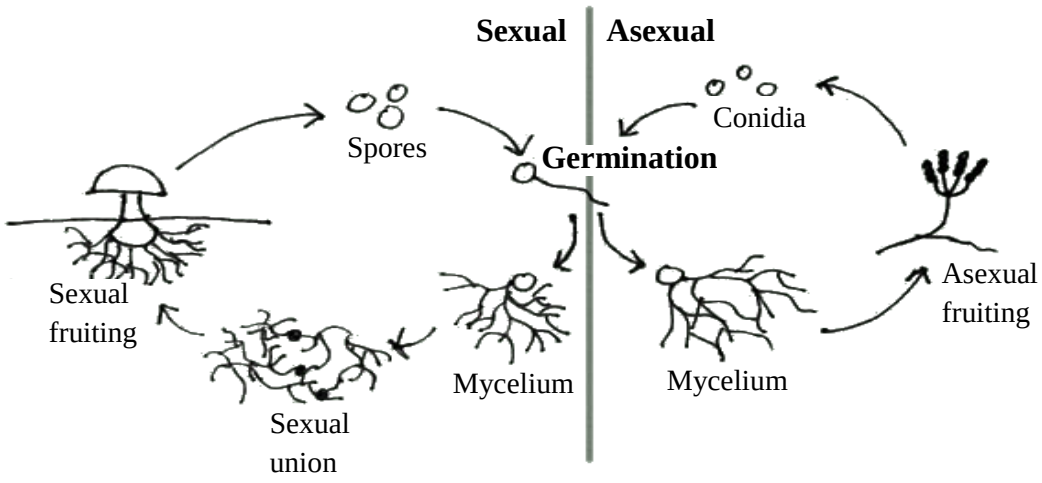


Figure 2.3 The life cycle of fungi.

2.3 Modes of action of antibiotics

Antimicrobial agents function as selective toxins that inhibit enzymes that are either unique to the prokaryotic cell or sufficiently different such that toxicity to the mammalian host is low. Most antimicrobials fall into one of five main categories,

based on their site of activity. They include inhibition of cell wall synthesis, protein synthesis, nucleic acid synthesis, metabolic pathway, or disruption of cell membrane integrity. The major mechanisms are given below, with examples of the more commonly used antimicrobial agents.

2.3.1 Inhibition of cell wall biosynthesis

The cell wall is an essential microbial structure responsible for the cell shape. In addition, the cell wall prevents cell lysis due the high cytoplasmic osmotic pressure and allows the anchoring of membrane components and extracellular proteins, such as adhesions (Guilhelmelli et al., 2013). The most commonly used antimicrobial agents that inhibit cell wall biosynthesis include β-lactam antibiotics such as penicillins and cephalosporins (Kotra and Mobashery, 1998).

2.3.2 Disruption of cell membrane integrity

Biologic membranes are composed basically of lipids, proteins and lipoproteins. The cytoplasmic membrane acts as a diffusion barrier for water, ions, nutrients and transport systems. Most health workers now believe that membranes are a lipid matrix with globular proteins randomly distributed to penetrate through the lipid bilayer. A number of antimicrobial agents can cause disorganization of the membrane. These agents can be divided into cationic, anionic, and neutral agents. The best-known compounds are polymyxin B and colistemethate (polymyxin E) (Neu and Gootz, 1996).

2.3.3 Inhibition of protein synthesis

Several classes of antimicrobial agents act by inhibiting bacterial protein synthesis (ribosome function). These include aminoglycosides, macrolides, tetracyclines, lincosamides, ketolides, streptogramins, oxazolidinones and

chloramphenicol (Hooper, 2001; Jacoby and Archer, 1991; Kohanski, Dwyer and Collins, 2010; Levy and Marshall, 2004; McKeegan, Borges-Walmsley and Walmsley, 2002). Microbial protein synthesis is directed by ribosomes in conjunction with cytoplasmic factors, which transiently bind to particles during the initiation phase, elongation phase and termination phases. Antimicrobial agents block different steps in bacterial protein synthesis by interfering with the function of either the cytoplasmic factors or the ribosomes (Cocito, Di Giambattista, Nyssen and Vannuffel, 1997).

2.3.4 Inhibition of nucleic acid synthesis

In general, antimicrobials that disrupt DNA and RNA synthesis do so by interfering with either nucleotide (e.g., sulfonamides) or nucleic acid (e.g., quinolones, rifamycins) biosynthetic processes in the cell. The compounds that inhibit enzyme function in nucleic acid synthesis typically act on RNA polymerase (rpoB) or DNA topoisomerases (gyrA, gyrB, parC, parE). The rifamycins, which include the antituberculosis drug rifampin, bind to the bacterial RNA polymerase, selectively inhibiting the initiation of bacterial transcription (Mc Dermott, Walker and White, 2003).

2.3.5 Inhibition of metabolic pathway

Trimethoprim and sulfonamides interfere with folic acid metabolism in the microbial cell by competitively blocking the biosynthesis of tetrahydrofolate, which acts as a carrier of one-carbon fragments and is necessary for the ultimate synthesis of DNA, RNA and bacterial cell wall proteins. Unlike mammals, bacteria and protozoan parasites usually lack a transport system to take up preformed folic acid from their environment. Most of these organisms must synthesize folic acid, although some are

capable of using exogenous thymidine, circumventing the need for folic acid metabolism (Neu and Gootz, 1996). Sulfonamides competitively inhibit the conversion of pteridine and p-aminobenzoic acid (PABA) to dihydrofolic acid by the enzyme pteridine synthetase. Sulfonamides have a greater affinity than PABA for pteridine synthetase. Trimethoprim has a tremendous affinity for bacterial dihydrofolate reductase (10,000 to 100,000 times higher than for the mammalian enzyme); when bound to this enzyme, it inhibits the synthesis of tetrahydrofolate (Neu and Gootz, 1996).

2.4 The opportunistic pathogens

Opportunistic pathogens are typically characterized in the medical literature as organisms that can become pathogenic following a perturbation to their host (Brown, Cornforth and Mideo, 2012). They are the normal flora that live on the surface or in deep layer of skin, saliva, oral mucosa, and conjunctiva and gastrointestinal. They normally are not invasive to the host but dependent on opportunities through breaks in the body barriers such as wounds, burns and depressed gastric acidity. The exposure risk includes person who have become weakened by some other bacteria, virus or other diseases. The exposure also includes patients of all ages receiving antibiotics or chemotherapy for a long time (Cedric et al., 2005).

Infectious diseases caused by opportunistic pathogens are a serious public health problem in the area where large numbers of people are in close localization, particularly hospitals. At least five percent of patients admitted to hospitals acquire nosocomial infections from opportunistic pathogens (Grabińska-Łoniewska, Wardzyńska, Pajor, Korsak and Boryń, 2007). The examples of opportunistic

pathogens which commonly cause the infectious diseases are *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Pseudomonas aeruginosa*.

Staphylococcus aureus is an opportunistic pathogen that can cause a variety of self-limiting to life-threatening diseases in humans. The bacteria are a leading cause of food poisoning, resulting from the consumption of food contaminated with enterotoxins. *Staphylococcus aureus* can cause a range of illnesses from minor skin infections such as pimples, impetigo, cellulitis, scalded skin syndrome and abscesses to life-threatening diseases such as pneumonia, meningitis, osteomyelitis, endocarditis, toxic shock syndrome and septicemia. *Staphylococcus aureus* is one of the most common causes of nosocomial infections, often causing postsurgical wound infections (Otto, 2009).

Staphylococcus epidermidis is a human skin commensally microorganisms which can become an opportunistic pathogen. This bacterium can cause severe infection after penetration of epidermal and mucosal barriers which frequently occurs in the hospital during the insertion of indwelling medical devices. The pathogenicity is its ability to adhere and form biofilms on the surfaces of the medical devices (Yao et al., 2005).

Pseudomonas aeruginosa is an opportunist Gram-negative rod that has a long and infamous association with burn infections. It grows well in the moist environment of a burn wound, producing a foul, green-pigmented discharge and necrosis. *Pseudomonas aeruginosa* establishes itself in vulnerable patients include the patients with cystic fibrosis or hospitalized in intensive care units. It has become a major cause of nosocomial infections worldwide (about 10% of all the infections in European Union hospitals) and a serious threat to public health (de Bentzmann and Plésiat, 2011).

Nowadays, many strains of opportunistic pathogens are found to be resistant to antibiotic drugs. The emergence of drug-resistance strains is due to the widespread use and misuse of antibiotics. Therefore, there is the need for the search of new effective drugs to replace the invalidated ones.

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

3.1.1 Test pathogenic bacterial strains

The pathogenic strains used in this study were purchased from American Type Culture Collection (ATCC), Department of Medical Sciences Thailand (DMST) and Thailand Institute of Scientific and Technological Research (TISTR). They were *Staphylococcus aureus* ATCC29213, *Staphylococcus aureus* TISTR1466, methicillin-resistant *Staphylococcus aureus* DMST20654 (MRSA), *Staphylococcus epidermidis* TISTR518, *Bacillus subtilis* TISTR008, *Bacillus cereus* TISTR687, *Escherichia coli* TISTR780, *Enterobacter aerogenes* TISTR1540, *Salmonella typhi* TISTR292, *Proteus mirabilis* TISTR100, *Candida albicans* TISTR5779, *Candida tropicalis* TISTR5174 and *Saccharomyces cerevisiae* TISTR5049.

3.1.2 Culture media

The following culture media were used in this study

- Starch Casein (SC) medium contained (per 1 liter) 10 g of soluble starch, 0.3 g of casein acid hydrolysate, 2 g of KHO_3, 2 g of NaCl, 2 g of K_2HPO_4, 0.05 g of $MgSO_4$, 0.002 g of $CaCO_3$, and 0.01 g of $FeSO_4$. The final pH adjusted to 7.0±0.2.

- International Streptomyces Project 2 (ISP2) medium contained (per 1 liter) 4 g of yeast extract, 10 g of malt extract and 4 g of dextrose. The final pH adjusted to 7.2±0.2.

- Nutrient (N) medium contained (per 1 liter) 15 g of peptone, 3 g of yeast extract, 1 g of dextrose and 6 g of NaCl. The final pH adjusted to 7.5±0.2.

- Sabouraud Dextrose (SD) medium contained (per 1 liter) 40 g of dextrose and 10 g of peptone. The final pH adjusted to 5.6±0.2.

- Yeast Extract Glucose (YEG) medium contained (per 1 liter) 1 g of yeast extract, 10 g of dextrose, 0.5 g of KNO_3 and 0.1 g of K_2HPO_4. The final pH adjusted to 7.0±0.2.

- Malt Extract (ME) medium contained (per 1 liter) 20 g of glucose, 20 g of malt extract and 1 g of peptone. The final pH adjusted to 5.4±0.2.

- Czepex Dox with 3% Glucose (CD) medium contained (per 1 liter) 30 g of glucose, 3 g of $NaNO_3$, 1 g of K_2HPO_4, 0.5 g of KCl, 0.5 g of $MgSO_4$ and 0.01 g of $FeSO_4$. The final pH adjusted to 7.3±0.2.

- Czepex Yeast Extract (CYE) medium contained (per 1 liter) 30 g of glucose, 5 g of yeast extract, 3 g of $NaNO_3$, 1 g of K_2HPO_4, 0.5 g of $MgSO_4$, 0.5 g of KCl and 1 ml of trace metal solution (1 g of $ZnSO_4$ and 0.05 of $CuSO_4$). The final pH adjusted to 7.3±0.2.

- Potato Dextrose (PD) medium (pH 5.1±0.2) was purchased from Hi-media, India. This medium contained (per 1 liter) 200 g of potato (infusion from) and 20 g of dextrose.

- Mueller Hinton (MH) medium (pH 7.2-7.4) was purchased from Himedia, India. This medium contained (per 1 liter) 300 g of beef extract, 17.5 g of casein acid hydrolysate and 1.5 g of starch.

All solid medium were added 15 g of agar into 1 liter of medium.

The sterilization of the medium was performed by autoclaving at 121ºC, 15 p.s.i for 15 minutes.

3.1.3 Antibiotics

- Chloremphenicol (Sigma-Aldrich, USA) stock solution was prepared by dissolving in sterile distilled water to final concentration of 25 mg/ml.

- Vancomycin (Amresco, USA) stock solution was prepared by dissolving in sterile distilled water to final concentration of 10 mg/ml.

- Tetracycline (Sigma-Aldrich, USA) stock solution was prepared by dissolving in sterile distilled water to final concentration of 10 mg/ml.

- Ketoconazole (Sigma-Aldrich, USA) stock solution was prepared by dissolving in sterile distilled water to final concentration of 10 mg/ml.

All the antibiotics stock solution were filter sterilized though a 0.22-0.45 µm pore filter (Corning®. Germany) and stored at -20ºC until used.

3.1.4 Buffers and solutions

- 10X TBE buffer for gel electrophoresis contained (per 1 liter) 108 g of Tris-HCl, 55 g of boric acid and 40 ml of 0.5 M EDTA pH 8.0.

- Lysis buffer composed of 400 mM Tris-HCl pH 8.0, 60 mM EDTA pH 8.0, 150 mM NaCl and 1% w/v of sodium dodecyl sulfate.

- TE buffer composed of 10 mM Tris-HCl and 0.1 mM EDTA pH 8.0.

- MaestroSafe^{TW}nucleic acid stains were purchased from MaestroGen, Taiwan.

3.1.5 Primers used for PCR amplification

The name of the oligonucleotides and their sequences are listed in Table 3.1

Table 3.1 List of primers used in this study.

Name	Sequences (5′-3′)	Sources
243F	GGATGAGCCGCGGCCTA	Monciardini, Sosio, Cavaletti, Chiocchini and Donadio, 2002
A3R	CCAGCCCCACCTTCGAC	Monciardini et al., 2002
ITS4	TCCTCCGCTTATTGATATGC	Gardes and Bruns, 1996
ITS5	GGAAGTAAAAGTCGTAACAAGG	Gardes and Bruns, 1996

3.1.6 Miscellaneous material

- GoTaq®Green master mix used for PCR amplification was purchased from Promega, USA.

- 100 bp DNA Ladder Ready to Load was used as marker. The marker was purchased from Solis Biodyne, Estonia.

3.1.7 Equipments

All the instruments used in this study are listed in Table 3.2

Table 3.2 List of instruments used in this study.

Name	Source
Autoclave	Tomy, USA
Hot air oven	Memmert, Schwabach
Incubator shaker	Appendorf, Germany
Spectrophotometer	PG Instrument, UK
Centrifuge machine	Hettich, Germany
pH meter	Sartorious, Germany
Laminar flow	Esco, Singapore
Vortex mixer	FINEPCR, Korea
Rotary evaporator	Buchi, Switzerland
Microscope (model CX31)	Olympus, USA
Thermal cycler	Biorad, USA

3.2 Methods

3.2.1 Study site and sample collection

The study site was located at the forest area in Suranaree University of Technology, Nakhon Ratchasima, Thailand. Soil samples were randomly collected at a depth of 10-15 cm from the surface of soil (during July 2014-May 2015). The soil were kept in polypropylene bags and transferred to the laboratory in icebox for isolation of microorganisms. The sampling sites of soil sample were shown in Figure 3.1 and 3.2

3.2.2 Isolation of soil microorganisms

One gram of soil samples was suspended in 99 ml sterile water and incubated at room temperature for 30 min with shaking. Soil suspension was serially diluted up to 10^{-5}. One hundred microliter portions of 10^{-3}, 10^{-4} and 10^{-5} dilutions were spreaded onto SCA plate for isolated actinobacteria strains and PDA plates

supplemented with 50 mg/l chloramphenicol for isolated fungi. The plates were incubated at room temperature for 7-14 days or until the colonies appeared. After incubation, the suspected microorganism colonies were sub-cultured to SCA plate or PDA plates without antibiotics, incubated for 5-7 days at room temperature and kept at 4ºC until further study.

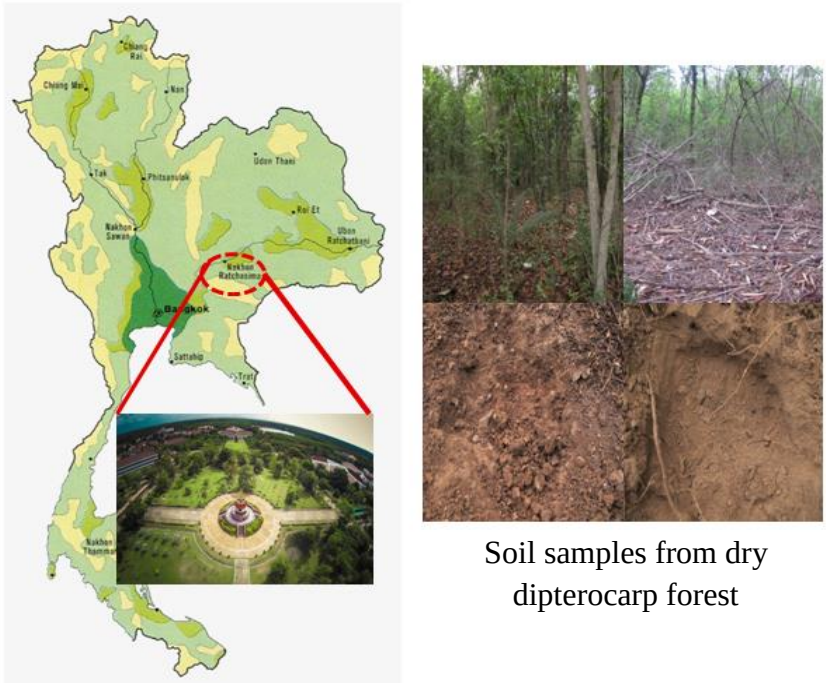


Figure 3.1 The map of sampling site. Suranaree University of Technology is located in Nakhon Ratchasima province, Thailand. Dry dipterocarp forest are found in this area.

Figure 3.2 Map of sampling site in Suranaree University of Technology. The asterisk represented the sampling sites of soil samples.

3.2.3 Preliminary screening of antimicrobial-producing soil isolates

The antimicrobial activities of soil isolates were determined by cross-streak method (Figure 3.3). The cell was inoculated on MHA or SDA by streaking at one side of a petri dish. MHA medium was used for bacterial sensitivity test while SDA medium was used for yeast sensitivity test. The plates were incubated at 28ºC for 5 days to allow the organisms to secrete antimicrobial substances into the agar. After incubation, the strains of test pathogenic bacteria (adjusted to 0.5 MacFarland) were streaked perpendicularly to the line of fungal colonies and incubated at 37ºC for 24-48 h. The zone of inhibition in millimeter against test pathogens was measured and recorded.

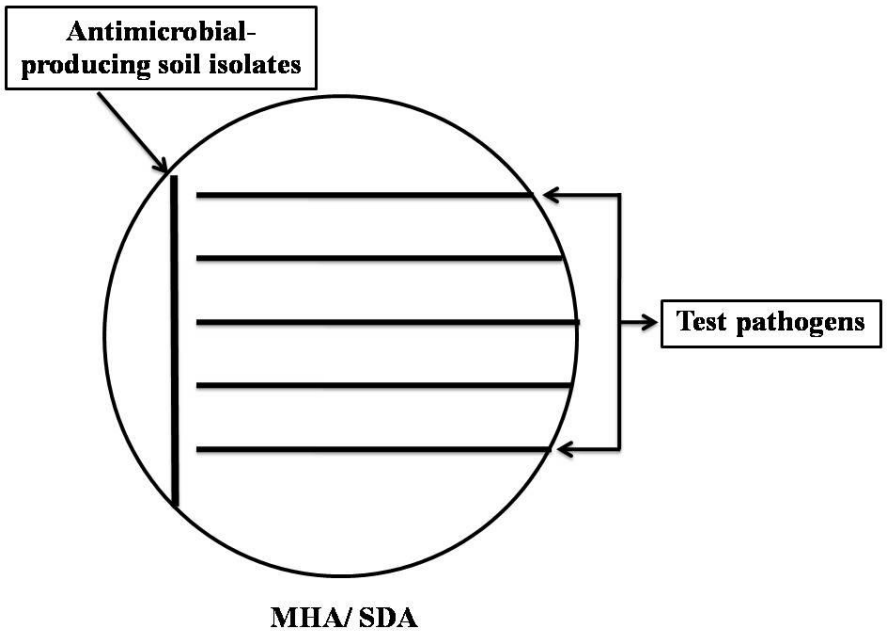


Figure 3.3 The cross-streak method.

3.2.4 Identification of antimicrobial-producing soil isolates

3.2.4.1 Preparation of DNA template

Genomic DNA was isolated from cell grown in 5 ml MHB medium and PDB medium at 28ºC for 3-5 days. The cell culture were centrifuged at 13,000 rpm for 5 min and the cell pellets were used for DNA extraction. The cell pellets were placed in a mortar in the presence of 500 µl lysis buffer and grounded with glass beads and pestle. The suspension was transferred into a 1.5 ml microcentrifuge tube. One hundred-sixty five microliter of 5 M NaCl was added. The suspension was mixed by inverting the tube several times and centrifuged at 13,000 rpm for 20 min. The supernatant was transferred to a new tube, and then mixed with 800 µl of chloroform: isoamyl alcohol (1:1). The suspension was mixed by gently inverting the tube until the solution become milky. The mixture was then centrifuged at 13,000 rpm for 20

min. The aqueous layer was removed and extracted with an equal volume of chloroform. The tube was centrifuged at 13,000 rpm for 20 min. The DNA was precipitated with two volume of absolute alcohol and centrifuged at 10,000 rpm for 10 min. The pellet was washed three times with 800 µl of 70% cold ethanol and air dried. The DNA was dissolved in 50 µl of TE buffer and stored at -20ºC. The supernatants were used as DNA template for PCR amplification of 16S rRNA gene.

3.2.4.2 PCR amplification of the internal transcribed spacer (ITS) region

The fungal genomic DNA was amplified by PCR with universal primers, ITS5 and ITS4 (Table 3.1). The 25 µl of PCR mixture is shown in Table 3.3. The thermal cycling conditions were as follows: initial denaturation at 95ºC for 5 min followed by 30 cycles of denaturation at 95ºC for 30 sec, annealing at 50ºC for 1 min, extension at 72ºC for 1 min and final extension at 72ºC for 7 min. The size of PCR product was examined by agarose gel electrophoresis. The amplified fragments were purified using NucleoSpin® Gel and PCR clean-up kit (MACHEREY-NAGEL, Germany).

3.2.4.3 PCR amplification of 16S rRNA gene

The genomic DNA of actinobacteria was used as DNA template for PCR of 16S rRNA gene. The 16S rRNA gene was amplified by using universal primers, 243F and A3R (Table 3.1). The 25 µl of PCR mixture is shown in Table 3.3. The amplification was performed in a thermal cycler according to the following conditions: initial denaturation 95ºC for 5 min followed by 30 cycles of denaturation at 95ºC for 1 min, annealing at 55ºC for 1 min, extension at 72ºC for 1 min and a final extension at 72ºC for 7 min. The size of PCR product was examined by agarose gel

electrophoresis. The amplified fragments were purified using NucleoSpin® Gel and PCR clean-up kit (MACHEREY-NAGEL, Germany).

Table 3.3 PCR reaction mixture.

Reaction mixture	**Volume (µl)**
Genomic DNA template	5
GoTaq® Green master mixed (2x)	12.5
Forward primer (10 µM)	1
Reverse primer (10 µM)	1
Distilled water	5.5
Final volume	**25**

3.2.4.4 The DNA sequencing

The purified PCR product was submitted for DNA sequencing at Macrogen Inc., Korea. The sequencing was obtained and compared against the EzBioCloud and UNITE databases using BLAST program.

3.2.4.5 Construction of phylogenetic tree

The sequences of 16S rRNA gene and ITS region were compared with known sequence from EzBioCloud database and UNITE database, respectively. The sequences were aligned with closest related strains by using ClustalW. The phylogenetic tree was constructed by neighbor-joining method using the Molecular Evolutionary Genetics Analysis software version 6.0 (MEGA 6.0). The tree topologies were evaluated by using bootstrap analysis (1,000 replications). The distances matrix between sequences was generated by using Kimura's two-parameter model.

3.2.5 Optimization of antimicrobial metabolites production from soil isolates

3.2.5.1 The effect of culture medium on antimicrobial activity

The antimicrobial-producing soil isolate were grown on ISP2 medium and incubated at 28ºC with 200 rpm condition for 3 days. After incubation, one milliliter of culture grown cell was transferred to 50 ml conical centrifuge tube containing 10 ml of 10 different culture medium, which were NB, MHB, ISP2, SDB, SCB, YEGB, PDB, MEB, CDB and CYE. The tube was incubated at 28ºC with 200 rpm condition for 5 days. After incubation, cell suspensions were centrifuged at 13,000 rpm. The cell-free supernatants were used for antimicrobial activity by agar-well diffusion method. One-hundred microliter of cell-free supernatant was applied into each well of plate lawn with test pathogens (adjusted to 0.5 McFarland). The plates were then incubated at 37ºC for 24-48 h. After incubation, the zone of inhibition in diameter (mm.) were observed and recorded.

3.2.5.2 The effect of incubation periods on antimicrobial activity

Soil isolates were grown on ISP2 medium and incubated at 28ºC with 200 rpm condition for 3 days. After incubation, twenty-five milliliter of culture grown cell was transferred to 500 ml-Erlenmeyer flask containing 250 ml of MHB medium and incubated at 28ºC with 200 rpm condition. Five milliliter of cell culture was collected every day for 10 days. Cell suspensions were centrifuged at 13,000 rpm. The cell-free supernatants were used for antimicrobial activity by agar-well diffusion method. One-hundred microliter of cell-free supernatant was applied into each well of plate lawn with test pathogens (adjusted to 0.5 McFarland). The plates were then incubated at 37ºC for 24-48 h. After incubation, the zone of inhibition in diameter (mm.) were observed and recorded.

3.2.6 Extraction of antimicrobial metabolites

3.2.6.1 The effect of solvent extraction on antimicrobial activity

The antimicrobial-producing actinobacteria isolate were grown on ISP2 media for 3 days. Ten milliliters of cell grown culture were transferred into a 250 ml-Erlenmeyer flask containing 100 ml of the MHB medium and incubated at 28ºC with 200 rpm shaking condition for 3 days. After incubation, the culture was filtrated through Whatman No.1 filter paper (Whatman™, GE Healthcare, UK). The filtrate was extracted with 3 different solvents, they were hexane, ethyl acetate and butane in the ratio of 1:1 (v/v) and shaken vigorously for 1 h. The organic solvent phase containing antimicrobial compounds was evaporated to high concentration in water bath at 55ºC. The antimicrobial activity of aqueous extract used by agar-well diffusion method. One-hundred microliter of aqueous extract was applied into each well of plate lawn with test pathogens (adjusted to 0.5 McFarland). The plates were then incubated at 37ºC for 24-48 h. After incubation, the zone of inhibition in diameter (mm.) were observed and recorded.

3.2.6.2 Preparation of crude extract

An antimicrobial-producing soil isolate was grown on ISP2 media for 3 days. Fifty milliliters of cell grown culture were transferred into a 1,000 ml-Erlenmeyer flask containing 500 ml of the MHB medium and incubated at 28ºC with 200 rpm shaking condition for 3 days. After incubation, the culture was filtrated through Whatman No.1 filter paper (Whatman™, GE Healthcare, UK). Ethyl acetate was added to the filtrated in the ratio of 1:1 (v/v) and shaken vigorously for 1 h. The organic solvent phase containing antimicrobial compounds was evaporated to dryness in water bath at 55ºC. The crude extract was used for antimicrobial activity test. And

the cell pellets was filtrated through Whatman No.1 filter paper (Whatman™, GE Healthcare, UK). The cell dry weight was measured after desiccation at 70ºC until constant weight.

3.2.7 Determination of the minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC)

The minimum inhibitory concentration (MIC) minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) of the crude extract were determined by broth macro-dilution method (CLSI, 2014). Using a test-tubes macrodilution test, 1000 µl of MHB medium was dispensed into all the test tubes. Crude extract was dissolved in dimethyl sulfoxide (DMSO) and diluted in MHB medium by the serial two-fold dilution method. The initial concentration of crude extract was 1024 to 0.125 µg/ml. The mixture of each tube was inoculated with 1000 µl of the suspension containing 1-5 × 10^6 CFU/ml of test pathogens in the mid-log phase. The final concentrations of test pathogens were approximately 5 × 10^5 CFU/ml for bacteria strains and approximately 2.5-5 × 10^5 CFU/ml for yeast strains. The tubes were then incubated at 37ºC for 18-20 h. The MIC value was recorded as the lowest concentration of the crude extract that inhibits visible growth of test organisms. The minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) was determined by carrying out a subculture of the tubes showing no growth on an agar plate without crude extract. The MBC and MFC is defined as the lowest concentration of an agent that inhibits 99.9% of the growth in subcultures (CLSI, 2006).

CHAPTER IV

RESULTS

4.1 Isolation of soil microorganisms

Twenty-one soil samples were randomly collected from Suranaree University of Technology, Thailand, during July 2014-May 2015. Soil samples were transferred by polyethylene bags and kept in ice box to the laboratory. Soil samples were used for the isolation of actinobacteria and fungal strains by serial dilution and spread plates on SCA and PDA medium. Several colonies were appeared on SCA and PDA medium after incubation at room temperature for 5-7 days. Total of 177 fungal strains and strains of 77 actinobacteria were picked based on difference of colony morphologies. They named PKF1, PKF2, PKF3…..PKF177 for fungal strains and given the named as PKA1, PKA2, PKA3…..PKA77 for actinobacteria strains. Two hundred-fifty four soil isolates were screened for their antimicrobial activity by cross-streak method.

4.2 Preliminary screening of antimicrobial-producing soil isolates

Preliminary screening of antimicrobial-producing soil isolates was performed by cross-streak method (Figure 4.1) using MHA and SDA medium. Total of 177 fungal strains and strains of 77 actinobacteria were screened for their antibacterial and antifungal activity against *Staphylococcus aureus* TISTR1466, methicillin-resistant

Staphylococcus aureus DMST20654 (MRSA), *Staphylococcus epidermidis* TISTR518, *Bacillus subtilis* TISTR008, *Bacillus cereus* TISTR687, *Escherichia coli* TISTR780, *Enterobacter aerogenes* TISTR1540, *Salmonella typhi* TISTR292, *Proteus mirabilis* TISTR100, *Candida albicans* TISTR5779, *Candida tropicalis* TISTR5174 and *Saccharomyces cerevisiae* TISTR5049. The results are shown in Table 4.1 and Table 4.2.

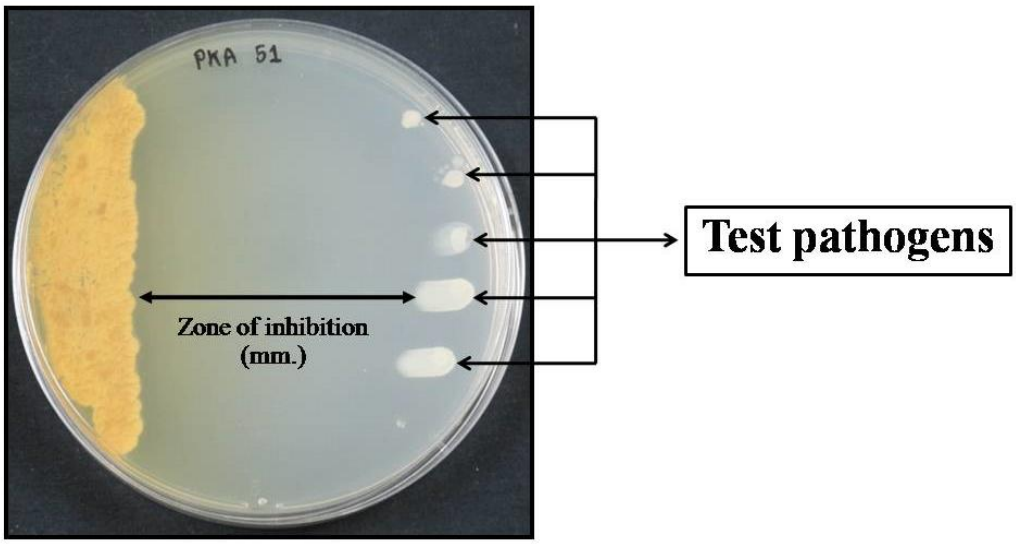


Figure 4.1 Preliminary antimicrobial activity screening of soil isolate was used by cross-streak method.

Table 4.1 Preliminary screening of antimicrobial activity from fungal isolates.

Isolates No.	Inhibition zone of antimicrobial activity (mm.)											
	Gram-Positive bacteria					Gram-Negative bacteria				Yeasts		
	***S. aureus* TISTR1466**	***S. aureus* DMST20654 (MRSA)**	***S. epidermidis* TISTR518**	***B. subtilis* TISTR008**	***B. cereus* TISTR687**	***E. coli* TISTR780**	***E. aerogenes* TISTR1540**	***S. typhi* TISTR292**	***P. mirabilis* TISTR100**	***C. albicans* TISTR5779**	***C. tropicalis* TISTR5174**	***S. cerevisiae* TISTR5049**
PKF6	16	-	20	21	-	-	-	-	-	-	-	-
PKF104	9	11	12	14	-	-	-	-	-	9	8	-
PKF105	-	-	-	-	-	-	-	-	-	20	20	15
PKF116	-	-	-	-	-	-	-	-	-	17	8	10
PKF121	5	10	20	14	6	-	-	-	-	10	5	3
PKF124	3	10	23	-	-	-	-	-	-	-	-	-
PKF125	7	12	25	13	14	-	-	-	-	-	-	-
PKF127	11	11	20	20	15	-	-	-	-	-	-	-
PKF145	-	-	-	-	-	-	-	-	-	5	5	10
PKF152	-	-	-	9	5	-	7	11	7	5	-	3
PKF161	5	5	15	7	5	-	-	-	-	-	-	5

- = No activity.

Table 4.2 Preliminary screening of antimicrobial activity from actinobacteria isolates.

Isolates No.	Inhibition zone of antimicrobial activity (mm.)											
	Gram-Positive bacteria					Gram-Negative bacteria				Yeasts		
	***S. aureus* TISTR1466**	***S. aureus* DMST20654 (MRSA)**	***S. epidermidis* TISTR518**	***B. subtilis* TISTR008**	***B. cereus* TISTR687**	***E. coli* TISTR780**	***E. aerogenes* TISTR1540**	***S. typhi* TISTR292**	***P. mirabilis* TISTR100**	***C. albicans* TISTR5779**	***C. tropicalis* TISTR5174**	***S. cerevisiae* TISTR5049**
PKA2	23	20	17	24	15	-	-	-	-	-	-	-
PKA20	10	15	7	-	7	-	-	-	-	-	-	-
PKA45	40	42	48	46	43	24	5-	25	23	40	20	41
PKA50	7	10	6	5	8	-	-	-	-	13	10	20
PKA51	40	43	45	47	40	25	7	32	39	39	19	41
PKA62	-	-	5	3	3	15	10	11	18	-	-	-

- = No activity.

Out of 254 soil isolates, 11 isolates, PKA2, PKA20, PKA45, PKA50, PKA51, PKA62, PKF6, PKF104, PKF105, PKF116, PKF121, PKF124, PKF125, PKF127, PKF, PKF145, PKF152 and PKF161, exhibited antimicrobial activity against test pathogens. The isolates could be divided into five groups based on spectra of their antimicrobial activity. Group I were PKA2, PKA20, PKF6, PKF124, PKF125 and PKF127. They showed antibacterial activity against only gram-positive bacteria. Group II were PKF105, PKF116 and PKF145, exhibited antifungal activity against only yeasts. Group III, PKA62 showed antibacterial activity against both gram-positive and gram-negative bacteria. Group IV were PKA50, PKF104, PKF121 and

PKF161. They showed antimicrobial activity against both gram-positive bacteria and yeasts. The final group consisted PKA45, PKA51, and PKF152, exhibited broad antimicrobial activity against gram-positive bacteria, gram-negative bacteria and yeasts. Moreover, 11 soil isolates, PKA2, PKA20, PKA45, PKA50, PKA51, PKF104, PKF121, PKF124, PKF125, PKF127 and PKF161, were effective against *S. aureus* DMST20654 (MRSA).

4.3 Identification of antimicrobial-producing soil isolates

The identification of antimicrobial-producing soil isolates was based on colony morphology and molecular technique. The colony morphology of fungal and actinobacteria isolates was visually inspected after growth on agar plates. The colony characteristics of fungal and actinobacteria isolates are summarized in Table 4.3-4.4.

Table 4.3 Colony morphology of eleven antimicrobial-producing fungal isolates. The fungal isolates were grown on PDA plate.

Isolates No.	Colony on agar plate	Characteristics
PKF6		White aerial mycelium, granular colony and rough-walled stipes
PKF104		Creamy white color, fluffy colony and the reverse side showed pale yellow

Table 4.3 Colony morphology of eleven antimicrobial-producing fungal isolates. The fungal isolates were grown on PDA plate (Continued).

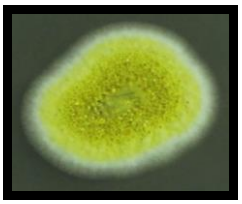
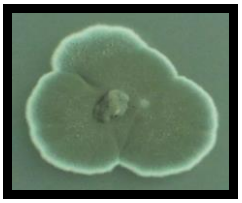
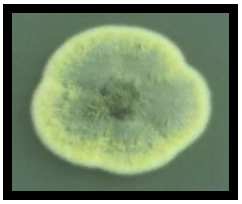
Isolates No.	Colony on agar plate	Characteristics
PKF105		Creamy white color, fluffy colony and radial grooved surface
PKF116		Yellow color with a white border, flat and fluffy colony
PKF121		Grayish green color with a white periphery and granular powdery colony
PKF124		Grayish green color with a yellow periphery and granular powdery colony
PKF125		Grayish green color with a white periphery, granular powdery colony and radial grooved surface

Table 4.3 Colony morphology of eleven antimicrobial-producing fungal isolates. The fungal isolates were grown on PDA plate (Continued).

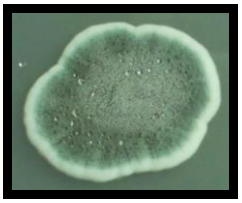
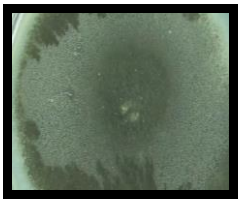
Isolates No.	Colony on agar plate	Characteristics
PKF127		Grayish green color with a white periphery and granular powdery colony
PKF145		White color, flat and suede-like colony
PKF152		Greenish yellow color with a yellowish periphery and granular powdery colony
PKF161		White mycelium, black conidia and granular colony

Table 4.4 Colony morphology of six antimicrobial-producing actinobacteria isolates. The actinobacteria isolates were grown on ISP2 agar plate.

Isolates No.	Colony on agar plate	Characteristics
PKA2		White substrate mycelium, white aerial mycelium and non-pigment
PKA20		Yellow substrate mycelium, White aerial mycelium and non-pigment
PKA45		White substrate mycelium, light brown aerial and produce yellow pigment
PKA50		Orange substrate mycelium, brown aerial mycelium and non-pigment
PKA51		White substrate mycelium, white aerial and produce yellow pigment

Table 4.4 Colony morphology of six antimicrobial-producing actinobacteria isolates. The actinobacteria isolates were grown on ISP2 agar plate (Continued).

Isolates No.	Colony on agar plate	Characteristics
PKA62		Yellow substrate mycelium, colorless aerial mycelium and non-pigment

The sequence analysis of ITS region was used for the identification of 11 antimicrobial-producing fungal strains in the genus and species level. To amplify the ITS region of these isolates, ITS5 and ITS4 primers were used. The partial ITS PCR products was purified and submitted for DNA sequencing (Figure 4.2). The amplified fragments were compared with nucleotide sequence from UNITE database. The sequence similarity of ITS region of 11 fungal isolates to the closest relative strains from the UNITE database was 99.2-100% identities (Table 4.5).

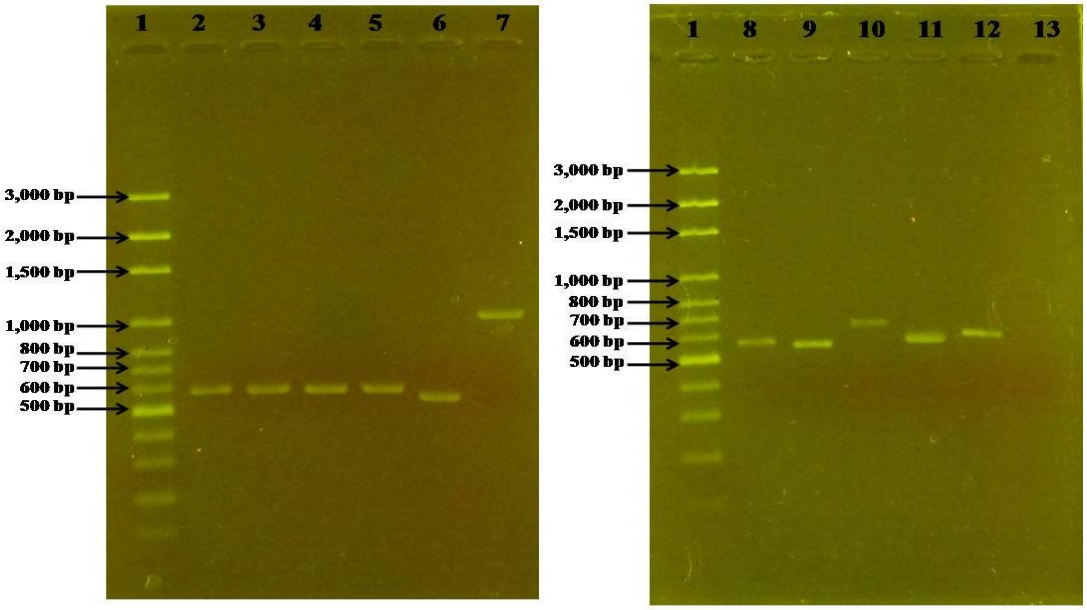


Figure 4.2 The PCR product of 11 antimicrobial-producing fungal strains on 1.2% agarose gel electrophoresis: lane 1, molecular weight marker; lane 2, PKF6; lane 3, PKF104; lane 4, PKF105; lane 5, PKF116; lane 6, PKF121; lane 7, PKF124; lane 8, PKF125; lane 9, PKF127; lane 10, PKF145, lane 11, PKF152, lane 12, PKF161 and lane 13, negative control.

Table 4.5 Closest relative strains of antimicrobial-producing fungi from UNITE database according to ITS region sequence similarity.

Isolates No.	Closest relative strains	Similarity (%)
PKF6	*Aspergillus flavus* EFB01	100
PKF104	*Clonostachys rogersoniana* B133	100
PKF105	*Aspergillus flavipes* IHBF2335	100
PKF116	*Aspergillus flavipes* IHBF2335	100
PKF121	*Penicillium citrinum* LTL46	100
PKF124	*Talaromyces allahabadensis* NRRL62157	99.8

Table 4.5 Closest relative strains of antimicrobial-producing fungi from UNITE database according to ITS region sequence similarity (Continued).

Isolates No.	Closest relative strains	Similarity (%)
PKF125	*Penicillium citrinum* HBhive04	100
PKF127	*Penicillium citrinum* LTL46	100
PKF145	*Aspergillus flavipes* IHBF2335	100
PKF152	*Talaromyces purpureogenus* SQU14109	99.8
PKF161	*Aspergillus niger* SF-6155	99.2

The ITS region sequence analysis was used to build a phylogenetic tree for 11 antimicrobial-producing fungal isolates with their closely related type strains from UNITE database (Figure 4.3). The neighbor-joining tree showed that PKF161, PKF6, PKF116 and PKF105, formed a phylogenetic clade with type strains from genera *Aspergillus* at high bootstrap value of 100% (Figure 4.3). PKF161 were closely related to *Aspergillus niger* with 100% identity (Table 4.5). Isolate PKF6 showed 100% similarity to *Aspergillus flavus* (Table 4.5). PKF145, PKF116 and PKF105 shared the highest similarities to *Aspergillus flavipes* with 100% identities (Table 4.5). Three fungal isolates, PKF127, PKF125 and PKF121, showed 100% similarity to *Penicillium citrinum* (Table 4.5) and they formed a monophyletic group at 100% bootstrap value (Figure 4.3). Isolates, PKF152, PKF124 and PKF104, were assigned to the same clade with their closest relative type strains obtained blast search (Figure 4.3). Fungal isolates, PKF152, PKF124 and PKF104, were identified as strains *Talaromyces purpureogenus*, *Talaromyces allahabadensis* and *Clonostachys rogersoniana*, respectively.

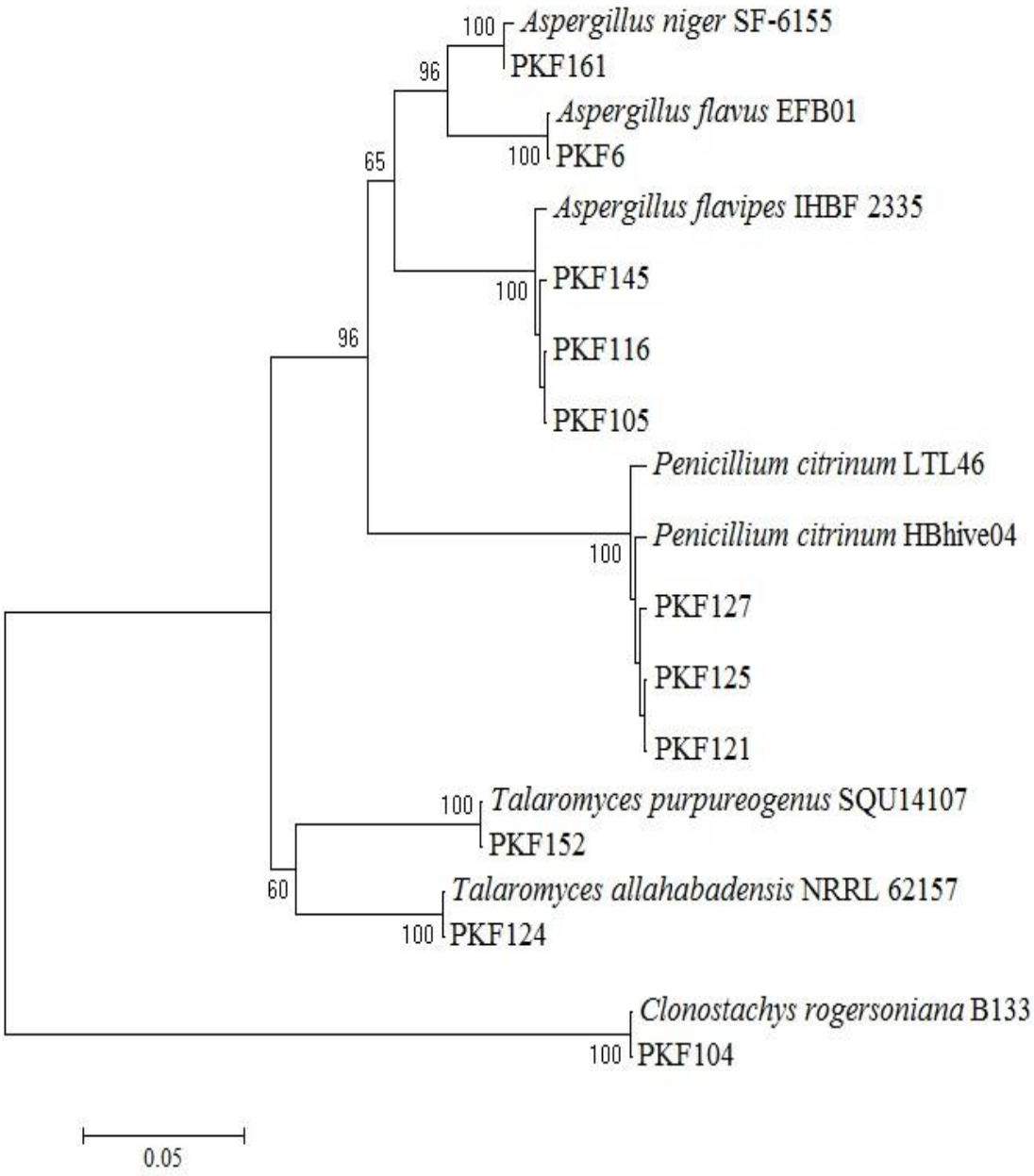


Figure 4.3 Phylogenetic tree showing evolutionary relationship of 11 antimicrobial-producing fungal isolates and their related taxa from UNITE database. The ITS region was aligned by using ClustalW. The neighbor-joining phylogenetic tree was generated by using MEGA 7.0. Numbers at the nodes indicate levels of bootstrap support based on 1,000 resampling, only ≥ 50% are shown. The scale bar indicates 0.05 substitutions per nucleotide position.

The identification of 6 antimicrobial-producing actinobacteria was based on 16S rRNA gene sequence analysis. The 16S rRNA was amplified by using 243F and A3R primers were used. The partial 16S rRNA PCR products was purified and submitted for DNA sequencing (Figure 4.4). The sequence of 16S rRNA gene was blasted and aligned with known strains from EzBioCloud database. The sequence similarity results of 6 isolates showed 99.8-100% identity to genera *Streptomyces* (Table 4.6).

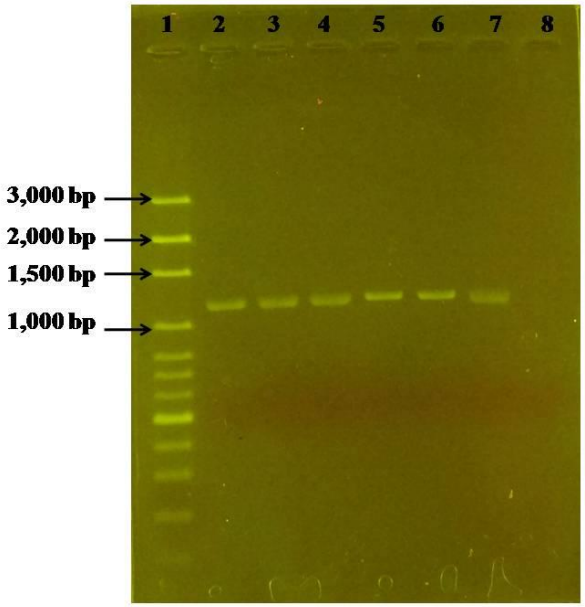


Figure 4.4 The PCR product of 6 antimicrobial-producing actinobacteria isolates on 1.2% agarose gel electrophoresis: lane 1, molecular weight marker; lane 2, PKA2; lane 3, PKA20; lane 4, PKA45; lane 5, PKA50; lane 6, PKA51, lane 7, PKA62 and lane 8, negative control.

Table 4.6 Closest relative strains of antimicrobial-producing actinobacteria from EzBioCloud database according to 16S rRNA sequence similarity.

Isolates No.	Closest relative strains	Similarity (%)
PKA2	*Streptomyces sioyaensis* NRRL B-5408	100
PKA20	*Streptomyces chattanoogensis* NRRL ISP-5002	99.8
PKA45	*Streptomyces sioyaensis* NRRL B-5408	99.9
PKA50	*Streptomyces luteosporeus* NBRC 14657	100
PKA51	*Streptomyces luteosporeus* NBRC 14657	100
PKA62	*Streptomyces lusitanus* NBRC 13464	99.9

The analysis of 16S rRNA sequences was used to determine taxonomic relationships of actinobacteria. The 16S rRNA sequences of 6 isolates and their closest relative strains obtained from EzBioCloud database were used to build a phylogenetic tree. The neighbor-joining tree was constructed using MEGA 7.0 program. The phylogenetic tree revealed that PKA2, PKA20 and PKA62, were formed a monophyletic clade with their closest relative type strains obtained blast search (Figure 4.5). Isolates, PKA2, PKA20 and PKA62, were classified as strains *Streptomyces sioyaensis*, *Streptomyces chattanoogensis* and *Streptomyces lusitanus*, respectively. PKA45 formed a distinct phyletic clade with *Streptomyces sioyaensis* and *Streptomyces chattanoogensis* at bootstrap value of 99% (Figure 4.5). It was closely related to *Streptomyces sioyaensis* with 99.9% identity (Table 4.6). Therefore, PKA45 could be identified as *Streptomyces sioyaensis*. Two isolates, PKA50 and PKA51, revealed 100% identities to sequences of type strain *Streptomyces*

luteosporeus (Table 4.6). The phylogenetic analysis exhibited they formed a distinct clade with *Streptomyces luteosporeus* at bootstrap value of 100% (Figure 4.5).

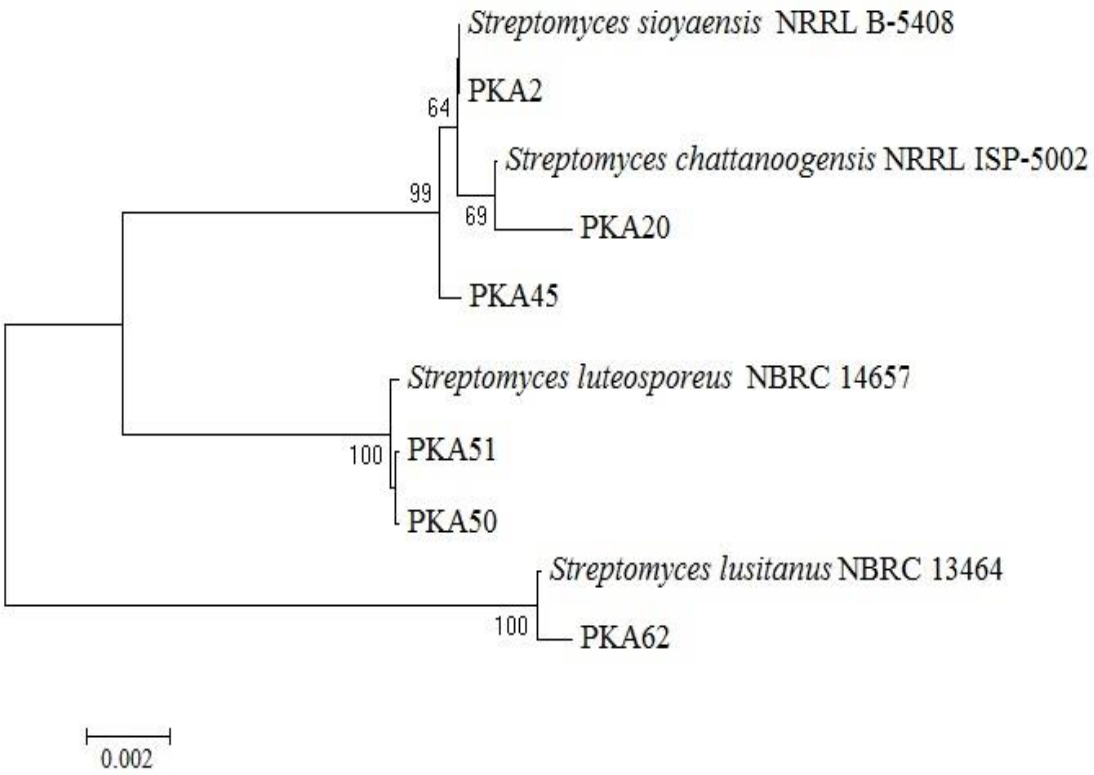


Figure 4.5 Phylogenetic tree showing evolutionary relationship of 6 antimicrobial-producing actinobacteria isolates and their related taxa from EzBioCloud database. The 16S rRNA gene sequence was aligned by using ClustalW. The neighbor-joining phylogenetic tree was generated by using MEGA 7.0. Numbers at the nodes indicate levels of bootstrap support based on 1,000 resampling, only ≥ 50% are shown. The scale bar indicates 0.002 substitutions per nucleotide position.

4.4 Optimization of antimicrobial metabolites production from soil isolates

Two isolates of antimicrobial-producing actinobacteria, PKA45 and PKA51 showed the highest antimicrobial activity against all the test pathogens including gram-positive bacteria, gram-negative bacteria and yeast. Thus, they were selected for optimal condition of antimicrobial activity against test pathogens by agar-well diffusion method. In this study, the antimicrobial activity of two actinobacteria isolates, PKA45 and PKA51 were tested by using 10 different culture media which were NB, MHB, ISP2, SDB, SCB, YEGB, PDB, MEB, CDB and CYE medium. After incubation, the supernatants of cell-free culture containing antimicrobial substances were collected and measured by agar-well diffusion method (Figure 4.6). Based on inhibition zone diameter measured from different culture media, PKA45 and PKA51 showed highest antimicrobial activity with MHB medium (Figure 4.7A-C and 4.8A-C) on 3rd day of incubation time (Table 4.7 and Table 4.8).

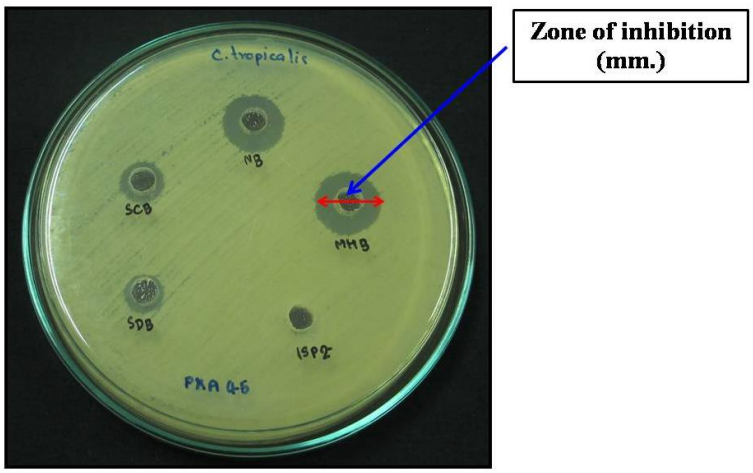


Figure 4.6 The antimicrobial activity against test pathogens by using agar-well diffusion method. Arrow shows the zone of inhibition.

(A)

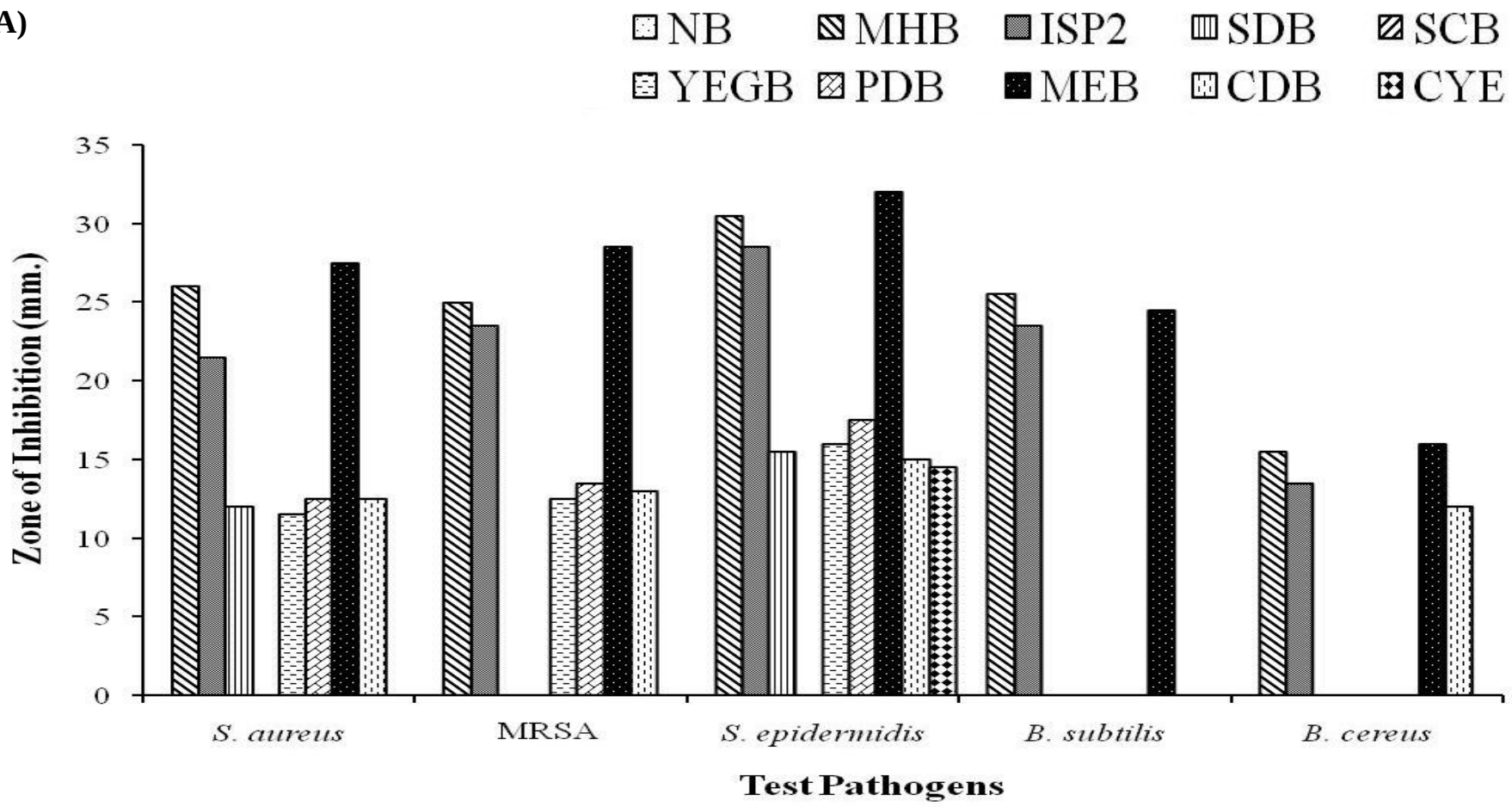


Figure 4.7A Effect of culture media on antimicrobial activity of PKA45 strain against gram-positive bacteria**.**

(B)

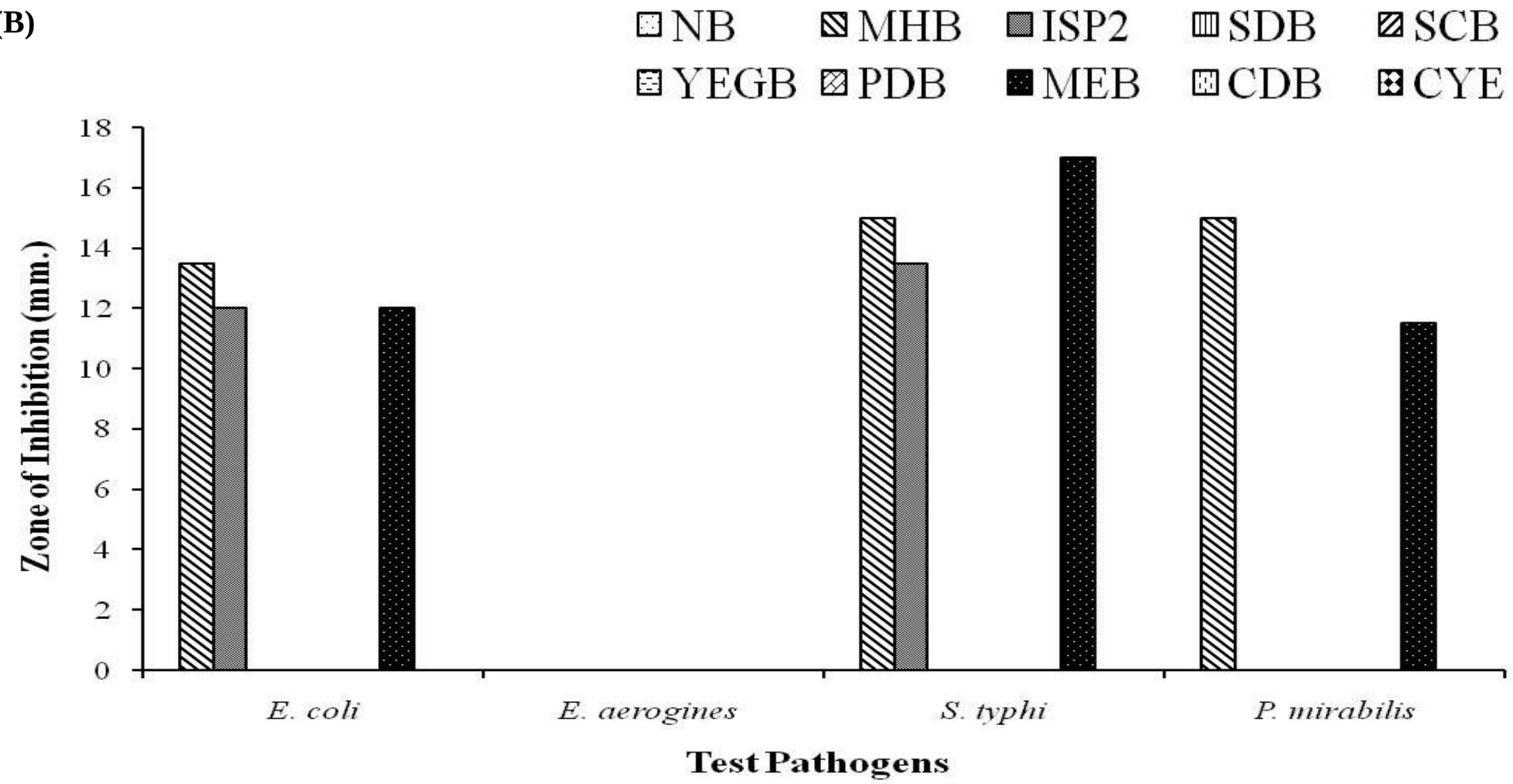


Figure 4.7B Effect of culture media on antimicrobial activity of PKA45 strain against gram-negative bacteria**.**

(C)

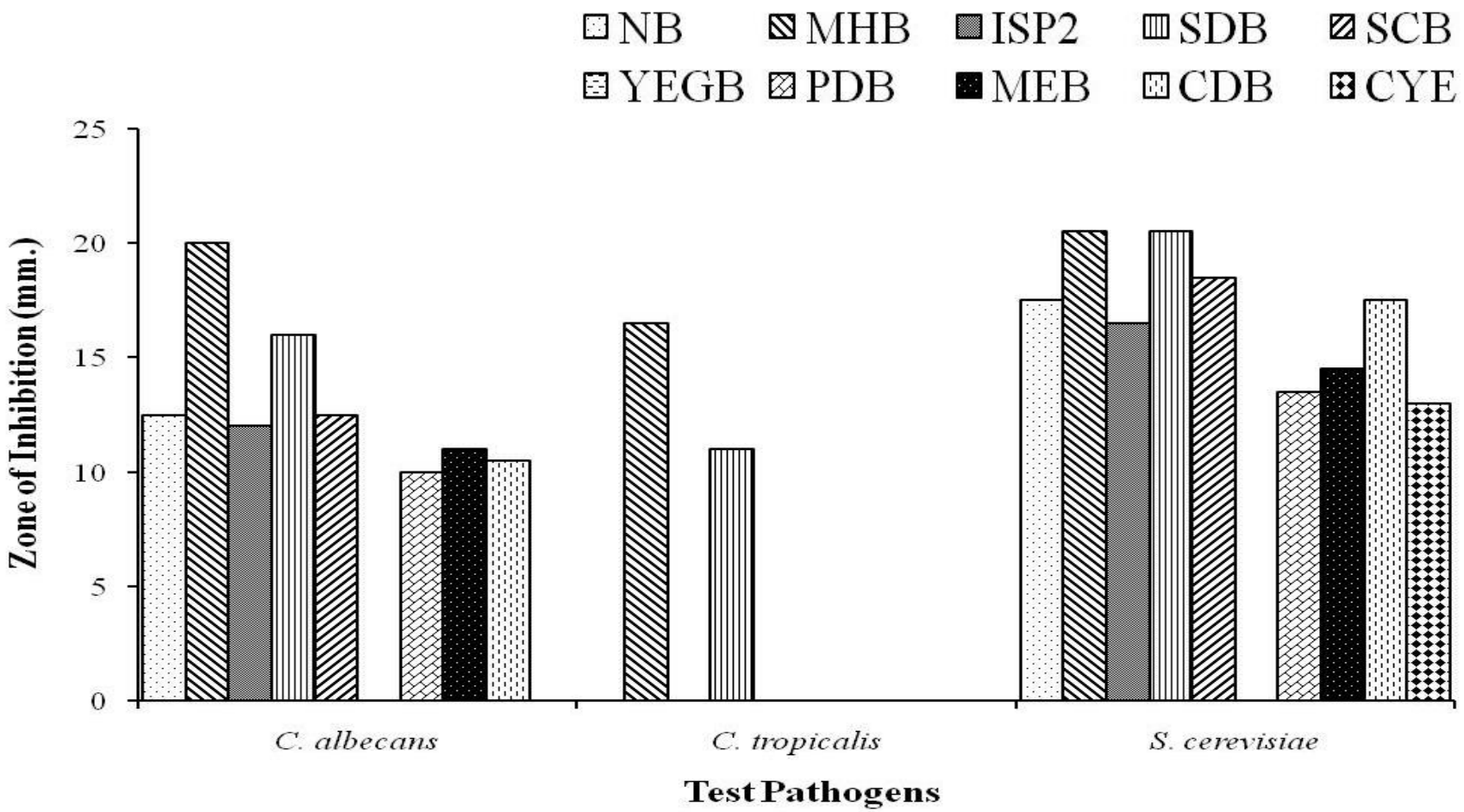


Figure 4.7C Effect of culture media on antimicrobial activity of PKA45 strain against yeasts**.**

(A)

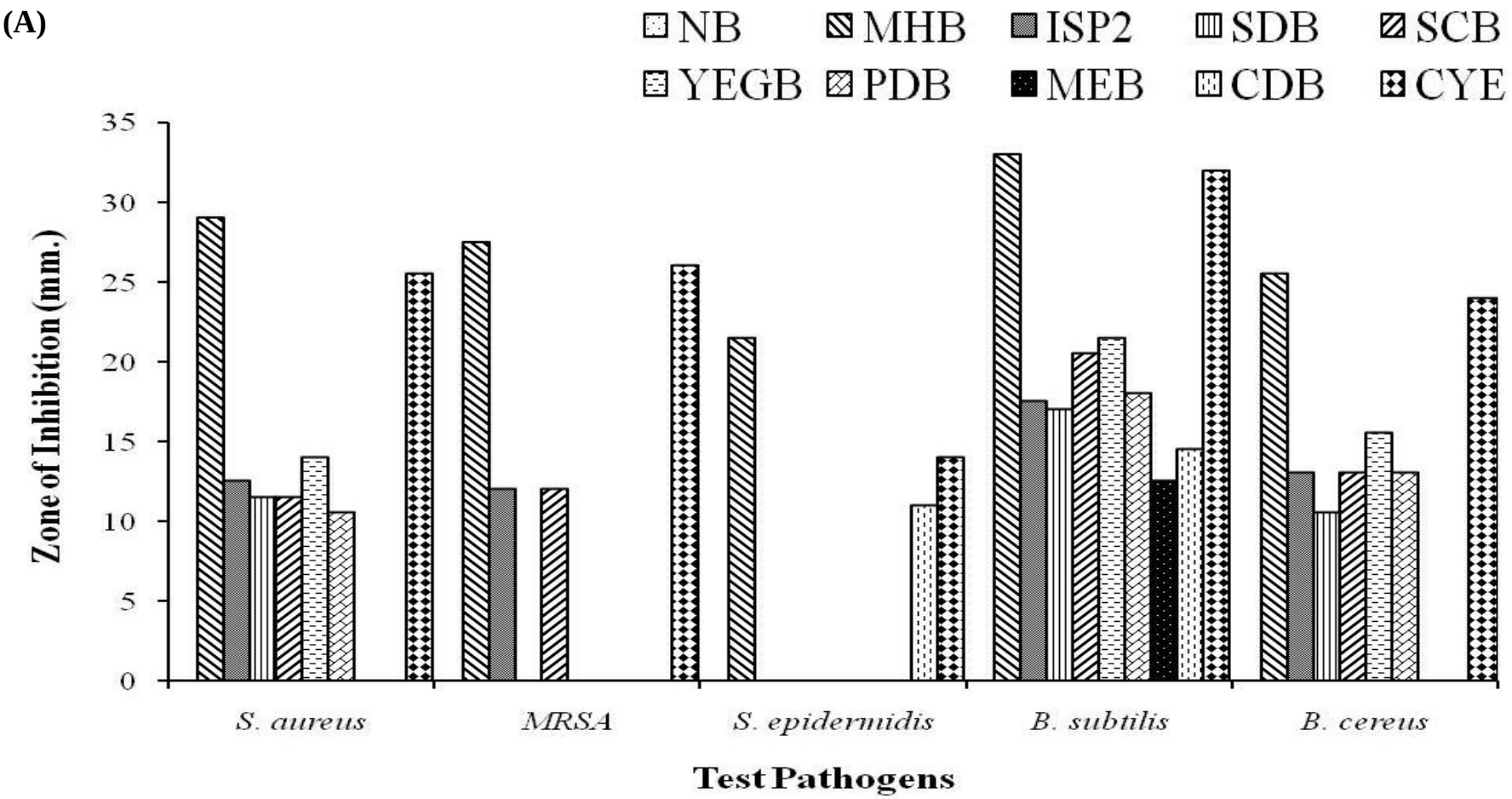


Figure 4.8A Effect of culture media on antimicrobial activity of PKA51 strain against gram-positive bacteria**.**

(B)

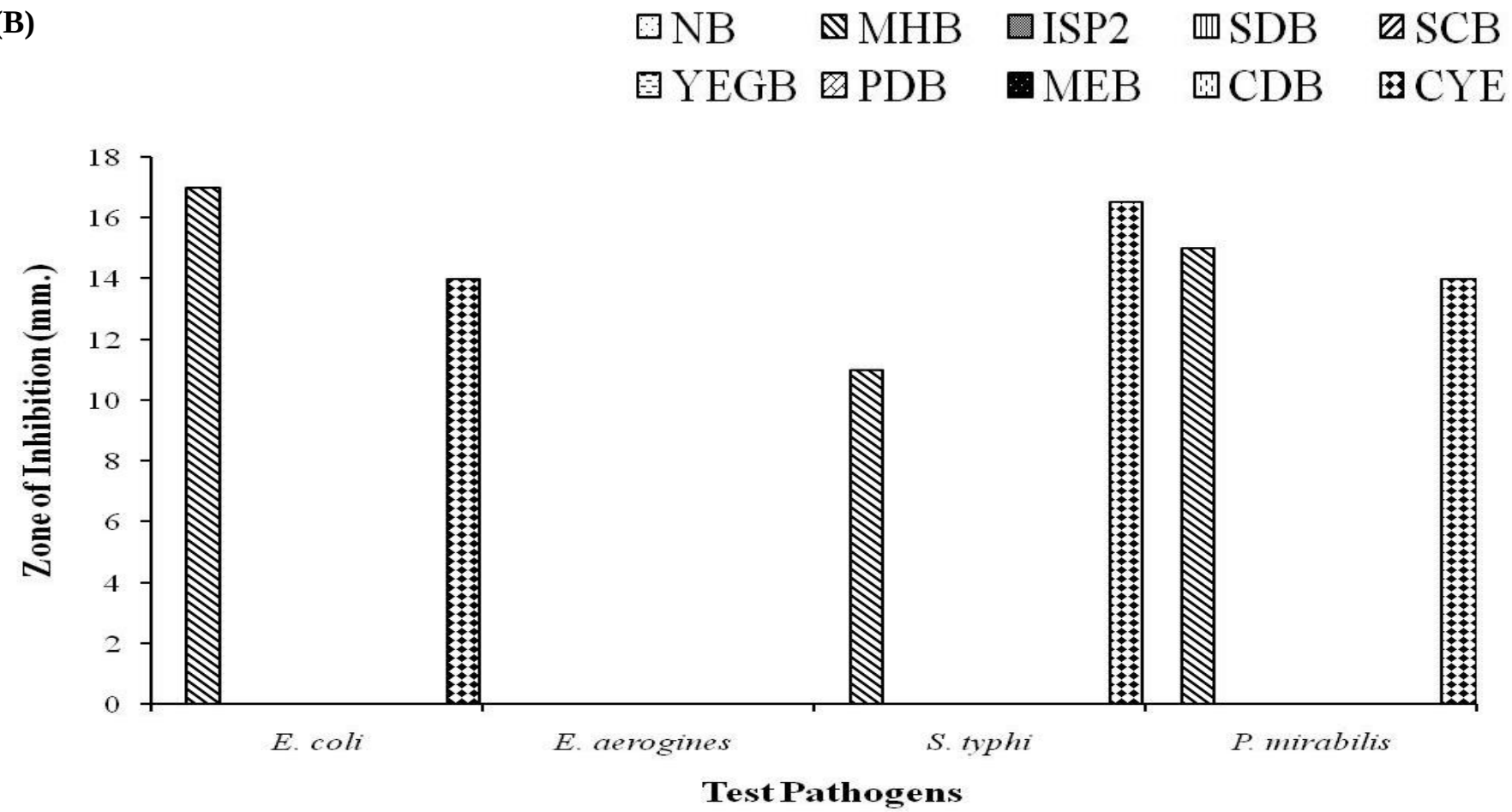


Figure 4.8B Effect of culture media on antimicrobial activity of PKA51 strain against gram-negative bacteria**.**

(C)

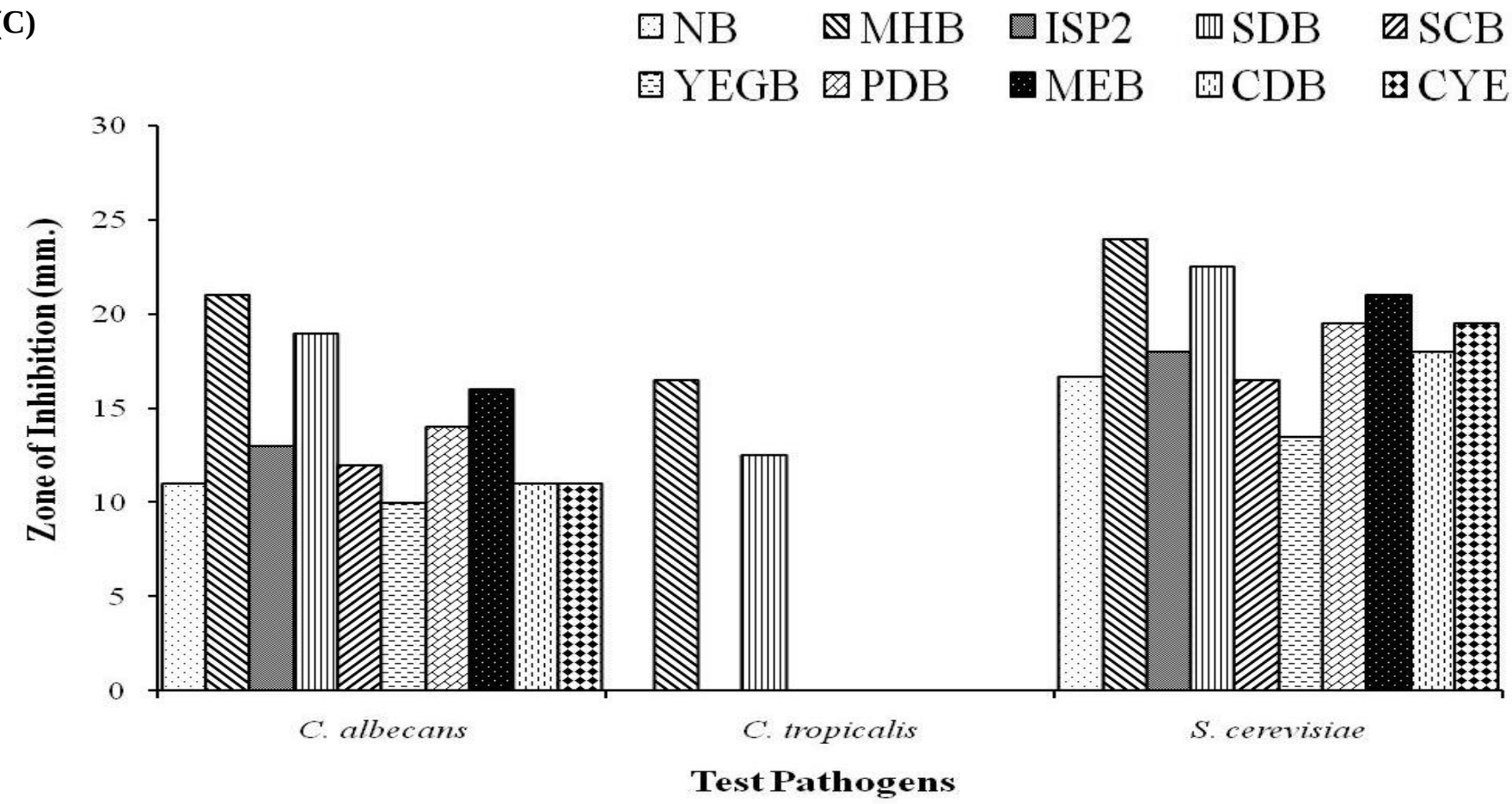


Figure 4.8C Effect of culture media on antimicrobial activity of PKA51 strain against yeasts**.**

Table 4.7 Effect of incubation periods on antimicrobial activity of PKA45 cultured in MHB medium.

Test pathogens	Zone of Inhibition (mm.)										
	D-0	**D-1**	**D-2**	**D-3**	**D-4**	**D-5**	**D-6**	**D-7**	**D-8**	**D-9**	**D-10**
S. aureus TISTR1466	0	13.5	22	23.25	24	18.5	19.5	18.5	14	0	0
S. aureus DMST20654 (MRSA)	0	0	20.75	22	21.25	16.75	13.5	13.5	0	0	0
S. epidermidis TISTR518	0	0	19.25	19.75	20.5	16	17.5	16	17	20	17
B. subtilis TISTR008	0	18	24	20	25.25	20.5	16	18	11	9.5	7.5
B. cereus TISTR687	0	11.5	19.25	20	18.5	15.25	0	0	0	0	0
E. coli TISTR780	0	0	14	14.5	14.5	7.5	0	14	0	0	0
E. aerogenes TISTR1540	0	0	0	0	0	0	0	0	0	0	0
S. typhi TISTR292	0	0	7	7.25	7.25	0	0	14	0	0	0
P. mirabilis TISTR100	0	0	13	13	12.75	0	0	13.5	0	0	0
C. albicans TISTR5779	0	20	24	24.5	24	22.25	24	19	22.5	23	22.5
C. tropicalis TISTR5174	0	14.5	17.5	18	17.75	16.25	17	15	17.5	17.5	16.5
S. cerevisiae TISTR5049	0	25.75	30	30	30	29	29.5	30	30.5	29.5	29

Table 4.8 Effect of incubation periods on antimicrobial activity of PKA51 cultured in MHB medium.

Test pathogens	Zone of Inhibition (mm.)										
	D-0	**D-1**	**D-2**	**D-3**	**D-4**	**D-5**	**D-6**	**D-7**	**D-8**	**D-9**	**D-10**
S. aureus TISTR1466	0	0	20.75	24	24.25	21	19.5	17.5	14.5	12	0
S. aureus DMST20654 (MRSA)	0	0	18.5	23.5	24.25	19	20.5	15	14	0	0
S. epidermidis TISTR518	0	0	21	24.25	24	20.5	22	22.5	23	23.5	22
B. subtilis TISTR008	0	12.75	24.5	27	27	25	21.5	19.5	18	16.5	12.5
B. cereus TISTR687	0	0	17	18.75	19	16.75	12	11	0	0	0
E. coli TISTR780	0	0	13.5	15	14.5	11.25	13	12	0	0	0
E. aerogenes TISTR1540	0	0	0	0	0	0	0	0	0	0	0
S. typhi TISTR292	0	0	6	6.75	6.5	5	0	0	0	0	0
P. mirabilis TISTR100	0	0	12	14.5	14.25	11.5	12	0	0	0	0
C. albicans TISTR5779	0	21	23	25.5	24	23.25	24	23.5	23	23.5	23
C. tropicalis TISTR5174	0	14.25	17.5	18.5	18.25	17.5	17	16	16	10	10
S. cerevisiae TISTR5049	0	25.5	30.25	30	30.5	29	29.5	29	28.5	28	28.9

4.5 Extraction of antimicrobial metabolites

The antimicrobial-producing actinobacteria isolates, PKA45 and PKA51 showed highest antimicrobial activity against test pathogens, they were selected for the preparation of crude extract. In this study, antimicrobial-producing isolates, PKA45 and PKF51 cultured in MHB medium. After incubation at 28ºC for 3 days with shaking condition, the cell-free culture supernatant were collected and extracted with 3 different solvents which were butane, hexane and ethyl acetate. The organic layer were collected and determined by agar-well diffusion. The crude extracts of PKA45 and PKA51 were extracted using ethyl acetate showed maximum antimicrobial activity against test pathogens (Table 4.9 and 4.10). The crude extracts from both isolates were yellow in color. The yield of crude extract from PKA45 and PKA51 were 46.93 and 50.74 mg/g of cell dry weight, respectively.

Table 4.9 Effect of solvents extraction on antimicrobial activity against test pathogens by PKA45 strain.

Test pathogens	Zone of Inhibition (mm.)					
	Butane Extract	**Butane**	**Ethyl acetate Extract**	**Ethyl acetate**	**Hexane Extract**	**Hexane**
S. aureus TISTR1466	19	10	32	0	0	0
S. aureus DMST20654 (MRSA)	15	9	30	0	0	0
S. epidermidis TISTR518	16	14	25	0	0	0
B. subtilis TISTR008	25	17	34	0	0	0
B. cereus TISTR687	19	11	30	0	0	0
E. coli TISTR780	16	15	11	0	0	0
E. aerogenes TISTR1540	26	24	14	0	0	0
S. typhi TISTR292	17	14	29	0	0	0
P. mirabilis TISTR100	33	12	38	0	0	0
C. albicans TISTR5779	27	0	24	0	0	0
C. tropicalis TISTR5174	36	0	43	0	0	0
S. cerevisiae TISTR5049	20	0	28	0	0	0

Table 4.10 Effect of solvents extraction on antimicrobial activity against test pathogens by PKA51 strain.

Test pathogens	Zone of Inhibition (mm.)					
	Butane Extract	**Butane**	**Ethyl acetate Extract**	**Ethyl acetate**	**Hexane Extract**	**Hexane**
S. aureus TISTR1466	19	10	32	0	0	0
S. aureus DMST20654 (MRSA)	18	9	30	0	0	0
S. epidermidis TISTR518	17	14	24	0	0	0
B. subtilis TISTR008	25	17	34	0	0	0
B. cereus TISTR687	20	11	30	0	0	0
E. coli TISTR780	16	15	10	0	0	0
E. aerogenes TISTR1540	26	22	13	0	0	0
S. typhi TISTR292	20	14	29	0	0	0
P. mirabilis TISTR100	34	12	37	0	0	0
C. albicans TISTR5779	27	0	23	0	0	0
C. tropicalis TISTR5174	38	0	41	0	0	0
S. cerevisiae TISTR5049	20	0	28	0	0	0

4.6 The minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) of crude extracts

The MIC values of crude extracts were determined by macro-dilution method. The MIC, MBC and MFC of PKA45 and PKF51 crude extracts against, *Staphylococcus aureus* TISTR1466, methicillin-resistant *Staphylococcus aureus* DMST20654 (MRSA) , *Staphylococcus epidermidis* TISTR518, *Bacillus subtilis* TISTR008, *Bacillus cereus* TISTR687, *Escherichia coli* TISTR780, *Enterobacter aerogenes* TISTR1540, *Salmonella typhi* TISTR292, *Proteus mirabilis* TISTR100, *Candida albicans* TISTR5779, *Candida tropicalis* TISTR5174 and *Saccharomyces cerevisiae* TISTR5049 are shown in Table 4.11. Tetracycline, vancomycin and ketoconazole were used as positive control. Standard strain, *Staphylococcus aureus* ATCC29213 was used as internal control.

The result showed that the MIC of PKA45 and PKA51 crude extract against *Staphylococcus aureus* TISTR1466, methicillin-resistant *Staphylococcus aureus* DMST20654 (MRSA), *Bacillus subtilis* TISTR008, *Bacillus cereus* TISTR687, *Escherichia coli* TISTR780, *Enterobacter aerogenes* TISTR1540, *Salmonella typhi* TISTR292, *Proteus mirabilis* TISTR100 were in range 8-1024 µl/ml. Whereas, the MBC of PKA45 and PKA51 crude extract against *Staphylococcus aureus* TISTR1466, methicillin-resistant *Staphylococcus aureus* DMST20654 (MRSA), *Bacillus subtilis* TISTR008, *Bacillus cereus* TISTR687, *Escherichia coli* TISTR780, *Enterobacter aerogenes* TISTR1540, *Salmonella typhi* TISTR292, *Proteus mirabilis* TISTR100 were in range 64->1024 µg/ml. The MIC of PKA45 and PKA51 crude

extract against *Candida albicans* TISTR5779, *Candida tropicalis* TISTR5174 and *Saccharomyces cerevisiae* TISTR5049 were in range 4-128 µg/ml. The MFC of PKA45 and PKA51 crude extract against *Candida albicans* TISTR5779, *Candida tropicalis* TISTR5174 and *Saccharomyces cerevisiae* TISTR5049 were in range 8-512 µg/ml.

The MIC and MBC of PKA45 and PKA51 crude extract exhibited high activity against *Staphylococcus aureus* DMST20654 (MRSA) when compared with tetracycline, while showed decrease activity against *Staphylococcus aureus* DMST20654 (MRSA) when compared with vancomycin.

PKA45 and PKA51 crude extract exhibited low values of MIC against *Candida albicans* TISTR5779, *Candida tropicalis* TISTR5174 and *Saccharomyces cerevisiae* TISTR5049 when compared with ketoconazole. Whereas, the MFC of PKA45 and PKA51 crude extract showed high activity against *Candida albicans* TISTR5779, and *Saccharomyces cerevisiae* TISTR5049 while, showed low activity against *Candida tropicalis* TISTR5174 when compared with ketoconazole.

Table 4.11 The MIC, MBC and MFC values (µg/ml) of crude extract from PKA45 and PKA51 against test pathogens.

Test pathogens	PKA45 (µg/ml)		PKA51 (µg/ml)		Tetracycline (µg/ml)		Vancomycin (µg/ml)		Ketoconazole (µg/ml)	
	MIC	MBC/ MFC	MIC	MBC/ MFC	MIC	MBC	MIC	MBC	MIC	MFC
S. aureus TISTR1466	13.3±4.6	170.7±73.9	10.7±4.6	85.3±37.0	0.2±0.1	10.7±4.6	-	-	-	-
S. aureus DMST20654 (MRSA)	10.7±4.6	256±0.0	10.7±4.6	85.3±37.0	16±0.0	256±0.0	1±0.00	5.3±2.3	-	-
B. subtilis TISTR008	42.7±18.5	170.7±73.9	21.3±9.2	64±0.0	4±0.0	42.7±18.5	-	-	-	-
B. cereus TISTR687	21.3±9.2	128±0.0	10.7±4.6	64±0.0	0.125±0.0	16±0.0	-	-	-	-
E. coli TISTR780	85.3±37.0	1024±0.0	85.3±37.0	1024±0.0	0.7±0.3	170.7±73.9	-	-	-	-
E. aerogenes TISTR1540	1024±0.0	>1024±0.0	1024.±0.0	>1024±0.0	2.7±1.2	256±0.0	-	-	-	-
S. typhi TISTR292	85.3±37.0	1024±0.0	85.3±37.0	1024±0.0	1.3±0.6	170.7±73.9	-	-	-	-
P. mirabilis TISTR100	85.3±37.0	512±0.0	85.3±37.0	512±0.0	53.3±18.5	1024±0.0	-	-	-	-
C. albicans TISTR5779	16±0.0	64±0.0	8±0.0	64±0.0	-	-	-	-	4±0.0	128±0.0
C. tropicalis TISTR5174	128±0.0	512±0.0	128±0.0	512±0.0	-	-	-	-	2±0.0	64±0.0
S. cerevisiae TISTR5049	8±0.0	16±0.0	4±0.0	8±0.0	-	-	-	-	0.125±0.0	64±0.0

Data are mean ± standard deviation (n=3).

CHAPTER V

DISCUSSION AND CONCLUSION

The soil in dry dipterocarp forest of Suranaree University of Technology, Nakhon Ratchasima, Thailand was selected for screening of antimicrobial-producing microorganisms and their antimicrobial properties. In the present study, a total of 177 fungal isolates and 77 actinobacteria strains were obtained from 23 soil samples. Actinobacteria and Fungi, among soil organisms, actively participate in organic matter decomposition liberating chemical nutrients and furthering plant growth. Microorganism numbers vary in and between different soil types and conditions. Actinobacteria apparently can grow in dry soil, due to spores of actinobacteria survived for long periods in dry soil (Fang et al., 2017). Their tolerance of high moisture tensions was greater than that of their own vegetative hyphae or the cells of non-sporing bacteria (Williams et al., 1972). While, most fungi prefer moist locations and they grow well in humidity and wet soil. Moisture can influence the distribution and spread of many fungi, as well as affecting the development, longevity, germination of fungal spores (Agrios, 1997).

Out of 177 fungal isolates, 11 isolates showed antimicrobial against various pathogens tested. Among 11 isolates, 4 (36.4%) isolates could inhibit bacterial pathogens, 3 (27.3%) isolates was possessed antiyeast activity, and 4 (36.4%) isolates inhibit both bacterial and yeast pathogens. Eleven fungal isolates were identified at generic level based on the colony morphology and molecular technique. Identification

of strains by both morphological and the sequence analysis of ITS region revealed that the isolates belonged to white, creamy white, yellow, green and black color series. Out of 11 isolates, 45% of isolates were assigned to the genus *Aspergillus* and the remaining was identified as *Penicillium* (27%), *Talaromyces* (18%) and *Clonostachys* (9%). The present study revealed that among the isolates, *Aspergillus* was the dominance genera. Frequency and dominance of *Aspergillus* among fungi in soil habitats were reported by several workers (Al-Sadi et al., 2017; do Nascimento Barbosa et al., 2016; Jena, Tayung, Rath and Parida, 2015; Vehra, Noor, Abdullah, Farooq and Qureshi, 2015).

The filamentous bacteria, especially actinobacteria are commonly found in soil habitats. In the present study determined that out of the 77 actinobacteria isolates, 6 isolates had antimicrobial activity. Among 6 isolates, 3 isolates showed antibacterial activity and 3 isolates showed both antibacterial activity and antiyeast activity. Ultimately, two isolates namely PKA45 and PKA51 had a broad-spectrum antimicrobial activity. The mechanism of this antimicrobial activity has been proposed of cell membrane disruption, which was found in both bacteria and yeast cells. Previous studies that have reported broad-spectrum antimicrobial agents exhibited excellent antimicrobial activity against both bacteria and yeast. These antimicrobial compounds were effective of damaging the cell membrane of bacteria and yeast (Axelsson, Chung, Dobrogosz and Lindgren, 1989; Lyu, Yang, Lyu, Dong, and Shan, 2016).

Six actinobacteria isolates were identified to the genera level based on the molecular and morphological characteristics. Identification of isolates by morphological and 16S rRNA gene sequence revealed that the isolates belonged to

colorless, white and brown color series. Six antimicrobial-producing actinobacteria were assigned to the genus *Streptomyces*. They were *S. sioyaensis, S. chattanoogensis, S. luteosporeus* and *S. lusitanus*. However, a scrutiny of the 16S rRNA phylogenetic tree suggests that some of the isolates may represent novel strains. Actinobacteria isolates PKA45 showed out of group from its recognize reference strain. Goodfellow and Dickenson (1985) observed that many organisms from natural habitats do not form tight clusters with recognized reference strains. This indicates their probability of being new strains. Therefore, PKA45 might be classified as a novel strain of *Streptomyces sioyaensis*. In Thailand, *Streptomyces sioyaensis* has been isolated from Wattle tree in Bangkok province. This strain exhibited antimicrobial activity against *Staphylococcus aureus*, *Bacillus cereus*, *Ralstonia solanacearum* and *Aspergillus niger* (Bunyoo, Duangmal, Nuntagij and Thamchaipenet, 2009). In this study, we were able to isolate antimicrobial-producing *Streptomyces sioyaensis* from soil collected in Nakhon Ratchasima province. To our best knowledge, this study constitutes the first antimicrobial activity of *Streptomyces sioyaensis* isolated from Thai soil.

Based on the results of preliminary screening of antimicrobial activity against test pathogens from antimicrobial-producing actinobacteria isolates, *Streptomyces sioyaensis* PKA45 and *Streptomyces luteosporeus* PKA51 exhibited high antimicrobial activity against all the test pathogens. Therefore, two strains of actinobacteria were selected for study based on their potential antimicrobial activity against all the pathogens tested. This study was designed to demined the optimize culture condition of bioactive metabolites by agar-well diffusion method. The cell-free culture supernatant of *Streptomyces sioyaensis* PKA45 and *Streptomyces*

luteosporeus PKA51 showed good antimicrobial activity against gram-positive bacteria, gram-negative bacteria and yeast with MHB medium on 3rd day of incubation time. Kathirresan, Balagurunathan, and Selvam (2005) and Oskey (2011) showed that the activity of actinobacteria isolates could be increased or decreased remarkably under different cultural conditions. Similarly, Thakur, Bora, Bordoloi, and Mazumdar (2009) reported that Thornton's medium on the sixth day of incubation increased bioactive metabolite production of *Streptomyces* sp.201. However, the cell-free culture supernatant of *Streptomyces sioyaensis* PKA45 and *Streptomyces luteosporeus* PKA51 showed that the antimicrobial activity against *Enterobacter aerogenes* TISTR1540 was lost. Due to, the cell-free supernatant may be contained a low concentration of active ingredients. Similarly, Charousová, Medo, Halenárová, and Javoreková (2017) reported that the strains of antimicrobial-producing actinomycetes isolated from soil may have a low concentration of active compound in liquid culture.

The antimicrobial efficacy of *Streptomyces sioyaensis* PKA45 and *Streptomyces luteosporeus* PKA51 were tested by using 3 different solvent extracts. Among the solvents used, ethyl acetate extracts showed maximum antimicrobial activity against all the pathogens tested. Similarly, various solvents were used for the extraction of antibiotic from actinobacteria by many workers using ethyl acetate (Ilić, Konstantinović and Todorović, 2005; Parthasarathi et al., 2012; Vijayakumar et al., 2012). The ethyl acetate compound from *Streptomyces sioyaensis* PKA45 and *Streptomyces luteosporeus* PKA51 were used for the MIC, MBC and MFC evaluation of antimicrobial activity against test pathogens by the broth dilution method. The results showed that the MIC_{index} values, being more than 4. Therefore, the crude

compounds could be categorized as a bacteriostatic. An antimicrobial agent is considered bacteriostatic if the MBC/MIC (MIC_{index}) ratio is >4 (Wald-Dickler, Holtom and Spellberg, 2017). However, both ethyl acetate compounds showed not better antimicrobial activity than the commercial drugs. The commercial drugs also known as a pure substance which is responsible for biological activities while crude compound contains both active and inactive ingredients. The low antimicrobial activity is probably due to the presence of active ingredient in crude compounds in very low quantities, which needed the use of the purified bioactive compounds of crude extracts.

In conclusion, 6 antimicrobial-producing actinobacteria and 11 antimicrobial-producing fungi were isolated from soil in Suranaree University of Technology, Thailand. These isolates were produced bioactive secondary metabolites, indicating that dry dipterocarp forest in Suranaree University of Technology are a valuable source of discovery for antimicrobial-producing microorganisms with promising potential to produce bioactive antimicrobial metabolites. Among them, antimicrobial-producing actinobacteria, PKA45 and PKA51 showed highest antimicrobial activity against test pathogens including methicillin-resistant *Staphylococcus aureus* (MRSA). Our future experiments are including the purification, identification and evaluation of active compounds. The active compounds of these strains might be further used for the treatment of MRSA infection.

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APPENDIX

APPENDIX

SEQUENCES OF ANTIMICROBIAL-PRODUCING SOIL ISOLATES

1. Sequences of antimicrobial-producing fungal strains

PKF6

GTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTACCGAGTGTA

GGGTTCCTAGCGAGCCCAACCTCCCACCCGTGTTTACTGTACCTTAGTTGC

TTCGGCGGGCCCGCCATTCATGGCCGCCGGGGGCTCTCAGCCCCGGGCCC

GCGCCCGCCGGAGACACCACGAACTCTGTCTGATCTAGTGAAGTCTGAGT

TGATTGTATCGCAATCAGTTAAAACTTTCAACAATGGATCTCTTGGTTCCG

GCATCGATGAAGAACGCAGCGAAATGCGATAACTAGTGTGAATTGCAGA

ATTCCGTGAATCATCGAGTCTTTGAACGCACATTGCGCCCCCTGGTATTCC

GGGGGGCATGCCTGTCCGAGCGTCATTGCTGCCCATCAAGCACGGCTTGT

GTGTTGGGTCGTCGTCCCCTCTCCGGGGGGGACGGGCCCCAAAGGCAGCG

GCGGCACCGCGTCCGATCCTCGAGCGTATGGGGCTTTGTCACCCGCTCTGT

AGGCCCGGCCGGCGCTTGCCGAACGCAAATCAAT

PKF104

GTAACAAGGTCTCCGTTGGTGAACCAGCGGAGGGATCATTACCGAGTTTA

CAACTCCCAAACCCATGTGAACATACCTATCGTTGCTTCGGCGGGATCGC

CCCGGGCGCCTTGTGTGCCCCGGATCCAGGCACCCGCCGGGGGACCTTAA

CTCTTGTTTTATTTAGAATCTTCTGAGTAGTTTTTACAAATAAATAAAAAC

TTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAA

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ACGCACATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTCTGAGCGTC

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CGCAAGGGACGGCCGGCCCCTAAATCTAGTGGCGGACCCGTCGTGGCCTC

CTCTGCGAAGTAGTAATATTCCGCATCGGAGAAGCGACGAGCCCCTGCCG

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CTGAACTTAA

PKF105

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ACTTTCAACAATGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGCGA

AATGCGATAACTAATGTGAATTGCAGAATTCAGTGAATCATCGAGTCTTT

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AATGCGATAACTAATGTGAATTGCAGAATTCAGTGAATCATCGAGTCTTT

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PKF121

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CCGACCCCC

PKF124

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GGGTACGGTAACAGACGATCTGGATACTTCTGCCTCCCGCAGAGACCATG

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CACTGGGGCCTCGCCCCGGTCGCCGGGGGGTTCTTGCCCCCGGGCCCGCG

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PKF125

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AGAACGCAGCGAAATGCGATAACTAATGTGAATTGCAGAATTCAGTGAAT

CATCGAGTCTTTGAACGCACATTGCGCCCTCTGGTATTCCGGAGGGCATGC

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PKF127

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PKF145

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GAACTTAAGCATATC

PKF152

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CTCTGTCACTCGCTCGGG

PKF161

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CGGCATCGATGAAGAACGCAGCGAAATGCGATAACTAATGTGAATTGCA
GAATTCAGTGAATCATCGAGTCTTTGAACGCACATTGCGCCCCCTGGTATT
CCGGGGGGCATGCCTGTCCGAGCGTCATTGCTGCCCTCAAGCCCGGCTTG
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GCGGCGGCACCGCGTCCGATCCTCGAGCGTATGGGGCTTTGTCACATGCT
CTGTAGGATTGGCCGGCGCC

2. Sequences of antimicrobial-producing actinobacteria strains

PKA2

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AGCGAGAGTGACGGTACCTGCAGAAGAAGCGCCGGCTAACTACGTGCCA
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TAAAGAGCTCGTAGGCGGCTTGTCGCGTCGGATGTGAAAGCCCGGGGCTT
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CACG

PKA20

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PKA45

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G

PKA50

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PKA62

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ACTCGACCCCATGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCATTGCT

GCGGTGAATACG

CURRICULUM VITAE

NAME: Miss Phimpha Khowangklang

DATE OF BIRTH: May 29, 1987

PLACE OF BIRTH: Nakhon Ratchasima, Thailand

EDUCATION: Huachiew Chalermprakiet University, 2007, Bachelor of Science in Industrial Microbiology

PROCEEDING: Khowangklang, P. and Nantapong, N. Filamentous Fungi PKF121 Isolated from Dry Dipterocarp Forest Soil in Northeast Thailand Produces Antimicrobial Agents Active against Methicillin-Resistant *Staphylococcus aureus*. 13th International Conference on Advances in Agricultural, Environmental, Biological and Medical Sciences (AAEBM-18). April 24-25, 2018 in Mercure Pattaya Ocean Resort, Pattaya, Thailand.