การประเมินฤทธิ์ต้านจุลินทรีย์ของเชื้อแอคติโนมัยสีท ที่แยกจากดินต่อเชื้อก่อโรคแบบฉวยโอกาส

นางสาวปัญจมาภรณ์ จันทเสนา

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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EVALUATION OF ANTIMICROBIAL ACTIVITY OF ACTINOMYCETES ISOLATED FROM SOIL AGAINST OPPORTUNISTIC PATHOGENS

Panjamaphon Chanthasena

A Thesis Submitted in Partial Fulfillment of the Requirements for the

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EVALUATION OF ANTIMICROBIAL ACTIVITY OF ACTINOMYCETES ISOLATED FROM SOIL AGAINST OPPORTUNISTIC PATHOGENS

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

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แบคทีเรียสายพันธุ์แอคติโนมัยสีท 123 ตัว ได้ถูกคัดแยกจากดินบริเวณป่าเต็งรังในเขตมหาวิทยาลัยเทคโนโลยีสุรนารี จังหวัดนครราชสีมา เชื้อเหล่านี้ได้ถูกนำมาทดสอบความสามารถในการสร้างสารต้านเชื้อก่อโรคแบบฉวยโอกาส จากเชื้อทั้งหมดที่แยกได้ เชื้อสายพันธุ์ PJ85 มีความสามารถในการสร้างสารต้านเชื้อก่อโรคแบบฉวยโอกาสแกรมบวกที่ใช้ในการทดสอบได้ดีที่สุด แบคทีเรียสายพันธุ์ PJ85 จึงถูกใช้ในการเตรียมสารสกัดหยาบ พบว่าสารสกัดจากเชื้อสายพันธุ์ PJ85 ให้ค่า MIC ต่อเชื้อ *Staphylococcus aureus* ATCC29213 *Staphylococcus aureus* TISTR1466 *Staphylococcus epidermidis* TISTR518 *Bacillus subtilis* TISTR008 และ *Bacillus cereus* TISTR687 เป็น 1 1 2 0.5 และ 0.25 ไมโครกรัมต่อมิลลิลิตร ตามลำดับ และให้ค่า MBC ต่อเชื้อ *Staphylococcus aureus* ATCC29213 *Staphylococcus aureus* TISTR1466 *Staphylococcus epidermidis* TISTR518 *Bacillus subtilis* TISTR008 และ *Bacillus cereus* TISTR687 เป็น 2 8 4 1 และ 2 ไมโครกรัมต่อมิลลิลิตร ตามลำดับ ผลการจำแนกชนิดโดยอาศัยลักษณะทางสัณฐานวิทยา ได้แก่ ลักษณะโคโลนี รูปร่างสปอร์ คุณสมบัติการติดสีแกรม และการวิเคราะห์ลำดับเบสบนยีน 16S rRNA พบว่า เชื้อแบคทีเรียสายพันธุ์ PJ85 มีความคล้ายคลึงกับเชื้อ *Streptomyces triostinicus* มากที่สุด และการศึกษาแผนภูมิวิวัฒนาการของยีน 16S rRNA พบว่า แบคทีเรียสายพันธุ์ PJ85 มีสายวิวัฒนาการที่แยกออกไปจากเชื้อสายพันธุ์ *Streptomyces triostinicus* และเชื้อในจีนัส *Streptomyces* สายพันธุ์อื่น ซึ่งมีความเป็นไปได้ว่าแบคทีเรียสายพันธุ์ PJ85 อาจจะเป็นเชื้อ *Streptomyces triostinicus* สายพันธุ์ใหม่ งานวิจัยนี้เป็นการรายงานการคัดแยกเชื้อ *Streptomyces triostinicus* จากดินในประเทศไทย ที่มีความสามารถในการต้านเชื้อแบคทีเรียได้ เป็นครั้งแรก

สาขาวิชาเภสัชวิทยา ลายมือชื่อนักศึกษา_____

ปีการศึกษา 2558 ลายมือชื่ออาจารย์ที่ปรึกษา_____

PANJAMAPHON CHANTHASENA : EVALUATION OF ANTIMICROBIAL ACTIVITY OF ACTINOMYCETES ISOLATED FROM SOIL AGAINST OPPORTUNISTIC PATHOGENS. THESIS ADVISOR : NAWARAT NANTAPONG, Ph.D. 63 PP.

SOIL ISOLATE/ ACTINOMYCETES/ *STREPTOMYCES TRIOSTINICUS*/ ANTIBIOTICS/ OPPORTUNISTIC PATHOGENS

The total of 123 Actinomycetes strains were isolated from dry dipterocarp forest soil in Suranaree University of Technology, Nakhon Ratchasima, Thailand. They were used for the screening of their antimicrobial activity against test opportunistic pathogens. Among all isolates, PJ85 exhibited highest antibacterial activity against Gram-positive bacteria. PJ85 was selected for the preparation of crude extract. The MIC values of PJ85 extract against *Staphylococcus aureus* ATCC29213, *Staphylococcus aureus* TISTR1466, *Staphylococcus epidermidis* TISTR518, *Bacillus subtilis* TISTR008 and *Bacillus cereus* TISTR687 were 1, 1, 2, 0.5 and 0.25 µg/ml, respectively. The MBC values against *Staphylococcus aureus* ATCC29213, *Staphylococcus aureus* TISTR1466, *Staphylococcus epidermidis* TISTR518, *Bacillus subtilis* TISTR008 and *Bacillus cereus* TISTR687 were 2, 8, 4, 1 and 2 µg/ml, respectively. The colony morphology, spore formation, Gram staining and 16S rRNA gene analysis showed that PJ85 was close affiliated with *Streptomyces triostinicus*. Phylogenetic tree analysis of 16S rRNA gene showed that PJ85 are not cluster with *Streptomyces triostinicus* and others known *Streptomyces* spp. It was represented a distinct phyletic line which could be suggested a novel strain.

To our best knowledge, this study constitutes the first antibacterial properties of *Streptomyces triostinicus* isolated from soil in Thailand.

School of Pharmacology Student's Signature_____

Academic Year 2015 Advisor's Signature_____

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Panjamaphon Chanthasena

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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
MIC	=	Minimum inhibitory concentration
ml	=	Milliliter
mm	=	Millimeter
PCR	=	Polymerase chain reaction
rpm	=	Round per minute
rRNA	=	Ribosomal ribonucleic acid
TBE	=	Tris-borate-EDTA
v/v	=	Volume by volume

CHAPTER I

INTRODUCTION

1.1 Background / Problem

Infectious disease caused by opportunistic pathogens have been a global healthcare problem for decades (Alanis, 2005). As a result, the search and development of a novel class of antibiotic drugs are required. Since, the discovery of penicillin-producing mold in 1928 by Alexander Fleming, microorganisms have become a target source for the production of antimicrobial agents. Soil microorganisms are the major resource for isolation of several important products such as antimicrobial drugs, anticancer drugs, herbicides and insecticides (Sanglier, Haag, Huck and Fehr, 1993; Jeya, Kiruthika and Veerapagu, 2013). A number of antibiotic drugs have been discovered from soil-inhabiting microorganisms which include fungi (20% of currently available antibiotics), actinomycetes (70%) and eubacteria (10%) (Berdy, 1974; Lechevalier, 1975). It has been shown that bacteria belonging to the order Actinomycetes are a potential source for bioactive secondary metabolites including antimicrobial agents. Actinomycetes are widely distributed groups in soil environments which play a major role in the recycling of organic matters and nutritional materials (Velayudham and Murugan, 2012). They are filamentous Gram-positive bacteria belonging to the phylum Actinobacteria. They represent one of the largest taxonomic units currently recognized within the domain Bacteria (Ventura et al., 2007).

Approximately 80% of the World's antibiotics are derived from Actinomycetes, mostly from the genera *Streptomyces* and *Micromonospora* (Arifuzzaman, Khatun and Rahman, 2010; George, George and Hatha, 2011).

After the discovery of penicillin during 1940-1962, several classes of antibiotics including *beta*-lactams, tetracyclines, aminoglycosides and macrolides were isolated and introduced to the clinical use (French, 2003; Nett, Ikeda and Moore, 2009). However, the finding of new compounds has substantially decreased since the late 1980s which may be due to the decline in the screening efforts (Watve, Tickoo, Jog and Bhole, 2001). Watve and co-workers estimated that only 1-3% of all known antimicrobial compounds produced by genus *Streptomyces* alone has been isolated so far. Thus, there is a vast majority of antibiotics left to be discovered (Watve, Tickoo, Jog and Bhole, 2001). Moreover, Shetty and colleagues have reported that only one fifth of the global soil has been used for the screening of antimicrobial-producing organisms. Thus, there are plenty of spaces on earth left for the search of the novel strains of antimicrobial-producing microorganisms (Shetty, Buddana, Tatipamula, Naga and Ahmad, 2014).

According to a World Bank report published in 2012, tropical forests cover approximately one third of Thailand's total land area (World Bank Group, 2015). However, there have been a few studies on Actinomycetes in Thai forest soil (Euanorasetr, Nilvongse, Tantimavanich, Nihira, Igarashi and Panbangred, 2010).

Dry dipterocarp forests are found throughout Suranaree University of Technology. Soil from dry dipterocarp forests have been known for its poor in nutrients and highly acidic which could establish somewhat extreme condition. An extreme environmental condition has been known to activate the protective mechanisms of soil-inhabiting

microorganisms by inducing the production of several defense compounds including antibiotics (Murphy, Maloney and Fenical, 2009). The present study attemped to isolate the antibiotic-producing Actinomycetes from dry dipterocarp forest soil in Suranaree University of Technology.

1.2 Research objectives

1.2.1 To isolate an antimicrobial-producing Actinomycetes from dry dipterocarp forest soil.

1.2.2 To evaluate an antimicrobial activity of crude extract produced by soil isolate Actinomycetes against opportunistic pathogens.

1.2.3 To identify the antimicrobial-producing soil isolates by morphological characteristics and 16S rRNA gene sequencing.

1.3 Research hypothesis

1.3.1 Dry diterocarp forest soil in Suranaree University of Technology is served as a potential source for screening and isolation of antimicrobial-producing Actinomycetes.

1.3.2 The crude extract produced from soil isolate Actinomycetes shows an antimicrobial activity against test pathogens.

1.4 Scope and limitations of study

This work involves the isolation of antimicrobial-producing Actinomycetes from soil collected in Suranaree University of Technology, Nakhon Ratchasima, Thailand. The isolate strains were used for screening of antimicrobial activity against opportunistic pathogens. The strain that shows highest antimicrobial activity was selected for the preparation of crude extract. The crude extract was used for determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) against test pathogens.

CHAPTER II

LITERATURE REVIEWS

2.1 A brief history of antibiotics

Antibiotics, also known as antimicrobial drugs, are medicines that can kill or inhibit the growth of bacteria which cause illness to humans and animals (Lihan, Choon, Hua and Wasli, 2014). Penicillin was the oldest available antibiotic that was first discovered in 1928 by Alexander Fleming. Fleming observed the inhibition of staphylococci on an agar plate contaminated with the *Penicillium* mold. In 1940s, penicillin was introduced to the clinical used and revolutionized the treatment of infectious caused by Gram-positive bacteria, especially staphylococci and streptococci. The discovery of penicillin has contributed the increasing of life span, leading to the effort of many researchers to isolate the antibiotic compounds from microorganisms. After the discovery of penicillin, new classes of antimicrobial agents such as *beta*-lactams, tetracyclines, aminoglycosides and macrolides were discovered.

In 1944, streptomycin was discovered by Selman Waksman. This antibiotic was derived from *Streptomyces griseus* isolated from soil in New Jersy, USA (Demain, 2006). Streptomycin was the first effective drugs to treat tuberculosis (Tripathi, Tewari, Dwivedi and Tiwari, 2005).

In 1945, the tetracycline-producing *Streptomyces aureofacians* was first discovered from soil in Missouri, USA by Benjamin Minge Duggar (Chopra and Roberts, 2001). Tetracycline is a broad-spectrum agent which is active against Gram-

positive bacteria, Gram-negative bacteria, chlamydia, mycoplasma and rickettsia. More recently, tetracycline has been used against eukaryotic protozoan parasites and the treatment of a variety of non-infectious conditions including acne (Eliopoulos and Roberts, 2003).

In 1947, chloromycetin was first discovered by Bukholder. It was derived from *Streptomyces venezuelae* isolated from soil sample in a mulched field near Caracas, Venezuela (Robertson, 2003). Chloromycetin is active against wide range of Gram-positive and Gram-negative bacteria (Parfitt, 1999).

In 1963, gentamicin was first discovered by Weinstein at Schering Corporation, Bloomfield, USA (Weinstein et al., 1963). Gentamicin is an aminoglycoside antibiotic produced by soil-bacteria, *Micromonospora purpure* and *Micromonospora echinospor*. It is active against wide range of bacterial infections caused by *Staphylococcus* spp., *Pseudomonas* spp., *Proteus* spp., *Serratia* spp., *Escherichia coli*, *Klebsiella pneumonia* and *Enterobacter aerogenes*. Gentamicin has been used in the treatment of respiratory tract infections, urinary tract infections, blood, bone and soft tissue infections.

The discovery of new antibiotic drugs were still increasing until late 1970s. It was only in 2000-2003 that linezolid and daptomycin belonging to the novel classes of antibiotics were introduced. The timeline of antibiotic drugs discovery is shown in Figure 2.1.

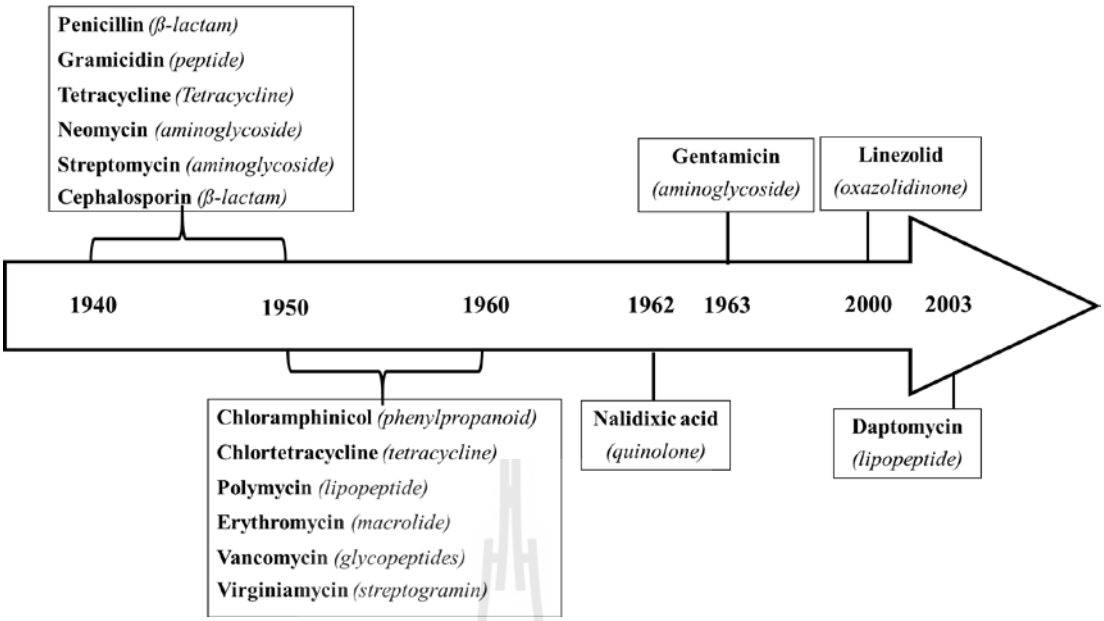


Figure 2.1 Developing of antimicrobial agents during the years 1940-2003 (Adapted from Wright, 2007).

2.2 Antibiotic-producing Actinomycetes

The antibiotic producers can be found in river, lakes and decaying plants, however; the majority of microorganisms that produce antibiotics inhabits in soil (Chandrashekhara, Nanjwade, Goudanavar, Manvi and Ali, 2010). Three quarter of all known antibiotics such as tetracycline, vancomycin and erythromycin are produced by Actinomycetes (Varghese, Nishamol, Suchithra, Jyothy and Hatha, 2012). The *Streptomyces* is the dominant genus that produces approximately 80% of all known antibiotics. The history of antibiotics derived from the genus *Streptomyces* have 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 1949, the first macrolide antibiotic, erythromycin was discovered by McGuire (Butler, 2008). Erythromycin was derived from *Streptomyces erythrecus* found in soil collected in Philippines. It was first marketed in 1952 under the brand name Ilosone to overcome penicillin-resistant *Staphylococcus aureus*.

In 1953, amphotericin was isolated from a strain of *Streptomyces nodosus* collected from soil in Venezuela (Mesa-Arango, Scorzoni and Zaragoza, 2012). It was used to combat life-threatening systemic fungal infections in patients whose immune system is compromised.

In 1955, vancomycin was isolated from *Streptomyces orientalis* at the Lilly laboratory (Levine, 2006). Vancomycin was effective against both aerobic and non-aerobic Gram-positive bacteria, especially resistant staphylococci by disrupting bacterial cell wall synthesis.

In 1957, kanamycin was isolated from soil bacterium, *Streptomyces kanamycesticus* by Hamao Umezawa in Tokyo (Umezawa, 1958). It was aminoglycoside antibiotic which was reserved for the treatment of penicillin-resistant staphylococcal and serious Gram-negative infections resistant to gentamicin.

In 1961, Shoji and Katakari isolated a peptide antibiotic, triostin A from soil bacterium, *Streptomyces triostinicus* (Shoji and Katagiri, 1961). It was active against Gram-positive bacteria and cytotoxic to mammalian cells in culture. The biological activity has been attributed to its binding to the DNA of susceptible cells through the quinoxaline chromopores.

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

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

Antibiotics	**Source**	**Spectrum**	**Mode of action**
Streptomycin	*S. griseus*	Gram-negative bacteria	Inhibition of protein synthesis
Cephalosporins	*S. clavuligerus*	Gram-positive and Gram-negative bacteria	Inhibition of cell wall synthesis
Chloramphenicol	*S. venezuelae*	Gram-positive and Gram-negative bacteria	Inhibition of protein synthesis
Neomycin	*S. fradiae*	Gram-positive and Gram-negative bacteria	Inhibition of protein synthesis
Tetracycline	*S. aureofaciens*	Gram-positive and Gram-negative bacteria	Inhibition of protein synthesis
Nystatin	*S. noursei*	*Candida* spp.	Induces membrane permeability
Clindamycin	*S. lincolnensis*	Gram-positive and Gram-negative bacteria (anaerobic bacteroides)	Inhibition of protein synthesis
Erythromycin, Azithromycin	*S. erythreus*	Gram-positive and Gram-negative bacteria not enterics, Neisseria, Legionella, Mycoplasma	Inhibition of protein synthesis
Amphotericin	*S. nodosus*	Fungi (Histoplasma)	Induces membrane permeability
Vancomycin	*S. orientalis*	Gram-positive bacteria *(Staphylococcus aureus*)	Inhibition of cell wall synthesis
Rifampicin	*S. mediterranei*	Gram-positive and Gram-negative bacteria (*Mycobacterium tuberculosis*)	Inhibition of bacterial RNA polymerase

Source: Todar, 2008.

Actinomycetes are aerobic, Gram-positive filamentous bacteria belonging to the phylum Actinobacteria (Stackebrandt, Rainey and Ward-Rainey, 1997). They are free living saprophytic bacteria widely distributed in soil which play an important role in decomposition of organic materials. Actinomycetes are the most distributed group of microorganisms in nature. They produce antibiotics to inhibit the growth of other microorganisms that inhabit the same environment. Hence, antibiotic-producing Actinomycetes can compete with other organisms and survive in nature for a long time (Oskay, Üsame and Cem, 2005). As a result, the numbers of Actinomycetes in soil often exceed one-million per gram.

Actinomycetes typically exhibit filamentous growth and produce spores. They form two kinds of branching mycelium including aerial mycelium and substrate mycelium on the culture. The filaments produced from Actinomycetes are much narrower than fungal hyphae. Cell of Actinomycetes are prokaryotic cells. They have a complex life cycle that includes the formation of spores and other cell types (Figure 2.2). Typically, a spore germinates under the right conditions to generate a substrate mycelium. This consists of a net of branching hyphae that grow and penetrate 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.

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).

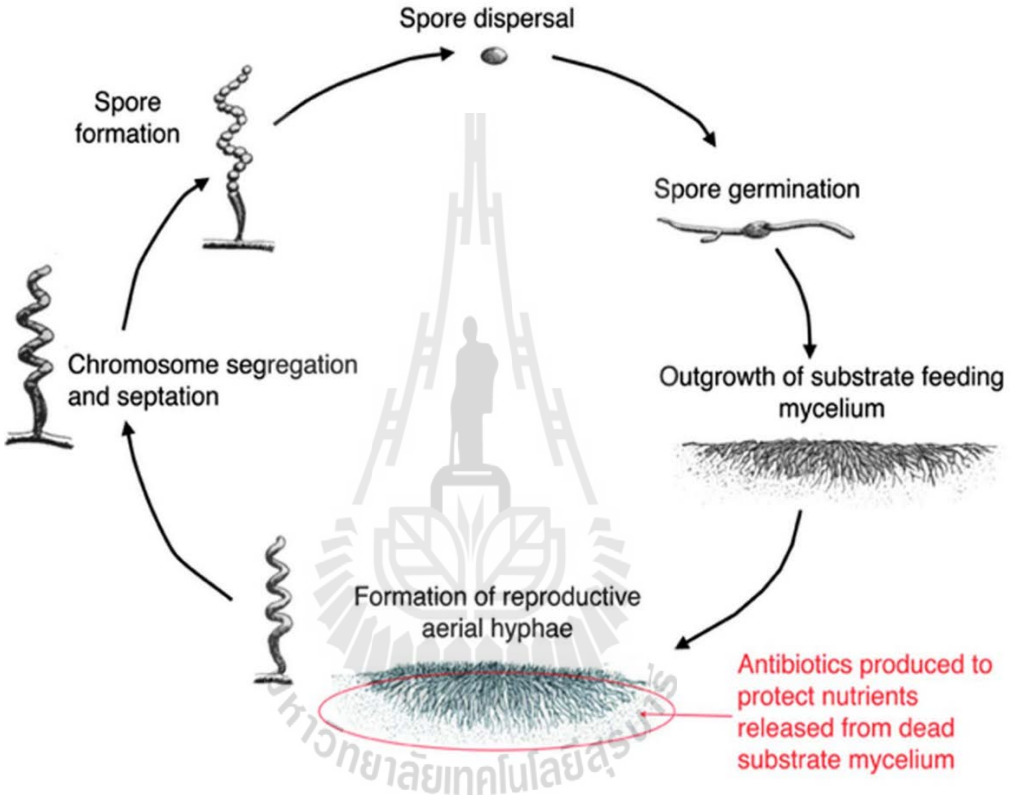


Figure 2.2 The life cycle of Actinomycetes.

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).

2.3 The opportunistic pathogens

Opportunistic pathogens are microbes that are incapable of causing disease in a healthy person but able to infect when the host defense mechanism are low. They are the normal flora that live on the surface or in deep layer of skin, saliva, oral mucosa, 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, Hazel, Richard, Ivan, Derek and Mark, 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 (Grabinska, Wardzynska, Pajor, Korsak and Boryn, 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 commensal 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, Sturdevant, Villaruz, Xu, Gao and Otto, 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 (Bentzmann and Plésiat, 2011).

Nowadays, many strains of opportunistic pathogens are found to be resist 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 pathogens

The pathogenic strains used in this study were obtained from American Type Culture Collection (ATCC) and Thailand Institute of Scientific and Technological Research (TISTR). They were *Staphylococcus aureus* ATCC29213, *Staphylococcus aureus* TISTR1466, *Staphylococcus epidermidis* TISTR518, *Bacillus subtilis* TISTR008, *Bacillus cereus* TISTR687, *Escherichia coli* TISTR780, *Enterobacter aerogenes* TISTR1540, *Pseudomonas aeruginosa* TISTR781, *Serratia marcescens* TISTR1354 and *Proteus mirabilis* TISTR100.

Escherichia coli JM109 (*rec*A1, *sup*E44, *end*A1, *hsd*R17, *gyr*A96, *rel*A1, *thi*, Δ(*lac-pro*AB), F′[*tra*D36, *pro*AB^{+}, *lac*I^{q} *lac*ZΔM15]) (Kwon, Georgellis, Lynch, Boyd and Lin, 2000) was used for transformation and plasmid construction.

3.1.2 Media

The following materials were purchased from Himedia, India.

Starch Casein (SC) medium contained (per 1 liter) 10 g of soluble starch, 0.3 g of casein acid hydrolysate, 2 g of KNO_3, 2 g of NaCl, 2 g of K_2HPO_4, 0.05 g of $MgSO_4$, 0.02 g of $CaCO_3$ and 0.01 g of $FeSO_4$. The final pH was adjust to 7.0.

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 was adjust to 7.2.

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 was adjust to 7.0.

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.

Luria-Burtani (LB) medium contained (per 1 liter) 5 g of yeast extract, 10 g of tryptone, 10 g of NaCl. The final pH was adjusted to 7.0.

All solid media were prepared by adding 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 min.

3.1.3 Antibiotics

Ampicillin (Bio Basic, Cannada) stock solution was prepared by dissolving in sterile distilled water to yield a final concentration of 10 mg/ml.

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

Tetracycline (Sigma-Aldrich, USA) stock solution was prepared by dissolving in sterile distilled water to yield a final concentration of 0.5 mg/ml.

All the antibiotic stock solutions were filter sterilized through a 0.2 µm pore filter (Corning®, Germany) and stored at -20ºC until used.

3.1.4 Buffers and solutions

3.1.4.1 Buffer for agarose gel electrophoresis

1x TBE buffer consisted of 89 mM Tris-HCl (pH 8), 89 mM boric acid and 2.5 mM EDTA.

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

3.1.5 Primers

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, Sosio, Cavaletti, Chiocchini and Donadio, 2002.
M13/pUC Forward	GTAAAACGACGGCCAGT	Helianti, Nurhayati and Wahyuntari, 2007.
M13/pUC Reverse	GCGGATAACAATTTCACACAGG	Helianti, Nurhayati and Wahyuntari, 2007.

3.1.6 Vector

The vector pGEM®-T Easy used in this study was purchased from Promega, USA. Map of this vector is shown in Figure 3.1.

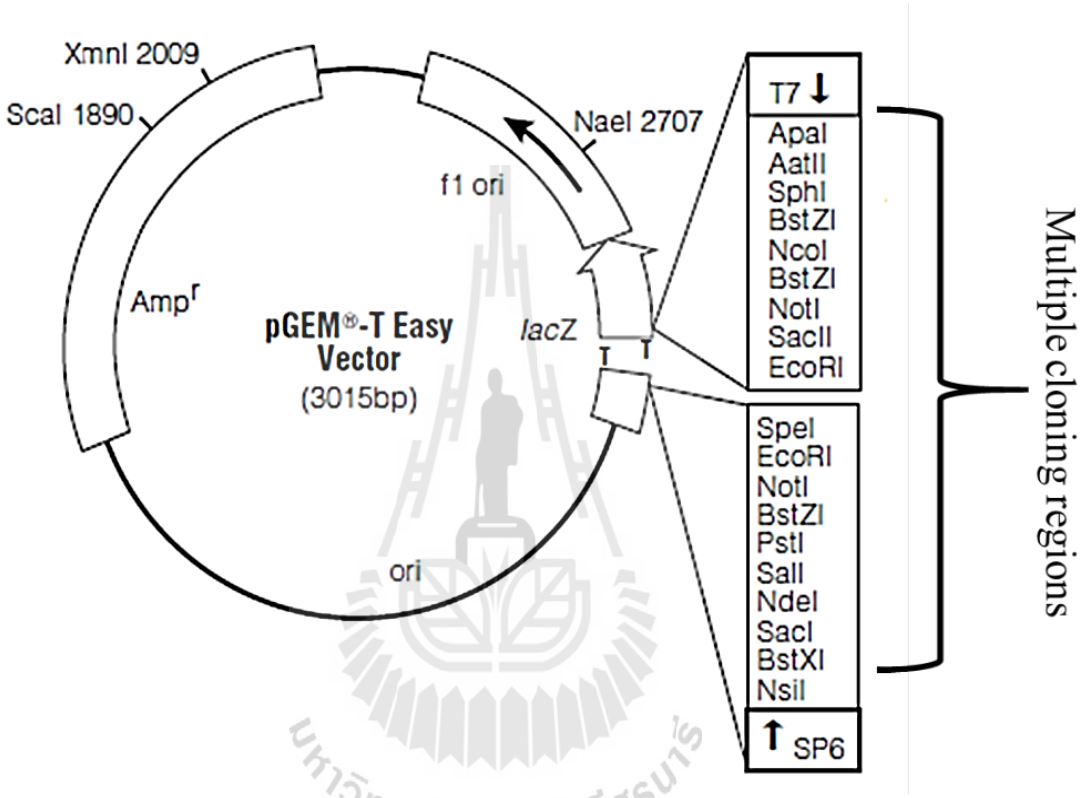


Figure 3.1 Map of pGEM®-T Easy vector.

The size of pGEM-T Easy vector is 3.015 kb. It contains the multiple cloning regions, β-gactosidase gene (*lac*Z), ampicillin-resistance gene (*amp*^{R}) and an *Escherichia coli* origin of replication.

3.1.7 Miscellaneous materials

5-Bromo-4-chloro-3-indolyl-*β*-D-galactoside (X-gal) was purchased from Bio basic, Canada.

λ-DNA digested with *Hin*d III was used as marker. The marker was purchased from Promega, USA.

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

3.1.8 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
Larminar 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 area and sample collection

The study site was located at the forest area in Suranaree University of Technology, Nakhon Ratchasima, Thailand (14.8729º N, 102.0237º E) during January 2012 to February 2013. Soil samples were randomly collected at a depth of 10-15 cm from the upper surface of soil using sterile technique.

3.2.2 Isolation of Actinomycetes

Isolation of Actinomycetes was performed by serial dilution and plating technique using Starch Casein Agar (SCA) medium. One gram of soil sample was suspended in 250 ml-Erlenmeyer flask containing 99 ml sterile water and incubated at room temperature without shaking for 30 min. The soil suspension was serially diluted and spread on SCA plate. The plates were incubated at 37ºC for 5 days or until the colonies appeared. The suspected Actinomycetes colonies were selected and purified by cross streak method. A single colony of Actinomycetes was inoculated into 5 ml of Starch Casein Broth (SCB) and incubated at 37ºC for 3 days. Three-day culture of Actinomycetes were kept in the presence of glycerol (15% v/v) at -80ºC for further study.

3.2.3 Primary screening of antimicrobial-producing soil isolates using perpendicular-streak plate

Soil isolates were screened for their antimicrobial activity by perpendicular-streak method (Figure 3.2). The isolate strains were inoculated on Mueller Hinton Agar (MHA) plate by single streaking at the center of a petridish. The plates were incubated at 37ºC for 5 days in order to allow the organisms to produce antimicrobial substances and release to an agar medium. The plates were then seeded with test pathogens (0.5

McFarland) by streaking perpendicular to the line of soil isolate colonies. The zone of inhibition against test pathogens of each isolate was observed after 24 h of incubation.

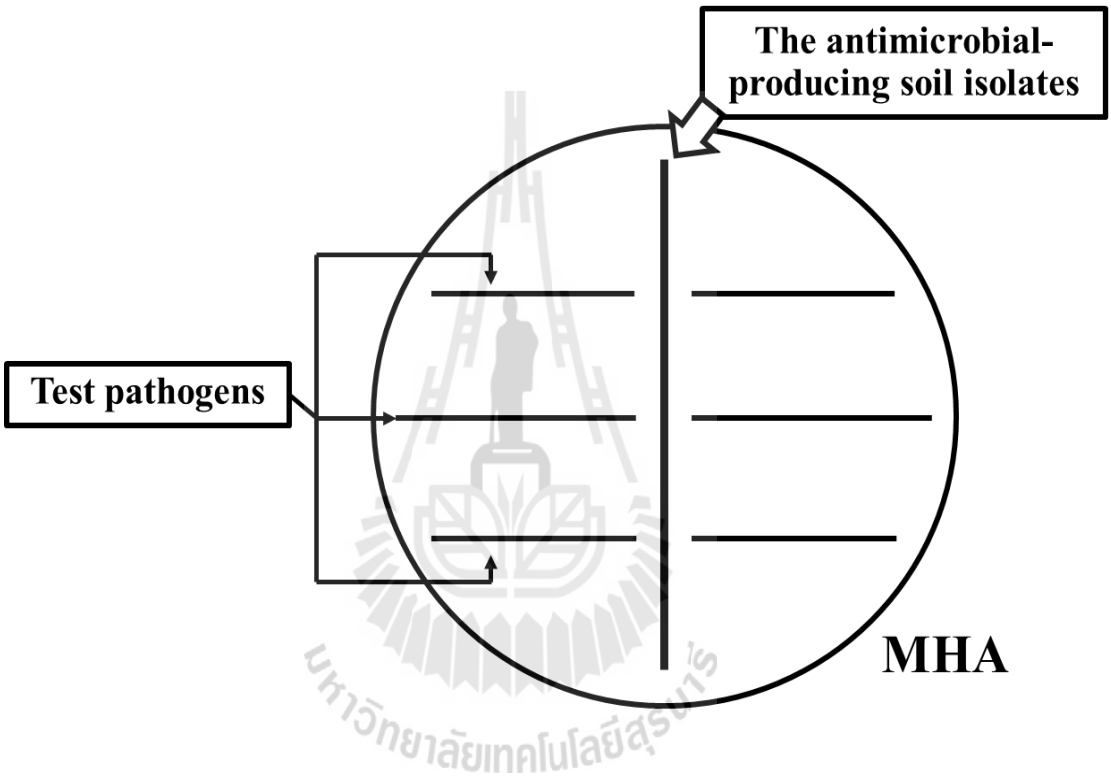


Figure 3.2 The perpendicular-streak method.

3.2.4 Secondary screening of antimicrobial-producing soil isolates using agar-well diffusion method

A single colony of the isolates was inoculated into 5 ml of culture media and incubated on a rotary shaker (200 rpm) at 37ºC for 3 days. After incubation, one milliliter of three-day culture was transferred to 500 ml-Erlenmeyer flask containing 100 ml of medium and incubated on a rotary shaker (200 rpm) at 37ºC. The cell culture were collected in aliquot every 24 h for 15 days. Cells suspension were centrifuged at 13,000 rpm. The cell-free supernatants were used for the secondary screening. Fifty microliter of cell-free supernatants were applied into each well of plate lawn with test pathogens (0.5 McFarland). The plates were then incubated at 37ºC for 24 h. After incubation, the zone of inhibition in diameter (mm) were observed and recorded.

3.2.5 Preparation of crude extract

An antimicrobial-producing soil isolate that showed highest activity in secondary screening method was grown on culture media for 3 days. Five milliliters of three-day culture were then transferred to a 1,000 ml-Erlenmeyer flask containing 500 ml of the ISP2 medium and incubated on a rotary shaker (200 rpm) at 37ºC for 9 days. After incubation, the culture was filtrated through Whatman No.1 filter paper (Whatman^{TM}, GE Healthcare, UK). Ethyl acetate was added to the filtrate in the ratio of 1:3 (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.

3.2.6 Determination of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of crude extract

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the crude extract were determined by broth macro-dilution method (Franklin et al., 2012). Using a test tubes macro-dilution test, 900 µl of MH broth was dispensed into all the test tubes. Crude extract was dissolved in 1% dimethyl sulfoxide (DMSO) and diluted in MH broth by the serial two-fold dilution method. The initial concentration of crude extract was 128 to 0.0625 µg/ml. The mixture of each tubes was inoculated with 100 µl of the suspension containing 5×10^6 CFU/ml of test pathogens in the mid-log phase. The final concentration of bacteria were approximately 5×10^5 CFU/ml. 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 inhibit visible growth of test organisms. The minimum bactericidal concentration (MBC) was determined by carrying out a subculture of the tubes showing no growth on an agar plate without crude extract. The MBC is defined as the lowest concentration of an agent that inhibits 99.9% of the growth in subcultures (Arthur, 1999).

3.2.7 Identification of antimicrobial-producing soil isolate by 16S rRNA gene sequencing

3.2.7.1 Preparation of DNA template

Genomic DNA was isolated from cell grown in 5 ml MH broth at 37ºC for 3 days. The cell culture were centrifuged at 13,000 rpm for 5 min and the cell pellets were used for DNA extraction. Five milligrams of cell pellets were mixed with 180 µl of 50 mM NaOH. The cell suspensions were incubated at 95ºC for 10 min. The 20 µl of 1M Tris-HCl was added into the mixture. Cells were pelleted by centrifugation

at 13,000 rpm for 5 min. The supernatants were used as DNA template for PCR amplification of 16S rRNA gene.

3.2.7.2 PCR amplification of 16S rRNA gene

The 16S rRNA gene was amplified by using specific 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 60 s, annealing at 55ºC for 60 s, extension at 72ºC for 60 s and a final extension at 72ºC for 7 min.

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**

3.2.7.3 Ligation of 16S rDNA into pGEM®-T easy vector

The amplified fragments were purified from 0.7% agarose gel by using NucleoSpin® Gel and PCR clean-up kit (Macherey-Nagel, Germany). The purified products were ligated to pGEM®-T easy vector. The ligation mixture (Table 3.4) was incubated at 4ºC overnight.

Table 3.4 Ligation mixture.

Reaction mixture	**Volume (µl)**
2x ligation buffer	5
pGEM® T easy vector (Figure 3.1)	1
Purified PCR product	X
Sterile water	10-(7+X)
T4 DNA ligase	1
Final volume	**10**

3.2.7.4 Preparation of *E. coli* competent cells

A single colony of *E. coil* JM109 was inoculated into 5 ml of LB broth and incubated at 37ºC overnight. One milliliter of overnight culture was transferred into 250 ml-Erlenmeyer flask containing 100 ml of LB broth. The culture was incubated at 37ºC with 200 rpm shaking speed until OD_{600} reached to 0.3-0.35. Then, the flask was placed on ice for 15-20 min and divided into pre-chilled sterile 50 ml conical tubes. Bacterial cells were harvested by centrifugation at 3,000 *g*, 4ºC for 10 min and washed with 10 ml of ice-cold 0.1 M $MgCl_2$. The suspension was centrifuged at 3,000 *g*, 4ºC for 10 min. The supernatants were discarded and the cell pellets were

gently resuspended in 10 ml of ice-cold 0.1 M $CaCl_2$. The suspensions were kept on ice for 60 min followed by centrifugation at 3,000 *g*, 4ºC for 10 min. The cell pellets were suspended in 2-3 ml of 0.1 M $CaCl_2$. The glycerol was added to the mixture to give a final concentration of 15% (v/v) glycerol. One-hundred microliters of cell suspensions were aliquoted into pre-chilled microcentrifuge tubes. The aliquots were dipped in liquid nitrogen and stored at -80ºC until used.

3.2.7.5 Transformation of recombinant DNA into *E. coli* JM109

The competent cells were thawed on ice. The DNA ligation was mixed with 100 µl of competent cells and incubated on ice for 1 h. The mixure was then heated at 42ºC for 60 sec and immediately cooled on ice for 2 min. Transformation mixture was transferred into 1 ml of LB broth and incubated on rotary shaker (200 rpm) at 37ºC for 1 h. The 100 µl of transformants were spread on LB agar containing 100 µg/ml of ampicillin and 80 µg/ml of X-gal. The plates were incubated at 37ºC overnight. The recombinant clones were selected by blue-white screening method (Sherwood, 2003). Transformants containing pGEM®-T easy vector exhibit a funtional *lacZ* gene encoded *β*-galactosidase. *β*-galactosidase is an enzyme that catalyzed the hydrolysis of X-gal to form blue pigments. The bacterial cells expressing *β*-galactosidase enzyme appear blue colony on the medium containing X-gal. The positive transforants harboring vector with an interrupted *lacZ* gene can not utilize X-gal and appear white colonies.

3.2.7.6 16S rRNA gene sequencing

The recombinant plasmid containing 16S rRNA gene was submitted to Macrogen, Korea for the sequencing. The M13/pUC forword and M13/pUC reverse

primers (Table 3.1) were used for the sequencing. The sequencing was obtained and compared against the GenBank DNA database using BLAST program (NCBI, USA).

3.2.8 Construction of phylogenetic tree

16S rRNA gene sequence of the isolates were compared against the GenBank DNA database using BLAST program. The sequences were aligned with closely-related species by using CLUSTAL W. The phylogenetic tree was constructed by neighbor-joining method using Molecular Evolutionary Genetics Analysis software version 6.0 (MEGA 6). The confidence level of each branch (1,000 replications) was tested by bootstrap analysis.

CHAPTER IV

RESULTS

4.1 Isolation of Actinomycetes

Total of 37 soil samples were collected from forest area around Suranaree University of Technology (Figure 4.1). Soil samples were randomly taken and aseptically transferred by sterile polyethylene bags to the laboratory. The sampling sites of soil samples were shown in Figure 4.2.

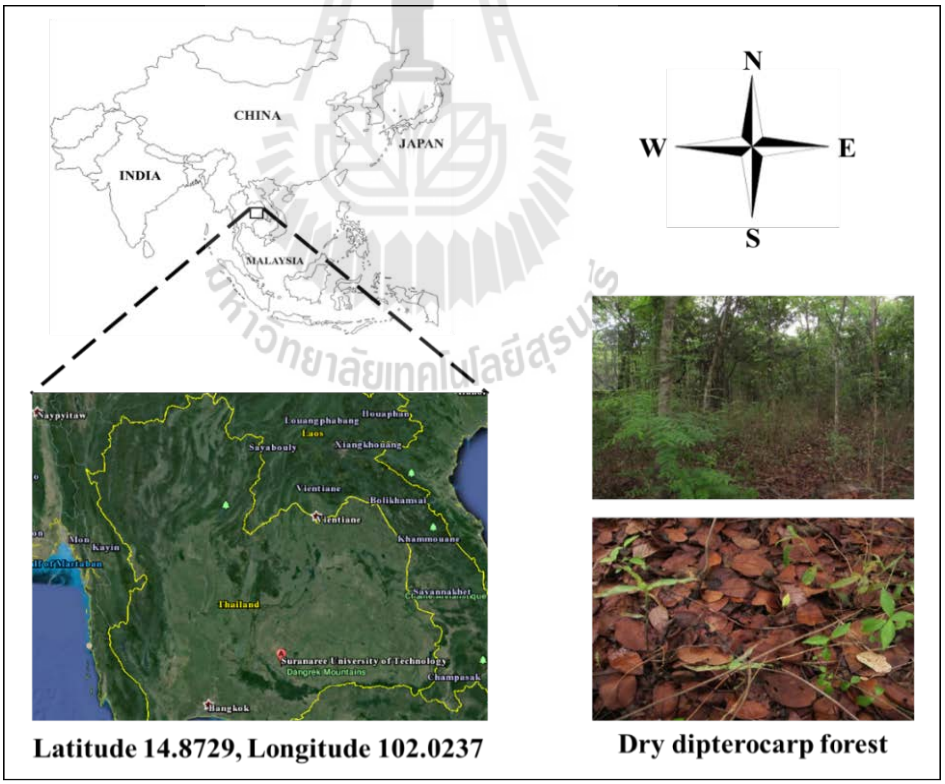


Figure 4.1 Map of the sampling site. Suranaree University of Technology is situated in Nakhon Ratchasima province, Thailand. Dry dipterocarp forests are found throughout this area.

Figure 4.2 Map of Suranaree University of Technology. The asterisk represented the sampling sites of soil samples.

Isolation of Actinomycetes was done by serial dilution and plating technique using SCA medium. Several colonies were appeared on SCA after incubation at 37ºC for 5 days. Colonies having characteristic features such as powdery or waxy appearance with convex, rough and color ranging from white, gray to pinkish and yellowish were isolated and screened for their antimicrobial activity. One hundred-twenty three isolates were obtained and given the name as PJ1 to PJ123.

4.2 Primary screening of antimicrobial-producing soil isolates

Primary screening of antimicrobial-producing soil isolates was performed by perpendicular-streak method using MHA medium. A total of 123 soil isolates were screened for their antibacterial activity against *Staphylococcus aureus* TISTR1466, *Bacillus cereus* TISTR687, *Pseudomonas aeruginosa* TISTR781, *Escherichia coli* TISTR780 and *Enterobacter aerogenes* TISTR1540 (Table 4.1).

Table 4.1 Primary screening of antibacterial activity of soil isolates.

Isolate name	Test organisms				
	Gram-positive bacteria		Gram-negative bacteria		
	S. aureus	*B. cereus*	*P. aeruginosa*	*E. coli*	*E. aerogenes*
PJ1	-	-	-	-	-
PJ2	-	-	-	-	-
PJ3	-	-	-	-	-
PJ4	-	-	-	-	-
PJ5	-	-	-	-	-
PJ6	-	-	-	-	-
PJ7	-	-	-	-	-
PJ8	-	-	-	-	-
PJ9	-	-	-	-	-
PJ10	-	-	-	-	-
PJ11	-	-	-	-	-
PJ12	-	-	-	-	-
PJ13	-	-	-	-	-
PJ14	-	-	-	-	-
PJ15	-	-	-	-	-
PJ16	-	-	-	-	-
PJ17	-	-	-	-	-
PJ18	-	-	-	-	-
PJ19	-	-	-	-	-
PJ20	-	-	-	-	-
PJ21	-	-	-	-	-
PJ22	-	-	-	-	-
PJ23	-	-	-	-	-
PJ24	-	-	-	-	-
PJ25	-	-	-	-	-
PJ26	-	-	-	-	-

Table 4.1 Primary screening of antibacterial activity of soil isolates (Continued).

Isolate name	Test organisms				
	Gram-positive bacteria		Gram-negative bacteria		
	S. aureus	*B. cereus*	*P. aeruginosa*	*E. coli*	*E. aerogenes*
PJ27	-	-	-	-	-
PJ28	-	-	-	-	-
PJ29	-	-	-	-	-
PJ30	-	-	-	-	-
PJ31	-	-	-	-	-
PJ32	-	-	-	-	-
PJ33	-	+	-	-	-
PJ34	-	-	-	-	-
PJ35	-	-	-	-	-
PJ36	+++	+++	-	+++	+++
PJ37	-	-	-	-	-
PJ38	-	-	-	-	-
PJ39	-	-	-	-	-
PJ40	-	-	-	-	-
PJ41	+	+	+	-	-
PJ42	-	-	-	-	-
PJ43	+	+	-	-	-
PJ44	-	-	-	-	-
PJ45	+	-	-	-	-
PJ46	+	-	-	-	-
PJ47	+	-	-	-	-
PJ48	-	-	-	-	-
PJ49	-	-	-	-	-
PJ50	-	-	-	-	-
PJ51	-	+	-	-	-

Table 4.1 Primary screening of antibacterial activity of soil isolates (Continued).

Isolate name	Test organisms				
	Gram-positive bacteria		Gram-negative bacteria		
	S. aureus	*B. cereus*	*P. aeruginosa*	*E. coli*	*E. aerogenes*
PJ52	++	+	-	-	-
PJ53	-	-	-	-	-
PJ54	-	-	-	-	-
PJ55	-	-	-	-	-
PJ56	-	-	-	-	-
PJ57	-	-	-	-	-
PJ58	-	-	-	-	-
PJ59	-	-	-	-	-
PJ60	-	-	-	-	-
PJ61	-	-	-	-	-
PJ62	-	-	-	-	-
PJ63	-	-	-	-	-
PJ64	-	-	-	-	-
PJ65	-	-	-	-	-
PJ66	-	-	-	-	-
PJ67	+	+	+	+	-
PJ68	-	-	-	-	-
PJ69	-	-	-	-	-
PJ70	-	-	-	-	-
PJ71	-	-	-	-	-
PJ72	+	+	+	+	-
PJ73	+	+	+	+	-
PJ74	-	+	-	-	-
PJ75	++	++	-	-	-
PJ76	-	+	-	-	-
PJ77	+	+	-	-	-

Table 4.1 Primary screening of antibacterial activity of soil isolates (Continued).

Isolate name	Test organisms				
	Gram-positive bacteria		Gram-negative bacteria		
	S. aureus	*B. cereus*	*P. aeruginosa*	*E. coli*	*E. aerogenes*
PJ78	++	+	-	-	-
PJ79	-	-	-	-	-
PJ80	-	-	-	-	-
PJ81	-	-	-	-	-
PJ82	-	-	-	-	-
PJ83	-	-	-	-	-
PJ84	-	-	-	-	-
PJ85	+++	+++	-	-	-
PJ86	-	-	-	-	-
PJ87	-	-	-	-	-
PJ88	+	+	-	-	-
PJ89	-	-	-	-	-
PJ90	+++	+++	-	-	-
PJ91	-	-	-	-	-
PJ92	-	-	-	-	-
PJ93	-	-	-	-	-
PJ94	-	-	-	-	-
PJ95	+++	+++	-	+++	+
PJ96	-	-	-	-	-
PJ97	-	-	-	-	-
PJ98	-	-	-	-	-
PJ99	-	-	-	-	-
PJ100	-	-	-	-	-
PJ101	-	-	-	-	-
PJ102	-	-	-	-	-
PJ103	-	-	-	-	-

Table 4.1 Primary screening of antibacterial activity of soil isolates (Continued).

Isolate name	Test organisms				
	Gram-positive bacteria		Gram-negative bacteria		
	S. aureus	*B. cereus*	*P. aeruginosa*	*E. coli*	*E. aerogenes*
PJ104	-	-	-	-	-
PJ105	-	-	-	-	-
PJ106	-	-	-	-	-
PJ107	+++	+++	-	-	-
PJ108	-	-	-	-	-
PJ109	-	-	-	-	-
PJ110	-	-	-	-	-
PJ111	-	-	-	-	-
PJ112	-	-	-	-	-
PJ113	-	-	-	-	-
PJ114	-	-	-	-	-
PJ115	-	-	-	-	-
PJ116	-	-	-	-	-
PJ117	-	-	-	-	-
PJ118	-	-	-	-	-
PJ119	-	-	-	-	-
PJ120	-	-	-	-	-
PJ121	+	-	-	-	ND
PJ122	-	-	-	-	-
PJ123	-	-	-	-	-

-: no activity; +: weak; ++: moderate; +++: strong

ND: not determine

Among them, PJ36, PJ85, PJ90, PJ95 and PJ107 showed high and broad spectrum antibacterial activity against test pathogens. These five isolates were then used for antibacterial activity test against *Staphylococcus epidermidis* TISTR518, *Bacillus subtilis* TISTR008, *Serratia marcescens* TISTR1354 and *Proteus mirabilis* TISTR100.

As shown in Figure 4.3, PJ85, PJ90 and PJ107 showed antibacterial activity against only Gram-positive bacteria, *Staphylococcus aureus* TISTR1466, *Staphylococcus epidermidis* TISTR518, *Bacillus subtilis* TISTR008 and *Bacillus cereus* TISTR687. On the other hand, PJ36 and PJ95 exhibited broad antibacterial spectrum against both Gram-positive and Gram-negative bacteria. PJ36 was active against *Staphylococcus aureus* TISTR1466, *Staphylococcus epidermidis* TISTR518, *Bacillus subtilis* TISTR008, *Bacillus cereus* TISTR687, *Escherichia coli* TISTR780, *Serratia marcescens* TISTR1354, *Proteus mirabilis* TISTR100 and *Enterobacter aerogenes* TISTR1540. Whereas, PJ95 exhibited antibacterial activity against *Staphylococcus aureus* TISTR1466, *Staphylococcus epidermidis* TISTR518, *Bacillus subtilis* TISTR008, *Bacillus cereus* TISTR687, *Escherichia coli* TISTR780, *Proteus mirabilis* TISTR100 and *Enterobacter aerogenes* TISTR1540. The results are summarized in Table 4.2.

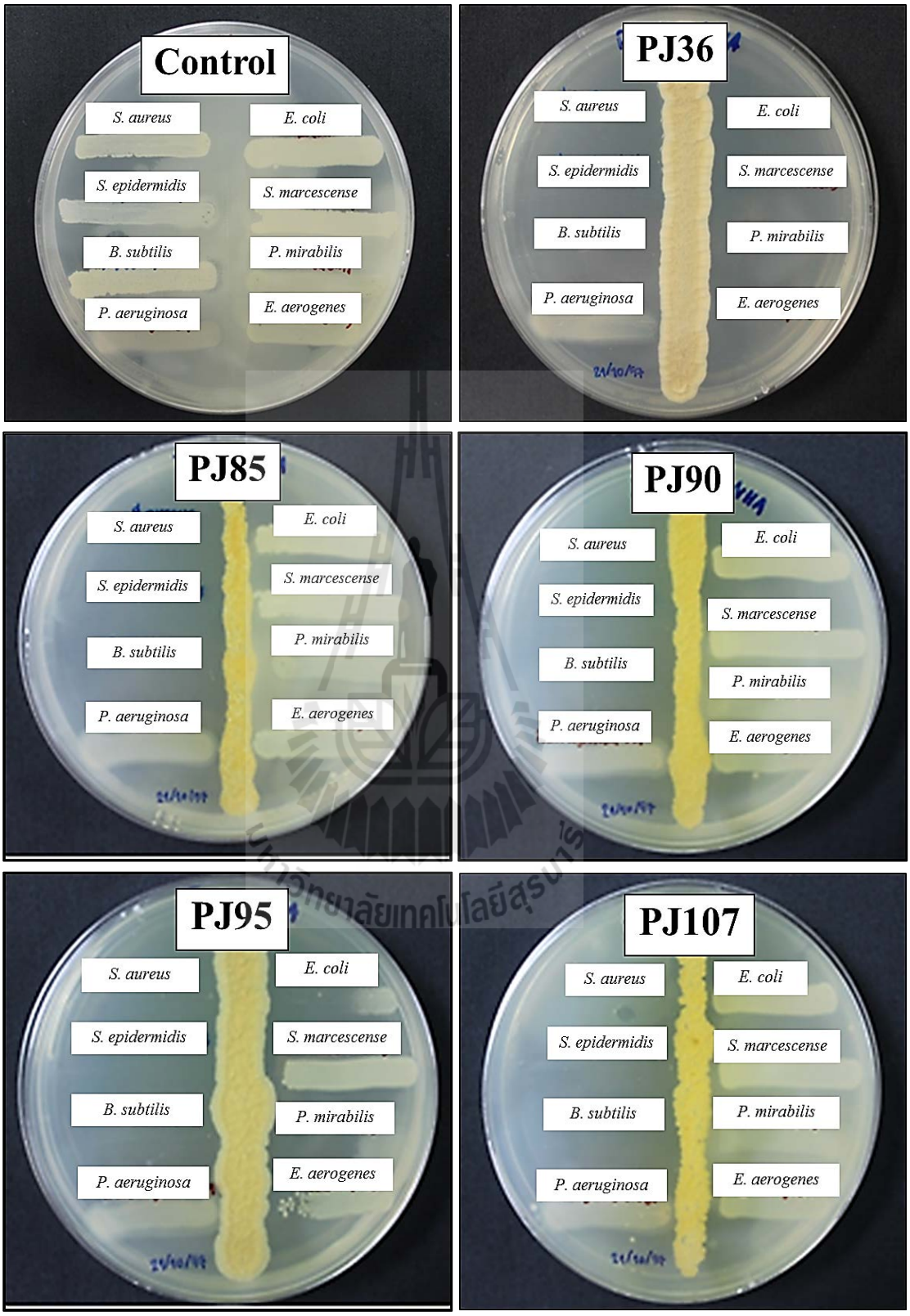


Figure 4.3 Antibacterial activity of PJ36, PJ85, PJ90, PJ95 and PJ107 against test pathogens compared with control plate.

Table 4.2. Antibacterial activity of antibiotic-producing soil isolates against test opportunistic pathogens.

Isolates	Zone of inhibition (mm)								
	Gram-positive bacteria				Gram-negative bacteria				
	S. aureus	***S. epidermidis***	***B. subtilis***	***B. cereus***	***P. aeruginosa***	***E. coli***	***S. marcescens***	***P. mirabilis***	***E. aerogenes***
PJ36	>30±0	>30±0	>30±0	>30±0	0	>30±0	>30±0	>30±0	>30±0
PJ85	27±0.6	26±0.6	>30±0	30±0	0	0	0	0	0
PJ90	25±0.3	25±0.9	>30±0	28±0.9	0	0	0	0	0
PJ95	>30±0	>30±0	>30±0	>30±0	0	18±1	0	18±1.0	5±0.8
PJ107	21±0.6	21±1	24±0	23±0.3	0	0	0	0	0

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

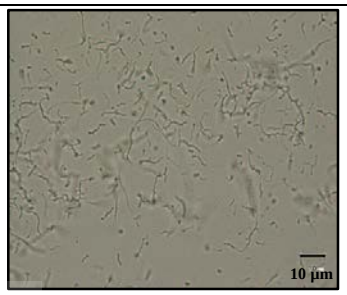
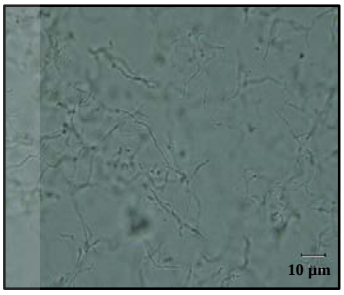
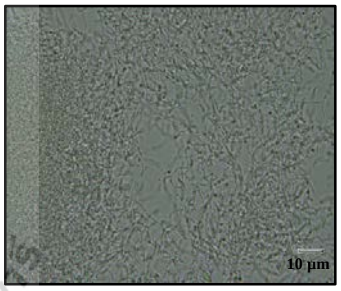
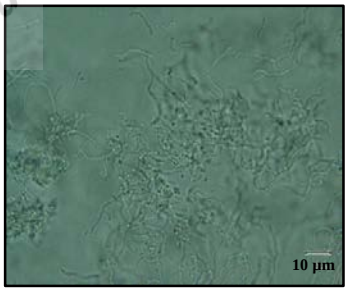
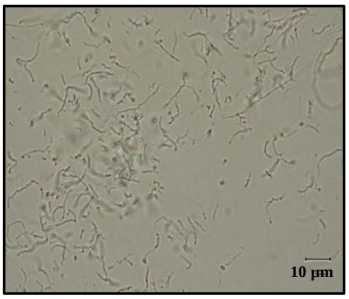
4.3 Identification of five antimicrobial-producing soil isolates

The colony morphology of isolates PJ36, PJ85, PJ90, PJ95 and PJ107 appeared rough, hard and powdery after cultured on SCA medium at 37ºC for 10 days (Table 4.3). These isolates showed Gram-positive filamentous bacteria and produced chain of spores. The macroscopic and microscopic (1,000x) morphologies of these antimicrobial-producing isolates were summarized in Table 4.4. These results indicated that PJ36, PJ85, PJ90, PJ95 and PJ107 might belong to the genus *Streptomyces* (Goodfellow et al., 2012).

Table 4.4 Morphological characteristics of antibacterial producing isolate strains.

Characteristics	**Antimicrobial-producing isolates**				
	PJ36	**PJ85**	**PJ90**	**PJ95**	**PJ107**
Color of colony	White	Yellow	Yellow	White	Yellow
Texture of colony	Hard	Powdery	Powdery	Powdery	Powdery
Color of mycelium	White	White	White	White	White
Gram staining	Positive	Positive	Positive	Positive	Positive
Spore morphology	Chain	Chain	Chain	Chain	Chain
Earthy odor	Present	Present	Present	Present	Present

Table 4.3 Macroscopic and microscopic morphologies of antimicrobial-producing isolates.

Isolates	Macroscopic	Microscopic
PJ36		
PJ85		
PJ90		
PJ95		
PJ107		

The identification of PJ36, PJ85, PJ90, PJ95 and PJ107 in the species level was done by 16S rRNA gene analysis. The 16S rRNA gene was amplified by using specific universal primers, 243F and A3R (Table 3.1). The PCR amplified 16S rRNA gene was cloned into *E. coli* JM109 and sequenced. The amplified fragments were compared to the nucleotide sequences of known species from NCBI GenBank database. The 16S rDNA sequences of PJ36, PJ85, PJ90, PJ95 and PJ107 were submitted to GenBank database. The GenBank accession numbers of these isolates are provided in Table 4.5.

The homology search by BLAST program revealed that 16S rDNA sequence of isolates PJ36 and PJ95 show similarities to *Streptomyces rimosus* and *Streptomyces luteosporeus*, respectively. Other remaining three isolates, PJ85, PJ90, PJ107 shared 99% resemblance to *Streptomyces triostinicus*.

Table 4.5 Phylogenetic affiliation and GenBank accession numbers of soil antibacterial-producing isolates.

Isolates	Identification (nearest match)	Similarity (%)	Accession numbers
PJ36	*Streptomyces rimosus* subsp. *paramomycinus* NBRC15454	99	KT795542
PJ85	*Streptomyces triostinicus* CKM7	99	KT795538
PJ90	*Streptomyces triostinicus* CKM7	99	KT795539
PJ95	*Streptomyces luteosporeus* NBRC14657	99	KT795540
PJ107	*Streptomyces triostinicus* CKM7	99	KT795541

4.4 Secondary screening of antimicrobial-producing soil isolates

The secondary screening was aimed to select the strain that showed highest antibacterial activity for the preparation of crude extract. In this study, the antibacterial activity of five isolates were tested by using four different media which were MH, YEG, SC and ISP2 broth. The supernatants of cell culture containing antimicrobial substances were collected and measured by agar-well diffusion method. Based on the inhibition zone diameter measured from different culture media, the cell culture on ISP2 broth showed the highest antibacterial activity compared with MH, YEG and SC broth (data not shown).

The results of antibacterial activity on ISP2 are shown in Figure 4.4. PJ85 showed maximum zone of inhibition against *Staphylococcus aureus* TISTR1466 (29±0.58 mm), *Staphylococcus epidermidis* TISTR518 (36±0 mm), *Bacillus subtilis* TISTR008 (30±2.08 mm) and *Bacillus cereus* TISTR687 (27±0.71 mm). Therefore, PJ85 was selected for the preparation of crude extract.

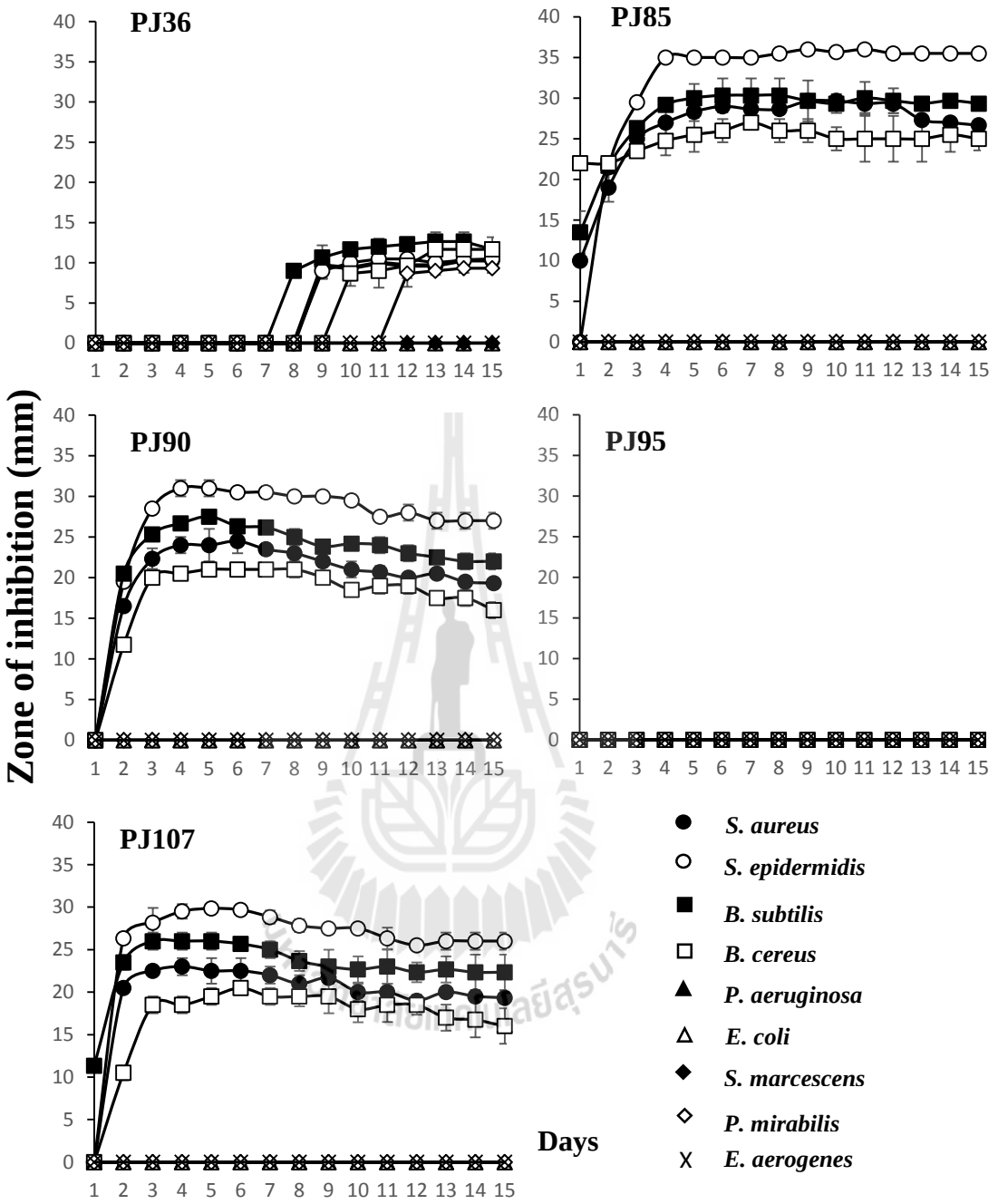


Figure 4.4 Zone of inhibition (mm) of antibacterial compounds produced from PJ36, PJ85, PJ90, PJ95 and PJ107 against test pathogens by agar-well difussion method. The error bars represents SD. Each value is a mean of n=3 experiments.

4.5 The preparation of crude extract from PJ85

Since, PJ85 showed highest antibacterial activity against test pathogens in the secondary screening, it was selected for the prepation of crude extract. The crude extract of PJ85 was extracted using ethyl acetate. The crude extract of PJ85 was yellowish-orange in color. The yield of PJ85 crude extract was 7.32 mg/g of cell dry weight (Fig. 4.5).

Figure 4.5 The ethyl acetate extract of PJ85.

4.6 Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the crude extract

The MIC values of PJ85 crude extract was determined by macro-dilution method. The MICs and MBCs of vancomycin, tetracycline and PJ85 crude extract against *Staphylococcus aureus* ATCC29213, *Staphylococcus aureus* TISTR1466, *Staphylococcus epidermidis* TISTR518, *Bacillus subtilis* TISTR008 and *Bacillus cereus* TISTR687 are shown in Table 4.6. Vancomycin and tetracycline were used as positive control. Standard strain of *Staphylococcus aureus* ATCC29213 was used as internal control. If the MICs of vancomycin and tetracycline against *Staphylococcus*

aureus ATCC29213 are within the range of 0.5 to 2 µg/ml and 0.12 to 1 µg/ml respectively, the results will be reliable (Table 4.6).

The result showed that the MICs of PJ85 crude extract against *Staphylococcus aureus* ATCC29213, *Staphylococcus aureus* TISTR1466, *Staphylococcus epidermidis* TISTR518, *Bacillus subtilis* TISTR008 and *Bacillus cereus* TI STR687 were 1, 1, 2, 0.5 and 0.25 µg/ml, respectively. Whereas, the MBCs of PJ85 crude extract against *Staphylococcus aureus* ATCC29213, *Staphylococcus aureus* TISTR1466, *Staphylococcus epidermidis* TISTR518, *Bacillus subtilis* TISTR008 and *Bacillus cereus* TISTR687 were 2, 8, 4, 1 and 2 µg/ml, respectively. The assay were carried out in triplicate in which the same MIC and MBC values were observed.

The MICs of PJ85 crude extract exhibited an improved activity against all Gram-positive test pathogens when compared with vancomycin. The MBCs of PJ85 crude extract showed a decrease activity against *Staphylococcus aureus* TISTR1466 while exhibited the same value against *Staphylococcus aureus* ATCC29213, *Staphylococcus epidermidis* TISTR518, *Bacillus subtilis* TISTR008 and *Bacillus cereus* TISTR687 when compared with vancomycin.

PJ85 crude extract exhibited the same value of MICs and MBCs against *Staphylococcus aureus* ATCC29213 when compared with tetracycline. *Staphylococcus aureus* TISTR1466 and *Bacillus subtilis* TISTR008 were susceptible to the crude extract with high antibacterial activity compared to tetracycline. On the other hand, an antibacterial activity of crude extract against *Staphylococcus epidermidis* TISTR518 was lower than tetracycline. An antibacterial activity of PJ85 crude extract and tetracycline against *Bacillus cereus* TISTR687 were similar.

Table 4.6 The MIC and MBC values (µg/ml) of crude extract from PJ85 against test pathogens.

Test pathogens	Vancomycin (µg/ml)		Tetracycline (µg/ml)		Crude extract (µg/ml)	
	MIC	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**
***S. aureus* ATCC29213**	2	2	1	2	1	2
***S. aureus* TISTR1466**	2	2	2	16	1	8
***S. epidermidis* TISTR518**	4	4	0.5	1	2	4
***B. subtilis* TISTR008**	1	1	8	16	0.5	1
***B.cereus* TISTR687**	2	2	0.25	0.5	0.25	2

CHAPTER V

DISCUSSION AND CONCLUSION

In this study, we attempted to isolate antimicrobial-producing Actinomycetes from dry dipterocarp forest soil in Suranaree University of Technology, Nakhon Ratchasima, Thailand. One-hundred twenty three soil isolates were screened for their antimicrobial activity. Only five isolates, PJ36, PJ85, PJ90, PJ95, PJ107 were exhibited high and broad spectrum antibacterial activity against test pathogens. The antibacterial activity of five isolates against Gram-positive bacteria was observed more often than that against Gram-negative bacteria. This frequency of activity against Gram-positive bacteria of soil Actinomycetes is similar to those reported by Basilio, Kokare, Valli and Deshmukh (Basilio et al., 2003; Kokare, Mahadik, Kadam and Chopade, 2004; Valli, Suvathi, Aysha, Nirmala, Vinoth and Reena, 2012; Deshmukh and Vidhale, 2015). It has been suggested that Gram-negative bacteria are more resistant to antibiotics than Gram-positive bacteria because the cell wall of Gram-negative bacteria possess an outer membrane. The outer membrane is compose of lipopolysaccharide that serve as a barrier for antibacterial drugs. Whereas, the cell wall of Gram-positive lack an outer membrane, thus Gram-positive bacteria are more susceptible than Gram-negative bacteria (Ilic et al., 2007; Gebreyohannes, Moges, Sahile and Raja, 2013).

Five antibacterial-producing Actinomycetes strains, PJ36, PJ85, PJ90, PJ95 and PJ107, were used for the secondary screening by agar-well diffusion method. The inhibition pattern from agar-well diffusion method was different from the co-culture method in which the activity against test Gram-negative bacteria were lost. The loss of antibacterial activity by PJ36 and PJ95 were observed in secondary screening. The loss of antibiotic activity of Actinomycetes was believed to be related to the nutrient limitations and differentiation processes of mycelium in submerged culture. Thus, the differences in cellular morphologies and culture conditions might affect the amount of antibiotic released from the cells (Manteca, Alvarez, Salazar, Yagüe and Sanchez, 2008).

Several reports have been shown that most of antimicrobial compounds were successfully extracted by ethyl acetate (Selvameenal, Radhakrishnan and Balagurunathan, 2009; Kiran, Subhan, Pirzada and Wahab, 2015; Barakat and Beltagy, 2015). In 2011, Deepa, Gowthami and Kumar isolated the antimicrobial-producing *Streptomyces* sp. PR01 from soil in Tamil Nadu, India. The antimicrobial compounds of *Streptomyces* sp. PR01 were extracted using different solvents such as n-butanol, n-hexane, ethyl acetate, petroleum ether, chloroform, benzene and xylene. The ethyl acetate extract of *Streptomyces* sp. PR01 showed the maximum zone of inhibition against *Microsporum* spp. (dermatophytic fungi) (Deepa, Gowthami and Kumar, 2011).

In 2013, Sharmin, Rahman, Anisuzzaman and Islam also reported that the ethyl acetate extract of *Streptomyces* sp. ANTS-1 exhibited strong antimicrobial activity against *Bacillus cereus*, *Streptococcus agalactiae*, *Pseudomonas aeruginosa* and *Escherichia coli* (Sharmin, Rahman, Anisuzzaman and Islam, 2013).

In this study, PJ85 was used for the extraction of the crude extract using ethyl acetate. The ethyl acetate extract of PJ85 showed the antibacterial activity against test pathogens which suggests the moderate-polar nature of antimicrobial substances of PJ85. The moderate degree of polarity is a feature of most antibiotics. If the compound was too polar it would not pass through the cellular membrane to attack the target sites inside the cells. On the other hand, if the compound was non-polar it would not dissolve in the cellular water in the concentrations high enough to inhibit microorganisms (Guardabassi, Jensen and Kruse, 2008).

Based on morphological characteristics and 16S rRNA gene analysis, isolate PJ85 could possibly be classified as *Streptomyces triostinicus*. In 1961, *Streptomyces triostinicus* ATCC21043 was first isolated from soil in Japan by Shoji and Kentagiri (Shoji and Katagiri, 1961). It produced an antitumor, triostin A, as its major secondary metabolites. Triostin A is a member of the quinoxaline antibiotics that binds DNA by bisintercalation. This compound has potent activity against Gram-positive bacteria. The MIC values of triostin A against *Staphylococcus aureus*, *Bacillus subtilis* and *Streptococcus faecalis* were 0.625, 0.0625 and 0.078 µg/ml, respectively.

Since, the discovery of *Streptomyces triostinicus* ATCC21043, many groups of researchers have been tried to improve the biosynthesis of triostin A derivatives (Yoshida and Katagiri, 1969; Cornish, Fox and Waring, 1983; Cornish, Fox, Santikarn, Waring and Williams, 1985). The isolation of novel *Streptomyces triostinicus* strains have never been reported until 2009.

In 2009, Singh and co-worker isolated the novel strain of *Streptomyces triostinicus* CKM7 from agricultural soil in Eastern Uttar Pradesh, India. This strain showed broad spectrum antibacterial and antifungal activities. Antibacterial and

antifungal activities of CKM7 were observed by agar-well diffusion method. Antimicrobial activity of CKM7 supernatants (100µl) were active against *Staphylococcus aureus*, *Bacillus subtilis*, *Candida albicans* and *Candida tropicalis* with the inhibition zone of 35 mm, 38 mm, 30 mm and 22 mm, respectively (Singh, Praveen, Khan and Tripathi, 2009). The active compound of CKM7 was identified as actinomycin V by using the Nuclear magnetic resonance (NMR) method. This is the first report of the isolation of actinomycin V from soil isolate *Streptomyces triostinicus* strain (Singh, Khan, Khan and Tripathi, 2009).

The phylogenetic relationship between PJ85 and known Actinomycetes was determined on the basis of their 16S rRNA gene sequence. The tree was constructed by neighbor-joining method with bootstrap 1,000 replicates. As shown in Figure 5.1, the phyletic line of *Streptomyces triostinicus* CKM7 was separated from triostin A-producing *Streptomyces triostinicus* ATCC21043. Our isolate, PJ85 also showed a distant relationship with *Streptomyces triostinicus* CKM7 and *Streptomyces triostinicus* ATCC21043. It could not be fitted into any known cluster as shown in Figure 5.1.

Goodfellow and Dickenson (1985) has been suggested that organisms from natural habitats that do not form tight clusters with recognized reference strains could be assigned to a new taxa. Therefore, PJ85 might be classified as a novel strain of *Streptomyces triostinicus*. However, the sufficient differences between this isolate and standard type strains needed to be compared in order to determine whether or not they could be classified as new strain. The purification of PJ85 bioactive compound are needed to be done. The purified bioactive compound will be further characterized by FTIR and/or NMR methods.

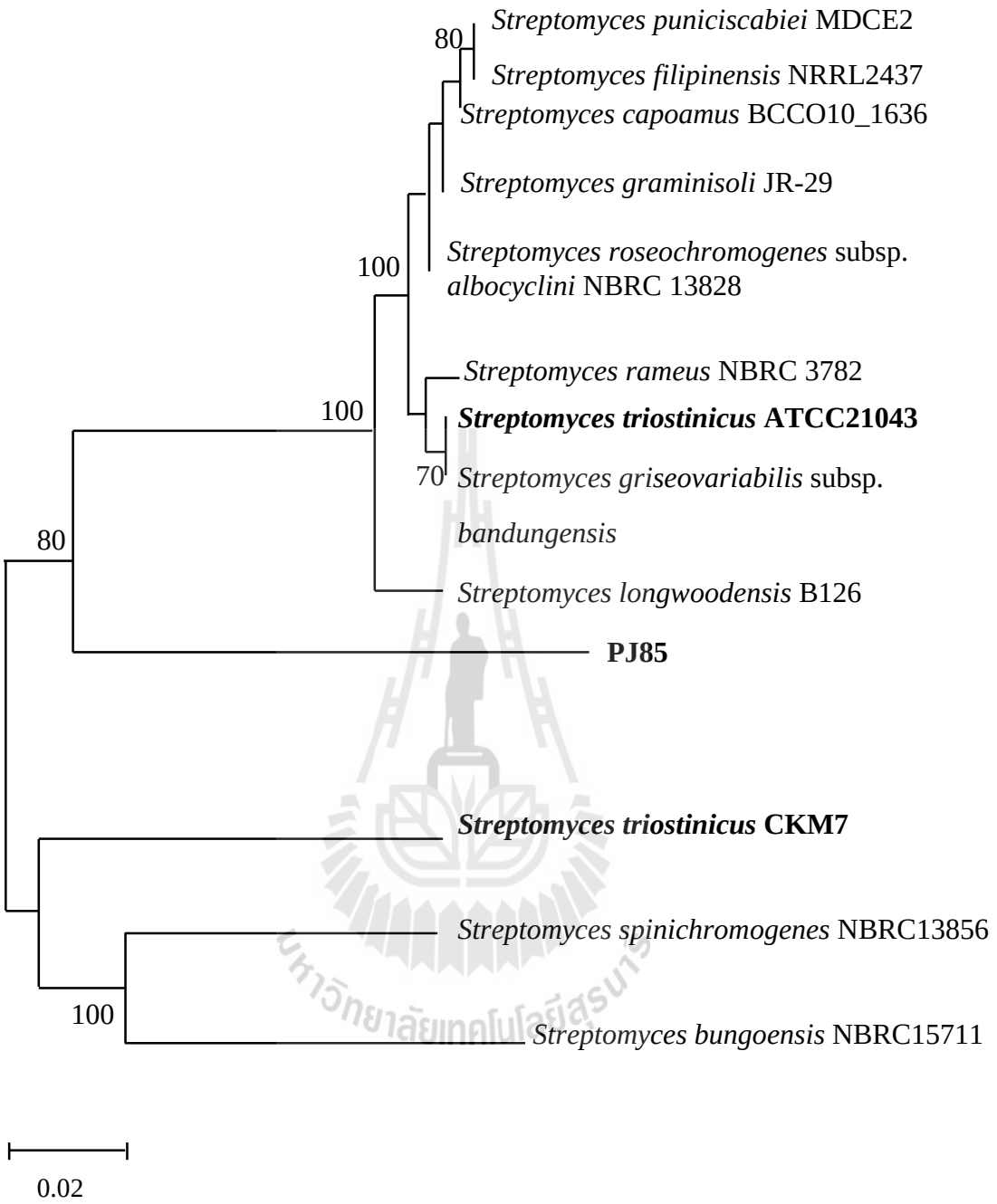


Figure 5.1 Phylogenetic tree showing evolutionary relationship of PJ85 and their related taxa. The 16S rRNA gene sequences were aligned by using CLUSTAL W. The neighbor-joining phylogenetic tree was generated by using the MEGA 6. Numbers at the nodes indicate levels of bootstrap support based on 1,000 resampling, only value ≥ 50% are shown. Bar, 0.02 nucleotide substitutions per position.

In Thailand, *Streptomyces triostinicus* has been isolated from soil in Chanthaburi province. This strain exhibited antifungal activity against plant pathogenic fungi, *Colletotrichum* spp. (Intra, Mungsuntisuk, Nihira, Igarashi and Panbangred, 2011). In this study, we were able to isolate antibacterial-producing *Streptomyces triostinicus* from soil in Nakhon Ratchasima province. To our best knowledge, this study constitutes the first antibacterial property of *Streptomyces triostinicus* isolated from Thai soil.

In conclusion, five antibacterial-producing Actinomycetes were isolated from the forest soil in Suranaree University of Technology. Among them, PJ85 exhibited highest antibacterial activity against test Gram-postitive bacteria. Based on morphological characteristics, 16S rDNA analysis and phylogenetic analysis, PJ85 might be classified as a new strain of *Streptomyces triostinicus*. However, the additional data are required in order to distinguish PJ85 from other known species. Our future experiments are include the purification, identification and evaluation of the active compound produced from PJ85.

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