CHARACTERIZATION AND EVALUATION OF ANTIMICROBIAL COMPOUNDS PRODUCED BY SOIL ACTINOMYCETES AGAINST DRUG-RESISTANT BACTERIA

Panjamaphon Chanthasena

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Microbiology

Suranaree University of Technology

Academic Year 2020

การศึกษาคุณสมบัติและประเมินฤทธิ์ของสารต้านจุลินทรีย์ที่สร้างโดยเชื้อแอคติโนมัยสีทที่แยกจากดินต่อเชื้อแบคทีเรียดื้อยา

นางสาวปัญจมาภรณ์ จันทเสนา

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต

สาขาวิชาจุลชีววิทยา

มหาวิทยาลัยเทคโนโลยีสุรนารี

ปีการศึกษา 2563

CHARACTERIZATION AND EVALUATION OF ANTIMICROBIAL COMPOUNDS PRODUCED BY SOIL ACTINOMYCETES AGAINST DRUG-RESISTANT BACTERIA

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

Thesis Examining Committee

(Assoc. Prof. Dr. Nuannoi Chudapongse)
Chairperson

(Asst. Prof. Dr. Nawarat Nantapong)
Member (Thesis Advisor)

(Asst. Prof. Dr. Duangkamol Maensiri)
Member

Wanwisa Limphirat
(Dr. Wanwisa Limphirat)
Member

(Dr. Pongrit Krubphachaya)
Member

(Assoc. Prof. Flt. Lt. Dr. Kontorn Chamniprasart)
Vice Rector for Academic Affairs and Internationalization

(Assoc. Prof. Dr. Worawat Meevasana)
Dean of Institute of Science

ปัญจมาภรณ์ จันทเสนา : การศึกษาคุณสมบัติและประเมินฤทธิ์ของสารต้านจุลินทรีย์ที่สร้างโดยเชื้อแอคติโนมัยสีทที่แยกจากดินต่อเชื้อแบคทีเรียดื้อยา (CHARACTERIZATION AND EVALUATION OF ANTIMICROBIAL COMPOUNDS PRODUCED BY SOIL ACTINOMYCETES AGAINST DRUG-RESISTANT BACTERIA)
อาจารย์ที่ปรึกษา : ผู้ช่วยศาสตราจารย์ ดร.นวรัตน์ นันทพงษ์, 94 หน้า.

การเพิ่มขึ้นของแบคทีเรียดื้อยาถือเป็นปัญหาสุขภาพทั่วโลก สายพันธุ์ดื้อยาเป็นอุปสรรคต่อการรักษาและการควบคุมโรคติดเชื้อ ทำให้ต้องมีการค้นหาและการพัฒนายาปฏิชีวนะชนิดใหม่ขึ้น ซึ่งสเตรปโตมัยสีทได้รับการยอมรับว่าเป็นแหล่งผลิตสารทุติยภูมิรวมถึงยาปฏิชีวนะ ดังนั้นวัตถุประสงค์ของงานวิจัยนี้จึงต้องการศึกษาลักษณะของสารปฏิชีวนะที่ได้จากเชื้อสายพันธ์ PJ85 ที่แยกได้จากดินบริเวณมหาวิทยาลัยเทคโนโลยีสุรนารี จังหวัดนครราชสีมา ประเทศไทย จากผลการจำแนกชนิดโดยอาศัยการวิเคราะห์ลำดับเบสบนจีน 16S rRNA และการศึกษาแผนภูมิวิวัฒนาการของจีน 16S rRNA พบว่า สายพันธุ์ PJ85 มีความคล้ายคลึงกับเชื้อ *Streptomyces actinomycinicus* RCU-197^{T} (JCM 30864^{T} TISTR 2208^{T} และ PCU 342^{T}) ที่แยกได้จากดินป่าพรุในจังหวัดระยอง ประเทศไทย มากที่สุด จากการศึกษาฤทธิ์ต้านจุลชีพของสายพันธุ์ PJ85 โดยวิธี perpendicular streak พบว่า สายพันธุ์ PJ85 สามารถยับยั้งเชื้อ *Staphylococcus aureus* DMST20651 ที่ดื้อต่อยาเมทิซิลิน (MRSA) ได้สูงสุด และ *Staphylococcus epidermidis* TISTR518 *Staphylococcus aureus* ATCC29213 *Bacillus subtilis* TISTR008 *Bacillus cereus* TISTR687 ตามลำดับ

สารออกฤทธิ์ต้านเชื้อจุลชีพที่ได้จาก PJ85 ถูกนำมาสกัดและวิเคราะห์ลักษณะสารด้วยเทคนิค liquid chromatography and mass spectrum (LC-MS) และ เทคนิคอินฟราเรดไมโครสเปกโทรสโกปีโดยใช้แสงซินโครตรอน ในการสกัดสารออกฤทธิ์นั้น เชื้อสายพันธุ์ PJ85 ถูกนำมาสกัดสารสกัดหยาบโดยใช้เอทิลอะซิเตต จากนั้น สารออกฤทธิ์ได้ถูกแยกจากสารสกัดหยาบโดยวิธี thin layer chromatography (TLC) และนำมาทดสอบการออกฤทธิ์ต้านจุลชีพด้วยเทคนิค contact bioautography จากการทดสอบดังกล่าว พบว่า สามารถแยกสารได้ 3 ชนิด คือ สารประกอบ 1 สารประกอบ 2a และ สารประกอบ 2b โดยสารที่ออกฤทธิ์ต้านจุลชีพได้นั้นมี 2 ชนิด คือ สารประกอบ 1 และ สารประกอบ 2a จากนั้น สารต้านจุลชีพทั้ง 2 ชนิด ได้ถูกนำมาวิเคราะห์เอกลักษณ์ด้วยวิธี LC-MS และ เทคนิคอินฟราเรดไมโครสเปกโทรสโกปีโดยใช้แสงซินโครตรอน

โดยสารประกอบ 1 ถูกระบุว่าเป็น actinomycin D ในขณะที่สารประกอบ 2a ถูกระบุว่าเป็นอนุพันธ์ของกรด caffeoylquinic ชื่อว่า tricaffeoyl quinic ที่มีความเกี่ยวข้องกับการย่อยสลายลิกนิน ซึ่งการศึกษานี้ถือเป็นครั้งแรกที่มีการรายงานถึงลักษณะของสารออกฤทธิ์ที่แยกได้จากเชื้อสายพันธุ์ *S. actinomycinicus*

สาขาวิชาปรีคลินิก ลายมือชื่อนักศึกษา ปัญญาภรณ์ จันทเสนา

ปีการศึกษา 2563 ลายมือชื่ออาจารย์ที่ปรึกษา

PANJAMAPHON CHANTHASENA : CHARACTERIZATION AND EVALUATION OF ANTIMICROBIAL COMPOUNDS PRODUCED BY SOIL ACTINOMYCETES AGAINST DRUG-RESISTANT BACTERIA. THESIS ADVISOR : ASST. PROF. NAWARAT NANTAPONG, Ph.D. 94 PP.

BACTERIAL SOIL ISOLATE/ ACTINOMYCETES/ *STREPTOMYCES SP.* PJ85/ ANTIBIOTICS/ CAFFEOYLQUINIC ACID/ *DRUG-RESISTANT MICROORGANISMS*

The increasing of antibiotic-resistant bacteria is a global health problem. The resistant strains have compromised the treatment and control of infectious diseases. As the result, the search and development of a novel class of antibiotic drugs are required. Streptomycetes have been recognized as a richest source of secondary metabolites including antibiotics. The aim of this study was to characterize the antimicrobial compounds of bacterial soil isolate PJ85 which was isolated from soil in Suranaree University of Technology, Nakhon Ratchasima province, Thailand. Based on 16S rRNA gene sequence and phylogenetic tree analysis, strain PJ85 possessed a high similarity to *Streptomyces actinomycinicus* RCU-197^{T} (JCM 30864^{T}, TISTR 2208^{T} and PCU 342^{T}) that have been isolated from soil of a peat swamp forest in Rayong province, Thailand. Perpendicular streak method was used for evaluation of antimicrobial activity of PJ85. The results revealed that methicillin-resistant *Staphylococcus aureus* DMST20651 (MRSA) was the most sensitive to PJ85 followed by *Staphylococcus*

epidermidis TISTR518, *Staphylococcus aureus* ATCC29213, *Bacillus subtilis* TISTR008 and *Bacillus cereus* TISTR687, respectively.

The active compounds of PJ85 were extracted and characterized by using liquid chromatography and mass spectrum (LC-MS) and synchrotron-fourier transform infrared (SR-FTIR) microspectroscopy. In order to extract active compounds, ethyl acetate was used as a solvent. The active compounds of PJ85 were then purified by using thin layer chromatography (TLC) technique and tested for antimicrobial activity by contact bioautography. The result showed that crude compound of PJ85 could be separate into 3 compounds including compound 1, compound 2a and compound 2b. Compound 1 and compound 2a that exhibited antimicrobial activity were then characterized by LC-MS and SR-FTIR microspectroscopy. Compound 1 was identified as actinomycin D while compound 2a was tricaffeoyl quinic acid, a derivative of caffeoylquinic acid involved in lignin degradation. To our best knowledge, this work provides the first report of characterization of active compounds derived from *S. actinomycinicus*.

School of Preclinical Sciences

Academic Year 2020

Student's Signature ปัญจกานต์ จันทลภ

Advisor's Signature

ACKNOWLEDGEMENTS

I would like to express my sincere thanks to all the people who directly or indirectly encourage and help me.

I gratefully acknowledge to the National Research Council of Thailand for research support plans to strengthen capacity and develop new researchers, according to the strategic direction research and innovation: the graduate-level year 2019. Laboratory facilities were supported by Suranaree University of Technology, Nakhon Ratchasima, Thailand.

My deepest appreciation is extended to my thesis advisor Asst. Prof. Dr. Nawarat Nantapong for her excellent guidance, caring, encourages and supports throughout my time as a Ph.D. student. I extend many thanks to my committee members, Assoc. Prof. Dr. Nuannoi Chudapongse, Asst. Prof. Dr. Duangkamol Maensiri, Dr. Wanwisa Limpirat and Dr. Pongrit Krubprachaya for their excellent advice and suggestions regarding this thesis. I would like to extend my sincere thanks to all staffs at Suranaree University of Technology, many thanks go to my colleagues and friends who are always willing to help in every circumstance.

My acknowledgement cannot be completed without expressing my extreme gratitude to my family who always being there for me.

Panjamaphon Chanthasena

CONTENTS

CONTENTS (Continued)

CONTENTS (Continued)

Page

CONTENTS (Continued)

Page

LIST OF TABLES

LIST OF FIGIRES

LIST OF FIGIRES (Continued)

Figure **Page**

LIST OF FIGIRES (Continued)

Figure **Page**

LIST OF ABBREVIATIONS

°C	=	Degree Celsius
h	=	Hour
g	=	Gram
min	=	Minute
sec	=	Second
μg/ml	=	Microgram per milliliter
μl	=	Microliter
mg/ml	=	Milligram per milliliter
mg/g	=	Milligram per gram
ng/μl	=	Nanogram per microliter
CFU	=	Colony forming unit
cm	=	Centimeter
nm	=	Nanometer
rpm	=	Round per minute
g	=	Gravity
m/z	=	Mass to charge ratio
psi	=	Pounds per square inch
OD	=	Optical density
v/v	=	Volume by volume
w/v	=	Weight by volume

CHAPTER I

INTRODUCTION

1.1 Background / Problem

Penicillin was first antibiotic that reported in 1928 by Alexander Fleming (Clardy, Fischbach and Currie, 2009; Zhao et al., 2016). The control of bacterial infections in World War II was successful by using penicillin (Baltz, 2007). Since then, many antibiotics were discovered and given to humans and animals for therapy and prophylaxis (Aminov, 2010). However, the resistance of microorganisms has emerged due to the widespread use of antibiotic drugs (Ventola, 2015).

Antibiotic resistance emerged in early 1942 when *Staphylococcus aureus* became resistant to penicillin (Ventola, 2015). Penicillin resistant *S. aureus* was then treated with methicillin in 1960 however the resistance to methicillin was soon developed (Enright et al., 2002). The first case of methicillin-resistant *S. aureus* (MRSA) was reported in 1962 in the United Kingdom (Ventola, 2015). Nowadays, MRSA infection is a global health concern since it causes high morbidity and mortality (Darabpour, Ardakani, Motamedi and Ronagh, 2012; Wangai, Masika, Maritim and Seaton, 2019; Chinnambedu et al., 2020). MRSA is capable to resist almost all antibiotic drugs (Kemung et al., 2018). Vancomycin has been identified as the last drug for the treatment of MRSA (Srinivasan, Dick and Perl, 2002; Kemung et al., 2018).

However, in 1997, the first report of vancomycin-intermediate *S. aureus* (VISA) strain was reported in Japan and later in several countries (Munita and Arias, 2016). Recently, many strains of bacteria including *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Acinetobacter baumannii* and *Enterobacter* spp. are adapting to resist to antibiotic drugs (Balsalobre et al., 2014). Therefore, there is the need of the novel potent antibiotic agents, particularly against antibiotics-resistant pathogens (Lee et al., 2014; Nguyen and Kim, 2015; Moghannem, El-Sherbiny and Kalaba, 2017; Sabaou et al., 2017).

The filamentous actinomycetes bacteria are an important source of antibiotics. It has been shown that the genus *Streptomyces* and *Micromonospora* produced around 80% of antibiotics use in the world (Singh, Sharma and Talukdar, 2014; Ravi, Vasantba, Bhoomi, Bonisha and Bhumika, 2015; El-Sherbini and Khattab, 2018; Subramani and Sipkema, 2019). However, there is only 3% of antibiotic drugs derived from *Streptomyces* have been isolated and identified. (Shetty, Buddana, Tatipamula, Naga and Ahmad, 2014; de Jesus Sousa and Olivares, 2016). Hence, there are an immense number of antimicrobial compounds remain to be discovered. This study was focused on identification of novel antimicrobial compounds produced by bacterial soil isolate *Streptomyces* sp. PJ85. The antimicrobial compounds of PJ85 were purified and characterized by using thin layer chromatography (TLC), liquid chromatography and mass spectrum (LC-MS) and synchrotron-fourier transform infrared (SR-FTIR) microspectroscopy.

1.2 Research objectives

1.2.1 To identify an antimicrobial-producing bacterial soil isolate actinomycetes.

1.2.2 To purify antimicrobial compounds of bacterial soil isolate actinomycetes.

1.2.3 To characterize and evaluate antimicrobial compounds of bacterial soil isolate actinomycetes.

1.3 Research hypothesis

Bioactive compounds extracted from bacterial soil isolate actinomycetes show antimicrobial activity against antibiotic-resistant and opportunistic pathogens.

1.4 Scope and limitations of study

The study associated with identification and evaluation of antimicrobial-producing *Streptomyces* that isolated from terrestrial soil in Suranaree University of Technology (SUT), Nakhon Ratchasima province, Thailand. In order to identification of antimicrobial-producing *Streptomyces*, the cultural characteristic and 16S rRNA gene analysis were used. Crude compounds were extracted and tested for antimicrobial activity against test pathogens. Test pathogens used in this study were Gram-positive bacteria (methicillin-resistant *Staphylococcus aureus* (MRSA) DMST20651, *Staphylococcus aureus* ATCC29213, *Staphylococcus epidermidis* TISTR518, *Bacillus subtilis* TISTR008 and *Bacillus cereus* TISTR687) and Gram-negative bacteria (*Enterobacter aerogenes* TISTR1540, *Escherichia coli* TISTR780, *Pseudomonas aeruginosa* TISTR781, *Proteus mirabilis* TISTR100, *Serratia marcescens* TISTR1354 and *Salmonella typhimurium* TISTR292). Active compounds were purification using TLC and were characterized by using spectral analysis including LC-MS and SR-FTIR microspectroscopy.

CHAPTER II

LITERATURE REVIEWS

2.1 The importance of antibiotics

Antibiotics are medicines that use for killing microorganisms which can cause disease to humans (Lihan, Choon, Hua and Wasli, 2014). Antibiotics contributed over years in bacterial infection control. The introduction of antibiotics in clinical use plays a major role in decreasing of mortality and morbidity. Patients who are receiving chemotherapy treatments, having complex surgeries, having chronic diseases are treated with antibiotics (Rossolini, Arena, Pecile and Pollini, 2014; Ventola, 2015). The expected life spans of people were extended by helping of antibiotics. The average age of people living in the United States in 1920 was expected to be 56.4 years old. Nowadays, the average U.S. life span is nearly 80 years (Ventola, 2015). Antibiotics have had similar beneficial effects worldwide. In developing countries, the morbidity and mortality from food-borne and other poverty related infections were decreased by using of the drugs.

The discovery of first antibiotic, penicillin, by Alexander Fleming in 1928 controls the bacterial infections of soldiers during World War II. However, shortly thereafter, penicillin resistance emerged when *S. aureus* became resistant to the penicillin in 1942. Therefore, the new β-lactam antibiotic methicillin was then developed and used to treat of penicillin-resistant *S. aureus*. Nevertheless, methicillin-resistant *S. aureus* (MRSA) was isolated in 1962 in the United Kingdom and in 1968 in the United States. The cure

of MRSA by developed antibiotics including quinolones, macrolides and aminoglycosides has eventually failure (Ventola, 2015; Vestergaard, Frees and Ingmer, 2019; Guo, Song, Sun, Wang and Wang, 2020). Then, in 1972, the treatment of MRSA and coagulase-negative staphylococci by vancomycin was introduced. However, the resistance of vancomycin in coagulase-negative staphylococci were reported in 1979.

Although, several novel antibiotic drugs were introduced to manage the resistance problem in 1940-1980, only a few of new semi-synthetic and synthetic drugs were discovered in the past two decades (Figure 2.1). The decreasing in the researching of microbial metabolite could be the result of human responsibility, scientific failure and increasing research costs (Bérdy, 2012). As a result, there have become a threat of the bacterial infection again.

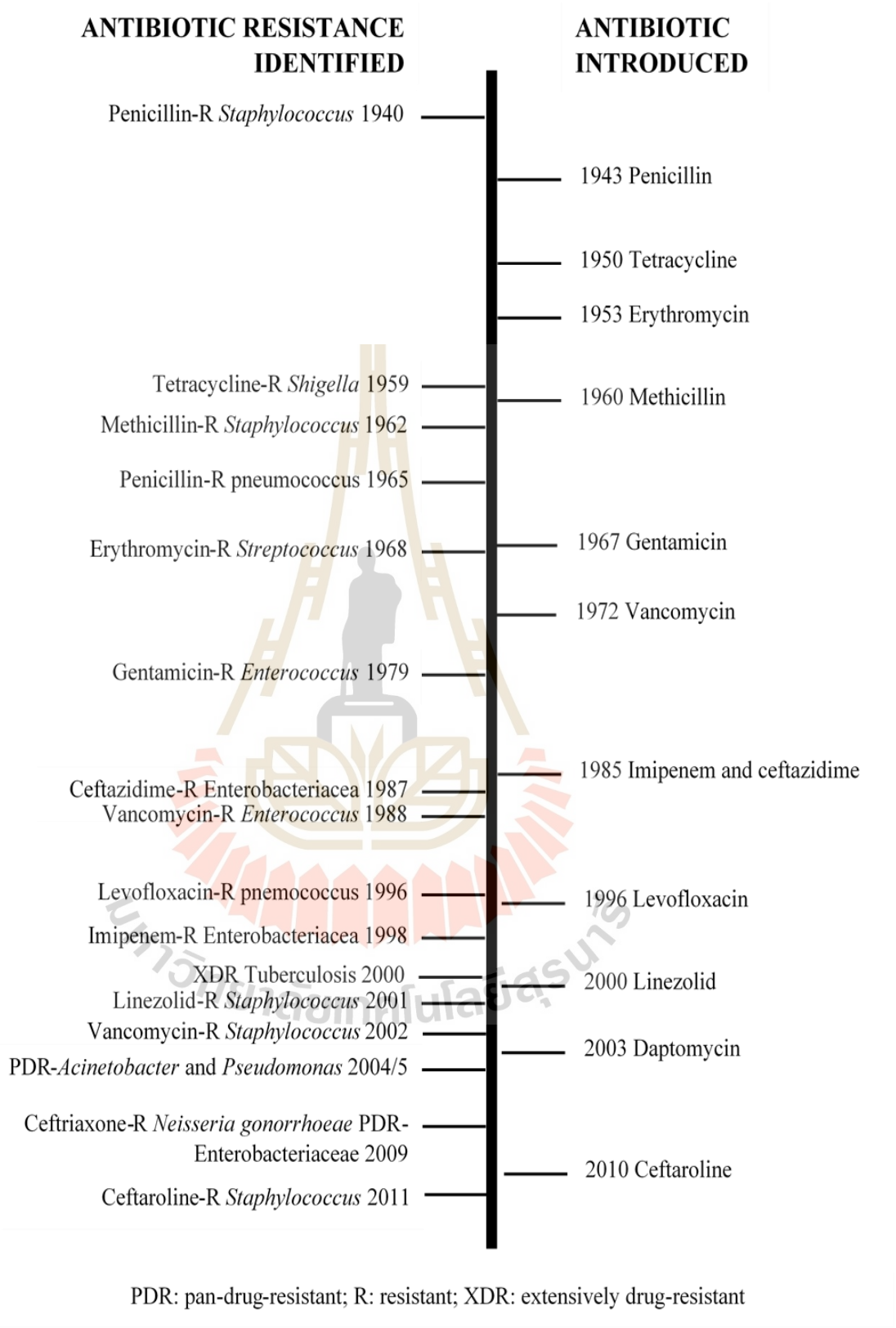


Figure 2.1 A timeline of antibiotic resistance (Ventola, 2015).

2.2 The source of antibiotics

Several habitats such as terrestrial soil, river, lakes and decaying plants are source of antibiotic producers. However, the majority of antibiotic-producing microorganisms inhabit in soil (Chandrashekhara, Nanjwade, Goudanavar, Manvi and Ali, 2010). It has been shown that actinomycetes produced three quarter of all known antibiotics (Varghese, Nishamol, Suchithra, Jyothy and Hatha, 2012). *Streptomyces* is the dominant genus that produces 80% of all known antibiotic. The number of actinomycetes produced bioactive metabolites are shown in Figure 2.2-2.7.

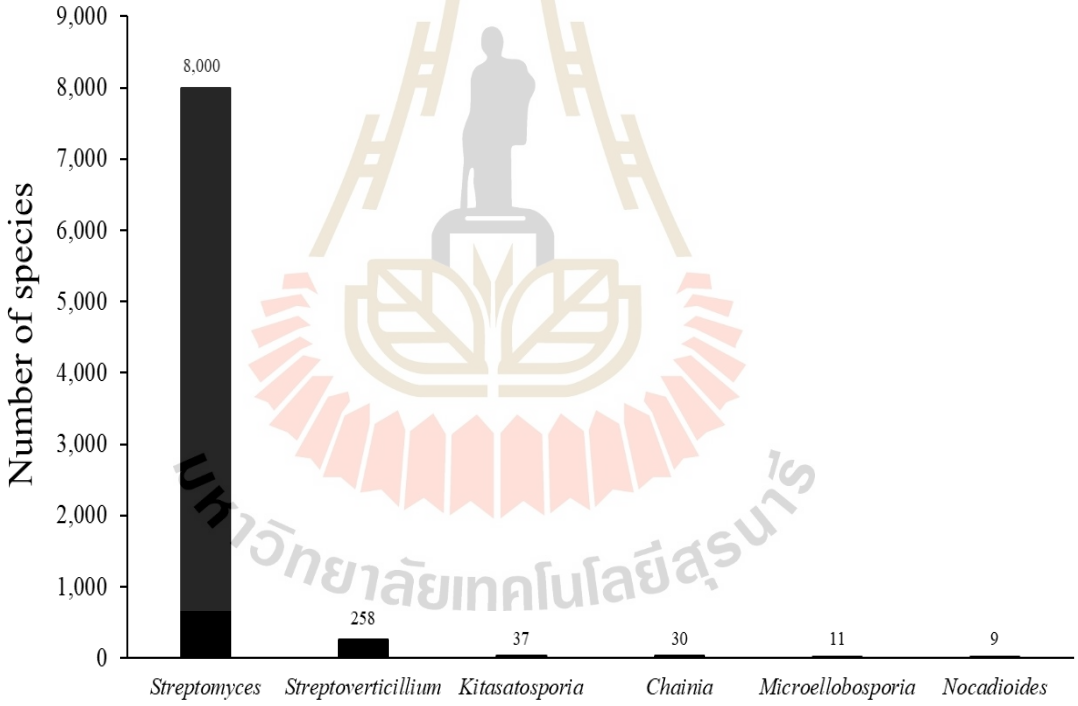


Figure 2.2 The number of actinomycetes belonging to family Streptomycetaceae produced bioactive metabolites.

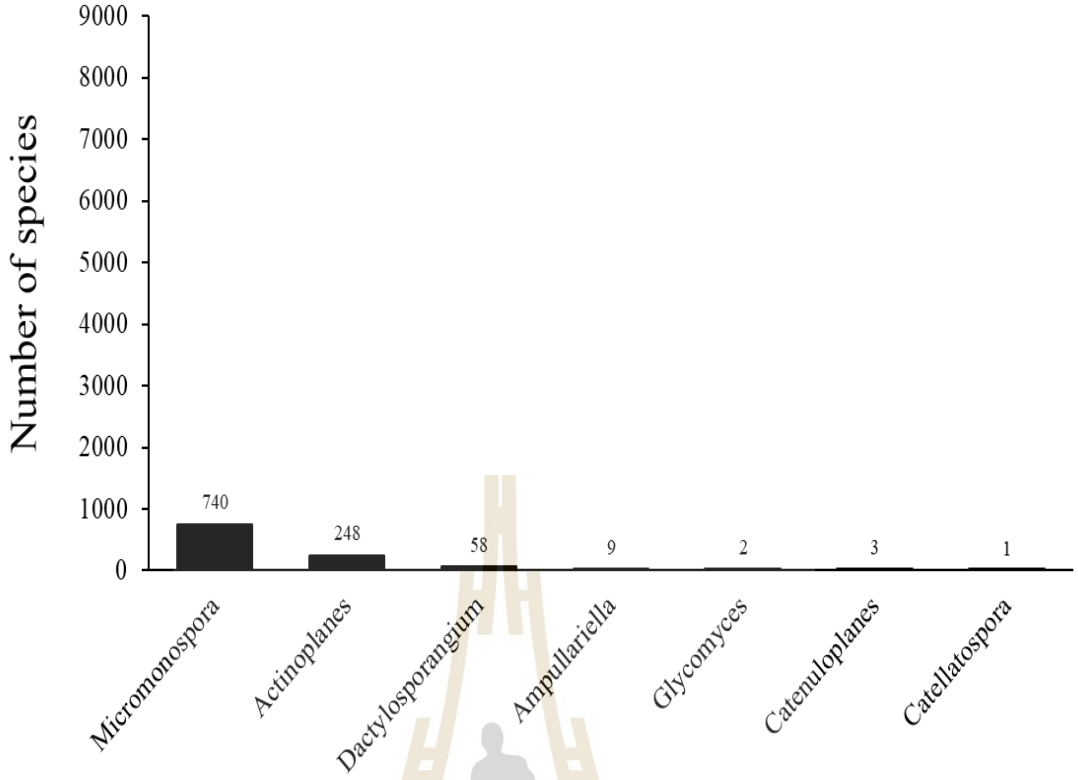


Figure 2.3 The number of actinomycetes belonging to family Micromonosporaceae produced bioactive metabolites.

Figure 2.4 The number of actinomycetes belonging to family Mycobacteriaceae produced bioactive metabolites.

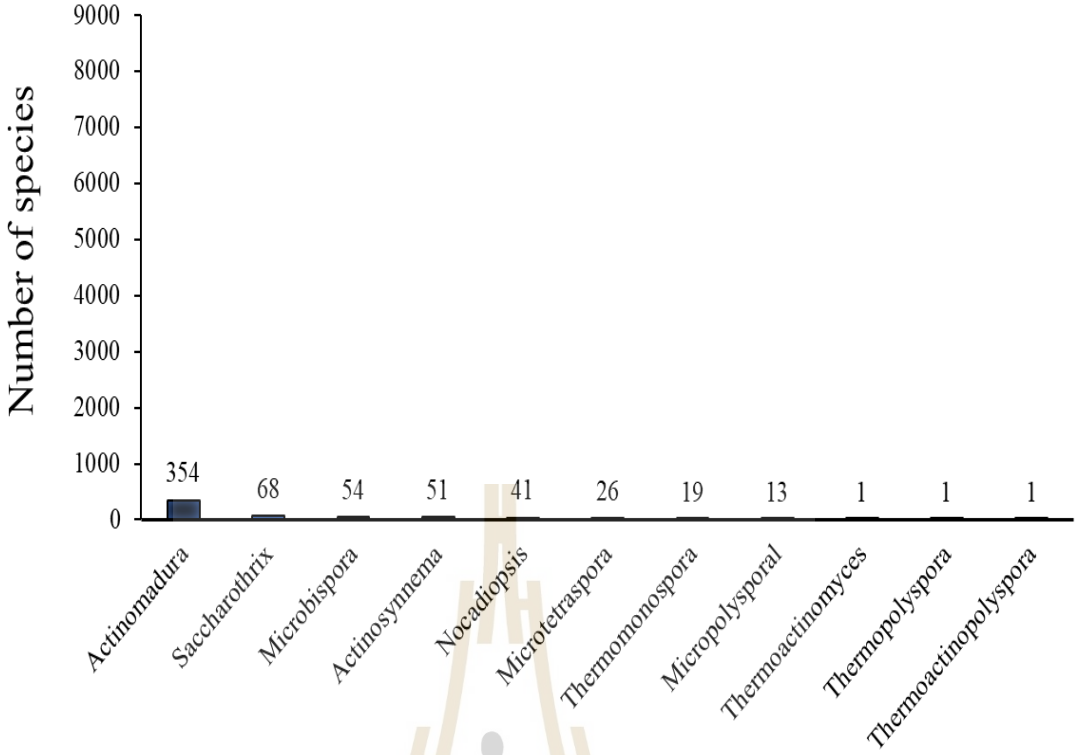


Figure 2.5 The number of actinomycetes belonging to family Thermomonosporaceae produced bioactive metabolites.

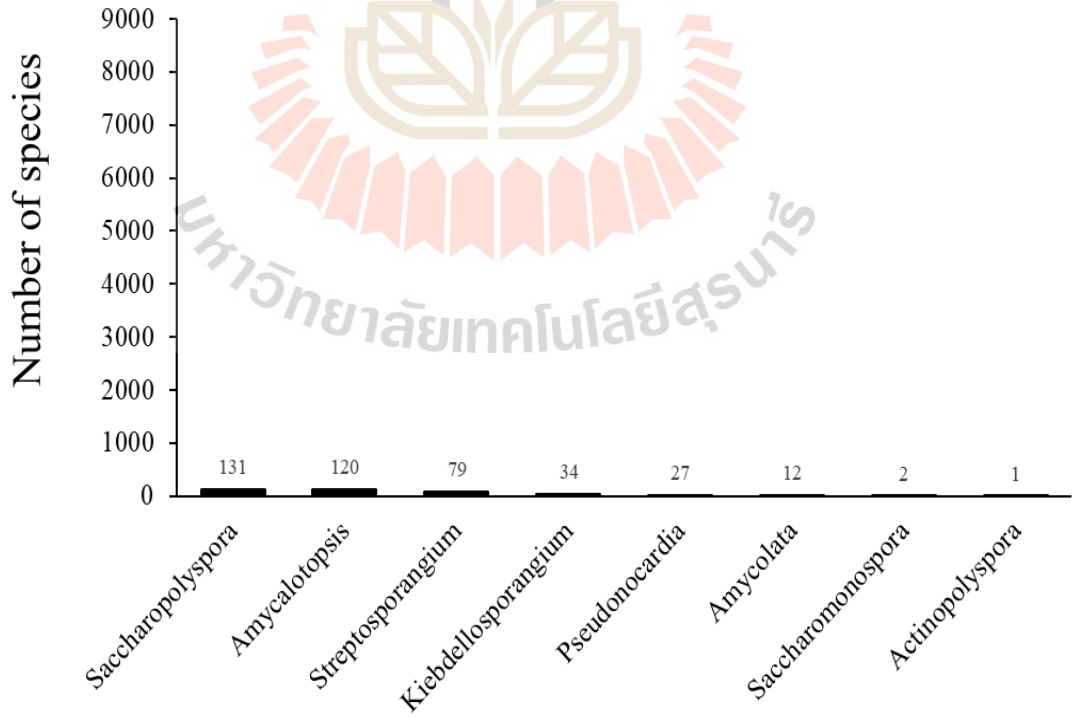


Figure 2.6 The number of actinomycetes belonging to family Pseudonocardiaceae produced bioactive metabolites.

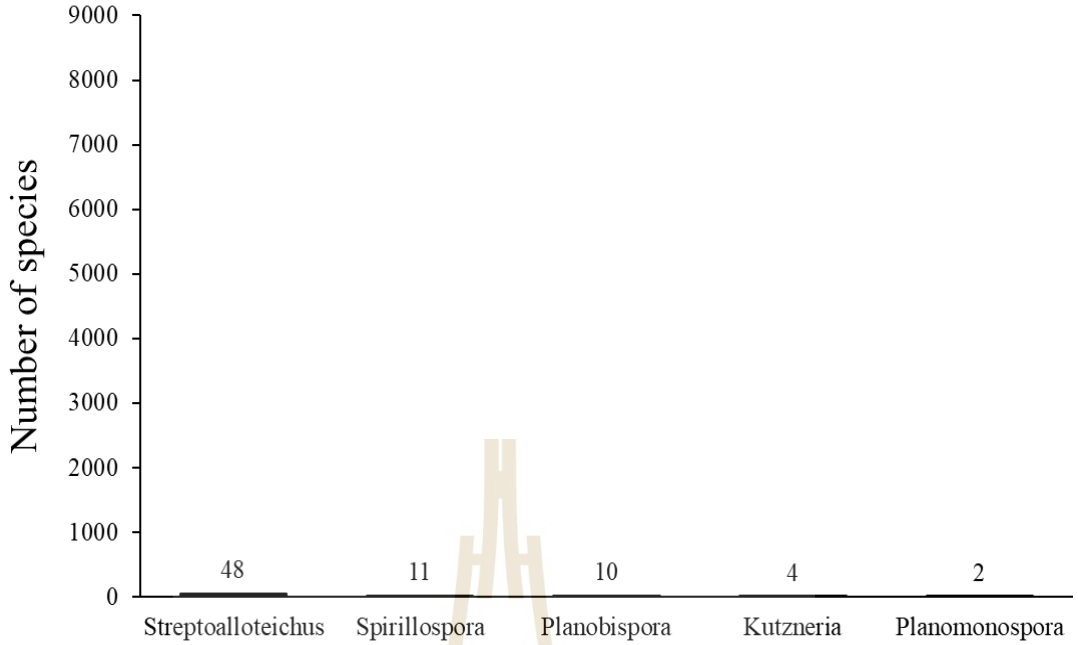


Figure 2.7 The number of actinomycetes belonging to family Streptosporangiaceae produced bioactive metabolites.

2.3 General overview of actinomycetes

Actinomycetes belonging to the phylum actinobacteria. They are aerobic, Gram-positive filamentous bacteria (Stackebrandt, Rainey and Ward-Rainey, 1997; Ventura et al., 2007). Actinomycetes found widely in soil habitat. They are free living saprophytic bacteria that play an important role in organic materials decomposition (Velayudham and Murugan, 2012). It has been reported that actinomycetes live as symbiosis to other organisms for example plants, fungi, insect and animals (Figure 2.8). The microorganisms that inhabit the same environment with actinomycetes are killed or inhibited the growth by bioactive substances that produced by actinomycetes. Thus, antibiotic-producing actinomycetes can survive in nature for long time by killing other organisms (Oskay, Üsame and Cem, 2005).

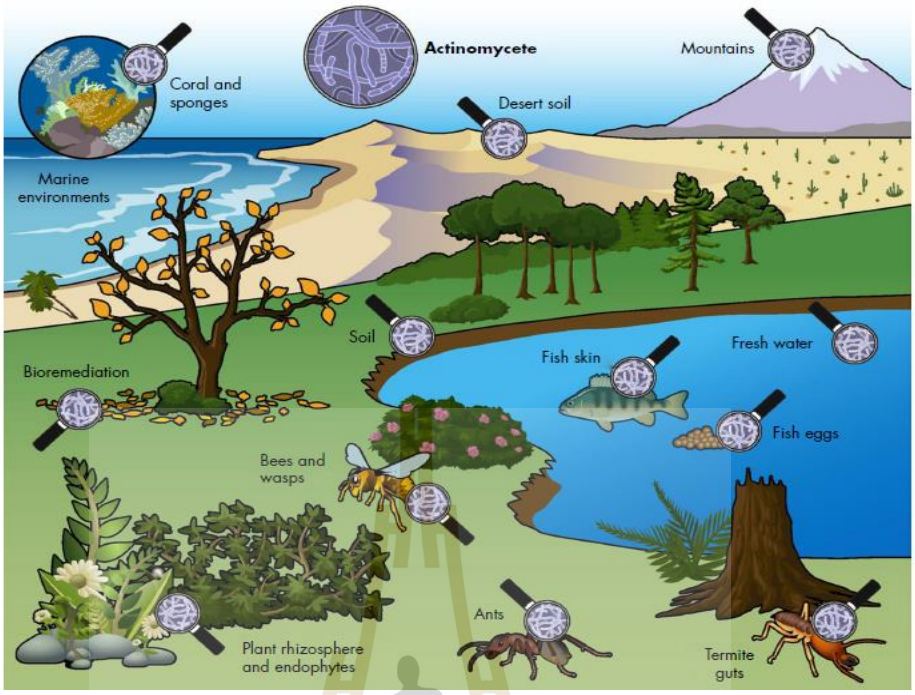


Figure 2.8 Actinomycetes ecology. The interaction between actinomycetes and other organisms are beneficial. Actinomycetes produce antibiotics to protect itself and host against pathogens in the same environment (Van der Meij, Worsley, Hutchings and Van Wezel, 2017).

Actinomycetes could produce filamentous spores in its life cycle. It produced two kind of aerial mycelium and substrate mycelium. Actinomycetes form filaments similar with fungal hyphae. However, the filamentous size of actinomycetes are narrower than the size of fungal hyphae. The life cycle of actinomycetes is complex which includes the formation of spores and other cell types (Figure 2.9). The life cycle starts with germination of spore to generate a substrate mycelium in right conditions. The mycelium forms a net of branching hyphae and penetrate into the substrate to reach nutrients. While, aerial mycelium start growing into the air when nutrients are scarce

(or in response to other signals). Finally, the chains of spores on the distal parts of aerial hyphae are generate (Kalakoutskii and Agre, 1976; Van der Meij et al., 2017).

In order to characterize actinomycetes, morphological characters such as the colony morphology, the color of mycelium and the presence or absence of spores are widely used (Anderson and Wellington, 2001). Several spores of actinomycetes are produced in order to dispersing and causing of colony color. Colony morphology of actinomycetes is typically velvety appearance which distinguishes them from other bacteria. In addition, soil actinomycetes have earth smell due to a volatile compound call geosmin which is produced by soil actinomycetes (Gerber and Lechevalier, 1965).

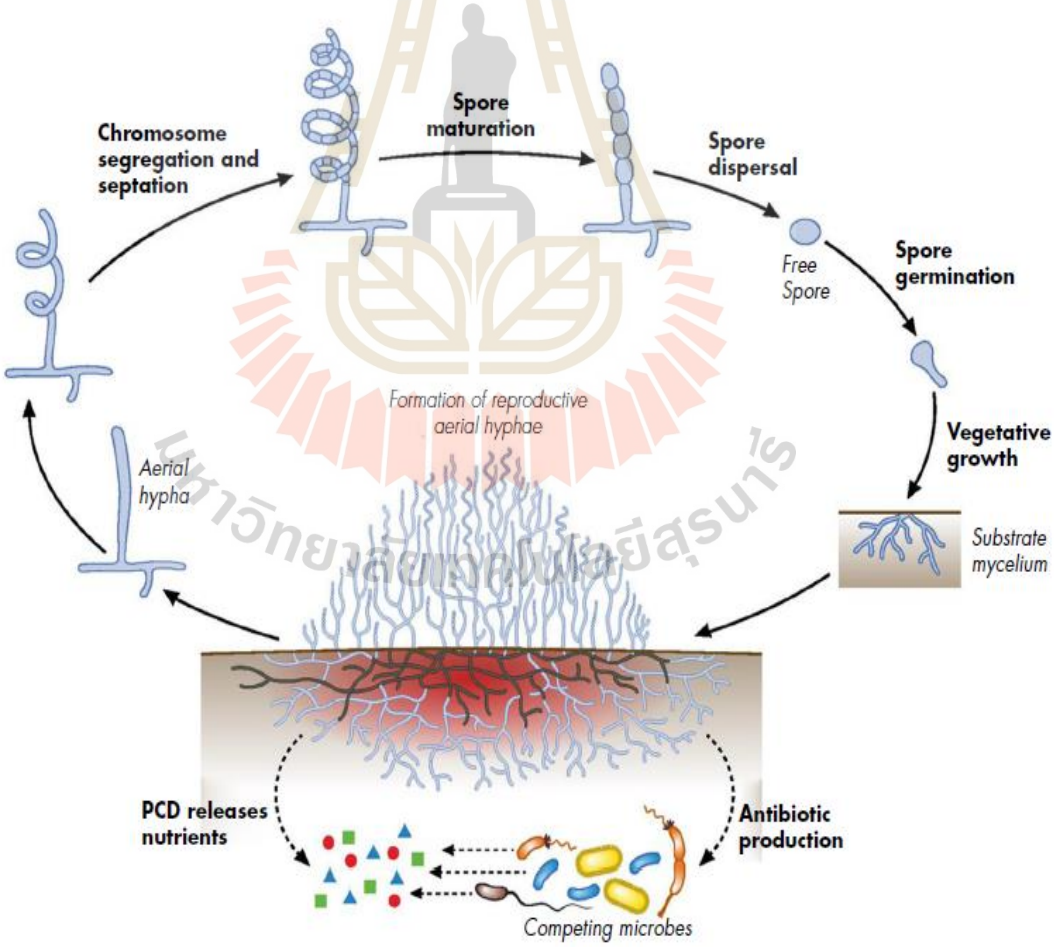


Figure 2.9 Actinomycetes life cycle.

2.3.1 Secondary metabolites of actinomycetes

The secondary metabolites of microbes are compounds produced from microorganisms in the late (stationary) growth phase. They have no function in growth, development and reproduction of microorganisms (Adegboye and Babalola, 2013). The secondary metabolites produced from microorganisms include antibiotics, antitumor agents, cholesterol-lowering drugs and others. Among the microorganisms, the order actinomycetes have been known to produce a wide variety of secondary metabolites. Secondary metabolites derived by actinomycetes can be antibiotics, anticancer, antiprotozoal, anticholesterol, antiaging, antihelminth, insecticides, herbicides and immunosuppressant (Adegboye and Babalola, 2013). Among, bioactive secondary metabolites obtained from actinomycetes, antibiotics are the most important products. Approximately 80% of clinical used antibiotic drugs are produced by actinomycetes, especially from *Streptomyces* and *Micromonospora* (Arifuzzaman, Khatun and Rahman, 2010; George, George and Hatha, 2010; Ravi et al., 2015). Among the actinomycetes, the filamentous *Streptomyces* play the major role of pharmaceutical field and biotechnology (de Lima Procópio, da Silva, Martins, de Azevedo and de Araújo, 2012; Adegboye and Babalola, 2013). Since, *Streptomyces* alone produce 75% of clinically usage antibiotics and they constituted 50% of soil actinomycetes (Kumar, Duraipandiyan and Ignacimuthu, 2014). Hence, actinobacteria, particularly genus *Streptomyces* is a target organism for the isolation and development of antimicrobial drugs. There are many reports of the discovery of antimicrobial compounds produced by actinobacteria.

The history of antibiotics obtained from actinomycetes began with the discovery of streptothricin and streptomycin in 1942 and 1943, respectively (Waksman, Reilly and

Johnstone, 1946; de Lima Procópio et al., 2012). These bioactive compounds, streptothricin and streptomycin, exhibit broad spectrum antimicrobial activity against both of Gram-positive and Gram-negative bacteria (Waksman et al., 1946).

In 1972, cephamycin, a new family of β-lactam antibiotics was discovered. It was produced by *Streptomyces lactamdurang* isolated from soil obtained from sources all around the world. Cephamycin exhibits broad antibacterial spectrum (Stapley et al., 1972).

In 1975, Maruyama and co-worker isolated *Streptomyces kurssanovii* from soil in Japan. *Streptomyces kurssanovii* produced antibacterial compound which was characterized as fumaramidmycin. Fumaramidmycin shows an antibacterial activity against both Gram-positive and Gram-negative bacteria (Maruyama et al., 1975).

In 1986, crisamicin A was discovered by Richard and co-worker. It was derived from *Micromonospora purpureachromogens* subsp. *halotolerans* isolated from a mud sample in Philippines. Crisamicin A was identified as a novel member of the isochromanequinone group of antibiotics that exhibited antibacterial activity (Nelson, Pope, Luedemann, Mcdaniel and Schaffner, 1986).

In 1989, *Streptomyces hygroscopicus* subsp. *luteolus* subsp. nov. was isolated from soil in Japan. The isolate produced a new antitumor agent, phospholine. Phospholine shows strong activity against murine leukemia cells and murine lymphoma cells (Ozasa et al., 1989).

In 2000, two novel angucyclinone antibiotics, simocyclinones D4 and D8, were introduced by Judith and co-workers. *Streptomyces antibioticus* Tü 6040 produced simocyclinones D4 and D8 which exhibit narrow antimicrobial spectrum against Gram-positive bacteria (Theobald, Schimana and Fiedler, 2000).

In 2000, Yasuhiro and co-worker introduced a new member of tellocarcin class of antibiotics named Aristostatins A and B. These compounds were derived from *Micromonospora* sp. TP-A0316 isolated from seawater in Japan. Aristostatins A and B show narrow spectrum antimicrobial activity against only Gram-positive (Igarashi et al., 2000).

In 2001, four novel cyclic homodecapetide antibiotics, stretocidins A, B, C and D were discovered by Alexandra and co-workers. Stretocidins A, B, C and D were derived from *Streptomyces* sp. Tü 6071. They were structurally related to tyeocidines and gramicidins of *Bacillus brevis* which show antimicrobial activity against Gram-positive bacteria (Höltzel et al., 2001).

In 2003, Marcelo and co-workers discovered a new bioactive compound, ripromycin. Ripromycin was produced by *Streptomyces* sp. Tü 6239 isolated from soil sample collected in Brazil. It exhibits antibiotic activity against only Gram-positive bacteria (Bertasso et al., 2003).

In 2005, a new antibacterial polyene, Sch 725424, was discovered. This compound was isolated from the culture of the genus *Kitasatospora*. The microbial metabolite Sch 725424 shows inhibitory activity against *S. aureus* with MIC values of 1-2 μg/ml and *Saccharomyces cerevisiae* with MIC value of 32 μg/ml (Yang et al., 2005).

In 2009, a novel benzoxazole antibiotic, caboxamycin, was introduced by Claudia and co-workers. Caboxamycin shows inhibitory activity against only Gram-positive bacteria. It was extracted from *Streptomyces* sp. NTK 937 which was isolated from an Atlantic Ocean deep-sea sediment. Mass spectrometry, NMR experiments and X-ray analysis were used to study the structure of caboxamycin. (Hohmann et al., 2009).

In 2010, a novel thiopeptide antibiotic was introduced by Kerstin and co-workers. Thiopeptide was produced by *Nocardiopsis* sp. which was isolated from sediments and sponges collected in Norway. It shows antibacterial and antifungal activities against vancomycin-resistant *Enterococcus faecium* and multidrug-resistant *Candida albicans,* respectively (Engelhardt et al., 2010).

In 2013, new napyradiomycins produced by *Streptomyces* sp. SCSIO 10428 that isolated from sediment sample in China were discovered. Napyradiomycins display antibacterial activity against *Staphylococcus* and *Bacillus* strains with MIC values ranging from 0.25 to 128 μg/ml (Wu et al., 2013).

In 2018, Iniyan and colleagues isolated ala-geninthiocin, a new broad spectrum thiopeptide antibiotic, from a marine *Streptomyces* sp. ICN19. Ala-geninthiocin exhibited potent antimicrobial activity against Gram-positive bacteria including *S. aureus*, *Bacillus subtilis*, *Mycobacterium smegmatis* and *Micrococcus luteus*. It has cytotoxicity against A549 human lung carcinoma cell with an IC50 value of 6 nM (Iniyan, Sudarman, Wink, Kannan and Vincent, 2018).

In 2019, desertomycin G with strong antibiotic activity against several clinically antibiotic resistant was reported. Desertomycin G was derived from marine actinomycete, *Streptomyces althioticus* MSM3, that isolated from the intertidal seaweed *Ulva* sp. collected in the Cantabrian Sea (Northeast Atlantic Ocean). It is strongly active against clinical isolate antibiotic resistant *Mycobacterium tuberculosis* (Brana et al., 2019).

Several secondary metabolites produced by actinomycetes are shown in the table 2.1.

Table 2.1 The important secondary metabolites produced by streptomycetes.

Antibiotic drugs	**Source**	**Chemical class**	**Application**
Actinomycin D	*Streptomyces* spp.	Peptide	Antitumor
Antimycin A	*Streptomyces* spp.	Macrolide	Telocidal
Avermectin	*S. avermitilis*	Macrolide	Antiparasitic
Bambermycin	*S. bambergiensis*	Substituted aminoglycoside	Growth promotant
Bialaphos	*S. hygroscopicus*	Peptide	Herbicidal
Bleomycin	*S. verticillus*	Glycopeptide	Antitumor
Candicidin	*S. griseus*	Polyene macrolide	Antifungal
Cephamycin C	*Nocardia lactamdurans*	B-lactam	Antibacterial
Chloramphenical	*S. venezuelae*	N-dichloroacyl phenylpropanoid	Antibacterial
Chlortetracycline	*S. aureofaciens*	Tetracycline	Antibacterial
Clavulanic acid	*S. clavuligerus*	β-lactam	Antibacterial
Cycloserine	*S. orchidaceus*	Substituted cyclic prptide	Antibacterial
Daptomucin	*S. rodeosporus*	Lipopeptide	Antibacterial
Daunorubicin	*S. peucetius*	Anthracycline	Antitumor
Desferrioxamine	*S. pilosus*	Peptide	Iron purging in iron overload

Table 2.1 The important secondary metabolites produced by streptomycetes (Continued).

Antibiotic drugs	Source	Chemical class	Application
Doxorubicin	*S. peuctius* var. *caesius*	Anthracycline	Antitumor
Erythromycin	*Sac. Erythraea*	Macrolide	Antibacterial
FK506	*S. hygroscopicus*	Macrolide	Immunosuppressant
Fortimicin	*Micromonospora olivoasterospora*	Aminoglycoside	Antibacterial
Fosfomycin	*Streptomyces* spp.	Phosphoric acid	Antibacterial
Gentamycin	*Micromonospora* spp.	Aminoglycoside	Antibacterial
Hygromycin B	*S. hygroscopicus*	Substituted aminoglycoside	Antihelminthic
Kanamycin	*S. kanamyceticus*	Aminoglycoside	Antibacterial
Lasalocid	*S. lasaliensis*	Polyether	Anticoccidal, growth promotant
Lincomycin	*S. lincolnensis*	Sugar-amide	Antibacterial
Milbemycin	*S. argilaceus*	Macrolide	Antiparasitic
Mithramycin	*S. argillaceus*	Aureolic acid	Antitumor
Mitomycin C	*S. caespitosus* *S. verticillatus*	Benzoquinone	Antitumor

Table 2.1 The important secondary metabolites produced by streptomycetes (Continued).

Antibiotic drugs	Source	Chemical class	Application
Monensin	*S. cinnamonensis*	Polyether	Anticoccidal, growth promotant
Natamycin	*S. nataensis*	Tetraene polyene	Antifungal
Neomycin	*S. fradiae*	Aminoglycoside	Antibacterial
Nikkomycin	*S. tendae*	Nucleoside	Antibacterial, insecticidal
Nocardicin	*N. uniformis*	B-lactam	Antibacterial
Nosiheptide	*S. actuosus*	Thiopeptide	Growth promotant
Novobiocin	*S. neveus*	Coumerin glycoside	Antibacterial
Nystatin	*S. noursei*	Polyene macrolide	Antifungal
Oleandomycin	*S. antibioticus*	Macrolide	Antibacterial
Oxytetracycline	*S. rimosus*	Tetracycline	Antibacterial
Paromomycin	*S. rimosus forma paramomycinus*	Aminoglycoside	Antibacterial
Phleomycin	*S. verticillus*	Glycopeptide	Antitumor
Polyoxins	*S. cacaoi* var. *asoensis*	Nucleosidepeptide	Antifungal
Rapamycin	*S. hygroscopicus*	Macrolide	Immunosuppressant

Table 2.1 The important secondary metabolites produced by streptomycetes (Continued).

Antibiotic drugs	Source	Chemical class	Application
Pristinamycin	*S. pristimaespiralis*	Peptidic macrolactone + polyunsaturated macrolactone	Antibacterial
Rifamycin	*Amycolatopsis mediterranei*	Ansamycin	Antibacterial
Ristocetin	*N. lurida*	Glycopeptide	Antibacterial
Salimomycin	*S. albus*	Polyether	Anticoccidal, growth promotant
Spectinomycin	*S. spectabilis*	Aminocyclitol	Antibacterial
Spinnosyns	*Sac. Spinosa*	Tetracyclic macrolide	Insecticidal
Spiramycin	*S. ambofaciens*	Macrolide	Antibacterial
Streptogramins	*S. graminofaciens*	Macrocyclic lactone	Antibacterial
Streptomycin	*S. griseus*	Aminoglycoside	Antibacterial
Teichoplanin	*Actinoplanes teichomyceticus*	Glycopeptide	Antibacterial
Tetracycline	*S. aureofaciens*	Tetracycline	Antibacterial

Source: Wanbanjob, 2008

2.4 Antibiotics-resistant microorganisms

According to the World Health Organization, the resistant of many pathogens have emerged because of the widespread use of antibiotics. Pathogens could resist to antibiotic drugs by several mechanisms for example inactivation of the drug by enzymes, increased efflux of the antibiotics, modification of the antibiotic targets and alteration of the site of action or cell wall (Shetty et al., 2014; Lin et al., 2015; Martens and Demain, 2017). Microbial strains resistant to antibiotics become a major cause of serious infections. Methicillin-resistant *S. aureus*, vancomycin-resistant enterococci, *E. coli*, *A. baumanni*, *P. aeruginosa* and *K. pneumoniae* are the most common resistant bacterial strains causing important community acquired infections and nosocomial infections (Subakaran, Joshua, Jansi and Prakash, 2015; Uzair et al., 2018).

Staphylococcus aureus is considered one of the most important pathogens, responsible for nosocomial infections (Novick, 2003; Schito, 2006). It is facultative anaerobe Gram-positive cocci, non-spore forming bacteria. *S. aureus* can cause an infection ranging from superficial lesions to life threatening septicemia (Kluytmans and Wertheim, 2005). The antibiotic resistance has emerged in 1942 when *S. aureus* became resistant to β-lactam antibiotics, penicillin, by producing penicillinase. Penicillinase is an enzyme that can disrupt the structure of penicillin by digesting the β-lactam ring and thus inactivate the drug action (Ventola, 2015). Methicillin was introduced in 1959 for treatment of the infection caused by penicillin-resistant *S. aureus* (Schito, 2006). In 1961, the first methicillin-resistant *S. aureus* has been reported in the United Kingdom (Enright et al., 2002). Since the first case of MRSA was proclaimed, the strain MRSA was rapidly spread to other countries and became a problem in the USA in the 1970s. (Enright et al., 2002; Robinson and Enright, 2004). Many drugs including

glycopeptides (vancomycin and teicoplanin), linezolid, tigecycline, daptomycin and new β-lactam antibiotics such as ceftaroline and ceftobiprole have been used against MSRA (Schito, 2006). However, MRSA has been identified as an important nosocomial infection causing organisms. Since, it is highly prevalent and potentially cause death of patients especially hospitalized patients with immunodeficiency (Datta and Huang, 2008). MRSA is capable of developing the new clones to resist to almost all currently available antibiotics except vancomycin (Enright et al., 2002).

Enterococci is the second leading cause of hospital acquired infections worldwide (Khan, Ahmad and Mehboob, 2015). They are facultative anaerobic Gram-positive enteric microbes. Enterococci cause a wide range of illness which are involved in the blood-borne infections and urinary tract infections (UTI) (Ventola, 2015). Enterococci are resistant to different classes of antibiotics particularly glycopeptide antibiotics (vancomycin and teicoplanin) (Khan et al., 2015). Vancomycin-resistant enterococci (VRE) are major therapeutic problem which caused high mortality rate of patients on health care associated infections (Eliopoulos and Gold, 2001; Ventola, 2015). Nowadays, a few antibiotic drugs are available to treat VRE. Antibiotics used against VRE include linezolid and quinupristin / dalfopristin.

Pseudomonas aeruginosa has emerged as the most common Gram-negative associated with serious hospital acquired infections, particularly within intensive care units. It is a facultative anaerobe Gram-negative bacterium which can cause pneumonia, bloodstream infection, urinary tract infection and surgical infection (Khan et al., 2015). *P. aeruginosa* has ability to develop resistance to anti-pseudomonal antibiotics through several mechanisms such as the restricted uptake of drug, drug modification and altered targets for antibiotics (Bălăşoiu, Bălăşoiu, Mănescu, Avramescu and Ionete, 2014;

Khan et al., 2015; Ventola, 2015). The increasing incidence of multidrug-resistant (MDR) *P. aeruginosa* has been recognized as a cause of treatment failure (Carmeli, Troillet, Eliopoulos and Samore, 1999).

The Centers for Disease Control and Prevention (CDC) divided the threatening of antibiotic-resistant bacteria based on level of concern into three categories which were urgent threats, serious threats and concerning threats (Ventola, 2015). The urgent threats include carbapenem-resistant *Acinetobacter*, *Candida auris*, *Clostridium difficile*, Carbapenem-resistant Enterobacteriaceae (CRE) and drug-resistant *Neisseria gonorrhoeae*. Several strains including multidrug-resistant *Acinetobacter*, drug-resistant *Campylobacter*, extended spectrum β-lactamase producing Enterobacteriaceae (ESBLs), vancomycin-resistant *Enterococci* (VRE), multidrug-resistant *P. aeruginosa*, drug-resistant nontyphoidal *Salmonella*, drug-resistant *S. typhimurium*, drug-resistant *Shigella*, methicillin-resistant *S. aureus* (MRSA), drug-resistant *Streptococcus pneumoniae* and drug-resistant tuberculosis are assigned in the serious threats. Other group is concerning threats including vancomycin-resistant *S. aureus* (VRSA), erythromycin-resistant Group A *Streptococcus* and clindamycin-resistant Group B *Streptococcus*.

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

3.1.1 Test organisms

The test microorganisms were Gram-positive bacteria (*S. aureus* ATCC29213, methicillin-resistant *S. aureus* (MRSA) DMST20651, *S. epidermidis* TISTR518, *B. subtilis* TISTR008 and *B. cereus* TISTR687) and Gram-negative bacteria (*E. coli* TISTR780, *E. aerogenes* TISTR1540, *P. aeruginosa* TISTR781, *S. marcescens* TISTR1354, *P. mirabilis* TISTR100 and *S. typhimurium* TISTR292). They were obtained from American Type Culture Collection (ATCC), Department of Medical Sciences Thailand (DMST) and Thailand Institute of Scientific and Technological Research (TISTR). All test microorganisms were inoculated in Mueller Hinton broth (MHB) (Himedia, India) at 37 °C for 18-24 h for antimicrobial testing.

3.1.2 Media

Mueller Hinton (MH) medium (per 1 liter) composed of beef extract 300 g, casein acid hydrolysate 17.5 g and starch 1.5 g (pH 7.2-7.4).

Luria-Burtani (LB) medium contained (g/l) yeast extract 5, tryptone 10 and NaCl 10. The medium pH was adjusted to 7.0.

International *Streptomyces* Project 2 (ISP-2) medium or yeast extract malt extract contained (per 1 liter) yeast extract (4 g), malt extract (10 g) and dextrose (4 g). The pH of medium was adjusted to 7.2.

Fifteen grams of agar were added into 1 liter of medium to prepare the solid medium.

All of media were sterilized by autoclaving in the conditions at 121ºC, 15 psi over pressure for 15 min.

3.1.3 Antibiotic drugs

The stock solution of antibiotics (10 mg/ml) including ampicillin (Bio Basic, Cannada), oxacillin (Sigma-Aldrich, USA), vancomycin (Amresco, USA) and tetracycline (Sigma-Aldrich, USA) were done by dissolving in sterile distilled. The filter size of 0.2 µm pore (Corning®, Germany) was used for sterilization of antibiotic drugs. Then, the sterilized antibiotics were stored at -20 ºC until used.

3.1.4 Reagents

3.1.4.1 Agarose gel electrophoresis

Tris-borate-EDTA (TBE) buffer was prepared for running of agarose gel electrophoresis. The working concentration is 1X that contained 89 mM Tris-HCl (pH 8.0), 89 mM boric acid and 2.5 mM EDTA.

The DNA was stained by using MaestroSafe^{TW} nucleic acid stain (MaestroGen, Taiwan).

3.1.5 Primers for 16S rRNA gene amplification

The 16S rRNA gene of antimicrobial-producing bacterial soil isolates were amplified by using universal primers. The sequences of primers used in this study are shown in Table 3.1.

Table 3.1 Primers used in this study.

Primers	Primer's sequences (5′-3′)	References
27F	AGAGTTTGATCCTGGCTCAG	Lane, 1991
1525R	AAAGGAGGTGATCCAGCC	Lane, 1991
M13 Forward	GTAAAACGACGGCCAGT	Macrogen, Korea
M13 Reverse	GCGGATAACAATTTCACACAGG	Macrogen, Kore

3.1.6 Vector

The plasmid used in this study for gene cloning is pTG19-T vector (Vivantis, Malaysia). The size of pTG19-T PCR cloning vector is 2.880 kb. Figure 3.1 is a map of pTG19-T vector.

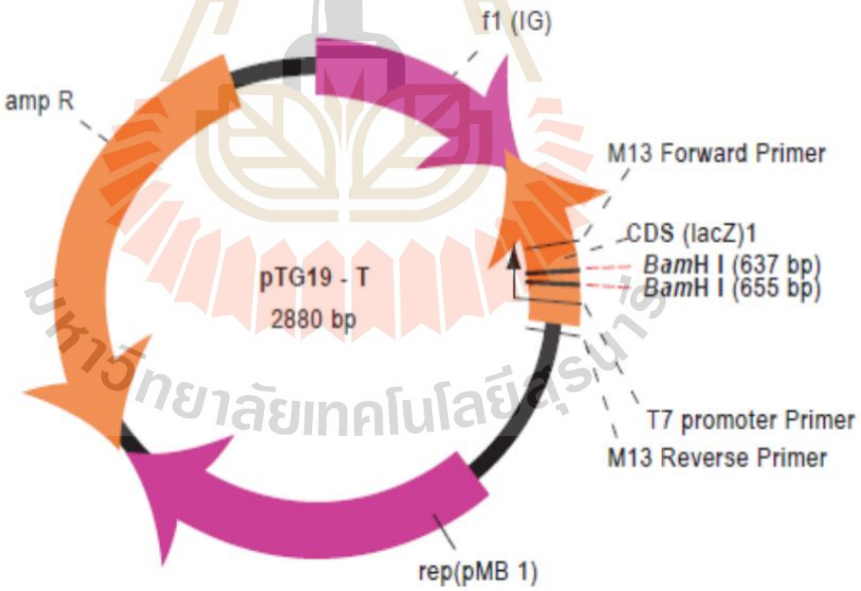


Figure 3.1 Genetic map of pTG19-T vector.

3.1.7 Materials for molecular technique

X-gal (5-Bromo-4-chloro-3-indolyl-*β*-D-galactoside) (Bio basic, Canada) was added to LB medium for blue-white screening method.

100 bp DNA ladder M consist of 12 fragments was use as DNA marker. It was purchased from Himedia, India.

The green master mix obtained from Promega, USA was used for amplification of 16S rDNA.

3.1.8 Instruments

The list instruments used in this study are shown in Table 3.2.

Table 3.2 Research instruments.

Name	Source
Larminar flow	Esco, Singapore
Rotary evaporator	Buchi, Switzerland
Synchrotron-FTIR microspectroscopy	Ettlingen, Germany
MicrOTOF-Q II spectroscopy	Bruker, England
Freeze dry	Labconco, USA
T80+ UV/VIS spectrometer	Thermo Scientific, USA
Thermo cycler	Thermo Scientific, USA
Electronic balance	Shimadzu, Japan
Microscope (model CX31)	Olympus, USA
Centrifuge machine	Hettich, Germany
pH meter	Sartorious, Germany
Vortex mixer	Finepcr, Korea
Hotplate magnetic stirrer	Merck, USA
Heat block	Hercuvan, UK

3.2 Methods

3.2.1 Characterization of antimicrobial-producing bacterial soil isolate

3.2.1.1 Cultural and morphological characteristics

The cultural morphology of antimicrobial-producing bacterial soil isolate was determined on ISP-2 medium. The color of aerial mycelium, substrate mycelium and diffusible pigments were observed and recorded.

3.2.1.2 DNA extraction and PCR Amplification of the 16S rDNA

In order to extract the genomic DNA of antimicrobial-producing bacterial soil isolate, bacterial soil isolate was cultured in 5 ml ISP-2 medium and incubated at 37 °C for 3 days. The cell pellets were collected by centrifugation at 13,000 rpm for 5 min, after incubation. Five mg of cell pellets were then placed in a mortar and mixed with 500 µl lysis buffers. The mixture was grounded with pestle and transferred into a new tube. One hundred-sixty-five microliter of 5 M NaCl was added and mixed by inverting. The tube was centrifuged at 13,000 rpm for 20 min. The supernatant was collected and added with 800 µl of chloroform:isoamyl alcohol (1:1) were added. The tube was mixed by inverting and 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 DNA pellet was washed three times with 800 µl of 70% cold ethanol and air dried. TE buffer (30-50 µl) was added in order to dissolve the genomic DNA. The genomic DNA was kept at -20 °C.

The amplification of 16S rDNA of bacterial soil isolate was done by using 27F and 1525R primers (Table 3.1). The PCR mixture in total volume of 25 µl is shown in Table 3.3. The thermal cycle obtained from Thermo Scientific, USA is instrument used to amplify DNA. The PCR conditions started with initial denaturation at 95 °C for 5 min followed by 25-30 cycles of denaturation at 95 °C for 1 min, annealing at 55 °C

for 1 min, extension at 72 °C for 1 min and a final elongation at 72 °C for 7 min (Figure 3.2).

Table 3.3 The mixture of PCR reaction.

PCR mixture	**Volume (µl)**
DNA template	5
2x Green master mixed	12.5
10 µM 27F	1
10 µM 1525R	1
DW	5.5
Final volume	**25**

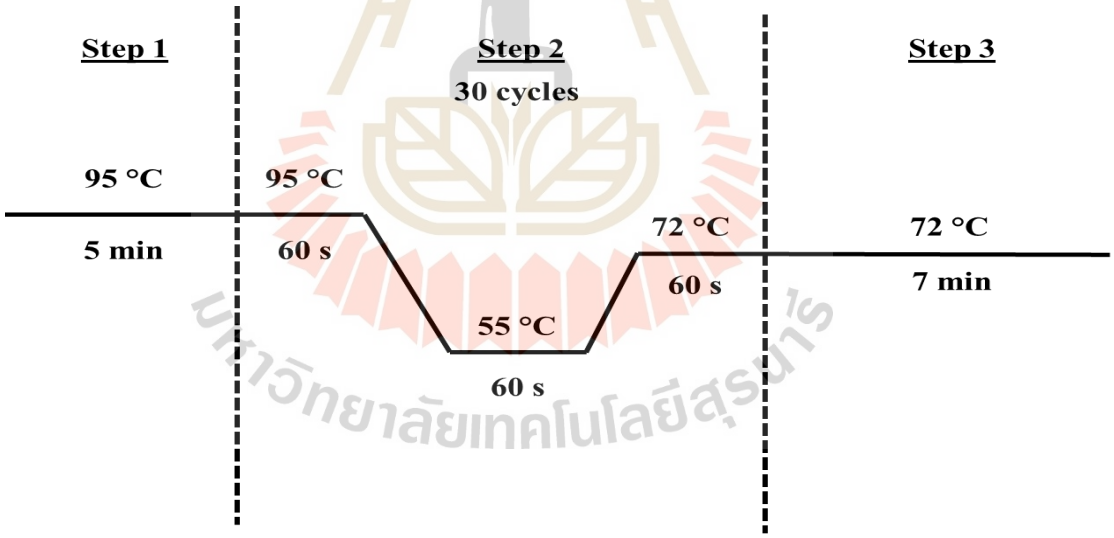


Figure 3.2 PCR conditions for amplification of 16 rRNA gene.

3.2.1.3 Cloning of 16S rDNA of bacterial soil isolates

The amplicons were purified by FavorPrep™ GEL/PCR Purification Kit (FAVORGEN, Taiwan). The purified PCR product was ligated to pTG19-T cloning vector and incubated at 22 °C for 1 h.

Table 3.4 Ligation of 16S rDNA and pTG19-T cloning vector.

Reaction mixture	**Volume (µl)**
10x buffer ligase	1
pTG19-T cloning vector (25 ng/μl)	2
Purified PCR product	X
DW	10-(4+X)
T4 DNA ligase (200 u/μl)	1
Total	**10**

3.2.1.4 The preparation of competent cells

Plasmid transformations and β-galactosidase assays were done by using the strain *Escherichia coli* JM109 (K-12, *endA1 gyr96 hsdR17* Δ(*lac-proAB*) *recA1 relA1 supE44 thi*-1 F′[$lacI^q$ *lacZ*ΔM15 $proAB^+$ *traD*36])]) (Kwon, Georgellis, Lynch, Boyd and Lin, 2000)

Escherichia coil JM109 was used for preparation of competent cells. Five milliliters of LB broth was used to grow *E. coli* JM109 at 37 °C for overnight. One milliliter of overnight culture was transferred into 100 ml of LB broth and incubated at 37 °C. The culture with OD_{600} around 0.3 was collected and placed on ice for 15-20. In order to harvest bacterial cells, the culture was centrifuged at 3,000-3,500 *g*, 4 °C for 10 min. Cell free supernatant was removed. Bacterial cells were washed by adding 10 ml of ice-cold 0.1 M $MgCl_2$ and centrifugation at 3,000 *g*, 4 °C for 10 min. Ten milliliter of ice-cold 0.1 M $CaCl_2$ was used to suspend the cell pellets. The suspensions were placed on ice for 1 h and centrifuged at 3,000 *g*, 4 °C for 10 min. The cell pellets were collected. Two-three milliliter of 0.1 M $CaCl_2$ containing 15% (v/v) glycerol was used

to suspend the cell pellets. Finally, 100 µl of cell suspensions were aliquoted and dipped in liquid nitrogen and stored at -80 °C.

3.2.1.5 Transformation

In order to transform the ligation into competent cells, the aliquot (100 µl) of competent cells were added with the recombinant DNA and incubated on ice for 1 h. The mixture was heated at 42 °C for 60 sec and cooled on ice for 2 min. One milliliter of LB broth was added with the miture and incubated at 37 °C, 200 rpm for 1 h. The plate (LB agar) containing ampicillin (100 µg/ml) and X-gal (80 µg/ml) was spread with 100 µl of transformantion mixture. The plates were incubated at 37 °C for 24 h. Blue-white screening method was used for selection of the recombinant clones (Padmanabhan, Mandi and Banerjee, 2011). *β*-galactosidase was encoded by *lac*Z gene of pTG19-T PCR cloning vector. The enzyme *β*-galactosidase catalyzes X-gal to obtain blue pigments. Therefore, the clones expressing *β*-galactosidase present blue colony on the LB agar containing X-gal. While, white colony could not metabolize X-gal to produce blue colony on LB agar. It is because of transformants harboring vector with an interrupted *lacZ* gene.

3.2.1.6 Sequencing of 16S rDNA

In order to study the sequencing of 16S rDNA, the recombinant plasmid was submitted to Macrogen, Korea. Two primers, M13 forword and M13 reverse, were used for amplification. The obtained sequencing was compared to the EzTaxon database (https://www.ezbiocloud.net/).

3.2.1.7 Phylogenetic tree analysis

16S rRNA gene sequence of bacterial soil isolate was compared against the EzTaxon database and determined sequence similarity. The CLUSTAL W

was used to align 16S rDNA sequence of bacterial soil isolate and its closely-related species. The analysis of phylogenetic tree was done by using Molecular Evolutionary Genetics Analysis (MEGA) version 7.0 with a neighbor-joining method (Kimura's two-parameter model). *Nocadia alba* YIM 30243 was used as an outgroup. The branch of phylogenetic tree was tested to estimate the confidence level by bootstrap analysis in 1,000 replications) (Felsenstein 1985).

3.2.2 Antimicrobial assay

3.2.2.1 Perpendicular streak method

Antimicrobial activity of bacterial soil isolate streptomycetes was done by using the perpendicular streak method (Arasu, Duraipandiyan, Agastian and Ignacimuthu, 2009; Al-Dhabi, Esmail, Duraipandiyan, Arasu and Salem-Bekhit, 2016; Singh et al., 2016) (Figure 3.3). Streptomycetes was inoculated on MHA (Hi-media, India) on one side of petri dish. The cultivation of streptomycetes at 37 °C for 5 days were performed to allow streptomycetes produce antimicrobial agents into an agar. Then, test pathogens were inoculated by streaking perpendicular (T-streak) to the line of streptomycetes colony and incubated at 37 °C overnight. The distance of inhibition between the colonies margin of streptomycetes and test microorganisms was observed for antimicrobial activity.

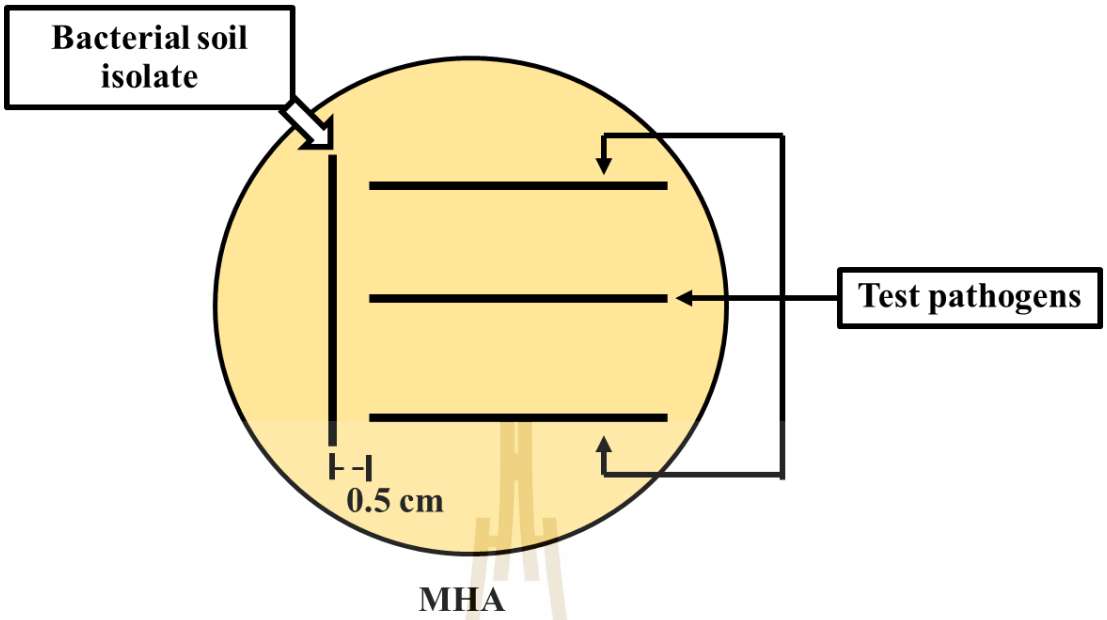


Figure 3.3 Streaking pattern for antimicrobial activity of bacterial soil isolate streptomycetes by perpendicular streak method.

3.2.2.2 Disc diffusion method

The antimicrobial activity of crude compounds was tested by using standard disc diffusion (Balouiri, Sadiki and Ibnsouda, 2016; Khatun, Haque and Islam, 2017). Six millimeter in diameter of filter paper discs containing crude compounds at 50 μg/disc were placed on MHA lawn with test microorganisms (0.5 McFarland standard). The antimicrobial activity was measured with the size of inhibition zone (mm) after incubation at 37 °C for 1 day.

3.2.2.3 Minimum inhibitory concentration (MIC)

In order to determine MIC values of crude compounds, the macro-dilution method was performed (Cockerill et al., 2012). An inoculum of test pathogens in the mid-log phase were transferred to a set of tubes containing serial two-fold

dilutions of crude compounds in liquid medium (256, 128, 64, 32, 16, 8, 4, 2, 1, 0.5, 0.25, 0.125, 0.0625 and 0.03125 μg/ml). The bacterial suspensions were added into each tube to give approximately 5.0×10^5 CFU/ml. The tubes were incubated at 37 °C for 16-18 h. The lowest concentration of crude compounds that inhibit visible growth of test organisms were recorded as MIC.

3.2.3 The study of incubation temperature and incubation period on growth and activity of antibacterial agents

The bacterial soil isolate was grown on ISP-2 broth for 14 Erlenmeyer flasks separately (20 ml in 50 ml Erlenmeyer flask) and incubated at 30 °C and 37 °C for 14 days. The biomass and cell-free supernatant of culture were harvested every day during 14-day period of incubation by filtration through Whatman No.1 filter paper (GE Healthcare, UK). The bacterial cells were dried with hot air oven at 60 °C for 5 days. The cell dry weight was recorded. The cell-free supernatants containing antimicrobial compounds were extracted by using ethyl acetate and used for a determination of antibacterial activity against test pathogens by using disc diffusion method.

3.2.4 Purification of active compounds

3.2.4.1 Preparation of crude compounds of bacterial soil isolate

The bacterial soil isolate was grown at 37 °C for 5 days. The culture was filtrated through filter paper. In order to extract the crude compounds, a fermented broth containing antimicrobial was mixed with ethyl acetate. The layer of ethyl acetate was collected and concentrated by using rotary evaporator at 45 °C. The crude compounds were obtained from freeze drying and used for determination of MIC values and purification.

3.2.4.2 Purification of antimicrobial compounds by TLC assay

The separation of active compounds from crude ethyl acetate compounds was conducted by TLC. Crude compounds were spotted on the TLC silica gel 60 F254 aluminum sheets (Merck, Germany) and left for drying. The TLC plate was placed in a developing tank that contain chloroform and hexane (9.5:0.5). The solvent was allowed 80% of TLC plate. After running the TLC, chromatogram was left to dry and visualized under UV light at 254 nm. The active compounds on chromatogram were identified by contact bioautography method.

3.2.5 Contact bioautography analysis

The bioautography analysis is usually used for the detection of antimicrobial activity of bioactive compounds separated on chromatogram (Kumar and Jadeja, 2018; Sharma and Manhas, 2019). The chromatogram was placed over MHA seeded with test pathogens (0.5 McFarland standard) and left for 30 min to allow the compounds from TLC sheet to diffuse into the agar medium. MHA plate was then incubated at 37 °C for 24 h. After the incubation, the bands of antimicrobial agent were indicated by zone of inhibition on the medium (Figure 3.4). The active band was scrapped from TLC plate and dissolved in methanol. The mixture was then centrifuged and filtrated in order to remove a residual silica. The supernatant containing antimicrobial compounds was used for characterization.

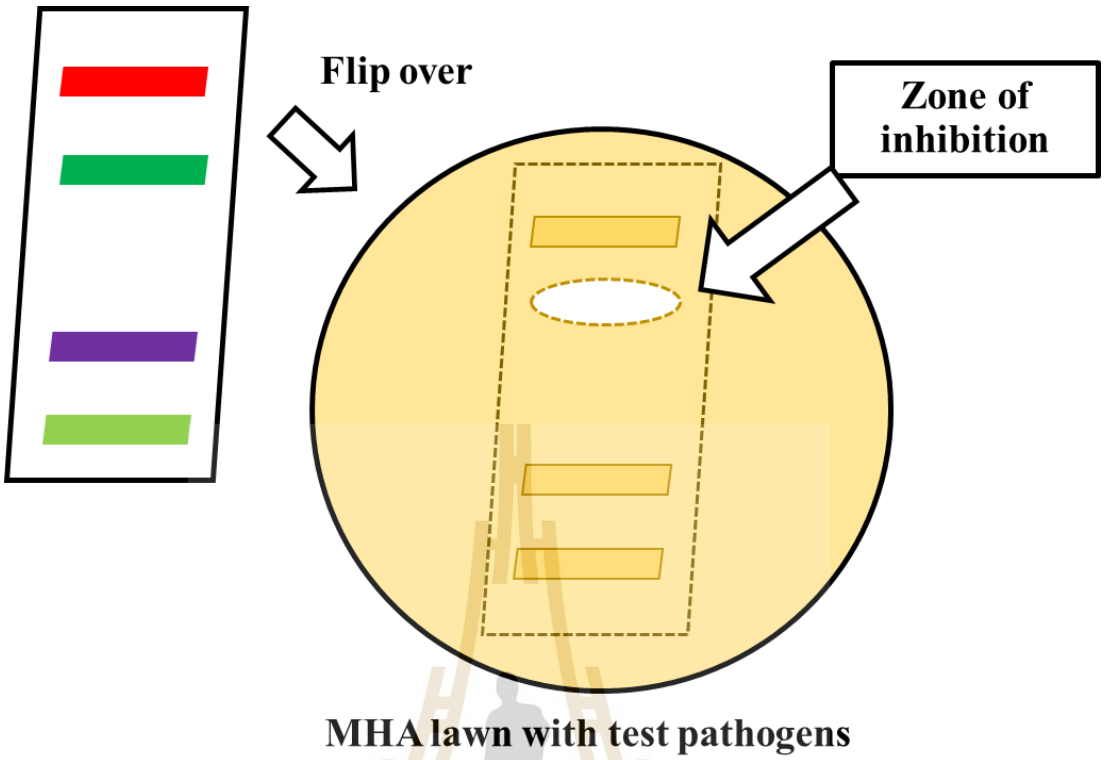


Figure 3.4 Contact bioautography of bioactive compounds produced by bacterial soil isolate against test pathogens.

3.2.6 Identification of active compounds

3.2.6.1 LC-MS analysis

The mass spectrum of the active compounds was assessed using LC-MS analysis. An active compound solution was subjected to the Q-TOF spectroscopy (micrOTOF-Q II, BRUKER, ENGLAND). Both of negative and positive scan modes of spectrometer were operated. The peak was analyzed and identified by matching the mass spectra with the mass bank database.

3.2.6.2 SR-FTIR microspectroscopy

The transmittance of infrared (IR) spectrum of active compounds was measured in transmission mode using the Diamond Anvil Cells with MCT detector.

The spectra were scanned in the range of 4000-600 cm^{-1} and spectral resolution of 4 cm^{-1} (Bruker Optics Ltd, Ettlingen, Germany). The functional groups of active compounds were done by study the frequencies of IR bands such as υ (C-N), υ (O-H), υ (CH), υ (C=C), υ (NH), υ (CO) and (CH) symmetric, asymmetric stretching and stretching.

3.2.6.3 UV-spectroscopy

The ultra violet (UV) and visible absorption spectra of the antimicrobial compounds were analyzed by using T80+UV/VIS spectrometer (Thermo Scientific, USA). The active compounds were dissolved in methanol and the spectrums were recorded at 200-800 nm range using UV-Probe software.

3.2.7 Statistical analysis

The experiments were done in triplicate. The statistical difference of the mean zone of inhibition of antimicrobial-producing bacterial soil isolate for individual test bacterium was carried out by using one-way analysis of variance (ANOVA) followed by post-hoc Fisher's LSD test at a significance level of $p < 0.05$. Significant difference between incubation temperature at 30 °C and 37 °C were compared by using Student's *t*-test. A $p < 0.05$ of *t*-test denoted the presence of a statistically significant difference.

CHAPTER IV

RESULTS

4.1 Identification of antimicrobial-producing bacterial soil isolate PJ85

According to the previous study, a total of 123 bacterial soil isolates were isolated from SUT. They were tested for antimicrobial activity against test pathogens including Gram-positive and Gram-negative bacteria. The result showed that strain PJ85 showed high antibacterial activity against test pathogens. Therefore, strain PJ85 obtained from previously study was used in this study.

Isolate PJ85 was Gram-positive, aerobic and filamentous in nature (Figure 4.2). Morphological observation of 14-day-old culture grown on ISP-2 agar medium revealed rich growth of aerial and vegetative hyphae with convex and wrinkled colony morphology. The color of aerial and vegetative hyphae was light and strong yellow, respectively. Strain PJ85 also produced strong yellow diffusible pigment after incubation for 14 days at 37 °C (Figure 4.1). The 16S rDNA of PJ85 was sequenced and compared to the reference sequences of the EzBiocloud database (https://www.ezbiocloud.net). The result revealed that 16S rRNA gene sequence of strain PJ85 (1493 nt) (Figure 4.3) was closely related to the members of the genus *Streptomyces*. The strain PJ85 shared the highest 16S rDNA sequence similarity to *Streptomyces actinomycinicus* RCU-197^{T} (98.90%), *Streptomyces echinatus* NBRC12763^{T} (97.80%), *Streptomyces graminisoli* JR-19^{T} (97.51%), *Streptomyces*

shenzhenensis 172115^{T} (97.30%) and *Streptomyces sasae* JR-39^{T} (97.23%) (Table 4.1). The nucleotide sequence of 16S rRNA gene of PJ85 was submitted to the GenBank (MK580459).

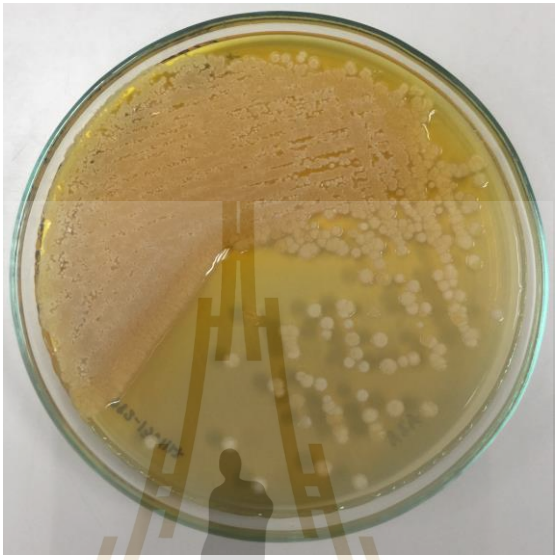


Figure 4.1 The colony morphology of strain PJ85 grown on ISP-2 agar medium after incubation at 37 °C for 14 days.

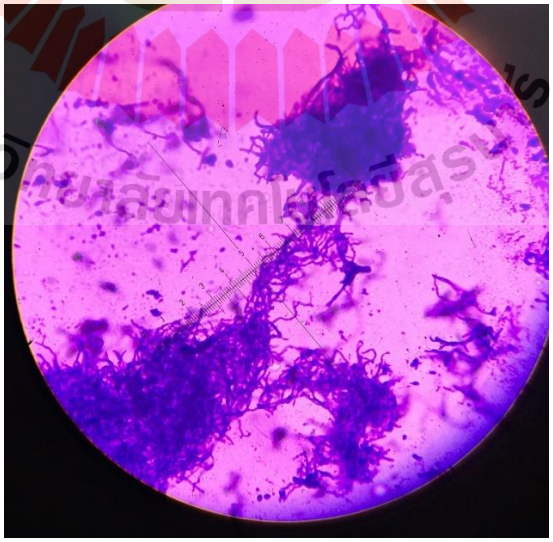


Figure 4.2 Microscopic morphology of PJ85 showing long filamentous, branched and Gram-positive bacilli under light microscope at 1000x magnification.

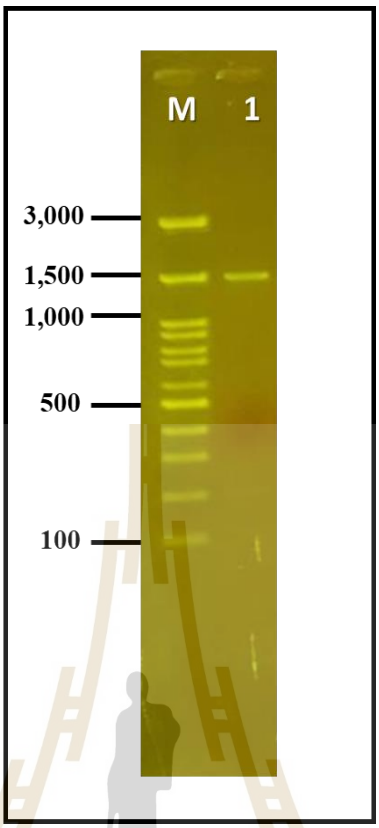


Figure 4.3 Agarose gel electrophoresis (0.8%) of PCR amplification product of 16S rRNA gene using primers, 27F and 1525R. Lane M: 100bp DNA ladder M (Himedia, India), Lane 1: PCR product of PJ85.

In order to study an evolutionary connections between PJ85 and other streptomycetes, a phylogenetic analysis based on 16S rRNA gene distance using the neighbor-joining method was constructed. In the neighbor-joining tree, PJ85 formed the same clade with its closest relative type strain obtained from EzBiocloud database (Figure 4.4). Strain PJ85 shared a node to *S. actinomycinicus* RCU-197^{T} with a bootstrap value 100%. Therefore, PJ85 could be classified as *S. actinomycinicus*.

Table 4.1 The closest relatives of 16S rRNA gene of bacterial soil isolate PJ85.

No.	Closest relative strains	Accession No.	Similarity (%)
1.	*Streptomyces actinomycinicus* RCU-197^T	LC069046	98.90
2.	*Streptomyces echinatus* NBRC12763^T	AB184126	97.80
3.	*Streptomyces graminisoli* JR-19^T	HQ267975	97.51
4.	*Streptomyces shenzhenensis* 172115^T	HQ660226	97.30
5.	*Streptomyces sasae* JR-39^T	HQ267987	97.23
6.	*Streptomyces neopeptinius* KNF 2047^T	EU258679	97.18
7.	*Kitasatospora psammotica* NBRC 13971^T	AB184554	97.16
8.	*Streptomyces rhizophilus* JR-41^T	HQ267989	97.09
9.	*Streptomyces caeruleatus* NRRL B-24802^T	KQ948975	97.08
10.	*Streptomyces gramineus* JR-43^T	HM748598	97.07
11.	*Streptomyces capoamus* JCM 4734^T	AB045877	97.02
12.	*Streptomyces bungoensis* DSM 41781^T	KQ948892	97.02
13.	*Streptomyces panaciradicis* 1MR-8^T	KF971876	97.02
14.	*Streptomyces lanatus* NBRC 12787^T	AB184845	96.95
15.	*Streptomyces filipinensis* NBRC 12860^T	AB184198	96.95
16.	*Streptomyces yaanensis* Z4^T	JQ307192	96.95
17.	*Streptomyces galbus* DSM 40089^T	X79852	96.94
18.	*Streptomyces hyaluromycini* NBRC 110483^T	BCFL01000051	96.94
19.	*Streptomyces regensis* NRRL B-11479^T	LFVR01000689	96.91

Table 4.1 The closest relatives of 16S rRNA gene of bacterial soil isolate PJ85. (Continued).

No.	Closest relative strains	Accession No.	Similarity (%)
20.	*Streptomyces longwoodensis* DSM 41677^T	KQ948572	96.88
21.	*Streptomyces durhamensis* NRRL B-3309^T	JNXR01000068	96.80
22.	*Streptomyces lucensis* NBRC 13056^T	AB184280	96.80
23.	*Streptomyces jiujiangensis* JXJ 0074^T	KF938657	96.80
24.	*Streptomyces gilvifuscus* T113^T	KM229362	96.79
25.	*Streptomyces olivaceoviridis* NBRC 13066^T	AB184288	96.73
26.	*Streptomyces canarius* NBRC 13431^T	AB184396	96.66
27.	*Streptomyces cyaneus* NRRL B-2296^T	AF346475	96.59
28.	*Streptomyces corchorusii* DSM 40340^T	KQ948396	96.59
29.	*Streptomyces humi* MUSC 119^T	LBMU02000042	96.59
30.	*Streptomyces achromogenes* subsp. *achromogenes* NBRC 12735^T	AB184109	96.52
31.	*Streptomyces cerasinus* SR3-134^T	LC128347	96.52
32.	*Streptomyces similanensis* KC-106^T	AB773850	96.52
33.	*Streptomyces violaceus* NRRL B-2867^T	KL569104	96.51
34.	*Streptomyces roseoviolaceus* ISP 5277^T	AJ399484	96.51
35.	*Streptomyces cyslabdanicus* K04-0144^T	AB915216	96.45
36.	*Streptomyces melanogenes* NBRC 12890^T	AB184222	96.45
37.	*Streptomyces niveiscabiei* S78^T	AF361786	96.45

Table 4.1 The closest relatives of 16S rRNA gene of bacterial soil isolate PJ85. (Continued).

No.	Closest relative strains	Accession No.	Similarity (%)
38.	*Streptomyces naganishii* NBRC 12892^T	AB184224	96.45
39.	*Streptomyces noboritoensis* NBRC 13065^T	AB184287	96.45
40.	*Streptomyces canus* DSM 40017^T	KQ948708	96.44
41.	*Streptomyces shaanxiensis* CCNWHQ 0031^T	FJ465151	96.41
42.	*Streptomyces variegatus* NRRL B-16380^T	JYJH01000110	96.38
43.	*Streptomyces crystallinus* NBRC 15401^T	AB184652	96.38
44.	*Streptomyces curacoi* DSM 40107^T	KQ948008	96.38
45.	*Streptomyces flavovariabilis* NRRL B-16367^T	JNXD01000071	96.38
46.	*Streptomyces recifensis* NBRC 12813^T	AB184165	96.38
47.	*Streptomyces aridus* H9^T	LT594571	96.36

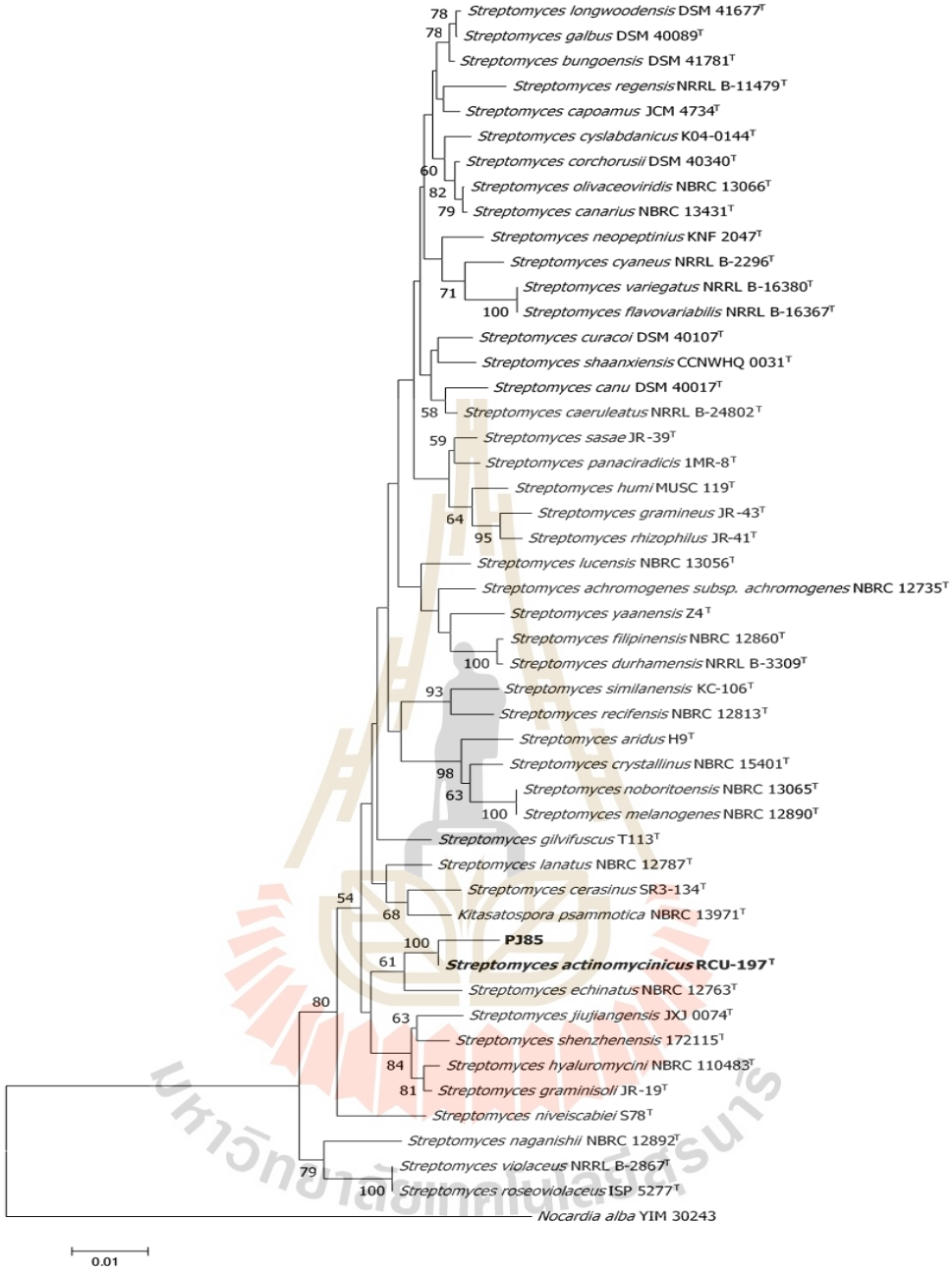


Figure 4.4 Neighbor-joining phylogenetic tree (Kimura's two-parameter model) based on relative 16S rDNA sequences showing the phylogenetic relationships among *Streptomyces* sp. PJ85 and its closest type strains. Numbers at nodes indicate percentages of bootstrap (1,000 replicates), only values greater than 50% are shown. The bar indicates a distance of 0.01 substitutions per nucleotide position.

4.2 Antibacterial activity of PJ85 by perpendicular streak method

Antibacteria activity of *Streptomyces* sp. PJ85 was tested against MRSA DMST20651, *S. aureus* ATCC29213, *S. epidermidis* TISTR518, *B. subtilis* TISTR008, *B. cereus* TISTR687, *E. coli* TISTR780, *E. aerogenes* TISTR1540, *P. aeruginosa* TISTR781, *S. marcescens* TISTR1354, *P. mirabilis* TISTR100 and *S. typhimurium* TISTR292 by perpendicular streak method. The result showed that *Streptomyces* sp. PJ85 exhibited antimicrobial activity against Gram-positive bacteria including MRSA while Gram-negative organisms were not affected.

The maximum value of inhibition zones (mm ± SD) of antibacterial activity of *Streptomyces* sp. PJ85 was found against MRSA DMST20651 (50.00 ± 0.00) followed by *S. epidermidis* TISTR518 (48.33 ± 2.89), *S. aureus* ATCC29213 (46.67 ± 0.58), *B. subtilis* TISTR008 (45.00 ± 3.00) and *B. cereus* TISTR687 (38.33 ± 1.15). The result of antimicrobial activity of *Streptomyces* sp. PJ85 against test pathogens by perpendicular streak method was shown in Table 4.2. From our results, the zone of inhibition of antimicrobial activity of PJ85 against MRSA DMST20651 was significantly higher than *B. subtilis* TIRTR008 and *B. cereus* TISTR687 ($p < 0.05$). Although antimicrobial activity of PJ85 against MRSA DMST20651 was not statistically different from *S. aureus* ATCC29213 and *S. epidermidis* TISTR518 ($p > 0.05$), strain PJ85 exhibited larger zone of inhibition than it did with *S. aureus* and *S. epidermidis* (Table 4.2).

Table 4.2 Antimicrobial activity of *Streptomyces* sp. PJ85 against test pathogens by perpendicular streak method.

Test pathogens	**Zone of inhibition (mm)**
MRSA DMST20651	50.00 ± 0.00^{a}
S. aureus ATCC29213	46.67 ± 0.58^{ab}
S. epidermidis TISTR518	48.33 ± 2.89^{ab}
B. subtilis TISTR008	45.00 ± 3.00^{b}
B. cereus TISTR687	38.33 ± 1.15^{c}

Data are means ± standard deviation (n=3); a, b and c: different letters represent significance (LSD, *p*-value < 0.05).

4.3 Incubation temperature modurate growth and antimicrobial activity of PJ85

The cultural temperature for growth and antimicrobial activity of PJ85 was studied on ISP-2 medium. Since PJ85 culture on ISP-2 exhibited highest antimicrobial activity compared to others medium (Chanthasena, 2015). In order to study the effect of incubation temperature on growth and antimicrobial activity, PJ85 was incubated at two temperature, 30 °C and 37 °C. The fermented broth of PJ85 was collected every day for 14 days. Cell biomass and cell free supernatant were separated by filtration. The cell pellet of PJ85 was dried to obtain cell dry weight while cell free supernatant was used to prepare ethyl acetate crude extracts. The result showed that the maximum growth was observed at 30 °C on day 3 of cultivation with biomass yield of 4.40 ± 0.25 mg/ml (Figure 4.5). The extracts were then used for a determination of antibacterial activity using disc diffusion method. The result revealed that an antimicrobial activity

of PJ85 crude extracts against Gram-positive bacteria was highest at day 5. An antimicrobial activity was stable from day 5 to day 9 of cultivation and begining to decrease at day 10 (Figure 4.6).

According to Student's *t*-test analysis (Table 4.3), antimicrobial activity of PJ85 crude extracts obtained from 37 °C grown cells possessed significant larger zone of inhibition against *B. subtilis* TISTR008 and *B. cereus* TISTR687 than that of 30 °C (two-tailed *t*-test, $p < 0.05$). Although an antibacterial activities against MRSA DMST20651, *S. aureus* ATCC29213 and *S. epidermidis* TISTR518 were not statistically different at both cultural temperatures, the activities were somewhat higher when cultured the cells at 37 °C than at 30 °C. Thus, the 37 °C cultivation temperature and 5 days incubation period were applied for a preparation of crude extracts in order to obtain a maximal yield of antibacterial activity.

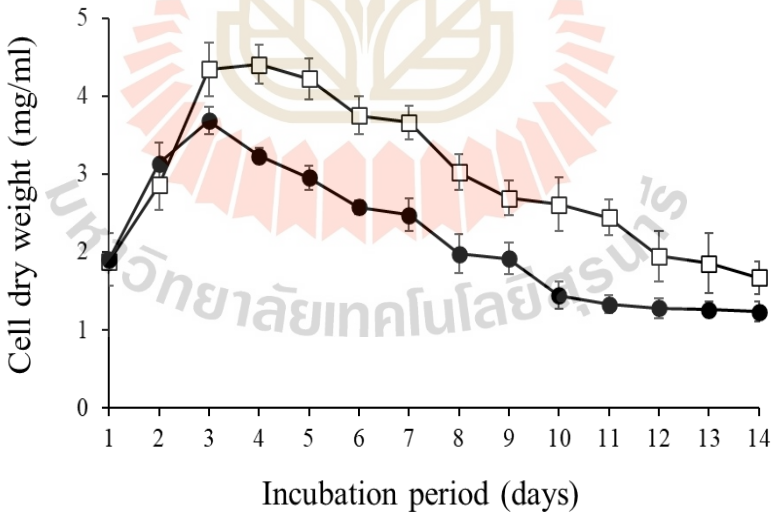


Figure 4.5 The effect of incubation temperature on growth of *Streptomyces* sp. PJ85 cultured with ISP-2 medium. Incubation temperatures: (□) 30 °C, (●) 37 °C.

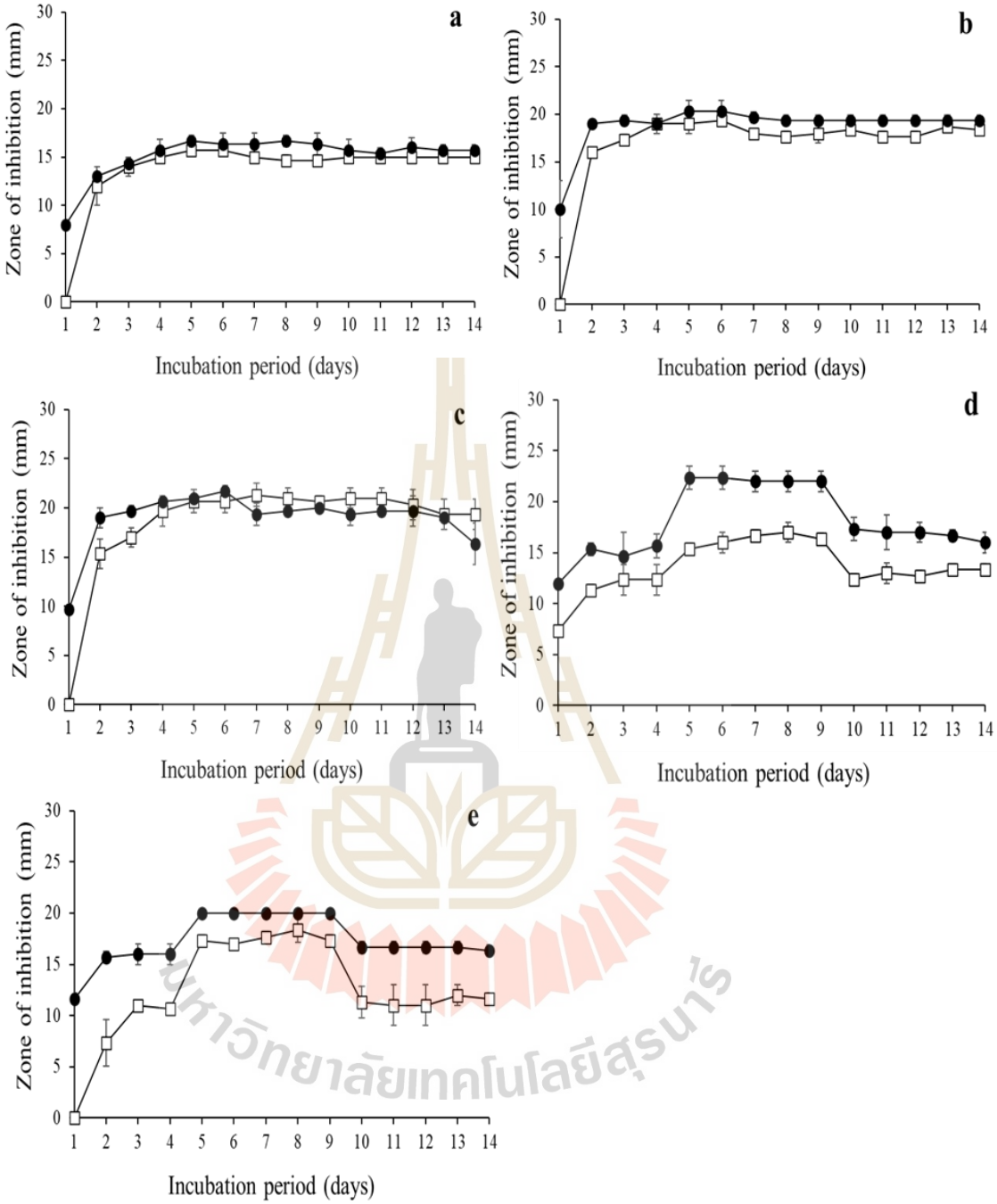


Figure 4.6 Effect of incubation temperature on the antimicrobial activity of *Streptomyces* sp. PJ85 cultured with ISP-2 medium against (a) MRSA DMST20651 (b) *S. aureus* ATCC29213 (c) *S. epidermidis* TISTR518 (d) *B. subtilis* TISTR008 (e) *B. cereus* TISTR687. Incubation temperatures: (□) 30 °C, (●) 37 °C.

Table 4.3 Inhibition zone diameter of crude ethyl acetate of *Streptomyces* sp. PJ85 collected at day 5 of cultivation.

Test microorganisms	Zone of inhibition (mm)		*p*-value
	at 30 °C	at 37 °C	
MRSA DMST20651	15.67±0.58	16.67±0.58	0.259
S. aureus ATCC29213	19.33±0.58	20.33±1.15	0.188
S. epidermidis TISTR518	21.33±1.15	21.67±0.58	0.757
B. subtilis TISTR008	17.00±1.00	22.33±1.15*	0.001
B. cereus TISTR687	18.33±1.15	20.00±0.00*	0.003

Each value represents mean ± SD of three independent experiments.

*: at confidence level of 95%, *p*-values < 0.05 is acceptable.

4.4 Crude compounds preparation and MIC values

The cultural conditions of PJ85 that yielded a highest antimicrobial activity was used to prepare the crude compounds. Therefore, PJ85 was inoculated in ISP-2 medium (400 ml in 1,000 ml Erlenmeyer flask) and incubated at 37 °C for 5 days. After incubation, cell free supernatant was collected to extract the crude compounds using ethyl acetate. The crude compounds of PJ85 was yellowish-orange in color (Figure 4.7). The yield of crude compounds was 246.54 ± 17.12 mg/g of cell dry weight.

The yellowish-orange crude ethyl acetate of PJ85 was used for an evaluation of minimum inhibitory concentration (MIC) by using broth two-fold macro-dilution method. MIC values of crude ethyl acetate of PJ85 against MRSA DMST20651, *S. aureus* ATCC29213, *S. epidermidis* TISTR518, *B. subtilis* TISTR008 and *B. cereus* TISTR687 were 2, 2, 16, 2 and 1 μg/ml, respectively (Table 4.4).

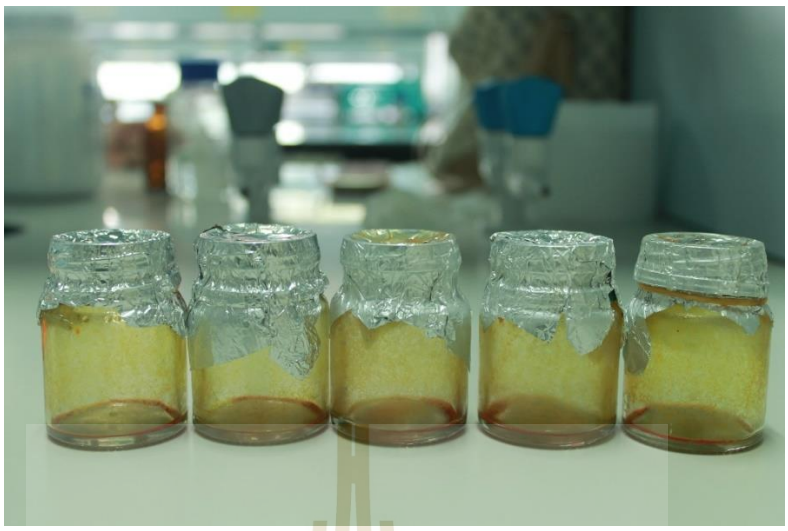


Figure 4.7 The crude compounds of PJ85 extracted by using ethyl acetate.

Table 4.4 The MIC values of crude ethyl acetate of PJ85 against test microorganisms.

Test pathogens	MICs (*μ*g/ml)			
	Crude	**Oxacillin**	**Vancomycin**	**Tetracycline**
MRSA DMST20651	2	512	2	32
S. aureus ATCC29213	2	0.125	1	0.5
S. epidermidis TISTR518	16	0.125	2	0.0625
B. subtilis TISTR008	2	0.25	0.5	4
B. cereus TISTR687	1	64	2	0.0312

Each value is a mean of n=3 experiments.

4.5 Purification of active compounds of *Streptomyces* sp. PJ85 by thin layer chromatography and bioautography analysis

The separation of antimicrobial metabolites present in yellowish-orange crude compounds was performed by using TLC. The mobile phase used to develop the plate

was chloroform:n-hexane (9.5:0.5, v/v). After running, the TLC plates were dried and used for detection of active bands on chromatogram by using contact bioautography. This assay has successfully been used to determine active spots with inhibitory effect on microbial growth (Rios, Recio and Villar, 1988; Dewanjee, Gangopadhyay, Bhattacharya, Khanra and Dua, 2015; Jumpathong, Nuengchamnong, Masin, Nakaew and Suphrom, 2019).

The result of bioautography revealed two active bands, compound 1 and compound 2, on TLC plate. These compounds exhibited antimicrobial activity against Gram-positive bacteria including MRSA (Figure 4.8). Based on LC-MS analysis, compound 1 was identified as actinomycin D. Actinomycin D has been known as a secondary metabolite produced by many streptomycetes including *S. actinomycinicus* RCU-197^{T}, the closest related strain of PJ85 (Figure 4.4). It has been reported that the sole antimicrobial agent produced by *S. actinomycinicus* RCU-197^{T} is actinomycin D (Tanasupawat, Phongsopitanun, Suwanborirux, Ohkuma and Kudo, 2016). However, an unidentified bioactive agent, compound 2, was detected from PJ85 crude extracts (Figure 4.8). Compound 2 showed antibacterial activity against several Gram-positive test pathogens. The result of antibacterial activity of compound 2 are shown in Table 4.5.

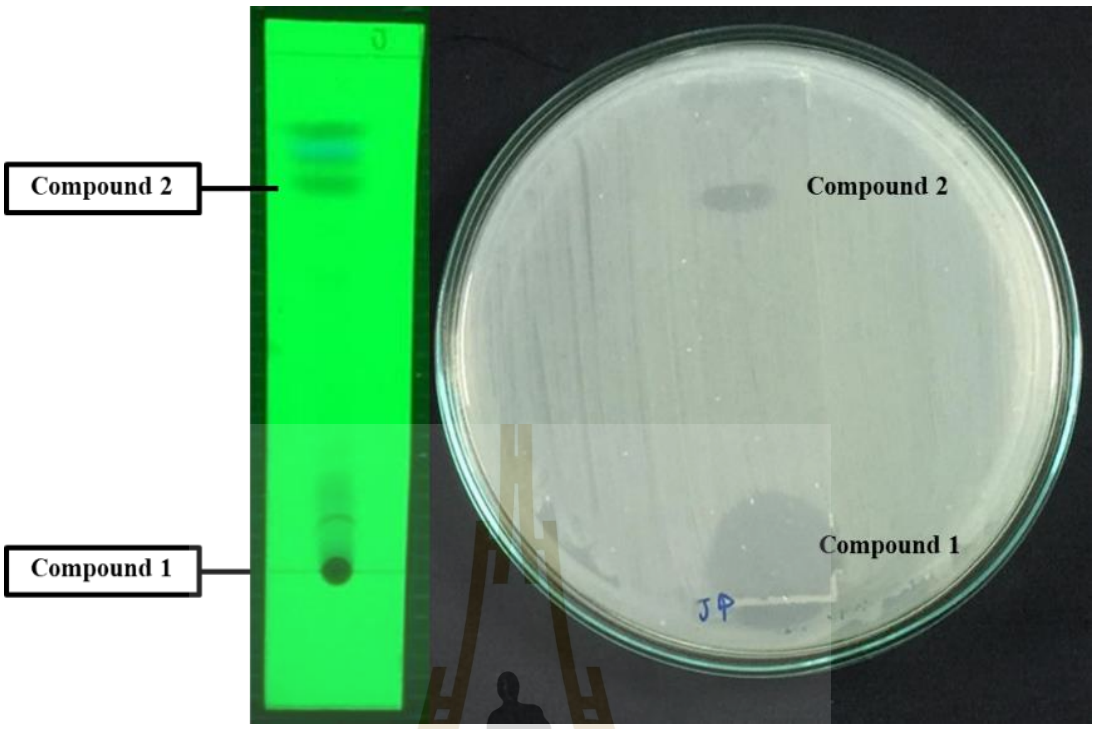


Figure 4.8 TLC-based bioautography of crude ethyl acetate of PJ85 exhibited antibacterial activity against MRSA DMST20651.

Table 4.5 Antibacterial activity of compound 2 against test microorganisms based on contact bioautography method.

Test microorganisms	Compound 2
MRSA DMST20651	+
S. aureus TISTR1466	+
S. epidermidis TISTR518	+
B. subtilis TISTR008	+

-: no activity; +: activity

In order to identify compound 2, the band consistent with compound 2 was carefully scrapped and used for re-purification by TLC. The solvent used for separation of compound 2 was chloroform:n-hexane:methanol (9.5:0.5:0.5 v/v). After ascending, the TLC plate was dried and observed visually under UV light. The result showed that compound 2 was separated to two bands, compound 2a and compound 2b, at Rf value 0.750 and 0.825, respectively (Figure 4.9). The antimicrobial activity of compound 2a and compound 2b were detected by using contact bioautography. The result revealed that only compound 2a showed antimicrobial activity. It should be noted that compound 2a displayed a weak signal on chromatogram though it showed a relatively high antibacterial activity against MRSA in bioautography (Figure 4.9). Compound 2a was then scrapped from TLC plate and used for characterization by LC-MS and SR-FTIR microspectroscopy.

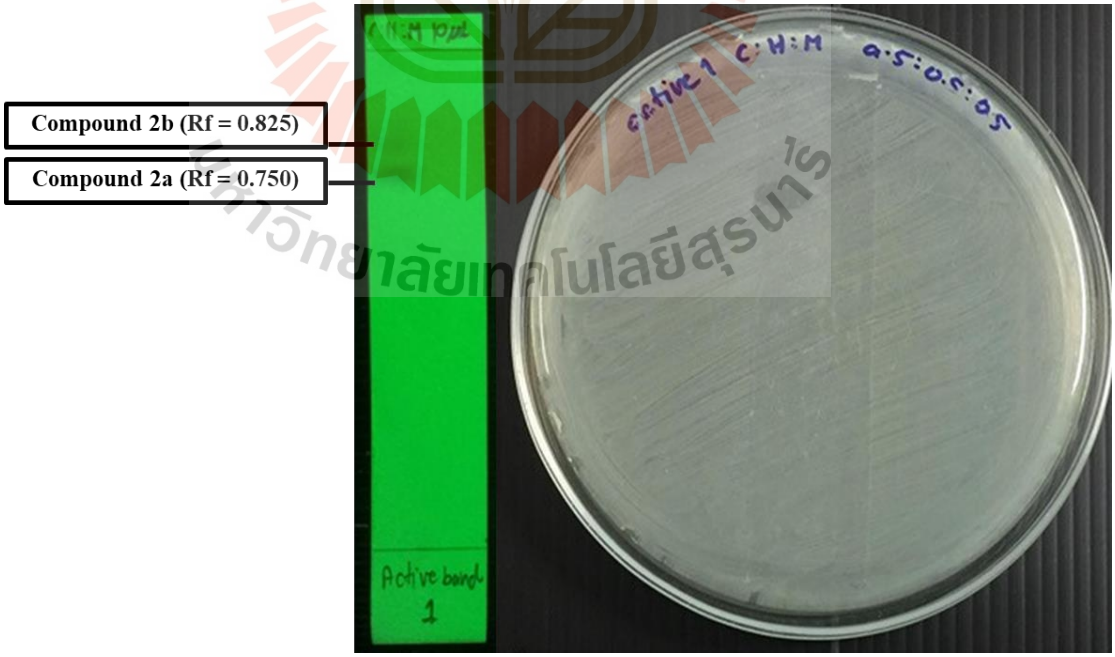


Figure 4.9 TLC-based bioautography of compound 2a exhibited antibacterial activity against MRSA DMST20651.

4.6 LC-MS analysis and UV-spectroscopy

LC-MS analysis was used for identification of antimicrobial compounds, compound 1 and compound 2a, produced by *Streptomyces* sp. PJ85. The MS peaks were analyzed and identified by matching the mass spectra with the MassBank Europe Mass Spectral Data Base (https://massbank.eu/MassBank/Search).

In order to identify compound 1, the MS spectrum at the retention time of 43.9 min with molecular ion peak at *m/z* 1255.6466 $[M+H]^+$ correlated to actinomycin D which was a major compound produced from the closest type strain, *S. actinomycinicus* RCU-197^T. The UV spectrum of compound 1 exhibited UV-VIS maxima at 225 nm and 446 nm which was similar to actinomycin D (240 nm and 445 nm) (Furukawa, Inoue and Asano, 1968). Therefore, compound 1 produced by PJ85 was identified as actinomycin D. The summarization of peak retention time, molecular weight and the nearest compounds hit for three major peaks of compound 1 were shown in Table 4.6.

Based on LC-MS analysis, compound 2a showed four major peaks at the retention time of 22.8, 34.3, 34.8 and 35.5 min (Figure 4.11a). The MS of these peaks showed molecular ions M^+ at *m/z* 679, 274, 274 and 290 of molecular weight, respectively. According to MassBank library search, the peak at the retention time of 22.8 min was matched to luteone 4',7-O-diglucoside, medicagenic acid base+O-HexA and tricaffeoyl quinic acid. Two molecular ion peaks at the retention time 34.3 min and 34.8 min were isomeric which correlated to chlormezanone, fenpropidin, lauryldiethanolamine, metazachlor OXA and modafinil. While, the peak at the retention time 35.5 min was corresponds to 6,7-dimethoxy-2-(1-piperazinyl)-4-quinazolinamine, adrafinil, atropine, benzoylecgonine and chlophedianol. The summarization of peak retention time, molecular weight and the nearest compounds hit for four major peaks were shown

in Table 4.7. However, there are reports on no antimicrobial activity of these compounds, except for tricaffeoyl quinic acid (Scholz, Heinrich and Hunkler, 1994). The data of molecular weight and antibacterial activity of compound 2a provided the evidence that this compound could be tricaffeoyl quinic acid.

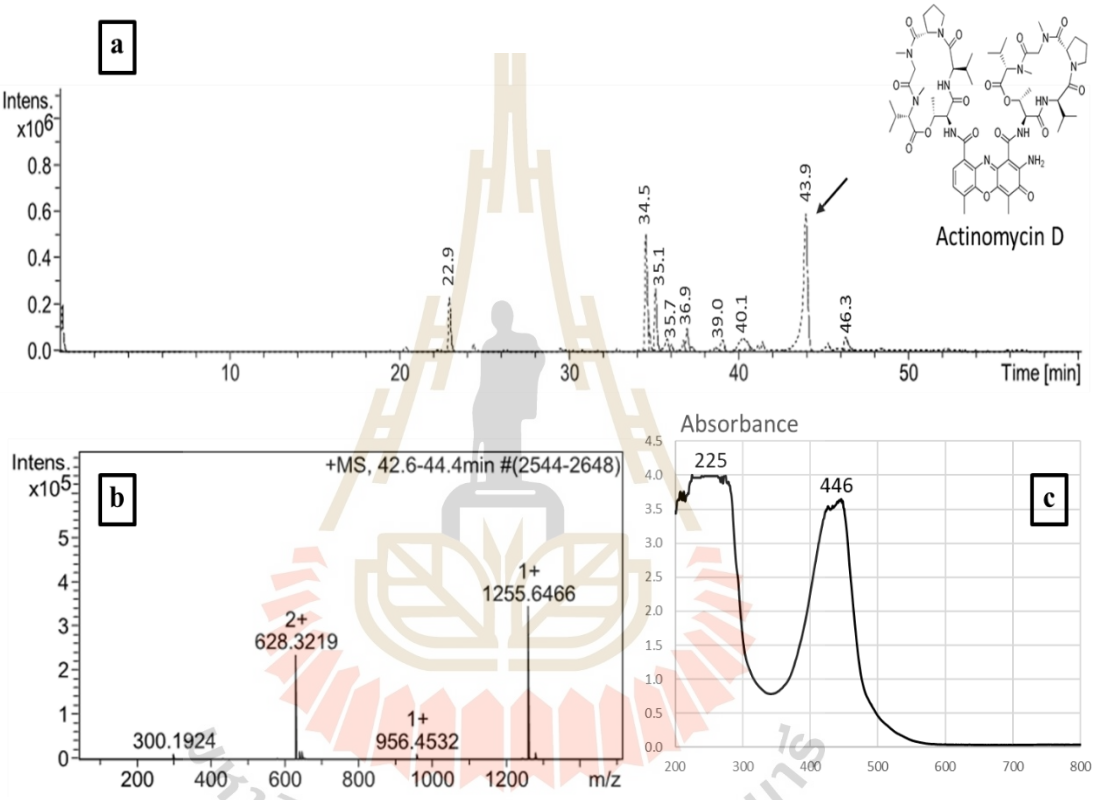


Figure 4.10 Liquid chromatography-mass spectrometry (LC-MS) analysis of compound 1 in positive ion mode. (a) LC-MS chromatogram of compound 1 of PJ85. (b) MS spectrum with the retention time 43.9 showing the ion clusters for $[M+H]^+$ at *m/z* 1255.6466 correlated to actinomycin D. (c) UV spectrum of compound 1 with the maximum at 225 and 446 nm.

Table 4.6 The nearest chemical compounds when match to MassBank database detected in compound 1 of *Streptomyces* sp. PJ85.

Retention time (min)	**Name of the compound**	**Chemical formula**	**Molecular weight (g/mol)**
22.9	Bryoamaride	$C_{36}H_{54}O_{12}$	678.82
22.9	Medicagenic acid base + O-HexA	$C_{36}H_{54}O_{12}$	678.80
34.5	Chlormezanone	$C_{11}H_{12}CINO_{35}$	273.74
34.5	Fenpropidin	$C_{19}H_{31}$	273.50
34.5	Lauryldiethanolamine	$C_{16}H_{35}NO_2$	273.45
34.5	Metazachlor OXA	$C_{14}H_{15}N_3O_3$	273.29
34.5	Modafinil	$C_{15}H_{15}NO_2S$	273.40
34.5	Tilidine	$C_{17}H_{23}NO_2$	273.37
34.5	Tolcapone	$C_{14}H_{11}NO_5$	273.24
43.9	Actinomycin D / Dactinomycin	$C_{62}H_{86}N_{12}O_{16}$	1255.42

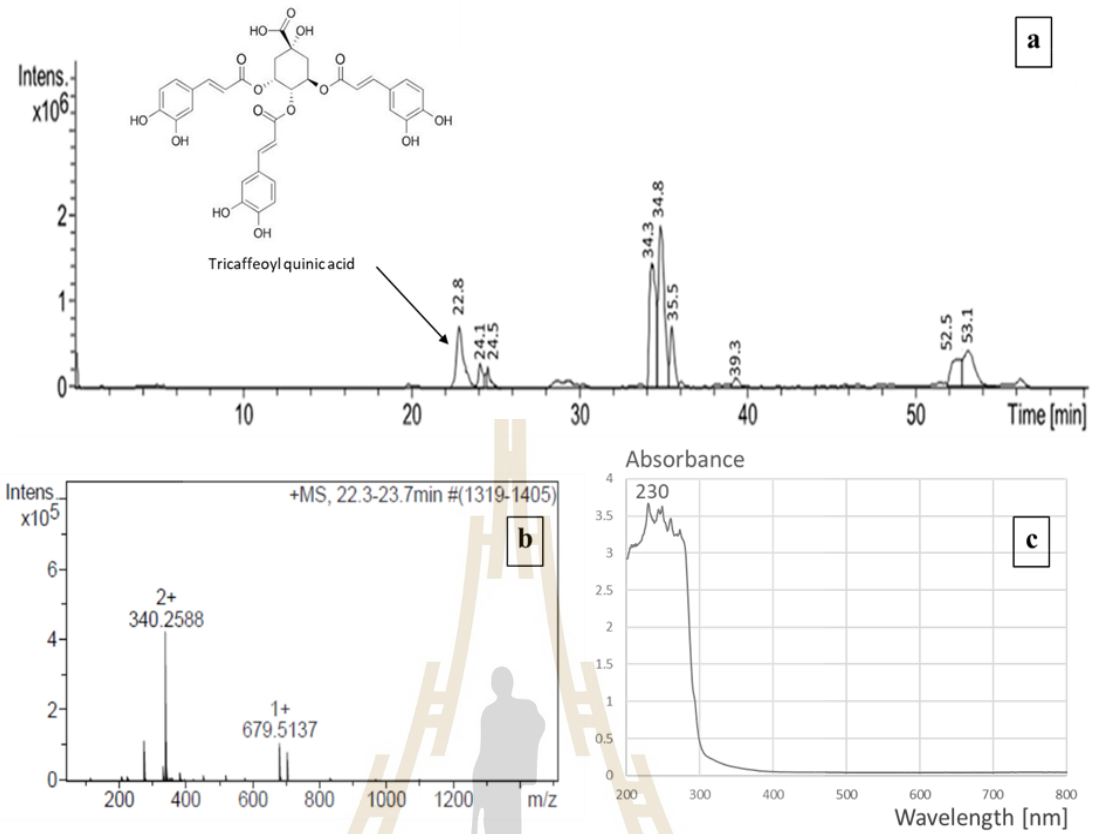


Figure 4.11 Liquid chromatography-mass spectrometry (LC-MS) analysis of compound 2a in positive ion mode. (a) LC-MS chromatogram of compound 2a of PJ85. (b) MS spectrum with the retention time 22.8 showing the ion clusters for $[M+H]^+$ at *m/z* 679.5137 correlated to tricaffeoyl quinic acid. (c) UV spectrum of compound 2a with the maximum at 230 nm.

Table 4.7 The nearest chemical compounds when match to MassBank database detected in compound 2a of *Streptomyces* sp. PJ85.

Retention time (min)	Name of the compound	Chemical formula	Molecular weight (g/mol)
22.8	Luteone 4',7-O-diglucoside	$C_{32}H_{38}O_{16}$	678.22
22.8	Medicagenic acid base+O-HexA	$C_{36}H_{54}O_{12}$	678.80
22.8	Tricaffeoyl quinic acid	$C_{34}H_{30}O_{15}$	678.60
34.3, 34.8	Chlormezanone	$C_{11}H_{12}ClNO_3S$	273.74
34.3, 34.8	Fenpropidin	$C_{19}H_{31}N$	273.50
34.3, 34.8	Lauryldiethanolamine	$C_{16}H_{35}NO_2$	273.45
34.3, 34.8	Metazachlor OXA	$C_{14}H_{15}N_3O_3$	273.29
34.3, 34.8	Modafinil	$C_{15}H_{15}NO_2S$	273.40
35.5	6,7-Dimethoxy-2-(1-piperazinyl)-4-quinazolinamine	$C_{14}H_{19}N_5O_2$	289.33
35.5	Adrafinil	$C_{15}H_{15}NO_3S$	289.40
35.5	Atropine	$C_{17}H_{23}NO_3$	289.40
35.5	Benzoylecgonine	$C_{16}H_{19}NO_4$	289.33
35.5	Chlophedianol	$C_{17}H_{20}ClNO$	289.80

4.7 SR-FTIR microspectroscopy

The funtional groups of compound 1 and compound 2a were examined by SR-FTIR microspectroscopy. The functional groups of compound 1 include amides, alkanes, cyclick alkanes and methyl group. IR spectrum showed the peaks at 2919 cm^{-1}, 1642 cm^{-1}, and 1448 cm^{-1} (Figure 4.12). Our data were in agreement with those observed by Furukawa and collegues (1968) when they tested the FTIR spectrum of actinomycin D (Figure 4.13) (Furukawa, Inoue and Asano, 1968).

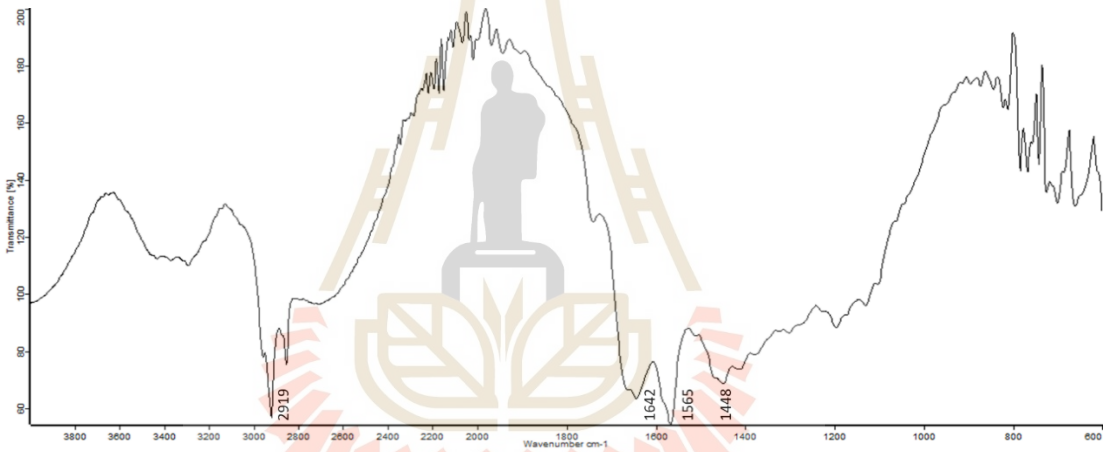


Figure 4.12 SR-FTIR analysis of compound 1 produced by *Streptomyces* sp. PJ85.

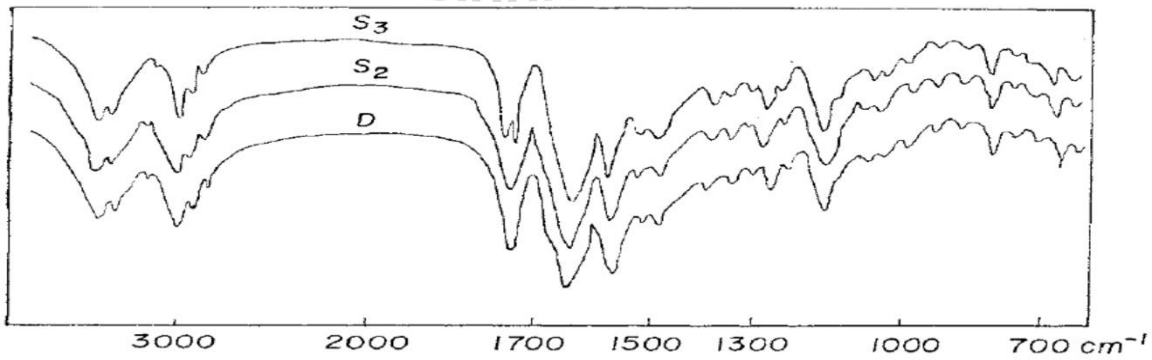


Figure 4.13 Infrared absorption spectra of S3 (actinomycin S3), S2 (actinomycin S2) and D (actinomycin D) (Furukawa, Inoue and Asano, 1968).

The FTIR spectroscopy spectra of compound 2a showed peaks corresponding to standard library spectra (Figure 4.14). Compound 2a in IR spectrum exhibited the peaks at 2924 and 2855 cm^{-1} referring to C-H stretching frequency. The peak at 1567 cm^{-1} indicates C=C stretching. The results of infrared (IR) spectrum revealed that compound 2a contains functional groups as alkane and cyclic alkene. These functional groups are also present in tricaffeoyl quinic acid. Thus, a result of SR-FTIR microspectroscopy supported that compound 2a might be classified as tricaffeoyl quinic acid.

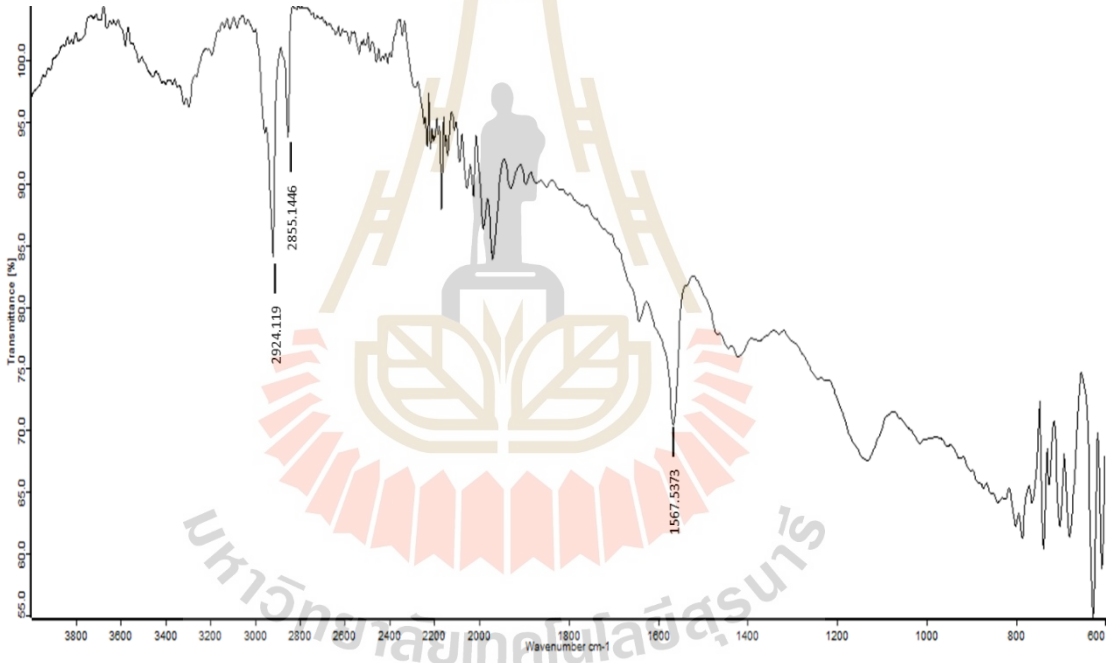


Figure 4.14 SR-FTIR analysis of compound 2a produced by *Streptomyces* sp. PJ85.

CHAPTER V

DISCUSSION AND CONCLUSION

Emergence of antibiotic resistance has been recognized as a worldwide health-threatening problem. Therefore, there is the need of novel therapeutics to replace an ineffective antimicrobial drug. Natural products are the main source of antimicrobial agents, the majority of which are produced by the genus *Streptomyces* (Roca et al., 2015; Ventola, 2015; Aslam et al., 2018). In this study, an antimicrobial-producing *Streptomyces* strain PJ85 was isolated from forest SUT soil. Molecular analysis was used to identify strain PJ85. The identification of microorganisms by molecular technique has many advantages such as rapid, less laborious, sensitive, specific and efficient (Lane 1991; Adzitey, Huda, Rusul and Ali, 2013; Wang and Salaza, 2016; Tan et al., 2018; Al-Dhabi et al., 2019). Based on the study of 16S rRNA gene sequence and phylogenetic relationship, strain PJ85 lies in a same clade with *S. actinomycinicus* RCU-197^{T} (JCM 30864^{T}). The 16S rRNA gene of PJ85 shared 98.90% sequence similarity with *S. actinomycinicus* RCU-197^{T}. Hence, it was identified as *S. actinomycinicus* PJ85. According to Stackebrandt and Goebel (1994), two strains were considered as belonging to different species if they shared 16S rRNA gene sequence similarity lower than 97% (Stackebrandt and Goebel, 1994; Rossi-Tamisier, Benamar, Raoult and Fournier, 2015; Beye, Fahsi, Raoult and Fournier, 2018)

S. actinomycinicus RUC-197^T was first discovered in 2016 (Tanasupawat et al., 2016). It was isolated from soil sample of a peat swamp forest in Rayong province, Thailand. Based on perpendicular-streak method, strain RCU-197^T was active against *Micrococcus luteus*, *S. aureus*, *B. subtilis*, *P. aeruginosa* and *Candida albicans* (Tanas upawat and Suwanborirux, 2014). To date there is no available reports regarding the isolation and characterization of antibacterial agents of *S. actinomycinicus*. In this study, *S. actinomycinicus* PJ85 was isolated and tested for antimicrobial activity against Gram-positive and Gram-negative bacteria. Strain PJ85 showed narrow antibacterial spectrum against Gram-positive bacteria including MRSA DMST20651, *S. aureus* ATCC29213, *S. epidermidis* TISTR518, *B. subtilis* TISTR008 and *B. cereus* TISTR687. The narrow spectrum antibacterial is considered as the activity of substances that acts against either Gram-positive or Gram-negative bacteria. While, broad spectrum antibacterial is effect both Gram-positive and Gram-negative bacteria (Ullah and Ali, 2017). Usually, the narrow spectrum antibacterial is considered ideal antibacterial and preferred over the broad-spectrum. The reason is that the narrow-spectrum antibiotics do not kill as many of the normal flora in the body as the broad-spectrum antibiotics (Ullah and Ali, 2017). Also, the narrow-spectrum antibiotic causes less resistance of the bacteria due to its effect only specific bacteria (Ullah and Ali, 2017).

Our result revealed that an antibacterial activity of PJ85 cultured at 37 °C was higher than 30 °C. The exhibition of higher antimicrobial activity of PJ85 at a slightly elevated temperature might be useful in industrial process. The advantages of industrial fermentation under a high temperature include faster reaction times and reduced cooling

costs for large-scale fermentation (James and Edwards, 1988). Thus, PJ85 could be a potential candidate for a production of low-cost industrial antibiotics.

In order to extract the crude compounds of PJ85, different solvents such as n-hexane, n-butanol, chloroform, ethyl acetate, ethanol and methanol were tested. However, ethyl acetate crude extracts showed highest antimicrobial activity compared to other solvents. It has been shown that ethyl acetate is a suitable solvent for an extraction of antimicrobial metabolites from *Streptomyces* spp. (Arusa et al., 2014; Shetty et al., 2014; Baskaran, Mohan, Sivakumar and Kumar, 2016). Edirisinghe et al. (1998) reported that bioactive compounds extracted by ethyl acetate exhibited a medium polarity (Edirisinghe, Perera and Bamunuarachchi, 1998). Thus, active metabolites contained in crude extracts of PJ85 could show a medium polarity. MIC value of PJ85 crude compounds against test microorganisms was determined by broth dilution method. The results indicated that MIC of crude extracts ranging from 1 to 16 μg/ml. The lowest MIC value, 1 μg/ml, was found against *B. cereus* TISTR687 while the highest 16 μg/ml MIC value was observed against *S. epidermidis* TISTR518. Crude compounds of PJ85 also inhibited the growth of MRSA DMST20651, *S. aureus* ATCC29213 and *B. subtilis* TISTR008 with 2 μg/ml MIC value.

The genus of *Streptomyces* has been known to produce several bioactive secondary metabolites possessing antimicrobial activity. There are substantial reports associated with the study of LC-MS for chemical analysis of *Streptomyces* spp. (Naumann, 2006; Dewanjee et al., 2015; Manimaran, Gopal and Kannabiran, 2017; Kumar and Jadeja, 2018; Sharma and Manhas, 2019; Maiti, Das, Sahoo and Mandal, 2020). For example, Awla and colleagues (2016) illustrated several antifungal agents such as pyrrolo [1, 2-a] pyrazine-1, 4-dione, hexahydro-3-(2-methylpropyl); pyrrolo [1,

2-a] pyrazine-1, 4-dione, hexahydro-3-(phenylmethyl); ergotamine; amicomacin; fungichromin; rapamycin and N-acetyl-D, L-phenylalanine produced from *Streptomyces* sp. UPMRS4 by using LC-MS (Awla, Kadir, Othman, Rashid and Wong, 2016). Bibi and colleagues (2017) reported the presence of different active compounds including sulfamonomethoxine, sulfadiazine, ibuprofen and metronidazole-OH in the culture extracts of *Streptomyces* sp. EA85 based on LC-MS analysis (Bibi, Naseer, Yasir, Al-Ghamdi and Azhar, 2017). In the present study, active compounds presented in ethyl acetate extract of *Streptomyces* sp. PJ85 were also identified by using LC-MS and SR-FTIR microspectroscopy. The results revealed that PJ85 produced two active compounds including actinomycin D and an unidentified compound 2a.

It has been shown that *S. actinomycinicus* RCU-197^{T}, the closest related strain of PJ85, produced only actinomycin D (Tanasupawat et al., 2016). Actinomycin D is one of the oldest chemotherapeutic drugs which has been used as an anti-tumor drug for treatment of childhood rhabdomyosarcoma and Wilms' tumor (Wei, Wang, Bie and Lu, 2017; Liu et al., 2019). Similar to those found in *S. actinomycinicus* RCU-197^{T}, PJ85 produced actinomycin D as a major product. However, strain PJ85 was able to generate a second active compound, compound 2a, that exhibited an antimicrobial activity against MRSA (Fig. 4.8). We proposed that compound 2a could be tricaffeoyl quinic acid.

The tricaffeoyl quinic acid has been known as a derivative of caffeoylquinic acid (CQA) which belongs to the generic group of chlorogenic acids. It can be isolated from plants such as *Pluchea symphytifolia* and *Pluchea indica* (Scholz et al., 1994; Arsiningtyas, Gunawan-Puteri, Kato and Kawabata, 2014). There was only a report of Buraimoh and colleagues (2017) that demonstrated the production of tricaffeoyl quinic

acid in microorganisms (Buraimoh, Ilori, Amunda, Isanbor and Michel, 2017). According to Buraimoh et al. (2017), tricaffeoyl quinic acid can be produced by *Streptomyces albogriseolus* AOB (KF977548) (Buraimoh et al., 2017). Strain AOB was used to study the production of chlorogenic acids by degradation of coniferyl alcohol, one of the major precursors of lignin. Medium containing coniferyl alcohol and lignin was used for culturing of *S. albogriseolus* AOB. Strain AOB could grow on lignin and coniferyl alcohol and produce tricaffeoyl quinic acid to the culture medium. It was suggested that coniferyl alcohol was utilized to caffeic acid, a substrate of tricaffeoyl quinic acid, by *S. albogriseolus* AOB (Buraimoh et al., 2017). The present study revealed that strain PJ85 might be able to produce tricaffeoyl quinic acid from ISP-2 medium. ISP-2 medium contains neither lignin nor coniferyl alcohol. However, the presence of lignin and caffeic acid, precursor of tricaffeoyl quinic acid, in malt extract have been reported (Gencheva, Dimitrov, Dobrev and Ivanova, 2012; Leitao et al., 2012). Malt extract is one of the major compositions of ISP-2 medium, thus ISP-2 might contain trace amount of lignin and caffeic acid which can be converted to tricaffeoyl quinic acid by PJ85 (Figure 5.1).

To date, only one report of tricaffeoyl quinic acid producing *Streptomyces* has been proclaimed (Buraimoh et al., 2017). This study demonstrated the first isolation and identification of tricaffeoyl quinic acid from *S. actinomycinicus*. Moreover, an article relating antibacterial activity against *E. coli*, *B. subtilis* and *M. luteus* of tricaffeoyl quinic acid has only been reported from plant (Scholz et al., 1994). To the best of our knowledge, this work demonstrated the first anti-MRSA activity of tricaffeoyl quinic acid produced from microbes. Due to several physiological and biological applications of tricaffeoyl quinic acid such as antibacterial, anti-nematode,

anti-inflammatory, anti-HIV and antioxidant (Scholz et al., 1994; Tamura et al., 2006; Arsiningtyas et al., 2014). The production of tricaffeoyl quinic acid through *Streptomyces* sp. PJ85 could play an important role in pharmaceutical industry. In the industry, the use of microorganisms in the large-scale manufacturing processes has many advantages over plants. Since the microbes are easier to culture and grow faster than plants, as such producing the required substance in a short time.

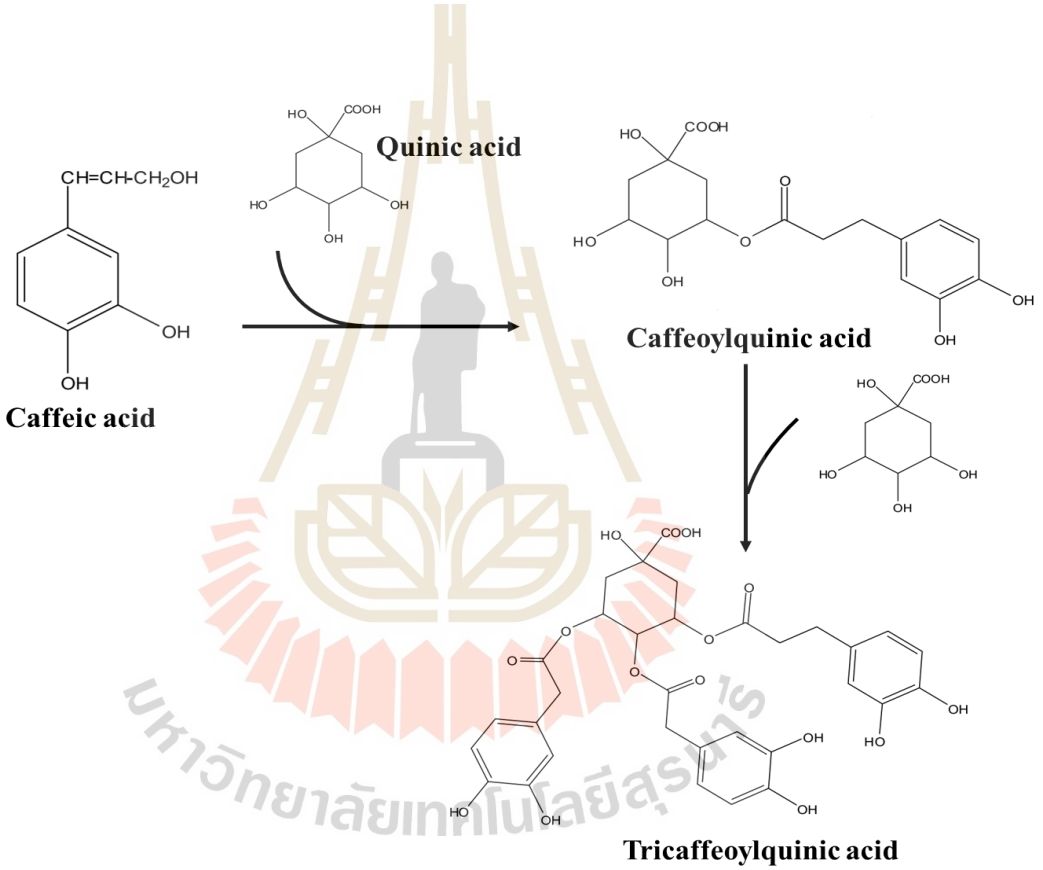


Figure 5.1 The proposed pathway for metabolic transformation of caffeic acid to tricaffeoyl quinic acid by antimicrobial-producing *Streptomyces* sp. PJ85.

REFERENCES

REFERENCES

Adegboye, M. and Babalola, O. (2013). Actinomycetes: a yet inexhaustive source of bioactive secondary metabolites. **Microbial Pathogens and Strategies for Combating Them: Science, Technology and Education, Formatex, Badajoz.** 786-795.

Adzitey, F., Huda, N., Rusul, G. and Ali, R. (2013). Molecular techniques for detecting and typing of bacteria, advantages and application to foodborne pathogens isolated from ducks. **3 Biotech.** 3: 97-107.

Al-dhabi, N.A., Esmail, G.A., Duraipandiyan, V., Arasu, M.V. and Salem-Bekhit, M.M. (2016). Isolation, identification and screening of antimicrobial thermophilic *Streptomyces* sp. Al-Dhabi-1 isolated from Tharban hot spring, Saudi Arabia. **Extremophiles**. 20(1).

Al-dhabi, N.A., Ghilan, A.-K. M., Esmail, G.A., Arasu, M.V., Duraipandiyan, V. and Ponmurugan, K. (2019). Bioactivity assessment of the Saudi Arabian Marine *Streptomyces* sp. Al-Dhabi-90, metabolic profiling and its in vitro inhibitory property against multidrug resistant and extended-spectrum beta-lactamase clinical bacterial pathogens. **Journal of Infection and Public Health**. 12: 549-556.

Aminov, R.I. (2010). A brief history of the antibiotic era: lessons learned and challenges for the future. **Frontiers in Microbiology**. 1(134): 1-7.

Anderson, A.S. and Wellington, E.M.H. (2001). The taxonomy of *Streptomyces* and related genera. **International Journal of Systematic and Evolutionary Microbiology**. 51: 797-814.

Arasu, M.V., Duraipandiyan, V., Agastian, P. and Ignacimuthu, S. (2009). In vitro antimicrobial activity of *Streptomyces* spp. ERI-3 isolated from Western Ghats rock soil (India). **Journal of Medical Mycology**. 19: 22-28.

Arifuzzaman, M., Khatun, M. and Rahman, H. (2010). Isolation and screening of actinomycetes from Sundarbans soil for antibacterial activity. **African Journal of Biotechnology**. 9(29): 4615-4619.

Arsiningtyas, I.S., Gunawan-Puteri, M.D.P.T., Kato, E. and Kawabata, J. (2014). Identification of α-glucosidase inhibitors from the leaves of *Pluchea indica* (L.) Less., a traditional Indonesian herb: promotion of natural product use. **Natural Product Research**. 2014.

Aslam, B., Wang, W., Arshad, M.I., Khurshid, M., Muzammil, S., Rasool, M.H., Nisar, M.A., Alvi, R.F., Aslam, M.A. and Qamar, M.U. (2018). Antibiotic resistance: a rundown of a global crisis. **Infection and Drug Resistance**. 11: 1645-1658.

Awla, H.K., Kadir, J., Othman, R., Rashid, T.S. and Wong, M-Y. (2016). Bioactive Compounds Produced by *Streptomyces* sp. Isolate UPMRS4 and Antifungal Activity against *Pyricularia oryzae*. **American Journal of Plant Sciences**. 7: 1077-1085.

Baskaran, R., Mohan, P.M., Sivakumar, K. and Kumar, A. (2016). Antimicrobial activity and phylogenetic analysis of *Streptomyces parvulus* dosmb-d105 isolated from the mangrove sediments of Andaman Islands. **Acta Microbiologica et Immunologica Hungarica**. 63: 27-46.

Bălăşoiu, M., Bălăşoiu, A., Mănescu, R., Avramescu, C. and Ionete, O. (2014). *Pseudomonas aeruginosa* resistance phenotypes and phenotypic highlighting methods. **Current Health Sciences Journal**. 40(2): 85-92.

Balouiri, M., Sadiki, M. and Ibnsouda, S.K. (2016). Methods for in vitro evaluating antimicrobial activity: A review. **Journal of Pharmaceutical Analysis**. 6: 71-79.

Balsalobre, L.C., Dropa, M. and Matté, M.H. (2014). An overview of antimicrobial resistance and its public health significance. **Brazilian Journal of Microbiology**. 45(1): 1-6.

Baltz, R.H. (2007). Antimicrobials from actinomycetes: back to the future. **Microbe**. 2: 125-133.

Bérdy, J. (2005). Bioactive Microbial Metabolites. **The Journal of Antibiotics**. 58(1): 1-26.

Bérdy, J. (2012). Thoughts and facts about antibiotics: Where we are now and where we are heading. **The Journal of Antibiotics**. 65: 385-395.

Bertasso, M., Holzenkaempfer, M., Zeeck, A., Stackebrandt, E., Beil, W. and Fiedler, H.P. (2003). Ripromycin and other polycyclic macrolactams from *Streptomyces* sp. Tü 6239. **The Journal of Antibiotics**. 56: 364-371.

Beye, M., Fahsi, N., Raoult, D. and Fournier, P.-E. (2018). Careful use of 16S rRNA gene sequence similarity values for the identification of *Mycobacterium* species. **New Microbes and New Infections**. 22: 24-29.

Bibi, F., Naseer, M.I., Yasir, M., Al-Ghamdi, A.A.K. and Azhar, E.I. (2017). LC-MS based identification of secondary metabolites from marine antagonistic endophytic bacteria. **Genetics and Molecular Research**. 17(1): gmr16039857.

Braña, A.F., Sarmiento-Vizcaíno, A., Pérez-Victoria, I., Martín, J., Otero, L., Juan José Palacios-Gutiérrez, J.J., Fernández, J., Mohamedi, Y., Fontanil, T., Salmón, M., Cal, S., Reyes, F., García, L.A. and Bianco, G. (2019). Desertomycin G, a New Antibiotic with Activity against *Mycobacterium tuberculosis* and Human Breast Tumor Cell Lines Produced by *Streptomyces althioticus* MSM3, Isolated from the Cantabrian Sea Intertidal Macroalgae Ulva sp. **Marine Drugs**. 17(2): 114.

Buraimoha, O.M., Ilori, M.O., Amunda, O.O., Isanbor, C. and Michel, F.C. (2017). The degradation of coniferyl alcohol and the complementary production of chlorogenic acids in the growth culture of *Streptomyces albogriseolus* KF977548 isolated from decaying wood residues. **Process Biochemistry**. 52: 22-29.

Carmeli, Y., Troillet, N., Eliopoulos, G.M. and Samore, M.H. (1999). Emergence of antibiotic-resistant *Pseudomonas aeruginosa*: comparison of risks associated with different antipseudomonal agents. **Antimicrobial Agents and Chemotherapy**. 43: 1379-1382.

Chandrashekhara, S., Nanjwade, B., Goudanavar, P., Manvi, F. and Ali, M.S. (2010). Isolation and characterization of antibiotic production from soil isolates by fermentation. **Research Journal of Pharmaceutical Dosage Forms and Technology**. 2(1): 32-36.

Chinnambedu, R.S., Marimuthu, R.R., Sunil, S.S., Amrose, P., Ramachandran, V. and Pachamuthu, B. (2020). Changing antibiotic resistance profile of *Staphylococcus aureus* isolated from HIV patients (2012–2017) in Southern India. **Journal of Infection and Public Health**. 13: 75-79.

Clardy, J., Fischbach, M.A. and Currie, C.R. (2009). The natural history of antibiotics. **Current Biology**. 19(11): R437-R441.

Cockerill, F.R., Wikler, M.A., Alder, J., Dudley, M.N., Eliopoulos, G.M., Ferraro, M.J., Hardy, D., Hecht, D., Hindler, J. and Patel, J. (2012). Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard. CLSI document M07-A9. 9. CLSI (Clinical and Laboratory Standard Institute), Pennsylvania, PA, USA.

Darabpour, E., Ardakani, M.R., Motamedi, H. and Ronagh, M.T. (2012). Isolation of a potent antibiotic producer bacterium, especially against MRSA, from northern region of the Persian Gulf. **Bosnian Journal of Basic Medical Sciences**. 12(2): 108-121.

Datta, R. and Huang, S.S. (2008). Risk of infection and death due to methicillin resistant *Staphylococcus aureus* in long-term carriers. **Clinical Infectious Diseases**. 47: 176-181.

De Jesus Sousa, J.A. and Olivares, F.L. (2016). Plant growth promotion by streptomycetes: ecophysiology, mechanisms and applications. **Chemical and Biological Technologies in Agriculture**. 3: 24.

de Lima Procópio, R.E., da Silva, I.R., Martins, M.K., de Azevedo, J.L. and de Araújo, J.M. (2012). Antibiotics produced by *Streptomyces*. **The Brazilian Journal of Infectious Diseases**. 16(5): 466-471.

Dewanjee, S., Gangopadhyay, M., Bhattacharya, N., Khanra, R. and Dua, T.K. (2015). Bioautography and its scope in the field of natural product chemistry. **Journal of Pharmaceutical Analysis**. 5: 75-84.

Edirisinghe, E.M.R.K.B., Perera, W.M.K. and Bamunuarachchi, A. (1998). Effect of Indian Gooseberry (Phynanthus emblica) on Fish Oil. **Antioxidation**. 163-171.

Eliopoulos, G.M. and Gold, H. (2001). Vancomycin-resistant enterococci: mechanisms and clinical observations. **Clinical Infectious Diseases**. 33: 210-219.

El-Sherbini, A. and Khattab, A. (2018). Induction of novel mutants of *Streptomyces lincolnensis* with high Lincomycin production. **Journal of Applied Pharmaceutical Science**. 8(2): 128-135.

Engelhardt, K., Degnes, K.F., Kemmler, M., Bredholt, H., Fjærvik, E., Klinkenberg, G., Sletta, H., Ellingsen, T.E. and Zotchev, S.B. (2010). Production of a new thiopeptide antibiotic, TP-1161, by a marine *Nocardiopsis* species**. Applied and Environmental Microbiology**. 76: 4969-4976.

Enright, M.C., Robinson, D.A., Randle, G., Feil, E.J., Grundmann, H. and Spratt, B.G. (2002). The evolutionary history of methicillin-resistant *Staphylococcus aureus* (MRSA). **Proceedings of the National Academy of Sciences**. 99(11): 7687-7692.

Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. **Evolution**. 39(4): 783-791.

Furukawa, M., Inoue, A. and Asano, K. (1968). Chemical studies on actinomycin S. II chemical structures of actinomycin S_2 and S_3. **The Journal of Anitibiotics**. 568-570

Gencheva, P., Dimitrov, D., Dobrev, G. and Ivanova, V. (2012). Hydrolysates from malt spent grain with potential application in the bioethanol production. **Journal of BioScience and Biotechnology**. 2012, SE/ONLINE: 135-141.

George, M., George, G. and Hatha, A.M. (2011). Diversity and antibacterial activity of actinomycetes from wetland soil. **The South Pacific Journal of Natural and Applied Sciences**. 28(1): 52-57.

Gerber, N. and Lechevalier, H. (1965). Geosmin, an earthy-smelling substance isolated from actinomycetes. **Applied Microbiology**. 13(6): 935-938.

Guo, Y., Song, G., Sun, M., Wang, J. and Wang, Y. (2020). Prevalence and Therapies of Antibiotic-Resistance in *Staphylococcus aureus***. Frontiers in Cellular and Infection Microbiology**. 10: 107.

Hohmann, C., Schneider, K., Bruntner, C., Irran, E., Nicholson, G., Bull, A.T., Jones, A.L., Brown, R., Stach, J.E. and Goodfellow, M. (2009). Caboxamycin, a new antibiotic of the benzoxazole family produced by the deep-sea strain *Streptomyces* sp. NTK 937. **The Journal of Antibiotics**. 62(2): 99-104.

Höltzel, A., Jack, R.W., Nicholson, G.J., Jung, G., Gebhardt, K., Fiedler, H.P. and Süssmuth, R. D. (2001). Streptocidins AD, Novel Cyclic Decapeptide Antibiotics Produced by *Streptomyces* sp. Tü 6071. **The Journal of Antibiotics**. 54: 434-440.

Igarashi, Y., Takagi, K., Kan, Y., Fujii, K., Harada, K.I., Furumai, T. and Oki, T. (2000). Arisostatins A and B, new members of tetrocarcin class of antibiotics from *Micromonospora* sp. TP-A0316. **The Journal of Antibiotics**. 53: 233-240.

Iniyan, A.M., Sudarman, E., Wink, J., Kannan, R.R. and Vincent, S.G.P. (2018). Ala-geninthiocin, a new broad spectrum thiopeptide antibiotic, produced by a marine *Streptomyces* sp. ICN19. **The Journal of Antibiotics**.

James, P.D.A. and Edwards, C. (1988). The Effects of Temperature on Growth and Production of the Antibiotic Granaticin by a Thermotolerant Streptomycete. **The Journal of General Microbiology**. 135: 1997-2003.

Jumpathong, J., Nuengchamnong, N., Masin, K., Nakaew, N. and Suphrom, N. (2019). Thin Layer Chromatography-Bioautography Assay for Antibacterial Compounds from *Streptomyces* sp. TBRC 8912, a Newly Isolated Actinomycin D Producer. **Chiang Mai Journal of Science**. 46(5): 839-849.

Kalakoutskii, L. and Agre, N.S. (1976). Comparative aspects of development and differentiation in actinomycetes. **Bacteriological reviews**. 40(2): 469-524.

Kemung, H.M., Tan, L.T.-H., Khan, T.M., Chan, K.-G., Pusparajah, P., Goh, B.-H. and Lee, L.-H. (2018). *Streptomyces* as a prominent resource of future anti-MRSA drugs. **Frontiers in Microbiology**. 9: 2221.

Khan, H.A., Ahmad, A. and Mehboob, R. (2015). Nosocomial infections and their control strategies. **Asian Pacific Journal of Tropical Biomedicine**. 5: 509-514.

Khatun, M.F., Haque, M.U. and Islam, M.A.U. (2017). Antibacterial and Cytotoxic Activities of Crude Ethyl Acetate Extract of *Streptomyces* sp. FEAI-1 Isolated From Soil Samples of Rajshahi, Bangladesh. **Bangladesh Journal of Pharmacology**. 20: 188-193.

Kluytmans, J. and Wertheim, H. (2005). Nasal carriage of *Staphylococcus aureus* and prevention of nosocomial infections. **Infection**. 33: 3-8.

Kumar, P.S., Duraipandiyan, V. and Ignacimuthu, S. (2014). Isolation, screening and partial purification of antimicrobial antibiotics from soil *Streptomyces* sp. SCA 7. **The Kaohsiung Journal of Medical Sciences**. 30: 435-446.

Kumar, R.R. and Jadeja, V.J. (2018). Characterization and partial purification of an antibacterial agent from halophilic actinomycetes *Kocuria* sp. strain rsk4. **BioImpacts**. 8(4): 253-261.

Kwon, O., Georgellis, D., Lynch, A.S., Boyd, D. and Lin, E. (2000). The ArcB sensor kinase of *Escherichia coli*: genetic exploration of the transmembrane region. **Journal of Bacteriology**. 182(10): 2960-2966.

Lane, D. (1991). 16S/23S rRNA sequencing. Nucleic acid techniques in bacterial systematics. 115-175.

Lee, L.-H., Zainal, N., Azman, A.-S., Eng, S.-K., Ab Mutalib, N.-S., Yin, W.-F. and Chan, K.-G. (2014). *Streptomyces pluripotens* sp. nov., a bacteriocin-producing streptomycete that inhibits meticillin-resistant *Staphylococcus aureus*. **International Journal of Systematic and Evolutionary Microbiology**. 64(9): 3297-3306.

Leitao, C., Marchioni, E., Bergaentzlé, M., Zhao, M., Didierjean, L., Miesch, L., Holder, E., Miesch, M. and Ennahar, S. (2012). Fate of polyphenols and antioxidant activity of barley throughout malting and brewing**. Journal of Cereal Science**. 55(3): 318-322.

Lihan, S., Choon, Y.K., Hua, N.K. and Wasli, M.E. (2014). Screening For Antimicrobial Activity Of Fungi In Soil Samples Collected From Kubah National Park. **International Journal of Scientific & Technology Research**. 3 (2).

Lin, J., Nishino, K., Roberts, M.C., Tolmasky, M., Aminov, R.I. and Zhang, L. (2015). Mechanisms of antibiotic resistance. **Frontiers in Microbiology**. 6(34): 1-3.

Liu, M., Jia, Y., Xie, Y., Zhang, C., Ma, J., Sun, C. and Ju, J. (2019). Identification of the Actinomycin D Biosynthetic Pathway from Marine-Derived *Streptomyces costaricanus* SCSIO ZS0073. **Marine Drugs**. 17(4): 240.

Maiti, P.K., Das, S., Sahoo, P. and Mandal, S. (2020). *Streptomyces* sp SM01 isolated from Indian soil produces a novel antibiotic picolinamycin effective against multi drug resistant bacterial strains. **Scientific Reports**. 10: 10092.

Manimaran, M., Gopal, J.V. and Kannabiran, K. (2017). Antibacterial activity of *Streptomyces* sp. VITMK1 isolated from mangrove soil of Pichavaram, Tamil Nadu, India. **Proceedings of the National Academy of Sciences, India Section B: Biological Sciences**. 87: 499-506.

Martens, E. and Demain, A.L. (2017). The antibiotic resistance crisis, with a focus on the United States. **The Journal of Antibiotics**. 70(5): 520-526.

Maruyama, H.B., Suhara, Y., Suzuki-Watanabe, J., Maeshima, Y., Shimizu, N., OguraHamada, M., Fujimoto, H. and Takano, K. (1975). A new antibiotic, fumaramidmycin. **The Journal of Antibiotics**. 28: 636-647.

Moghannem, S.A., El-Sherbiny, G.M. and Kalaba, M.H. (2017). Isolation and identification of *Streptomyces baarnensis* MH-133 produce bioactive metabolite inhibiting multidrug resistant bacteria (MDRB). **World Journal of Pharmaceutical and Medical Research**. 3(6): 64-75.

Munita, J.M. and Arias, C.A. (2016). Mechanisms of antibiotic resistance. **Microbiology Spectrum**. 4(2): 481-511.

Naumann, D. (2006). Infrared spectroscopy in microbiology. Encyclopedia of Analytical Chemistry: Applications, Theory and Instrumentation. **Biomedical Spectroscopy**.

Nelson, R.A., Pope Jr, J.A., Luedemann, G.M., Mcdaniel, L.E. and Schaffner, C.P. (1986). Crisamicin a, a new antibiotic from *Micromonospora*. **The Journal of Antibiotics**. 39: 335-344.

Nguyen, T.M. and Kim, J. (2015). *Streptomyces olivicoloratus* sp. nov., an antibiotic-producing bacterium isolated from soil. **International Journal of Systematic and Evolutionary Microbiology**. 65(10): 3262-3270.

Novick, R.P. (2003). Autoinduction and signal transduction in the regulation of *Staphylococcal virulence*. **Molecular microbiology**. 48: 1429-1449.

Oskay, A.M., Üsame, T. and Cem, A. (2005). Antibacterial activity of some actinomycetes isolated from farming soils of Turkey. **African Journal of Biotechnology**. 3(9): 441-446.

Ozasa, T., Suzuki, K., Sasamata, M., Tanaka, K., Kobori, M., Kadota, S., Nagai, K., Saito, T., Watanabe, S. and Iwanami, M. (1989). Novel antitumor antibiotic phospholine. **The Journal of Antibiotics**. 42: 1331-1338.

Padmanabhan, S., Mandi, N. and Banerjee, S. (2011). Screening of bacterial recombinants: strategies and preventing false positives: **INTECH** Open Access Publisher.

Ravi, R., Vasantba, J., Bhoomi, M., Bonisha, T. and Bhumika, C. (2015). Antibacterial potentials of actinomycetes isolated from Gujarat. **International Journal of Pharmaceutical Sciences Review and Research**. 30(1): 78-83.

Rios, J., Recio, M. and Villar, A. (1988). Screening methods for natural products with antimicrobial activity: A review of the literature. **Journal of Ethnopharmacology**. 23(2-3): 127-149.

Robinson, D. and Enright, M. (2004). Multilocus sequence typing and the evolution of methicillin-resistant *Staphylococcus aureus*. **Clinical Microbiology and Infection**. 10: 92-97.

Roca, I., Akova, M., Baquero, F., Carlet, J., Cavaleri, M., Coenen, S., Cohen, J., Findlay, D., Gyssens, I., Heure, O., Kahlmeter, G., Kruse, H., Laxminarayan, R., Liébana, E., López-Cerero, L., MacGowan, A., Martins, M., Rodríguez-Baño, J., Rolain, J.-M., Segovia, C., Sigauque, B., Taconelli, E., Wellington, E. and Vila1, J. (2015). The global threat of antimicrobial resistance: science for intervention. **New Microbes and New Infections**. 6: 22-29.

Rossi-Tamisier, M., Benamar, S., Raoult, D. and Fournier, P.-E. (2015). Cautionary tale of using 16S rRNA gene sequence similarity values in identification of human-associated bacterial species. **International Journal of Systematic and Evolutionary Microbiology**. 65: 1929-1934.

Rossolini, G.M., Arena, F., Pecile, P. and Pollini, S. (2014). Update on the antibiotic resistance crisis. **Current Opinion in Pharmacology**. 18: 56-60.

Sabaou, N., Bijani, C., Zitouni, A., Pont, F., Mathieu, F. and Badji, B. (2017). *Streptomyces* sp. AT37 isolated from a Saharan soil produces a furanone derivative active against multidrug-resistant *Staphylococcus aureus*. **World Journal of Microbiology and Biotechnology**. 33(105).

Scholz, E., Heinrich, M. and Hunkler, D. (1994). Caffeoylquinic Acid and Some Biological Activities of *Pluchea symphytifolia*. **Planta Medica**. 60(4): 360-364.

Sharma, M. and Manhas, R.K. (2019) Purification and characterization of actinomycins from *Streptomyces* strain M7 active against methicillin resistant *Staphylococcus*

aureus and vancomycin resistant *Enterococcus*. **BMC Microbiology**. 19(44): 1-12.

Shetty, P.R., Buddana, S.K., Tatipamula, V.B., Naga, Y.V.V. and Ahmad, J. (2014). Production of polypeptide antibiotic from *Streptomyces parvulus* and its antibacterial activity. **Brazilian Journal of Microbiology**. 45(1): 303-312.

Schito, G. (2006). The importance of the development of antibiotic resistance in *Staphylococcus aureus*. **Clinical Microbiology and Infection**. 12: 3-8.

Singh, L.S., Sharma, H. and Talukdar, N.C. (2014). Production of potent antimicrobial agent by actinomycete, *Streptomyces sannanensis* strain SU118 isolated from phoomdi in Loktak Lake of Manipur, India. **BMC microbiology**. 14(278).

Singh, V., Haque, S., Singh, H., Verma, J., Vibha, K., Singh, R., Jawed, A. and Tripathi, C. (2016). Isolation, screening, and identification of novel isolates of actinomycetes from India for antimicrobial applications. **Frontier in microbiology**. 7(1921).

Srinivasan, A., Dick, J.D. and Perl, T.M. (2002) Vancomycin resistance in staphylococci. **Clinical Microbiology Reviews**. 15(3): 430-438.

Stackebrandt, E. and Goebel, B.M. (1994). Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. **International Journal of Systematic Bacteriology.** 44: 846-849.

Stackebrandt, E., Rainey, F.A. and Ward-Rainey, N.L. (1997). Proposal for a new hierarchic classification system, Actinobacteria classis nov. **International Journal of Systematic Bacteriology**. 47(2): 479-491.

Stapley, E., Jackson, M., Hernandez, S., Zimmerman, S., Currie, S., Mochales, S., Mata, J., Woodruff, H. and Hendlin, D. (1972). Cephamycins, a new family of β-lactam antibiotics I. Production by Actinomycetes, including *Streptomyces lactamdurans* sp. n. **Antimicrobial agents and chemotherapy**. 2: 122-131.

Subakaran, M., Joshua, S.A., Jansi, M. and Prakash, S.G. (2015). Isolation and identification of antibacterial compound producing *Streptomyces parvulus* ICN698 from a Wetland Ecosystem of Kanyakumari**. Indian Journal of Pharmacy and Pharmacology**. 2: 217-221.

Subramani, R. and Sipkema, D. (2019). Marine Rare Actinomycetes: A Promising Source of Structurally Diverse and Unique Novel Natural Products. **Marine drugs**. 17(5): 249.

Tamura, H., Akioka, T., Ueno, K., Chujyo, T., Okazaki, K.-I. King, P.J. and Robinson, W.E. (2006). Anti-human immunodeficiency virus activity of 3,4,5-tricaffeoylquinic acid in cultured cells of lettuce leaves. **Molecular Nutrition and Food Research**. 50: 396-400.

Tan, L.T.-H., Chan, K.-G., Chan, C.K., Khan, T.M., Lee, L.-H. and Goh, B.-H. (2018). Antioxidative potential of a *Streptomyces* sp. MUM292 isolated from mangrove soil. **BioMed Research International**. 2018.

Tanasupawat, S. and Suwanborirux, K. (2014). Taxonomy and Antimicrobial activity of Actinomycetes from Peat Swamp Forest Soil in Rayong Province (Research report). Bangkok: Chulalongkorn University.

Tanasupawat, S., Phongsopitanun, W., Suwanborirux, K., Ohkuma, M. and Kudo, T. (2016). *Streptomyces actinomycinicus* sp. nov., isolated from soil of a peat

swamp forest. **International Journal of Systematic and Evolutionary Microbiology**. 66: 290-295.

Theobald, U., Schimana, J. and Fiedler, H.P. (2020). Microbial growth and production kinetics of *Streptomyces antibioticus* Tü 6040. **Antonie van Leeuwenhoek**. 78: 307-313.

Ullah, H. and Ali, S. (2017). Classification of Anti-Bacterial Agents and Their Functions. Antibacterial agents, Chapter 1.

Uzair, B., Menaa, F., Khan, B.A., Mohammad, F.V., Ahmad, V.U., Djeribi, R. and Menaa, B. (2018). Isolation, purification, structural elucidation and antimicrobial activities of kocumarin, a novel antibiotic isolated from actinobacterium *Kocuria marina* CMG S2 associated with the brown seaweed Pelvetia canaliculata. **Microbiological Research**. 206: 186-197.

Varghese, R., Nishamol, S., Suchithra, R., Jyothy, S. and Hatha, A.M. (2012). Distribution and antibacterial activity of Actinomycetes from shola soils of Tropical Montane Forest in Kerala, South India. **Journal of Environment**. 1: 93-99.

Van Der Meij, A., Worsley, S.F., Hutchings, M.I. and Van Wezel, G.P. (2017). Chemical ecology of antibiotic production by actinomycetes. **FEMS Microbiology Reviews**. 41: 392-416.

Velayudham, S. and Murugan, K. (2012). Diversity and antibacterial screening of actinomycetes from Javadi Hill forest soil, Tamilnadu. **India. Journal of Microbiology Research**. 2(2): 41-46.

Ventola, C.L. (2015). The antibiotic resistance crisis: part 1: causes and threats. **Pharmacology & Therapeutics**. 40(4): 277-283.

Ventura, M., Canchaya, C., Tauch, A., Chandra, G., Fitzgerald, G.F., Chater, K.F. and Van Sinderen, D. (2007). Genomics of Actinobacteria: tracing the evolutionary history of an ancient phylum. **Microbiology and Molecular Biology Reviews**. 71(3): 495-548.

Vestergaard, M., Frees, D. and Ingmer, H. (2019). Antibiotic Resistance and the MRSA Problem. **Microbiology Spectrum**. 7(2): GPP3-0057-2018.

Yang, S.W., Chan, T.-M., Terracciano, J., Patel, R., Loebenberg, D., Chen, G., Patel, M., Gullo, V., Pramanik, B. and Chu, M. (2005). New antibiotic Sch 725424 and its dehydration product Sch 725428 from *Kitasatospora* sp. **The Journal of Antibiotics**. 58: 192-195.

Waksman, S.A., Reilly, H.C. and Johnstone, D.B. (1946). Isolation of streptomycin producing strains of *Streptomyces griseus*. **Journal of Bacteriology**. 52(3): 393-397.

Wanbanjob, G. (2008). Investigation of bioactive compounds from endophytic Actinomycetes. M.Sc. Silpakorn University, Thailand.

Wang, Y. and Salaza, J.K. (2016). Culture-Independent Rapid Detection Methods for Bacterial Pathogens and Toxins in Food Matrices. **Comprehensive Reviews in Food Science and Food Safety**. 15: 183-205.

Wangai, F.K., Masika, M.M., Maritim, M.C. and Seaton, R.A. (2019). Methicillin-resistant Staphylococcus aureus (MRSA) in East Africa: red alert or red herring? **BMC Infectious Diseases**. 19 (596).

Wei, Z., Xu, C., Wang, J., Lu, F., Bie, X. and Lu, Z. (2017). Identification and characterization of *Streptomyces flavogriseus* NJ-4 as a novel producer of actinomycin D and holomycin. **PeerJ**. 5, e3601.

Wu, Z., Li, S., Li, J., Chen, Y., Saurav, K., Zhang, Q., Zhang, H., Zhang, W., Zhang, W. and Zhang, S. (2013). Antibacterial and cytotoxic new napyradiomycins from the marine-derived *Streptomyces* sp. SCSIO 10428. **Marine drugs**. 11: 2113-2125.

Zhao, F., Qin, Y.-H., Zheng, X., Zhao, H.-W., Chai, D.-Y., Li, W., Pu, M.-X., Zuo, X.-S., Qian, W. and Ni, P. (2016). Biogeography and adaptive evolution of *Streptomyces* strains from saline environments. **Scientific Reports**. 6: 1-9.

APPENDICES

APPENDIX A

SEQUENCE OF ANTIMICROBIAL-PRODUCING BACTERIAL SOIL ISOLATE

PJ85

CGATGAACCACTTCGGTGGGGATTAGTGGCGAACGGGTGAGTAACACGTG

GGCAATCTGCCCTGCACTCTGGGACAAGCCCTGGAAACGGGGTCTAATAC

CGGATATGAGCCGGAACCGCATGGTTCTGGTTGTAAAGCTCCGGCGGTGC

AGGATGAGCCCGCGGCCTATCAGCTTGTTGGTGAGGTAATGGCTCACCAA

GGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACT

GAGACACGGCCCAGACCCCTACGGGAGGCAGCAGTGGGGAATATTGCAC

AATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTT

CGGGCTGTAAACCTCTTTCAGCAGGGAAGAAGCGAGAGTGACGGTACCTG

CAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAG

GGCGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGCT

TGTCACGTCGATTGTGAAAGCCCGAGGCTTAACCTCGGGTCTGCAGTCGA

TACGGGCTAGCTAGAGTGTGGTAGGGGAGATCGGAATTCCTGGTGTAGCG

GTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGATCTC

TGGGCCATTACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGAT

TAGATACCCTGGTAGTCCACGCCGTAAACGGTGGGAACTAGGTGTTGGCG

ACATTCCACGTCGTCGGTGCCGCAGCTAACGCATTAAGTTCCCCGCCTGG

GGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCA

CAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACC

AAGGCTTGACATACACCGGAAACGTCTGGAGACAGGCGCCCCCTTGTGGT

CGGTGTACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTG

GGTTAAGTCCCGCAACGAGCGCAACCCTTGTTCTGTGTTGCCAGCATGCCC

TTCGGGGTGATGGGGACTCACAGGAGACCGCCGGGGTCAACTCGGAGGA

AGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACA

CGTGCTACAATGGCCGGTACAAAGAGCTGCGATACCGTGAGAAATCAGTA

GCGAATTCAAGCGGCCGGTCTGCAGTTCGACGATATGGGGTCTGCAGCTC

GACCCCATGACGTCGGAGTCGCATAGTAATCGCAGATCAGCATTGCTGCG

GTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACGTCACGAAAGT

TGGTAACACCCGAAGCCGGTGGCCCAACCCCTTGTGGGAGGGAGCTGTCG

AAGGTGGGACTAGCGATTGGGACGAAGTCGTAACAAGGTAGCCGTACCG

GAAGGTGCGGCTGGATCACCTCCTTTAATCTTGGGGATCCTCTAGAGTCG

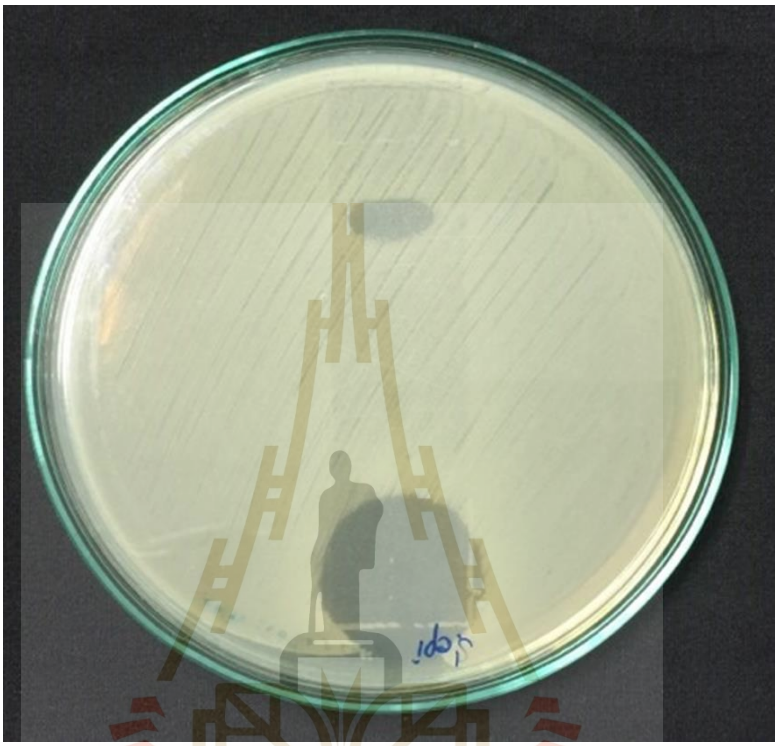
A

APPENDIX B

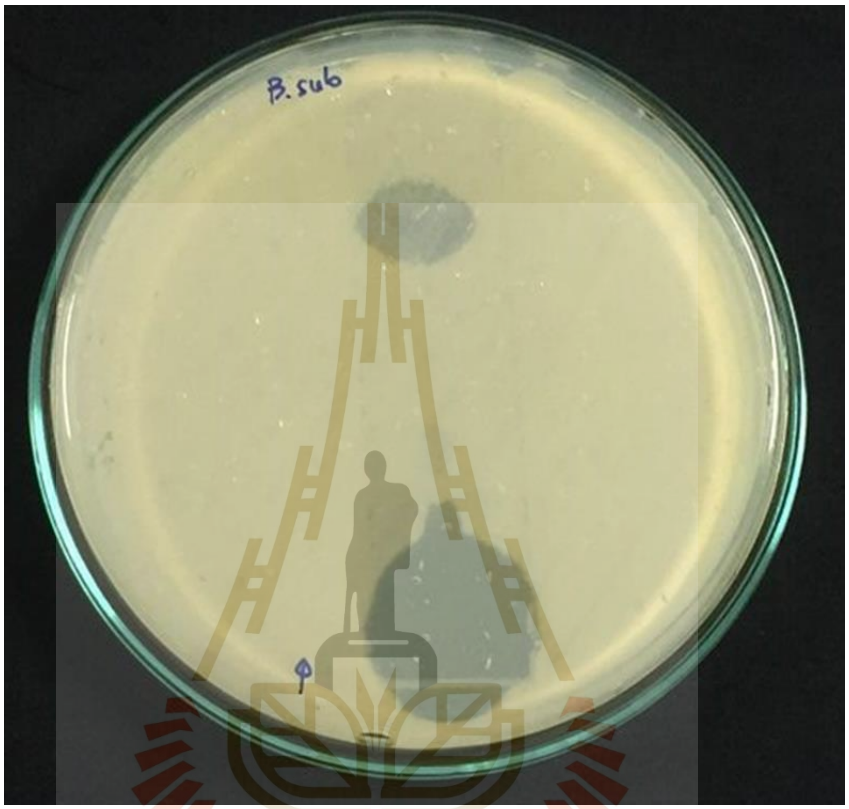
ANTIMICROBIAL OF COMPOUND 2 BASED ON CONTACT BIOAUTOGRAPHY

A1 TLC-based bioautography of compound 2 showed antibacterial activity against *S. aureus* TISTR1466.

A2 TLC-based bioautography of compound 2 showed antibacterial activity against *S. epidermidis* TIRTR518.



A3 TLC-based bioautography of compound 2 showed antibacterial activity against *B. subtilis* TIRTR518.



APPENDIX C

PREPARATION OF REAGENTS

C1 10X TBE buffer

	g/liter
Tris-base (MW. 121.1)	108 g
Boric acid	55 g
0.5M EDTA, pH 8.0	20 ml

Dissolve the ingredients in distilled water and bring up to volume 1,000 ml. Working solution in the gel and the buffer is 1X.

C2 TE buffer

	g/liter
Tris-base (MW. 121.1)	1.22
EDTA	0.4

Dissolve the ingredients with 500 ml of distilled water. Adjust the pH to 8.0 and bring up to volume 1,000 ml. Sterilize by autoclaving at 121 °C for 15 min.

C3 Lysis buffer (1M Tris-HCl (pH 8.0), 0.5M EDTA (pH8.0), 5M NaCl, 10% SDS)

1M Tris-HCl, pH 8.0

Dissolved Tris-hydroxymethyl aminomethane 12.11 g with 80 ml of distilled water. Adjust the pH to 8.0. Bring to a final volume of 100 ml with distilled water. Sterilize by autoclaving at 121 °C for 15 min.

0.5M EDTA, pH8.0

Dissolved disodium ethylenediamine tetraacetate 18.61 g with 80 ml of distilled water. Adjust the pH to 8.0. Bring to a final volume of 100 ml with distilled water. Sterilize by autoclaving at 121 °C for 15 min.

5M NaCl

Dissolved NaCl 29.22 g with 80 ml of distilled water. Bring to a final volume of 100 ml with distilled water. Sterilize by autoclaving at 121 °C for 15 min.

10% SDS

Dissolved SDS 10 g with 80 ml of distilled water. Bring to a final volume of 100 ml with distilled water. Sterilize by autoclaving at 121 °C for 15 min.

C4 0.1M MgCl2

	g/liter
$MgCl_2.6H_2O$	20.3

Dissolve the ingredients with 500 ml of distilled water. Bring up to volume 1,000 ml with distilled water. Sterilize by autoclaving at 121 °C for 15 min.

C5 0.1M CaCl2

	g/liter
$CaCl_2.2H2O$	14.7

Dissolve the ingredients with 500 ml of distilled water. Bring up to volume 1,000 ml with distilled water. Sterilize by autoclaving at 121 °C for 15 min.

C6 5M NaCl

	g/liter
NaCl	292.2

Dissolve the ingredients with 500 ml of distilled water. Adjust the volume to 1,000 ml with distilled water. Sterilize by autoclaving at 121 °C for 15 min.

CURRICULUM VITAE

Name: Miss Panjamaphon Chanthasena

Date of birth: May 29, 1984

Place of birth: Nakhon Ratchasima, Thailand

Education:

2015-Present	Ph.D. Candidate (Microbiology), School of Preclinical Sciences, Institute of Science, Suranaree University of Technology, Nakhon Ratchasima, Thailand
2011-2015	Master of Science (Biomedical Sciences), Institute of Science, Suranaree University of Technology, Nakhon Ratchasima, Thailand
2003-2006	Bachelor of Science in Medical Technology, Huahiew Chalermprakiet University, Samut Prakan, Thailand