ISOLATION AND CHARACTERIZATION OF ANTIMICROBIAL COMPOUNDS PRODUCED BY *STREPTOMYCES* SP. PKA51 AGAINST ANTIBIOTIC-RESISTANT BACTERIA

PHONGSAKORN GANTA

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การแยกและการศึกษาคุณสมบัติของสารต้านจุลชีพที่สร้างโดยเชื้อสเตรปโตมัยซีสสายพันธุ์ PKA51 ต่อเชื้อแบคทีเรียดื้อยาปฏิชีวนะ

นายพงศกร กันทะ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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ISOLATION AND CHARACTERIZATION OF ANTIMICROBIAL COMPOUNDS PRODUCED BY *STREPTOMYCES* SP. PKA51 AGAINST ANTIBIOTIC-RESISTANT BACTERIA

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

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พงศกร กันทะ : การแยกและการศึกษาคุณสมบัติของสารต้านจุลชีพที่สร้างโดยเชื้อสเตรปโตมัยซีสสายพันธุ์ PKA51 ต่อเชื้อแบคทีเรียดื้อยาปฏิชีวนะ (ISOLATION AND CHARACTERIZATION OF ANTIMICROBIAL COMPOUNDS PRODUCED BY *STREPTOMYCES* SP. PKA51 AGAINST ANTIBIOTIC-RESISTANT BACTERIA)
อาจารย์ที่ปรึกษา : ผู้ช่วยศาสตราจารย์ ดร.นวรัตน์ นันทพงษ์, 98 หน้า.

คำสำคัญ: สารออกฤทธิ์ทางชีวภาพ, *Streptomyces*, เชื้อจุลินทรีย์ดื้อยาปฏิชีวนะ

การใช้ยาปฏิชีวนะอย่างแพร่หลายทางการแพทย์นำไปสู่การพัฒนาแบคทีเรียที่ดื้อต่อยาปฏิชีวนะ การเกิดขึ้นอย่างรวดเร็วของแบคทีเรียที่ดื้อยาปฏิชีวนะเป็นปัญหาด้านสาธารณสุขทั่วโลก เนื่องจากทำให้การรักษาล้มเหลว เกิดการเจ็บป่วยและการเสียชีวิตในมนุษย์ ดังนั้นการค้นหาสารต้านจุลชีพที่มีศักยภาพจากแหล่งธรรมชาติจึงเป็นสิ่งสำคัญ สารต้านจุลชีพจำนวนมากถูกสร้างโดยเชื้อจุลินทรีย์ โดยเฉพาะอย่างยิ่งจาก streptomycetes แบคทีเรียเส้นใยที่อยู่ในสกุล *Streptomyces* เป็นที่รู้กันว่าเป็นแหล่งของยาปฏิชีวนะที่มีศักยภาพ ในปี พ.ศ. 2560 เชื้อ *Streptomyces* sp. PKA51 ถูกแยกได้จากดินป่าเต็งรังที่มหาวิทยาลัยเทคโนโลยีสุรนารี ประเทศไทย ในการศึกษานี้จากผลการวิเคราะห์ลำดับเบสของ 16S rDNA และการวิเคราะห์แผนภูมิสายวิวัฒนาการ พบว่าสามารถระบุสายพันธุ์ PKA51 ได้เป็นเชื้อ *Streptomyces luteosporeus* สายพันธุ์ PKA51 แสดงฤทธิ์ต้านจุลชีพแบบกว้างต่อเชื้อแบคทีเรียแกรมบวก เชื้อแบคทีเรียแกรมลบ และเชื้อก่อโรคดื้อยาปฏิชีวนะ ดังนั้น จุดมุ่งหมายของการศึกษานี้คือการแยกและระบุลักษณะสารประกอบต้านจุลชีพที่ผลิตโดยสายพันธุ์ PKA51 สารออกฤทธิ์ทางชีวภาพของ PKA51 ถูกทำให้บริสุทธิ์โดยใช้เทคนิคโครมาโทรกราฟีแบบแผ่นบาง (TLC) และ คอลัมน์โครมาโทรกราฟี (CC) จากผล CC พบสารประกอบออกฤทธิ์ทางชีวภาพสองชนิดต้านเชื้อ methicillin-resistant *Staphylococcus aureus* (MRSA) DMST20654 จากผลการศึกษา อัลตราไวโอเลตและวิสิเบิลสเปกโทรสโคปี, โครมาโทรกราฟีของเหลว-แมสสเปกโตรเมตรี (LC-MS) และ นิวเคลียร์แมกเนติกเรโซแนนซ์สเปกโตรสโคปี (NMR) พบว่าสารประกอบออกฤทธิ์ทางชีวภาพทั้ง 2 ชนิด ถูกระบุว่าเป็น thiolutin ($C_8H_8N_2O_2S_2$) และ thiolutin dioxide ($C_8H_8N_2O_4S_2$) ตามลำดับ สารประกอบออกฤทธิ์ทางชีวภาพได้รับการประเมินฤทธิ์ต้านจุลชีพต่อเชื้อ MRSA methicillin-resistant *Staphylococcus epidermidis* (MRSE) *E. coli* 2026 ทางคลินิก และ *K. pneumoniae* 1617 ทางคลินิก จากผลการศึกษาพบว่า thiolutin มีประสิทธิภาพในการยับยั้งการเจริญของเชื้อ MRSA DMST20654 ได้สูงสุด ที่ค่าความเข้มข้นต่ำสุดในการยับยั้ง (MIC) และความเข้มข้นต่ำสุดในการฆ่าเชื้อแบคทีเรีย (MBC) ที่ 4 และ 8 ไมโครกรัมต่อมิลลิลิตร ตามลำดับ

นอกจากนี้ thiolutin dioxide ยังแสดงศักยภาพสูงสุดในการต้านจุลชีพต่อเชื้อ MRSE โดยมีค่า MIC และ MBC ที่ 4 ไมโครกรัมต่อมิลลิลิตร ตามความรู้ที่ดีที่สุดของเรา นี่เป็นรายงานฉบับแรกที่สามารถแยกและศึกษาคุณลักษณะของ thiolutin dioxide ที่ได้จากเชื้อ *S. luteosporeus* การค้นพบนี้นำไปสู่การพัฒนาสารต้านจุลชีพที่มีศักยภาพสำหรับการใช้งานทางเภสัชกรรม

สาขาวิชาปรีคลินิก ลายมือชื่อนักศึกษา พงศกร ภันทะ

ปีการศึกษา 2564 ลายมือชื่ออาจารย์ที่ปรึกษา

PHONGSAKORN GANTA : ISOLATION AND CHARACTERIZATION OF ANTIMICROBIAL COMPOUNDS PRODUCED BY *STREPTOMYCES* SP. PKA51 AGAINST ANTIBIOTIC-RESISTANT BACTERIA. THESIS ADVISOR: ASST. PROF. NAWARAT NANTAPONG, Ph.D. 98 PP.

Keyword: Bioactive compound, *Streptomyces*, Antibiotic-resistant bacteria

The widespread use of antibiotics in medicine has led to the development of antibiotic-resistant bacteria. The rapid occurrence of antibiotic-resistant bacteria is a global public health concern since it causes treatment failure, morbidity, and mortality in humans. Thus, the discovery of potent antimicrobial compounds from natural sources is required. Several numbers of antimicrobial compounds are produced by microorganisms, especially from streptomycetes. The filamentous bacteria of the genus *Streptomyces* have been known as potential sources of antibiotics. In 2017, *Streptomyces* sp. PKA51 was isolated from dry dipterocarp forest soil at Suranaree University of Technology, Thailand. Based on 16S rDNA and phylogenetic tree, the strain PKA51 can be identified as *Streptomyces luteosporeus*. The strain PKA51 showed broad-spectrum antimicrobial activity against Gram-positive bacteria, Gram-negative bacteria, and drug-resistant pathogens. The aim of this study was to isolate and identify antimicrobial compounds produced by the strain PKA51. Bioactive compounds of PKA51 were purified by using thin-layer chromatography (TLC), and column chromatography (CC). The CC results revealed two active compounds against methicillin-resistant *Staphylococcus aureus* (MRSA) DMST20654. Based on UV-Vis spectroscopy, liquid chromatography-mass spectrometry (LC-MS), and nuclear magnetic resonance (NMR) spectroscopy, the two bioactive compounds were identified as thiolutin ($C_8H_8N_2O_2S_2$) and thiolutin dioxide ($C_8H_8N_2O_4S_2$), respectively. Bioactive compounds were examined for antimicrobial activity against MRSA DMST20654, methicillin-resistant *Staphylococcus epidermidis* (MRSE), clinical isolate *Escherichia coli* 2026, and clinical isolate *Klebsiella pneumonia* 1617. The result showed that thiolutin was the most effective against MRSA DMST20654 with minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of 4 and 8

ug·ml^{-1}, respectively. Furthermore, thiolutin dioxide exhibited the most potent antimicrobial activity against MRSE with MIC and MBC values of 4 ug·ml^{-1}. To our best knowledge, this is the first report on isolation and characterization of thiolutin dioxide from *S. luteosporeus*. This finding leads to the development of potential antimicrobial agents for pharmaceutical applications.

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Student's Signature พงศกร กันทะ
Advisor's Signature

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LIST OF ABBREVIATIONS

AIA	= Actinomycetes isolation agar
CFU	= Colony forming unit
cm	= Centimeter
DI water	= Deionized water
DNA	= Deoxyribose nucleic acid
ISP	= International *Streptomyces* Project Medium
MDR	= Multi-drug resistant
MHA	= Mueller-Hinton Agar
MRSA	= Methicillin-resistant *Staphylococcus aureus*
MRSE	= Methicillin-resistant *Staphylococcus epidermidis*
ESBL	= Extend spectrum beta-lactamase
WHO	= World health organization
nm	= Nanometer
mm	= Millimeter
PCR	= Polymerase chain reaction
rRNA	= Ribosomal ribonucleic acid
°C	= Degree Celsius
h	= Hour
g	= Gram
mg	= Milligram
min	= Minute

LIST OF ABBREVIATIONS (Continued)

AMU	= Atomic mass unit
Sec	= Second
µg	= Microgram
µl	= Microliter
ml	= Milliliter
rpm	= Round per minute
TBE	= Tris-borate-EDTA
m/z	= Mass to charge ratio
v/v	= Volume by volume
w/v	= Weight by volume
OD	= Optical density
λ	= Lambda
MIC	= Minimum inhibitory concentration
MBC	= Minimum bactericidal concentration
MCS	= Multiple cloning site

CHAPTER I

INTRODUCTION

1.1 Background / Problem

The discovery of penicillin, world's first antibiotic, by Sir Alexander Fleming was reported in early 1928. Since then, several antibiotics have been continuously discovered and developed for the treatment of infectious diseases. However, the widespread use of antibiotics has led to the development of antibiotic-resistant organisms (Chandra and Kumar, 2017; Gaynes, 2017; Gurung et al., 2009; Walsh, 2003). Antibiotic-resistant bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *S. aureus* (VRSA), multidrug-resistant *Mycobacterium tuberculosis* (MDR-MTB) extended-spectrum beta-lactamase (ESBL) (*Enterococcus faecium*, *Escherichia coli*, *Salmonella enterica*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacter* sp.), and carbapenem-resistant Enterobacteriaceae are a serious public health concern worldwide. (Li and Webster, 2018; Selvameenal et al., 2009; Walsh, 2003). According to the World Health Organization (WHO), MRSA and ESBL are particularly harmful antibiotic-resistant bacteria due to their high morbidity and fatality rates. As a result, the demand for antimicrobial compounds has grown due to their potential application in the pharmaceutical industry. Antibiotics are the most important category of fermentation products, with the highest demand. In the biotechnological process, microbial fermentation is used the for economic production of antibiotics by submerged-state fermentation (Shyamkumar et al., 2014).

It has been demonstrated that a wide range of bioactive compounds are isolated from soil microorganisms (Bérdy, 2005). Thus, soil microorganisms have become a major source of novel antimicrobial compounds. A number of antibiotics have been reported from soil microorganisms, 70% from actinobacteria and 30% from filamentous

fungi. It has been reported that more than 12,000 commercial antibiotic drugs are produced by the genus *Streptomyces* (Nandhini et al., 2018; Rathod et al., 2018). Therefore, *Streptomyces* spp. are an important natural source of novel antimicrobial compounds (Bunyapaiboonsri et al., 2016; Chandra and Kumar, 2017; Gaynes, 2017).

The members of the genus *Streptomyces* are Gram-positive filamentous bacteria with high C+G content (>55%) in their genome. They produce layers of aerial mycelium with branches. Aerial mycelium can be divided into a long chain of spores through cell division. *Streptomyces* spp. can be found in diverse habitats of soil, freshwater, marine water, and extreme environment (Barka et al., 2015; Gebreyohannes et al., 2013; Hasani et al., 2014; Flärdh and Buttner, 2009). In general, *Streptomyces* spp. have the ability to produce a wide variety of primary and secondary metabolites which are commercially valuable as enzymes, amino acids, bioactive compounds, antibacterial agents, antifungal agents, antiparasitic agents, anticancer compounds, insecticides, pesticides, and herbicides (Al-Dhabi et al., 2016; Thumar et al., 2010).

To address the issue of antibiotic-resistant bacteria, it is critical to search for a novel antibiotic against drug-resistant strains. Thus, the aim of this study is to isolate and identify the antimicrobial compounds of soil isolate *Streptomyces* sp. PKA51. The bioactive compounds of strain PKA51 were purified and characterized. Bioactive agents were also evaluated for antimicrobial activity against antibiotic-resistant bacteria.

1.2 Research objectives

1.2.1 To identify a soil isolate, *Streptomyces* sp. PKA51.

1.2.2 To evaluate the antimicrobial activity of crude extract of *Streptomyces* sp. PKA51 against drug-resistant bacteria.

1.2.3 To purify and characterize bioactive compounds of *Streptomyces* sp. PKA51.

1.2.4 To evaluate the antimicrobial activity of purified bioactive compounds.

1.3 Research hypothesis

Purified bioactive compounds of *Streptomyces* sp. PKA51 exhibit antimicrobial activity against tested pathogens.

1.4 Scope and limitations of the study

This research involved the identification and evaluation of *Streptomyces* sp. PKA51. The strain PKA51 was isolated from terrestrial soil, Suranaree University of Technology, Nakhon Ratchasima province, Thailand (Khowangklang, 2017). The antimicrobial-producing *Streptomyces* was identified by cultural characteristics and 16s rRNA gene sequence. The *Streptomyces* sp. PKA51 was used for the extraction of crude compound. The antimicrobial activity of crude compound against Gram-positive and Gram-negative pathogens were determined by agar well diffusion and contact bioautography. The crude compounds were purified by thin-layer chromatography and column chromatography. The purified bioactive compounds were characterized by using UV-Visible spectroscopy, liquid chromatography-mass spectrometry (LC-MS), and nuclear magnetic resonance (NMR) spectroscopy. The MIC and MBC of purified bioactive compounds were also investigated.

CHAPTER II

LITERATURE REVIEWS

2.1 Streptomycetes

Streptomycetes are aerobic, chemoorganotrophic, Gram-positive filamentous bacteria with high cytosine and guanine (C + G) content in the genome. Streptomycetes belong to the Phylum: Actinobacteria, Class: Actinobacteria, Order: Streptomycetales, Family: Streptomycetaceae, and Genus: *Streptomyces*. Streptomycete colonies usually grow with substrate mycelial and produce conidia from reproductive structures called aerial mycelial. Aerial mycelium is emerged upward from the surface of the colony into the air (Figure 2.1). They could produce geosmin and siderophore in terrestrial environments. Geosmin is a volatile organic compound which causes earth smell and musty odors in soil (Bhatti et al., 2017; Flärdh and Buttner, 2009; Li et al., 2016; Sharma and Manhas, 2020; Usha Nandhini et al., 2018).

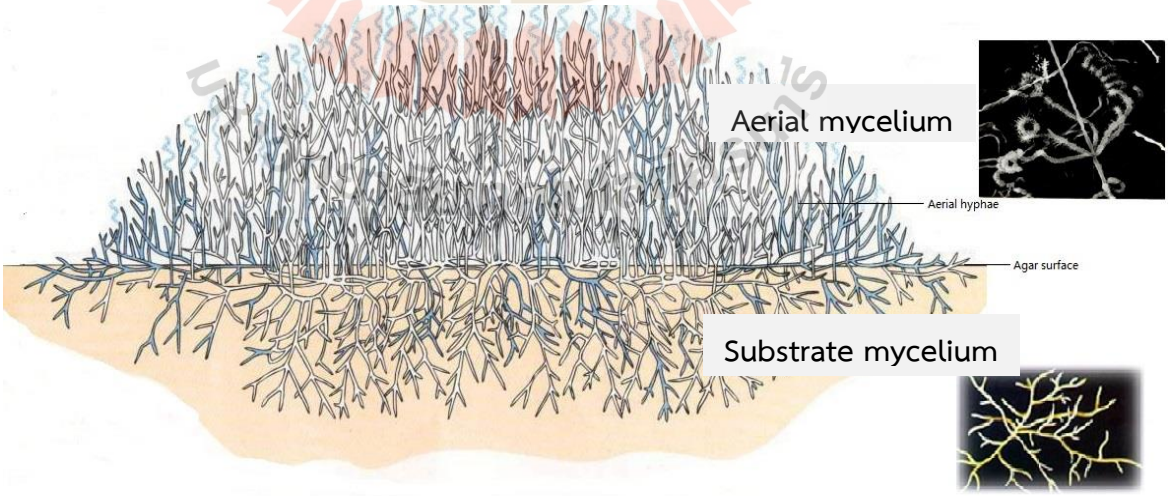


Figure 2.1 The cross-section of a streptomycetes colony showing substrate/vegetative mycelium, aerial mycelium, and conidia, which develops upward from the surface of a colony (Li et al., 2016).

Streptomycetes can be found in diverse environments, including terrestrial soil, freshwater, marine water, and unexplored habitat (Arifuzzaman et al., 2010; Chandra and Kumar, 2017; Gebreyohannes et al., 2013; Gurung et al., 2009; Kumar et al., 2014). It has been reported that streptomycetes can be found in extreme environments such as Tharban hot spring, Mount Everest, Himalayan region, Egyptian desert, Saudi Arabia desert, Indian saline desert, volcanic cave, the lake of Ghana, and mangrove soil (Al-Dhabi et al., 2016; Gurung et al., 2009; Hozzein et al., 2011; Ser et al., 2015; Nithya et al., 2018; Radhakrishnan et al., 2007; Radhakrishnan et al., 2010; Selim et al., 2021; Tawiah et al., 2012; Thumar et al., 2010). Most of streptomycetes live as a symbiosis with fungi, animals, plants, insects, coral, and sponges (Figure 2.2). The interactions between streptomycetes and other hosts give benefit for the production of secondary metabolites such as, protection from other microorganisms and degradation of the chemical composition of organic compound polymers (lignocellulose). As a result, streptomycetes can survive in nature for a long period (Meij et al., 2017).

The most important characteristic of streptomycetes is the ability to create secondary metabolites with antimicrobial properties. Based on the number of species identified, one of the major genera of actinomycetes is *Streptomyces*. It has been shown that nearly two-thirds of the naturally occurring antimicrobial substances are derived from *Streptomyces* sp. (Kumar et al., 2019; Sharma and Manhas, 2019). *Streptomyces* spp. can grow in various environments. It has the ability to produce an aerial mycelium layer that can differentiate into a chain of spores.

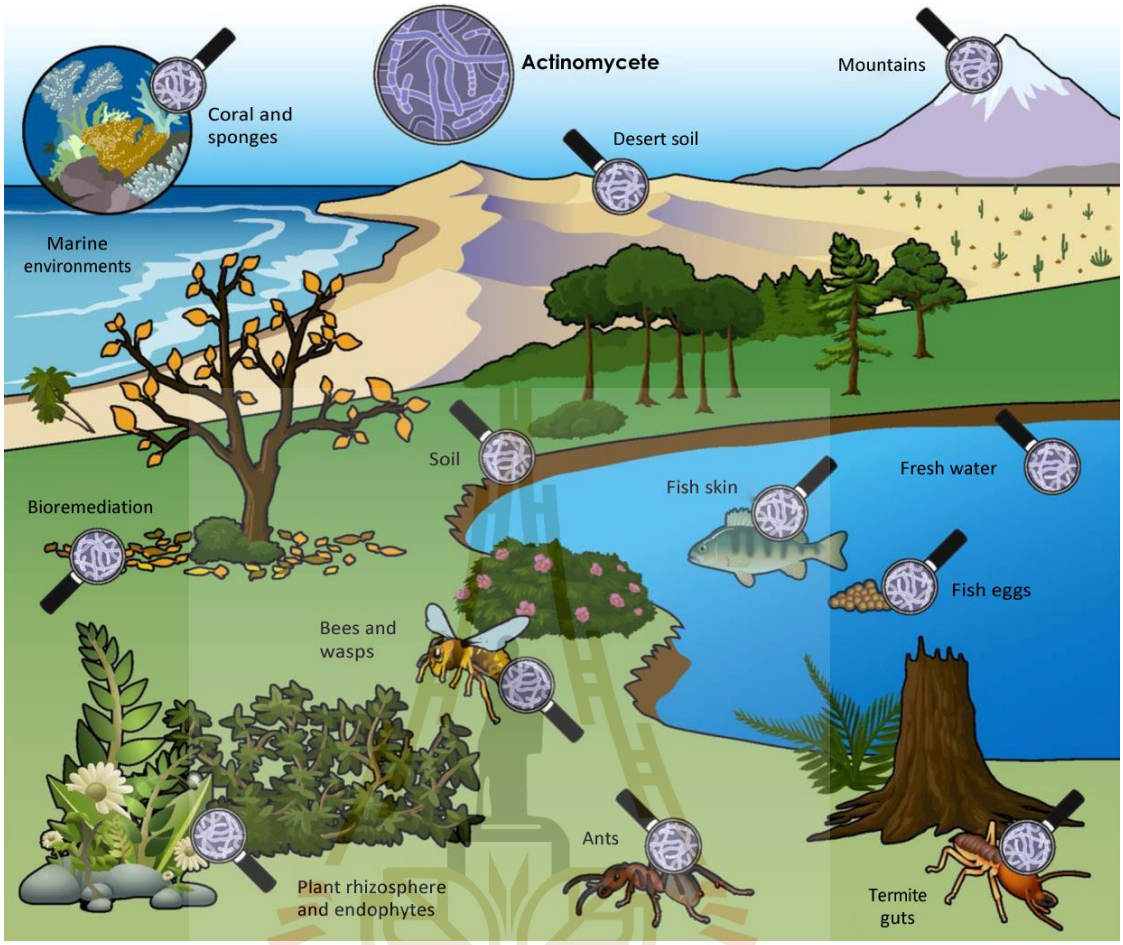


Figure 2.2 Ecology and distribution of streptomycetes in environments. Streptomycetes live as a symbiosis with other microorganisms and organisms such as coral, sponges, mycorrhiza, plants, insects, and fish scales (Meij et al., 2017).

The life cycle of *Streptomyces* begins when environmental conditions are favorable such as carbon source, nitrogen source, temperature, oxygen, pH, and moisture. Spores fall on the appropriate environment and germinate (formation of germ tube). Germ tubes are developed into mycelium by tip elongation and branching, called substrate mycelium. To obtain nutrients, the tip can secrete exo-enzymes to break down the chemical composition of organic compounds (chitin, cellulose, lignocellulose). When nutrients are scarce, autolytic degradation via programmed cell death occurs to provide nutrients for the development of aerial mycelium. Substrate mycelium starts to differentiate into aerial mycelium. The process of sporulation begins

at the tips of aerial mycelium. Chromosome segregation, septation, and cell division occur during the formation of reproductive aerial mycelium, resulting in a lengthy chain of spores. Each spore develops into a mature spore containing a single chromosome. To penetrate the surface tension of the agar medium and grow into the air, the mycelium was covered with a hydrophobic sheath. Finally, the reproductive aerial mycelium differentiates to form chains of spores (Figure 2.3) (Flärdh and Buttner, 2009; Hasani et al., 2014; Meij et al., 2017).

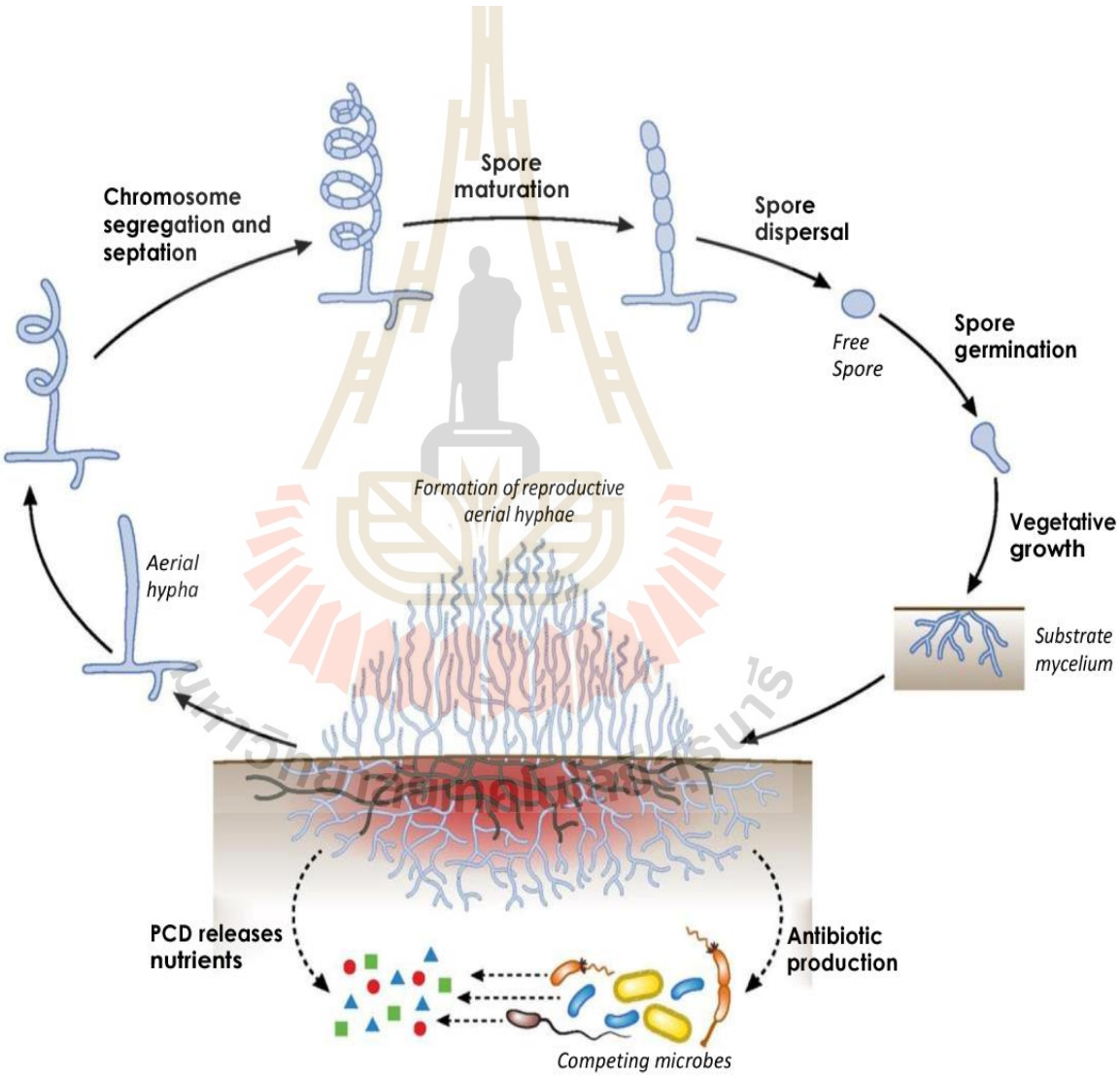


Figure 2.3 The life cycle of filamentous *Streptomyces* (Meij et al., 2017).

2.2 Secondary metabolites and antibiotics

Secondary metabolites are small organic molecules produced by an organism that does not directly participate in cell growth. The excretion of secondary metabolites is not related to the development of cell growth and reproduction of microorganisms. Most of the microbes such as bacteria, filamentous fungi, unicellular algae, and plankton could produce several types of secondary metabolites. Secondary metabolites derived from microorganisms have various biological properties including bioactive compounds, antibacterial agents, antifungal agents, antiparasitic agents, anticancer compounds, insecticides, pesticides, herbicides, toxic substances, metal-transporting agents, sex hormones, bacterial pigments, immunomodulators, growth factors, and immunosuppressive agents. These substances are usually produced during the late exponential phase and stationary phase of cell growth (Al-Dhabi et al., 2016; Gokulan et al., 2014; Jakubiec-Krzesniak et al., 2018). Secondary metabolites prevent streptomycetes from other microorganisms and increase their survival rate. Secondary metabolites can impede the growth of other microorganisms (Hasani et al., 2014; Meij et al., 2014).

The most unique characteristic of the genus *Streptomyces* is the ability to create antibacterial secondary metabolites. Antimicrobial compounds or antibiotics have been used to prevent infectious diseases in human, livestock, veterinary, and crop production (Chandra and Kumar, 2017; Gebreyohannes et al., 2013). Most *Streptomyces* can produce a wide variety of antibiotics including aminoglycosides (streptomycin), anthracyclines, beta-lactams, glycopeptides, kanamycin, viomycin, macrolides (erythromycin), tetracyclines, chloramphenicol, neomycin, nystatin, ivermectin, amphotericin, rifamycin, polyenes, non-polyenes, and cycloheximide (Basavaraj et al., 2010; El-nagger et al., 2017).

About 22,500 secondary metabolites have been discovered so far (Bérdy, 2005; Radhakrishnan et al., 2010). Approximately, 12,000 substances of antibiotics are produced by actinomycetes especially from the genus *Streptomyces* (70%), non-actinomycete bacteria, and filamentous fungi (30%) (Basavaraj et al., 2010; Hasani et al., 2014; Selvameenal et al., 2009). *Streptomyces* spp. is thought to synthesize over

100,000 different compounds (Arifuzzaman et al., 2010; Al-Dhabi et al., 2016; Dasari et al., 2012; Rathod et al., 2018). It has been reported that less than 0.1% of global soil has been screened for antimicrobial compounds in the last 50 years (Shetty et al., 2014). As a result, several antimicrobial substances are yet unidentified.

2.3 A brief history of antibiotics discovery and resistance

The world's first antibiotic "penicillin" was found in 1928s by Sir Alexander Fleming, a microbiologist at St. Mary's Medical School of London University. On an agar plate, Fleming observed that there have no bacteria growing in the clear zone around the fungus colonies. Fleming noticed that organic substances excreted by fungal colonies might suppress bacterial growth. After that, the fungal colony was isolated and identified as *Penicillium notatum*, later reclassified as *P. rubens* (Houbraken et al., 2011). In 1942s, the bioactive compound was purified and identified as Penicillin. In the 1945s, the purification procedure eventually results in penicillin antibiotic mass production and distribution. Penicillin was introduced into medical used and revolutionized the treatment of infectious diseases caused by Gram-positive pathogens such as staphylococci and streptococci (*Streptococcal meningitis*) (Aminov, 2014; Chandra and Kumar, 2017; Durand et al., 2019; Gaynes, 2017). The history of antibiotics produced by the genus *Streptomyces* began with the identification of streptothricin and streptomycin between the years 1942 and 1943 (Hasani et al., 2014). During the years 1940-1970, various types of antibiotics were screened for their antimicrobial activities and introduced to clinical use. Several types of antibiotics were discovered from various microorganisms (Table 2.1).

Table 2.1 List of antibiotics produced by various microorganisms.

Antibiotic	Microorganism/source	Discovery year
Penicillin	*Penicillium notatum*	1929
Griseofulvin	*Penicillium griseojitlvum*	1939
Tyrothricin	*Bacillus brevis*	1939
Actinomycin	*Streptomyces fradiae*	1940
Streptomycin	*Streptomyces griseus*	1944
Antimycin A	*Streptomyces kitazawensis*	1945
Bacitracin	*Bacillus subtilis*	1945
Cephalosporins	*Streptomyces clavuligerus*	1945
Chlortetracycline	*Streptomyces aureofaciens*	1945
Chloramphenicol	*Streptomyces venezualae*	1947
Neomycin	*Streptomyces fradiae*	1949
Nystatin	*Streptomyces noursei*	1949
Oxytetracycline	*Streptomyces rimosus*	1949
Paromomycin	*Streptomyces rimosus*	1950
Novobiocin	*Streptomyces niveus*	1950
Mytomycin C	*Streptomyces lavendulae*	1950
Viomycin	*Streptomyces vinaceus*	1951
Tetracyclines	*Streptomyces aureofaciens*	1952
Erythromycin	*Streptomyces erythraeus*	1952
Spiramycin	*Streptomyces ambofaciens*	1952
Amphotricin B	*Streptomyces nodosus*	1953
Cycloserine	*Streptomyces garyphalus*	1954
Oleandomycin	*Streptomyces antibioticus*	1954
Cycloserin	*Streptomyces orchidaccus*	1956
Vancomycin	*Streptomyces orientalis*	1956
Kanamycin	*Streptomyces kanamyceticus*	1957
Rifamycin	*Streptomyces mediterranei1*	1957
Polyoxins	*Streptomyces cacaoi*	1960

Table 2.1 List of antibiotics produced by various microorganisms (Continued).

Antibiotic	Microorganism/Source	Discovery year
Spectinomycin	*Streptomyces spectabilis*	1962
Streptogramin	*Streptomyces graminofaciens*	1962
Gentamycin	*Micromonospora purpurea*	1963
Lincomycin	*Streptomyces lincolensis*	1964
Candicidin	*Streptomyces griseus*	1965
Tobramycin	*Streptomyces tenebrarius*	1965
Clindamycin	*Streptomyces lincolensis*	1966
Monensin	*Streptomyces cinnamonensis*	1967
Actinomycin D	*Streptomyces antibioticus*	1968
Fosfomycin	*Streptomyces fradiae*	1969
Ribostamycin	*Streptomyces ribosidificus*	1970
Bambermycin	*Streptomyces bambergiensis*	1974
Clavulanic acid	*Streptomyces clavuligerus*	1974
Nikkomycin	*Streptomyces tendae*	1976
Thienamycin	*Streptomyces cattleya*	1976
Lasalocid	*Streptomyces lasaliensis*	1977
Dekamycin	*Streptomyces fradiae*	1977
Avermectic	*Streptomyces avermitilis*	1978
Daptomycin	*Streptomyces roseosporus*	1980
Aureofacin	*Streptomyces oreofaciens*	1981
Oligomycin	*Streptomyces diastachromogenes*	1987
Salimomycin	*Streptomyces albus*	1987
Retapamulin	Fungi	2007
Ceftobiprole Medocaril	Fungi	2008
Ceftaroline fosamil	Fungi	2010

Source: Chandra and Kumar, 2017; Hasani et al., 2014; wanbanjob, 2008

Many antibiotics have been introduced to treat various infectious diseases. Millions of people worldwide use antibiotics in agriculture and medicine. According to World Health Organization (WHO), Infectious Disease Society of America, and Centers for Disease Control and Prevention (CDC), the widespread use and misuse of antibiotics has led to the development of antibiotic-resistant bacteria. More than two million people worldwide have been reported to be infected with antibiotic-resistant bacteria. It has been estimated that antibiotic resistance is responsible for 25,000 and 23,000 deaths each year in the USA and EU (Aminov, 2010; Dahal and Chaudhary, 2018; Uzair et al., 2018). The emergence of bacterial resistance to various antibiotics is a serious medical problem that leads to the failure of antibiotic treatment in humans (Gurung et al., 2009). Pathogenic bacteria could resist to antibiotics by four main mechanisms include (1) limiting uptake of a drug by reduced membrane permeability, (2) inactivation of antibiotics, (3) modification of a drug target, and (4) increased efflux pump (figure 2.4) (Aminov, 2010; Blair et al., 2015; Dahal and Chaudhary, 2018; Hasani et al., 2014; Lambert, 2002; Reygaert, 2018). Recently, antibiotic-resistant bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *S. aureus* (VRSA), vancomycin-resistant *Enterococci* (VRE), multidrug-resistant *Mycobacterium tuberculosis* (MDR-MTB), extended-spectrum beta-lactamase producing bacteria (ESBL) (*Enterococcus faecium*, *Escherichia coli*, *Klebsiella pneumoniae*, *Salmonella enterica*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacter* sp.) are developing resistant to almost all antibiotics (Chanthasena and Nantapong, 2016; Dahal and Chaudhary, 2018; Kaur et al., 2015; Rajivgandhi et al., 2018; Selvameenal et al., 2009). MRSA and ESBL (*K. pneumoniae*, *E. coli*, and *P. aeruginosa*) are considered as dangerous antibiotic-resistant bacteria because they can cause morbidity and mortality in humans and animals (Kemung et al., 2018; Singh et al., 2018).

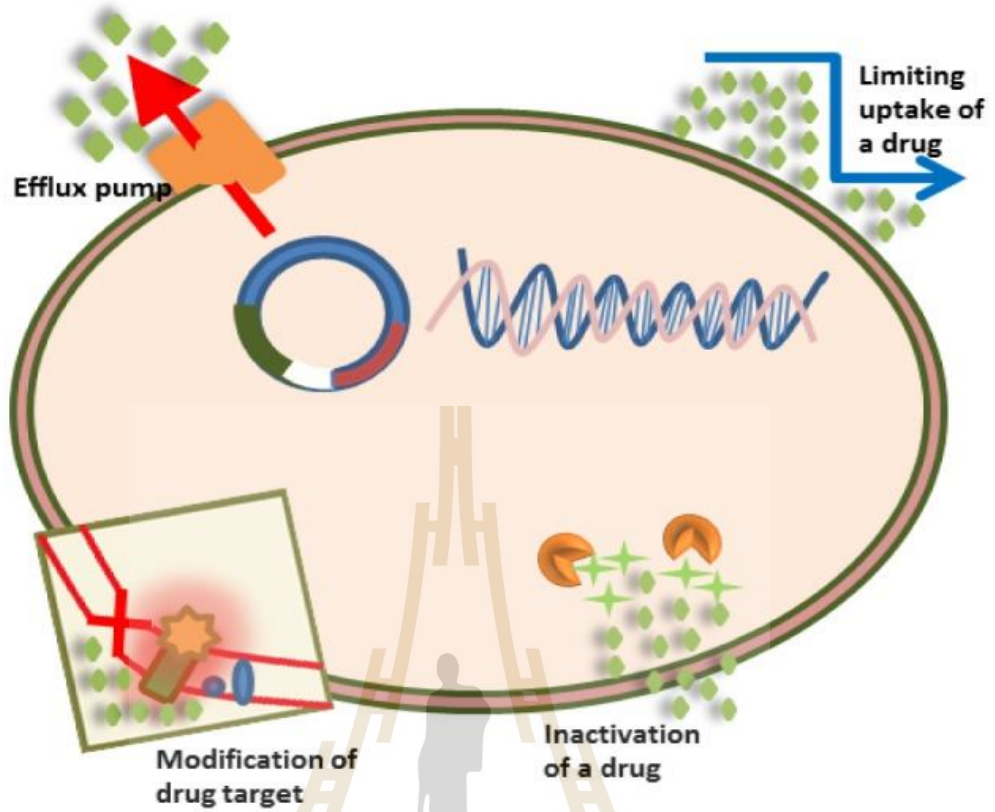


Figure 2.4 An overview of the antimicrobial resistance mechanisms (Reygaert, 2018).

The antibiotic resistance has emerged in the late 1940s when resistance to penicillin (beta-lactam antibiotics) had appeared in *S. aureus*. The penicillinase enzyme (beta-lactamase) produced by the resistance strain degrades the beta-lactam ring, making antibiotics ineffective. In 1950, to address the issue of penicillin-resistant *S. aureus*, penicillinase-stable methicillin was developed for clinical use. Methicillin was then used to treat infectious diseases caused by beta-lactam-resistant *S. aureus*. In 1951, methicillin-resistant *S. aureus* (MRSA) was first isolated in the UK. It has been reported to resist almost all beta-lactams and other antibiotic classes. The PBP2 gene encodes the PBP2 enzyme involved in cell wall formation that has a low binding affinity for beta-lactam antibiotics, reducing the inhibitory effect of antibiotics (Saga and Yamaguchi, 2009). Many cases of MRSA infections have been reported in hospitals around the world and become a significant global public health concern. In 1961, vancomycin was first isolated from *Streptomyces orientalis* and became the most potent antibiotic to treat MRSA infection (Kemung et al., 2018; Walsh, 2003). However,

S. aureus began to develop resistance to vancomycin, resulting in the development of vancomycin-intermediate *S. aureus* (VISA). VISA was first reported in Japan in the 1990s. On the other hand, vancomycin-resistant enterococci (VRE) were first isolated in the US in 1986. VRE (*Enterococcus faecalis* and *Enterococcus faecium*) demonstrated high-level resistance to vancomycin. In the US, VRE has been shown to transfer resistance genes to VISA by horizontal gene transfer (HGT), and vancomycin-resistant *S. aureus* (VRSA) emerged in 1996. Since then, the combination of antibiotics such as quinupristin/dalfopristin, and linezolid/daptomycin were used to treat MRSA, VRE, and VRSA infections (Sharma and Manhas, 2019; Walsh, 2003). Despite the fact that numerous antibiotics were discovered and introduced into clinical use during the twentieth century, only a few antibiotics are currently effective due to an increase in the number of bacteria acquiring antibiotic resistance (Figure 2.5). According to the US Food and Drug Administration (FDA), only nine new antibiotics have been approved for clinical use during the 2000s (Table 2.2). The decreasing number of drugs approved for clinical use suggested that the discovery of antimicrobial agents is urgently needed (Ventola, 2015).

Therefore, the emergence and spread of antimicrobial resistance is a global public health concern. Isolation and characterization of a novel antibiotic are required to combat the problem of antibiotic-resistant bacteria.

Table 2.2 Antibiotics approved by FDA since 2005.

Antibiotics	Drug class	Type of patients	Year approved
Tigecycline (Tygacil, Pfizer)	Tetracycline	>18 years	2005
Doripenem (Doribax. Shionogi)	Carbapenem	Adult patients	2007
Telavancin (Vibativ, Theravance Biopharma)	Glycopeptide	Adult patients	2008
Ceftaroline (Teflaro, Cerexa)	Cephalosporin	Adult patients	2010
Tedizolid (Sivextro, Cubist Pharmaceuticals)	Oxazolidinone	Adult patients	2014
Dalbavancin (Dalvance, Durata)	Glycopeptide	Adult patients	2014
Oritavancin (Orbactiv, Medicines)	Glycopeptide	Adult patients	2014
Ceftolozane/tazobactam (Zerbaxa, Cubist Pharmaceuticals)	Cephalosporin/beta-lactamase inhibitor	Adult patients	2014
Ceftazidime/avibactam (Avycaz, Cerexa Inc.)	Cephalosporin/beta-lactamase inhibitor	Adult patients	2015

Source: Ventola, 2015

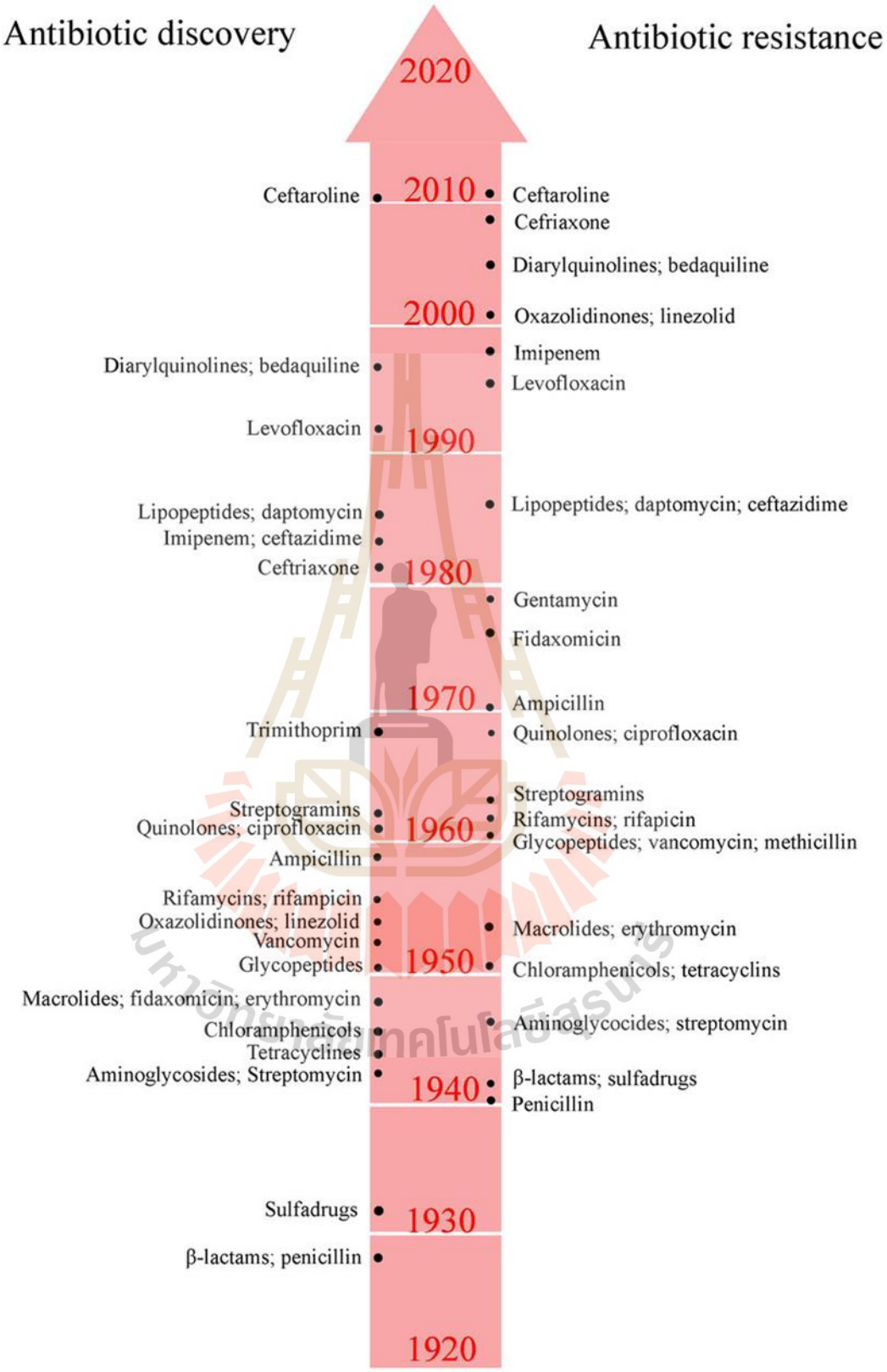


Figure 2.5 Timeline of antibiotics discovery and resistance (Dahal et al., 2018).

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

3.1.1 Bacterial strains

The test pathogens used in this research were Gram-positive bacteria: *Bacillus subtilis* TISTR008, *Bacillus cereus* TISTR684, *Staphylococcus epidermidis* TISTR518, *Staphylococcus aureus* ATCC1466, methicillin-resistant *S. aureus* (MRSA) DMST20654, and methicillin-resistant *S. epidermidis* (MRSE). The test pathogens were Gram-negative bacteria: *Escherichia coli* TISTR780, *Enterobacter aerogenes* TISTR1540, *Serratia marcescens* TISTR1354, *Salmonella* Typhimurium TISTR292, *Proteus mirabilis* TISTR100, *Pseudomonas aeruginosa* TISTR1287, clinical isolate *E. coli* 2026, and clinical isolate *Klebsiella pneumoniae* 1617. These microorganisms were obtained from the American Type Culture Collection (ATCC), Thailand Institute of Scientific and Technological Research (TISTR), Department of Medical Sciences Thailand (DMST), and Suranaree University of Technology hospital. All inoculums of test pathogens were prepared by growing cells in Luria-Bertani (LB) broth or Mueller Hinton broth (MHB) (Himedia, India) at 37 °C for 18-24 h.

3.1.2 Growth medium

In this study, the following growth media were used:

Luria-Bertani (LB) medium, Bennet's agar, Czapek's agar, Starch casein agar (SCA), Mueller-Hinton (MH) medium, International *Streptomyces* Project (ISP) medium including tryptone yeast extract agar (ISP-1), yeast malt agar (ISP-2), oatmeal agar (ISP-3), inorganic salt starch agar (ISP-4), glycerol asparagine agar base (ISP-5), peptone yeast extract iron agar (ISP-6), tyrosine agar (ISP-7), nitrate agar (ISP-8), and carbon utilizing agar (ISP-9). All chemicals and trace elements used are detailed in Appendix A.

3.1.3 Antibiotics

The following antibiotics were used in this study:

- Ampicillin (Bio Basin Inc, Canada)
- Vancomycin (Sigma-Aldrich, Germany)
- Tetracycline (Sigma-Aldrich, United Kingdom)

The stock solution of 512 $\mu g \cdot ml^{-1}$ of vancomycin and tetracycline was prepared by dissolving in sterile DI water. The sterile syringe filter with a 0.20 μm pore size (Corning® I Sigma-Aldrich, Germany) was used for filtration and sterilization of antibiotics. During this research, all antibiotics were stored as dry powders at 2-8 °C.

3.1.4 Chemical compounds

All chemical compounds used in this study were analytical reagent (AR) grade:

- Acetic acid glacial 99.7% from Loba Chemie, India
- Acetone from Carlo Erba™, United Kingdom
- Acetonitrile from RCI Labscan, Ireland
- Benzene from Loba Chemical, India
- Butan-1-ol from Carlo Erba™, United Kingdom
- Chloroform from RCI Labscan, Ireland
- Dichloromethane 99.5% from Loba Chemie, India
- Dimethyl sulfoxide from Fisher Scientific, United Kingdom
- Ethyl alcohol 95%, from Fisher Scientific, United Kingdom
- Ethyl acetate, from RCI Labscan, Ireland
- Glycerol 99.5% from Loca Chemie, India
- Iso-amyl alcohol from VWR Chemicals BDH®, France
- Methanol from RCI Labscan, Ireland
- n-Hexane 95% from RCI Scan, Ireland
- Isopropanol from Qrëc, Newzealand

3.1.5 Buffers

- Lysis buffer containing 400 mM Tris-HCl (pH 8.0), 60 mM EDTA (pH 8.0), 150 mM NaCl, 1% (w/v) sodium dodecyl sulfate (SDS).

- 10X TBE buffer (stock solution) for gel electrophoresis containing 108 g Tris-borate, 55 g of boric acid, 900 ml of distilled water, 40 ml of 0.5 M EDTA solution (pH 8.3), and adjust final volume to 1,000 ml.

- TE buffer containing 10 mM Tris-HCL, 0.1 mM EDTA (pH 8.0)

3.1.6 Primers

The universal primers were used to amplify the 16s rRNA gene of the stain PKA51. The following primers were used:

- 27 Forward, sequence 5′-AGAGTTTGATCCTGGCTCAG-3′
- 1525 Reverse, sequence 5′-AAAGGAGGTGATCCAGCC-3′
- M13 Forward, sequence 5′-GTAAAACGACGGCCAGT-3′
- M13 Reverse, sequence 5′-GCGGATAACAATTTCACACAGG-3′

3.1.7 Cloning vector

The pTG19-T PCR cloning vector (Vivantis, Malaysia) was used for 16s rRNA gene cloning. The cloning vector was designed for cloning PCR products with single-stranded 3′-dA overhangs. The map and multiple cloning site (MCS) of the pTG19-T PCR cloning vector is shown in Figure 3.1.

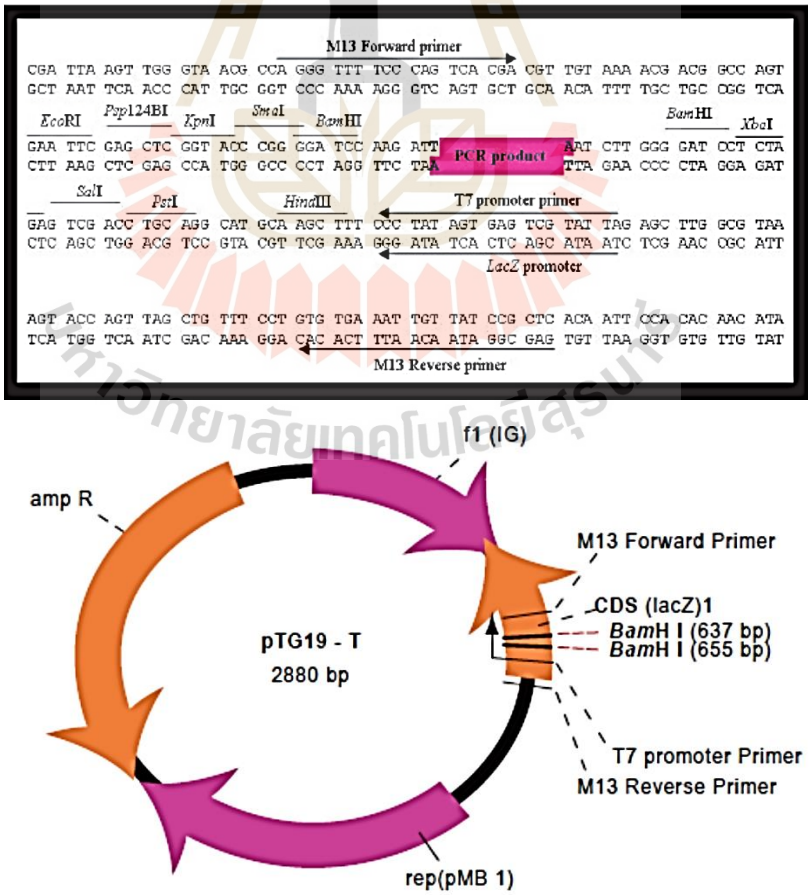


Figure 3.1 Map of pTG19-T PCR cloning vector.

3.1.8 Miscellaneous materials

- GoTaq® Green Master Mix from Promega, USA, was used for PCR amplification.

- Safe-Green™ nucleic acid stains from Canada, was used for visualization of nucleic acids in agarose gel electrophoresis.

- One MARK B 1,000 bp DNA ladder from Bio-Helix (GeneDirex), Taiwan, was used as a DNA marker.

- 100 bp DNA ladder M from Himedia, India, was used as a DNA marker.

- UltraPure™ DNase, RNase, Free Distilled Water was purchased from Thermo Fisher Scientific, USA.

- T4 DNA ligase purchased from Vivantis, Malaysia.

- X-gal was purchased from Bio basic, Canada. X-gal solution is added to the agar medium for screening the clones containing recombinant DNA in the blue-white screening technique.

- FavorPrep™ GEL/PCR purification Minikit was purchased from Favorgen®, Taiwan. It was used for gel or PCR product purification.

- FavorPrep™ Plasmid extraction kit was purchased from Favorgen®, Taiwan. It was used for plasmid purification.

- TLC Silica gel 60 F_{254} from Merck, Germany, was used for the purification of bioactive metabolites.

- Silica gel 60 from Merck, Germany.

3.1.9 Instruments and tools

All the instruments used in this study are listed in Table 3.1.

Table 3.1 List of instruments used in this study.

Instruments	Source
Autoclave Sterilizers	Tomy, USA
Biosafety cabinet	Esco, Singapore
Centrifuge machine	Hettich, Germany
Electronic balance	Shimadzu, Japan
Fume hood	S.K. powerable, Thailand

Table 3.1 List of instruments used in this study (Continued).

Instruments	Source
Gel electrophoresis systems	Cleaver scientific, United Kingdom
Heat box	Hercuvan, United Kingdom
Hot air oven	Memmert, Germany
Hotplate magnetic stirrer	IKA, China
Incubator shaker	Appendorf New Burnswick™, Germany
LC/MS instruments	Bruker, Germany
Microcentrifuge	Denville, Canada
Microscope	Olympus, USA
NMR spectrometer	Bruker, Switzerland
PCR thermal cycler	Thermo Fisher Scientific, USA
pH meter	Sartorius, Germany
Rotary evaporator	Buchi, Switzerland
UV/ VIS spectrometer T80^{+}	Thermo Fisher Scientific, USA
UV cabinet	CAMAG, Germany
Vortex mixer	FinePCR, South Korea

3.2 Methods

3.2.1 Isolation and maintenance of soil isolate

The strain PKA51 was isolated from terrestrial soil, Suranaree University of Technology, Nakhon Ratchasima province, Thailand. (Khowangklang, 2017). PKA51 was isolated by serial dilution plate technique using starch casein agar (SCA: soluble starch 10 g; casein 0.3 g; KNO_3 2.0 g; NaCl 2.0 g; K_2HPO_4 2.0 g; $MgSO_4{\cdot}7H_2O$ 0.05 g; $CaCO_3$ 0.02 g; $FeSO_4{\cdot}7H_2O$ 0.01 g; agar 15 g; H_2O 1.0 L; pH 7.2) (Dasari et al., 2012). PKA51 is maintained on International *Streptomyces* Project medium no. 2 agar (ISP-2: yeast extract 4.0 g; malt extract 10 g; glucose 4 g; agar 15 g; H_2O 1.0 L; pH 7.2) at 4 °C during the study. The strain PKA51 was kept as a glycerol stock (40% v/v) at -80 °C for further study.

3.2.2 Identification of the strain PKA51

3.2.2.1 Cultural and morphological identification

The cultural and morphological characteristics of the strain PKA51 were observed on Bennet's agar, Czapek's agar, Starch casein agar (SCA), various types of International *Streptomyces* Project (ISP) medium including tryptone yeast extract agar (ISP-1), yeast malt agar (ISP-2), oatmeal agar (ISP-3), inorganic salt starch agar (ISP-4), glycerol asparagine agar base (ISP-5), peptone yeast extract iron agar (ISP-6), tyrosine agar (ISP-7), nitrate agar (ISP-8), and carbon utilizing agar (ISP-9). The growth, diffusible pigment, aerial, and substrate mycelium colors were observed. Morphological characteristics of branch mycelium were observed under a field emission scanning electron microscope (SEM) (JEOL JSM 7800F model, Japan).

3.2.2.2 Genomic DNA extraction

Molecular identification of the strain PKA51 was performed by 16s rRNA gene analysis. The strain PKA51 was cultured on 10 ml of ISP-2 medium and incubated at 28 °C, 200 rpm for 3 days or until mycelia are formed. The cell pellets and mycelium were harvested by centrifuged at 4 °C, 9000 rpm for 5 min. The genomic DNA was extracted from cell pellets and mycelium by using the modified phenol-chloroform method (Khowangklang, 2017). Five mg of cell pellets were placed in a sterile mortar and mixed with 500 µl of lysis buffer and grinded with glass beads until fine. Then, the mixer was transferred into a new 1.5 ml microcentrifuge tube. Then, 165 µl of 5 M NaCl was added to the mixture and mixed by inverting the tube. The mixer was centrifuged at 4 °C, 13,000 rpm for 10 min. In a new microcentrifuge tube, 400 µl of chloroform and 400 µl of isoamyl alcohol were added to the supernatant. By carefully inverting the tube, the color of the mixer became milky. After that, the mixer was centrifuged at 4 °C, 13,000 rpm for 10 min. The upper layer was transferred into a clean microcentrifuge tube, which was then inverted to mix with an equal volume of chloroform. The sample was centrifuged at 4 °C, 13,000 rpm for 5 min. The supernatant was transferred into a new tube and a two-volume of 95% ethanol was added to precipitate the genomic DNA. The tube was mixed by inverting and centrifuged at 4 °C, 13,000 rpm for 5 min. The supernatant was discarded. After that, DNA pellets were washed three times with 500 µl of 70% ice-cold ethanol and centrifuged at 4 °C, 13,000

rpm for 1 min. The remaining supernatant was discarded. The genomic DNA was dried in a heat box (Hercuvan, UK) at 45 °C and then dissolved in 30 µl of TE buffer. Using agarose gel electrophoresis, the genomic DNA was quantified qualitatively. The genomic DNA was stored at -20 °C for further PCR amplification.

3.2.2.3 16S rRNA gene amplification

The genomic DNA was used as a template for amplification of the 16S rRNA gene by using universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1525R (5'-AAGGAGGTGATCCAGCC-3'). The PCR amplification was performed by using a thermal cycler (Px2 Thermo Fisher Scientific, USA). The PCR conditions consist of an initial denaturation at 95 °C for 5 min. Twenty-five cycles of denaturation at 95 °C for 60 s, primer annealing at 55 °C for 30 s, extension at 72 °C for 90 s, and a final extension at 72 °C for 7 min. Figure 3.2 depicts a description of PCR conditions. A total volume of 75 µl of PCR mixtures is shown in Table 3.2. The PCR amplification products were validated on agarose gel (0.7%, w/v) in 1X TBE buffer. The amplified fragment length of 1,500 bp was purified from agarose gel by using FavorPrep™ GEL/PCR Purification Kit (FAVORGEN, Taiwan).

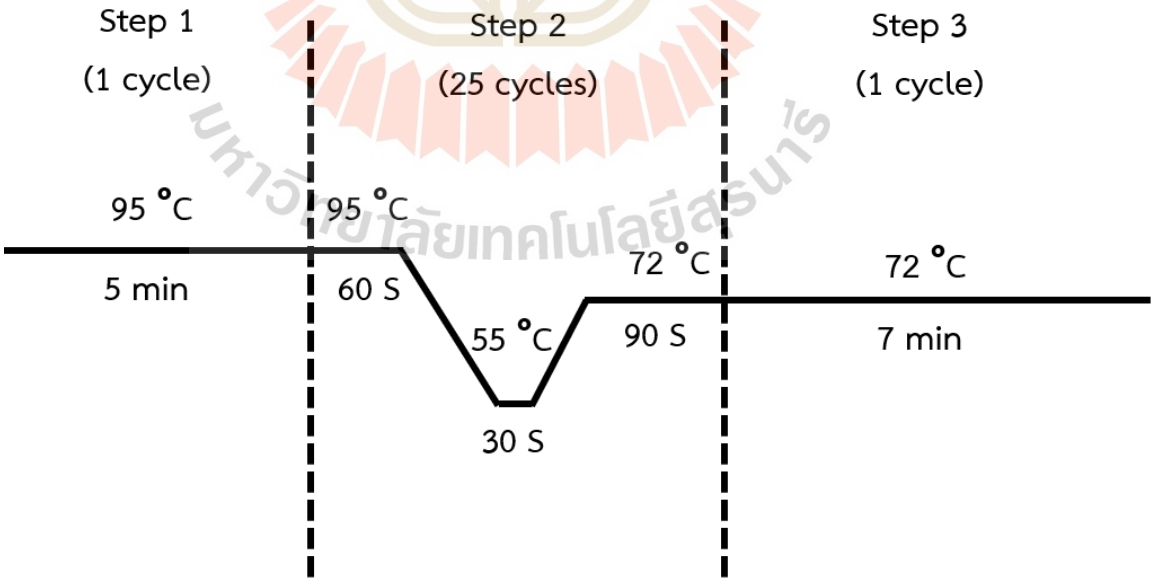


Figure 3.2 Description of PCR conditions.

Table 3.2 Compositions of the PCR reaction.

Chemicals	Volume (µl)
Genomic DNA template	15
GoTaq® Green Master Mix	37.5
27 Forward primer (10 µM)	2.5
1525 Reverse primer (10 µM)	2.5
UltraPure™ DNase, RNase, Free Distilled Water	17.5
Total volume	**75**

3.2.2.4 Preparation of *E. coli* competent cells

Competent cells were prepared from *Escherichia coli* JM109 by using the calcium chloride ($CaCl_2$) method. A single colony of *E. coli* JM109 was picked and aseptically transfer into 5 ml of LB broth. The colony was grown at 37 ℃, 200 rpm for 24 h. After incubation, 1 ml of the culture was transferred to 500 ml Erlenmeyer flask containing 100 ml of LB broth and incubated at 37 ℃, 200 rpm until the OD_{600nm} reached 0.3. After that, the bacterial cells were centrifuged at 4 ℃, 5000 rpm for 10 min. The cell pellets of *E. coli* JM109 were resuspended in 10 ml of ice-cold $MgCl_2$ solution (0.1 M) and incubated on ice for 30 min. The tube was centrifuged at 4 ℃, 3000 rpm for 5 min, then discarded the supernatant. The cell pellets were then gently resuspended in ice-cold $CaCl_2$ solution (0.1 M) and incubated on ice for 1 h. The tube was centrifuged at 4 ℃, 3000 rpm for 5 min, then discarded the supernatant. Finally, the cell pellets were collected and resuspended in 2 ml of ice-cold $CaCl_2$ solution (0.1 M) containing glycerol (15 %, v/v), and incubated on ice for 1 h. After incubation, each 100 µl of *E. coli* JM109 competent cells were immersed in liquid nitrogen and kept at -80 ℃.

3.2.2.5 Construction of recombinant plasmid containing 16s rRNA gene and transformation

The purified fragment of 16s rRNA gene was ligated to a TA cloning vector (pTG19-T PCR cloning vector, vivantis). A total volume of 10 µl of ligation mixtures is shown in Table 3.3. The microcentrifuge tube was incubated at 16 ℃ for 24 h. After that, the recombinant plasmid was transformed into *E. coli* JM109. One-hundred

milliliters of *E. coli* JM109 competent cells were mixed with ligation mixture and incubated on ice for 1 h. The mixture was heated at 42 °C for 60 sec before being placed on ice for 2 min. One milliliter of LB broth was added to the mixture and incubated for 1 h at 37 °C, 200 rpm. Then 100 µl of suspension was spread on LB agar containing ampicillin (100 $\mu g \cdot ml^{-1}$) and X-gal (80 $\mu g \cdot ml^{-1}$). The plate was incubated at 37 °C for 24 h. The recombinant clones were selected by the blue-white colony screening method. This method allows recombinant clones to be distinguished by the color of colony. Transformants of white colonies containing the 16S rRNA of the strain PKA51 were extracted and purified by FavorPrep™ Plasmid DNA Extraction Kit (FAVORGEN, Taiwan).

Table 3.3 Chemicals used for ligation of 16s rRNA gene.

Chemicals	Volume (µl)
pTG19-T cloning vector (25 $ng \cdot ml^{-1}$)	2
Purified PCR product	3
Ligase buffer (10X)	1
T4 DNA ligase (200 $u \cdot \mu l^{-1}$)	1
Ultrapure nuclease-free water	3
Total volume	**10**

3.2.2.6 DNA sequencing

Purified recombinant plasmids were sent for sequencing at ATCG company limited, Thailand. The recombinant plasmid containing the 16S rRNA gene of the strain PKA51 was sequenced by using universal primers M13F (-40) (5'-GTTTTCCCAGTCACGAC-3') and M13R (-40) (5'-CAGGAAACAGCTATGAC-3'). Nucleotide sequence obtained from 16S rRNA gene was compared with other related species maintained in the EzBiocloud database (http://www.ezbiocloud.net) through BLAST search. The nucleotide sequence of the strain PKA51 was deposited in National Center for Biotechnology Information (NCBI) database.

3.2.2.7 Phylogenetic tree analysis

To determine the genetic relationship between the strain PKA51 and closely related species. Multiple sequence alignments of relatively nucleotide sequences from the EzTaxon database were aligned by using ClustalW. The phylogenetic tree was constructed by using Molecular Evolutionary Genetics Analysis (MEGA) software version X. Bootstrap values are the results of 1000 repetitions of the analysis.

3.3 Preparation of crude compound

The crude extract was prepared by submerged state fermentation. Soil isolate PKA51 was inoculated into ISP-2 broth and incubated at 28 °C, 200 rpm for 3 days. Then, the seed culture was transferred into a 1,000 ml Erlenmeyer flask containing 400 ml of MHB medium and incubated at 28 °C, 200 rpm, for 5 days. After incubation, the fermented broth was centrifuged at 4 °C, 9,000 rpm for 5 min. Then, the culture medium was filtrated to remove bacterial cells by Whatman No.1 filter paper (Whatman™, GE Healthcare, UK). The cell pellets were collected and air-dried in a hot air oven (Memmert, Germany) at 65 °C for 3 days. The cell-free supernatant containing antimicrobial compounds was collected and extracted with an equal volume of ethyl acetate (1:1, v/v). The mixture of ethyl acetate and cell-free supernatant were shaken at 28 °C, 250 rpm for 1 h. The ethyl acetate phase was collected by a separatory funnel. After that, the organic solvent phase was evaporated under reduced pressure to dryness. Cell dry weight was used to calculate the yield of a crude compound by the following formula:

Yield = (mg of crude compound) / (g of cell dry weight)

The crude compound was used to test antimicrobial activities by agar well diffusion. Bioactive compounds were purified by thin-layer chromatography (TLC) and column chromatography.

3.4 Antimicrobial assay

3.4.1 Perpendicular cross streak method

The antimicrobial activity of the strain PKA51 was tested preliminarily by using the perpendicular cross streak method (Figure 3.3) against bacterial pathogens (Al-

Dhabi et al., 2016; Singh et al., 2016). The test pathogens were prepared in sterile 0.85% normal saline solution (NSS) and adjusted the turbidity to 0.5 McFarland standard. The strain PKA51 was inoculated in a straight line on Mueller Hinton Agar (MHA) (Hi-media, India). The plates were incubated at 28 °C for 5 days. After incubation, test pathogens were streaked perpendicular to the PKA51 colony. The plates were incubated at 37 °C for 24 h. The antimicrobial activity of PKA51 was observed by the inhibition zone. The susceptibility of the microorganism to the antimicrobial agent is recorded by the area of inhibited bacterial growth.

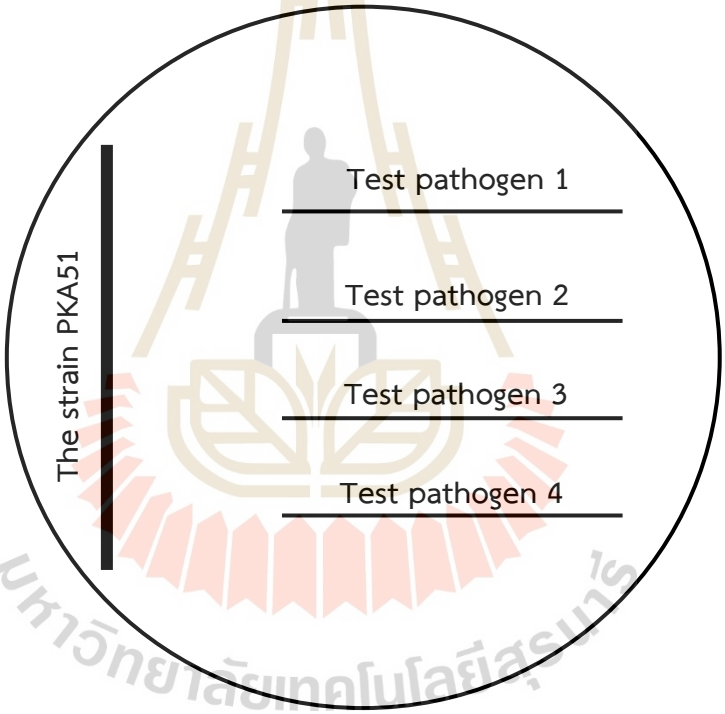


Figure 3.3 Streaking pattern for preliminary antimicrobial activity by perpendicular cross streak method.

3.4.2 Agar well diffusion method

The antimicrobial activity of PKA51 crude extract was determined by the agar well diffusion method (Figure 3.4). The bacteria inoculum was prepared with normal saline solution (0.85%, w/v) and compared to the turbidity with the 0.5 McFarland standard. Then, Mueller Hinton agar was swabbed by the cotton swab of test

pathogens. Agar plugs were removed by using a sterile toothpick. One hundred microliters of a crude extract (1.0 mg·well^{-1}) and ethyl acetate (negative control) were loaded into the well. The plates were left for 1 h to allow the crude compound to diffuse into the medium. The plates were incubated at 37 °C for 24 h or until the bacteria grows. The antimicrobial activity of crude compounds was observed by measuring the diameter of the inhibition zone (mm).

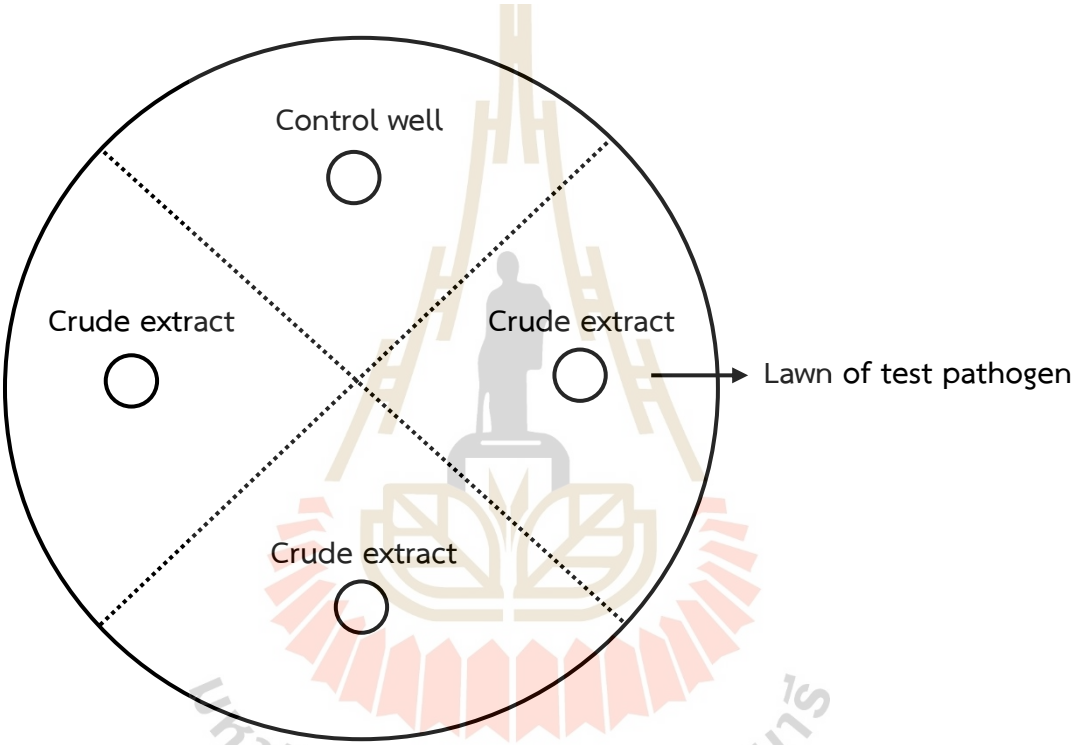


Figure 3.4 Agar well diffusion method for determination of antimicrobial activity of crude extract PKA51 against test pathogens.

3.4.3 Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The broth microdilution method on 96-well plates was used to determine the minimum inhibitory concentration (MIC) of purified bioactive compounds against antibiotic-resistant bacteria (CLSI, 2012; Hussain et al., 2018; Sharma and Manhas, 2019). Each pathogen was streaked onto Mueller Hinton Agar (MHA) and incubated at 37 °C

for 20-24 h. After incubation, the bacteria inoculum was prepared by adjusting the turbidity in Mueller Hinton Broth (MHB) until the OD_{625nm} reached 0.08-0.13 (1×10^8 CFU·ml^{-1}). Then, the final inoculum (turbidity 5×10^5 CFU·ml^{-1}) was prepared by transferring 100 µl of bacterial suspension into 9.9 ml of MHB. The stock solution (1 mg·ml^{-1}) of bioactive compound was prepared in 1% dimethyl sulfoxide (DMSO) diluted with MHB. The different concentrations of bioactive compound and standard drugs (vancomycin, and tetracycline) were made by serial two-fold dilution method (256, 128, 64, 32, 16, 8, 4, 2, 1, 0.5, 0.25, 0.125, 0.0625 and 0.03125 µg·ml^{-1}). Each well was inoculated with 100 µl of an inoculum. Growth control well consisted of 100 µl of test pathogen and 100 µl of MHB, negative control consisted of 100 µl of test pathogen and 1% DMSO in MHB, and sterility control contained MHB only. The plate was incubated at 37 °C for 20-24 h. After incubation, optical density (OD_{600nm}) was measured by using a microplate reader (Thermo Scientific MULTISCAN GO). The MIC value is the lowest concentration of bioactive agent that inhibits visible bacterial growth. Further, minimum bactericidal concentration (MBC) was determined by sub-spotting the last clear MIC culture samples onto the MHA plate. Inoculated plates were incubated at 37 °C for 24 h (Nithya et al., 2018; Rajivgandhi et al., 2018). After incubation, the concentration which show no bacterial growth was defined as the MBC value.

3.5 Purification of antimicrobial compounds

3.5.1 Partial purification of bioactive compounds by TLC

Thin-layer chromatography is used for the separation of mixture compounds using silica gel coated onto an aluminum sheet. The crude compound was applied to the bottom edge of TLC plates (Merck, TLC Silica gel 60 F254). The spot was left to dryness. Then, the chromatogram was immersed and developed in a developing chamber using chloroform and methanol (9.9: 0.1, v/v) as a mobile phase. When the solvent reaches 0.5 cm from the top of the plate, the TLC plate was removed from the developing chamber. After that, the chromatogram was left to dry and visualized under UV light (λ_{254nm}). The location of bioactive compounds was indicated by the contact bioautography technique (Taddei et al., 2006).

3.5.2 Detection of bioactive compound by contact bioautography

The antimicrobial activity of bioactive compounds was determined by contact bioautography assay (Figure 3.5). The developed chromatogram was sterile by exposure under UV for 30 min. The chromatogram was placed onto the medium agar seeded with a suspension of MRSA DMST20654 (turbidity equal to 0.5 McFarland standard). The chromatogram was left for 30 min to allow the compounds to diffuse into medium agar. Then, the chromatogram was removed from the agar plate. The plate was incubated at 37 °C for 24 h or until the bacteria grow. After incubation, the position of a clear zone on the agar plate indicates bioactive compounds. The bioactive bands in TLC showing inhibition zone were scraped and redissolved in ethyl acetate (Kumar and Jadeja, 2018). The partial purified bioactive compounds were further purified by column chromatography.

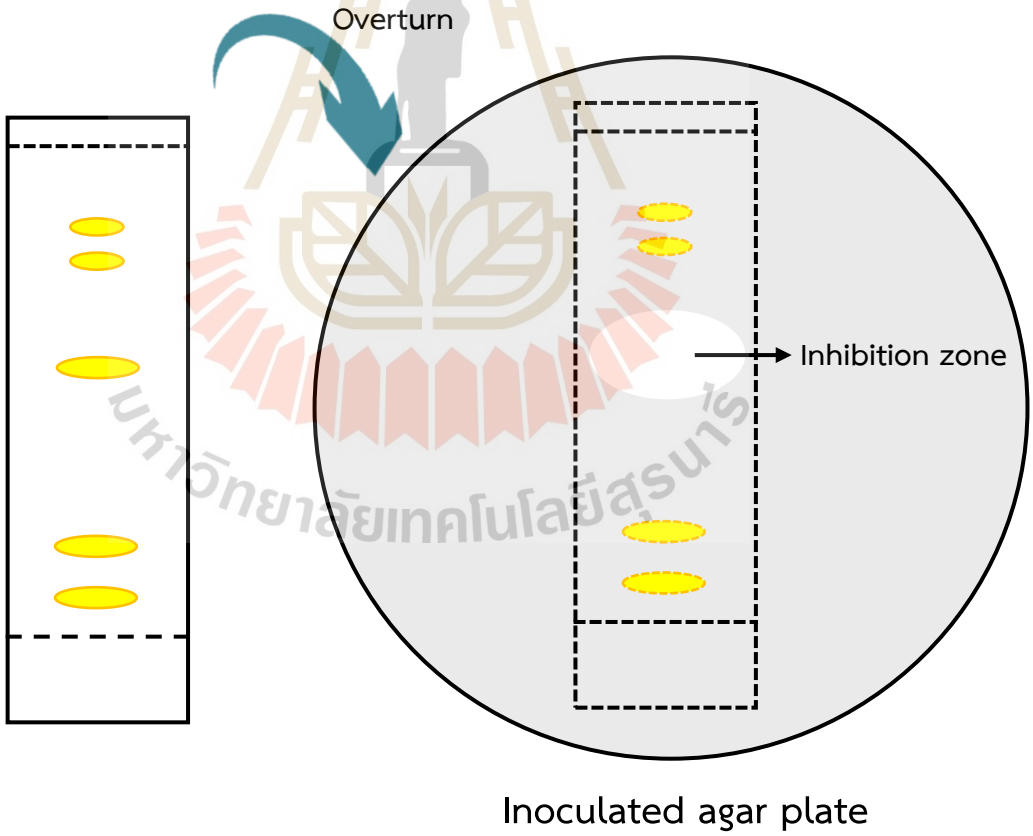


Figure 3.5 Contact bioautography for the detection of bioactive compounds inhibited MRSA DMST20654.

3.5.3 Column chromatography

Purification of bioactive compounds was conducted by column chromatography. The silica gel slurry was prepared by mixing silica powder (60-120 mesh size) with dichloromethane (DCM). A glass column was packed with silica gel slurry up to the two-third level of the column. After that, partial purified bioactive compounds were subjected to column chromatography (35.0 × 1.0 cm) using silica gel as a stationary phase. Then, column was pre-eluted with n-hexane followed by step-wise of chloroform and n-hexane (v/v; 50:50, 60:40, 70:30, 80:20, 90:10, 100:0). The purity of all fractions was screened by using TLC under UV light (λ_{254nm}). The collected fractions showing single bands (similar Rf value) were tested for antimicrobial activity against MRSA DMST20654 by contact bioautography. Fractions showing antimicrobial activity were collected for further study. The chemical structure of bioactive compounds was elucidated by spectroscopic analysis.

3.6 Characterization of bioactive compounds

The purified bioactive compounds were characterized by using spectroscopic analysis which includes UV-Visible spectroscopy, Liquid chromatography-mass spectrometry (LC-MS), and Nuclear magnetic resonance (NMR) spectroscopy.

3.6.1 UV-Visible spectroscopy

Ultraviolet and visible (UV-Vis) absorption spectra of the bioactive compounds were recorded by a UV-Visible spectrophotometer (T80^{+}, Shimadzu). The UV-Vis absorption spectra were scanned in a wavelength between 200-800 nm. The absorption spectra of UV light and visible light can be absorbed by chromophores such as aromatic rings, dienes, polyenes, conjugated ketones, or some bioactive ingredients (El-Naggar et al., 2017). The maximal wavelength of UV light absorption by a chemical compound resulted in a distinct spectrum.

3.6.2 Liquid chromatography-mass spectrometry (LC-MS)

Mass spectrums of the bioactive compounds were performed by liquid chromatography-mass spectrometry (LC-MS). The Dionex Ultimate 3000 UHPLC system (Dionex, USA) coupled with electrospray ionization (ESI) tandem mass spectrometer (micrOTOF-Q II) (Bruker, Germany) was used for this analysis. The injection volume for all samples was 10 µl. The column Zorbax SB-C18 (250 mm × 4.6 mm, 3.5 Micron

(Agilent Technologies, USA)) was used for separation and thermostated at 35 ℃. The mobile phase included deionized water containing 0.1 % formic acid as solvent A, and acetonitrile containing 0.1 % formic acid as solvent B, with a flow rate of 0.8 ml/min. The gradient elution was performed as followed: starting with 20 % solvent B and holding until 5 min, increasing to 40 % solvent B at 20 min, then increasing to 80% solvent B at 40 min and holding for 3 min, reducing to 20 % solvent B in 2 min and holding until the run ending at 50 min. The eluted components were ionized by an electrospray ionization (ESI) source and were detected in the mass scanning mode, in the range of 50 *m/z* to 1,500 *m/z* at negative ion polarity. A 10 mM sodium formate was used as an external standard for accurate mass calibration and injected into the ESI source before each sample by a syringe pump at a flow rate of 60 µl/min. The nebulizer gas (Nitrogen gas) was 2 Bar, drying gas was 8 L/min, dry heater temperature was 180 ℃, and capillary voltage was 4.5 kV. The mass spectrum peak was identified by matching mass spectra with MassBank databases (https://massbank.eu/MassBank and https://mona.fiehnlab.ucdavis.edu). The LC-QTOF data were collected and processed by Compass 1.3 software (Bruker, Germany). The molecular formula was predicted based on accurate mass.

3.6.3 Nuclear magnetic resonance (NMR) spectroscopy

The chemical structure was elucidated by using nuclear magnetic resonance (NMR) spectroscopy. The ^{1}H-, ^{13}C-NMR, and 2D spectra were measured in dimethyl sulfoxide ($DMSO_{d6}$) on a 500 MHz NMR Bruker spectrometer equipped with a CPP BBO 500 CryoProbe.

3.7 Statistical analysis

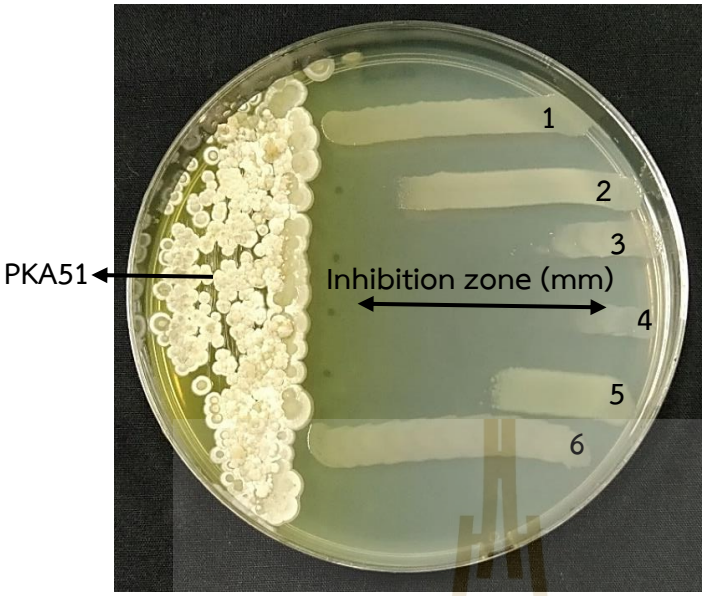
The results were statistically analyzed and presented as means ± standard deviation (SD) for triplicate samples.

CHAPTER IV

RESULTS

4.1 Preliminary antibacterial screening of soil isolates

The strain PKA51 used in this study was kindly received from Miss Phimpha Khowangklang. This strain was isolated from terrestrial soil in Suranaree University of Technology, Nakhon Ratchasima province (Khowangklang, 2017). In this study, soil isolate PKA51 was evaluated for antibacterial activity against *B. subtilis* TISTR008, *B. cereus* TISTR684, *S. epidermidis* TISTR518, *S. aureus* ATCC1466, *E. coli* TISTR780, *S.* Typhimurium TISTR292, *E. aerogenes* TISTR1540, *S. marcescens* TISTR1540, *P. mirabilis* TISTR100, methicillin-resistant *S. aureus* DMST20654 (MRSA), clinical isolate methicillin-resistant *S. epidermidis* (MRSE), clinical isolate *K. pneumoniae* 1617, and clinical isolate *E. coli* 2026 (Figure 4.1). The results obtained from the perpendicular cross streak method showed that the strain PKA51 could produce diffusible substances with broad-spectrum antimicrobial activity against Gram-positive, Gram-negative, and antibiotic-resistant bacteria. Strain PKA51 effectively inhibited the growth of almost all species of test pathogens except *E. aerogenes* TISTR1540, *S. marcescens* TISTR1354, and clinical isolate *K. pneumoniae* 1617. The results of the preliminary antimicrobial activity screen were summarized in Table 4.1. The result also showed that strain PKA51 exhibited strong antibacterial activity against antibiotic-resistant bacteria including MRSA DMST20654, clinical isolate MRSE, and clinical isolate *E. coli 2026.* Therefore, soil isolate PKA51 was selected for further study.



1: *A. baumannii*

2: *K. pneumoniae* 1617

3: MRSA DMST20654

4: Clinical isolate MRSE

5: Clinical isolate *E. coli* 2026

6: *P. aeruginosa* N90PS

Figure 4.1 Antimicrobial activity of strain PKA51 against test pathogens by perpendicular cross streak method.

Table 4.1 Antimicrobial activity of the strain PKA51.

	Test pathogens	Inhibition zone (mm)
Gram-Positive	*B. subtilis* TISTR008	41.67 ± 3.78
	B. cereus TISTR684	40.00 ± 0.00
	S. epidermidis TISTR518	41.00 ± 2.82
	S. aureus ATCC1466	39.33 ± 0.47
Gram-Negative	*E. coli* TISTR780	28.33 ± 2.36
	S. Typhimurium TISTR292	30.67 ± 0.94
	E. aerogenes TISTR1540	4.33 ± 1.89
	S. marcescens TISTR1354	3.33 ± 2.36
	P. mirabilis TISTR100	37.00 ± 1.41
Antibiotic-resistant bacteria	*S. aureus* DMST20654 (MRSA)	41.67 ± 1.89
	Clinical isolate MRSE	42.00 ± 0.00
	Clinical isolate *K. pneumoniae* 1617	12.00 ± 0.00
	Clinical isolate *E. coli* 2026	30.00 ± 0.00

Data are means ± standard deviation (n = 3)

4.2 Identification of soil isolate PKA51

The species identification of the strain PKA51 is based on cultural, and morphological characteristics. The strain PKA51 was cultured on ISP-2 agar and incubated at 28 °C for 14 days. After incubation, the morphology of aerial mycelium was observed under a light microscope (400X). The microscopic images showed branching mycelium and a large number of disposable spores (Figure 4.2a - 4.2b). The mycelium has a smooth surface visualized under a field emission scanning electron microscope (SEM) (Figure 4.2c – 4.2d).

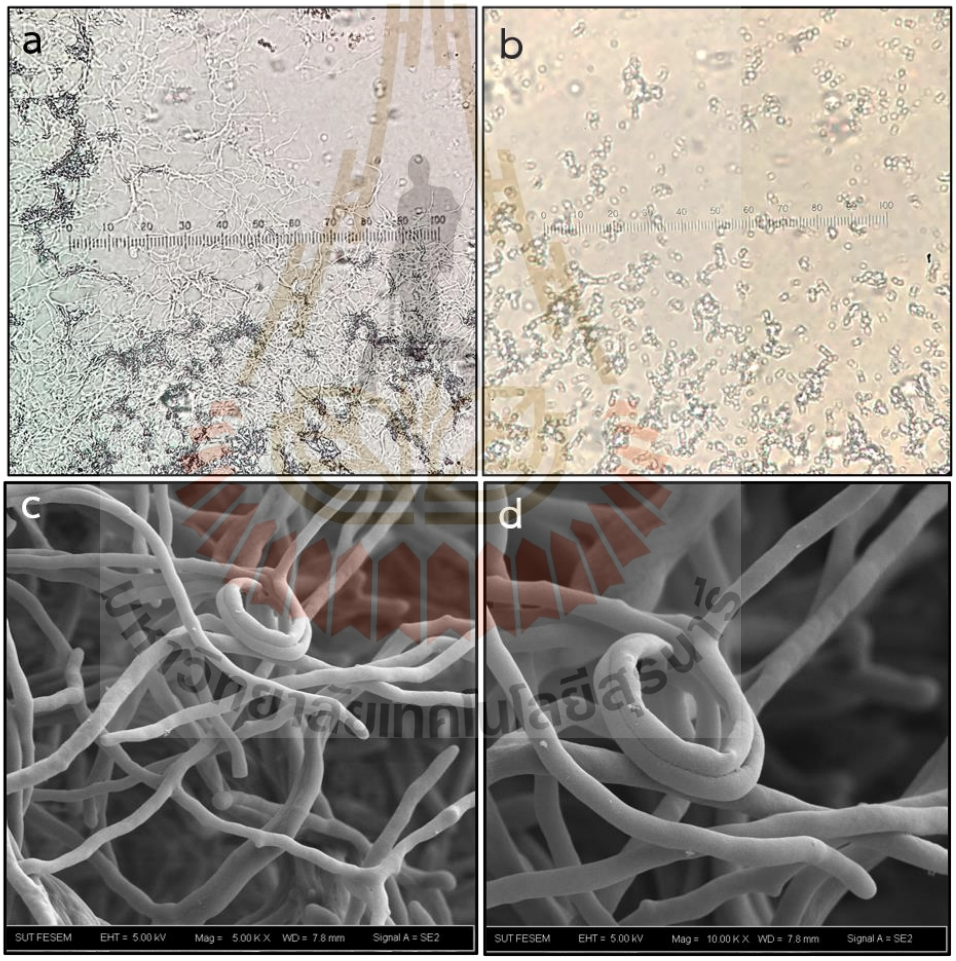


Figure 4.2 Morphological characteristics of the strain PKA51. a: branching mycelium under a light microscope (400×), b: a large number of disposable spores (400×), c: mycelium under a scanning electron microscope (5000×), and d; SEM (10000×).

Colony morphology of PKA51 were observed after 14 days of cultivation on several media (ISP-1 to ISP-9, SCA, Czapek's agar, Bennet's agar) (Figure 4.3). Abundant growth was observed on the ISP-1 to ISP-8 and Bennet's agar while moderate growth was observed on ISP-9 and SCA. The strain PKA51 exhibited poor growth on czapek's agar. The color of colonies was white on ISP-1, ISP-2, ISP-4, ISP-6, ISP-8, ISP-9, czapek's agar, and bennet's agar. The color of colonies was grey on ISP-3. The color of colonies was pale grey on ISP-5, ISP-7, and SCA. The strain PKA51 could produce diffusible pigment diffuse into surrounding medium on all media but non-diffusible pigment on czapek's agar. The colors of diffusible pigment on various media ranged from bright yellow to mild yellow to black. The result of cultural characteristics and diffusible pigments are shown in Table 4.2.

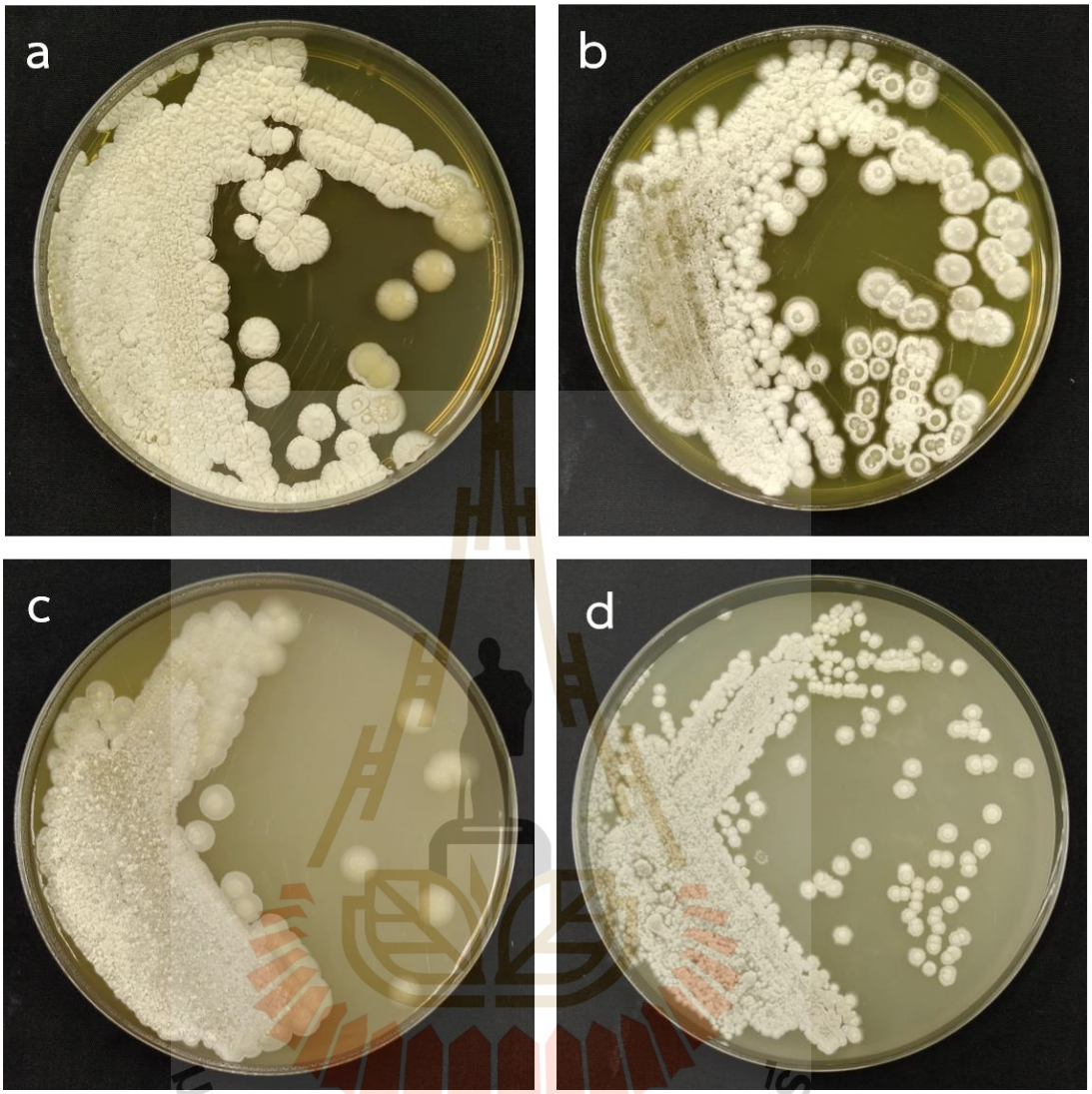


Figure 4.3 Colony morphology of PKA51 on various agar media, a: International *Streptomyces* Project no. 1 (ISP-1), b: ISP-2, c: ISP-3, d: ISP-4, e: ISP-5, f: ISP-6, g: ISP-7, h: ISP-8, i: ISP-9, j: Starch casein agar SCA), k: Czapek's agar, and l: Bennet's agar.

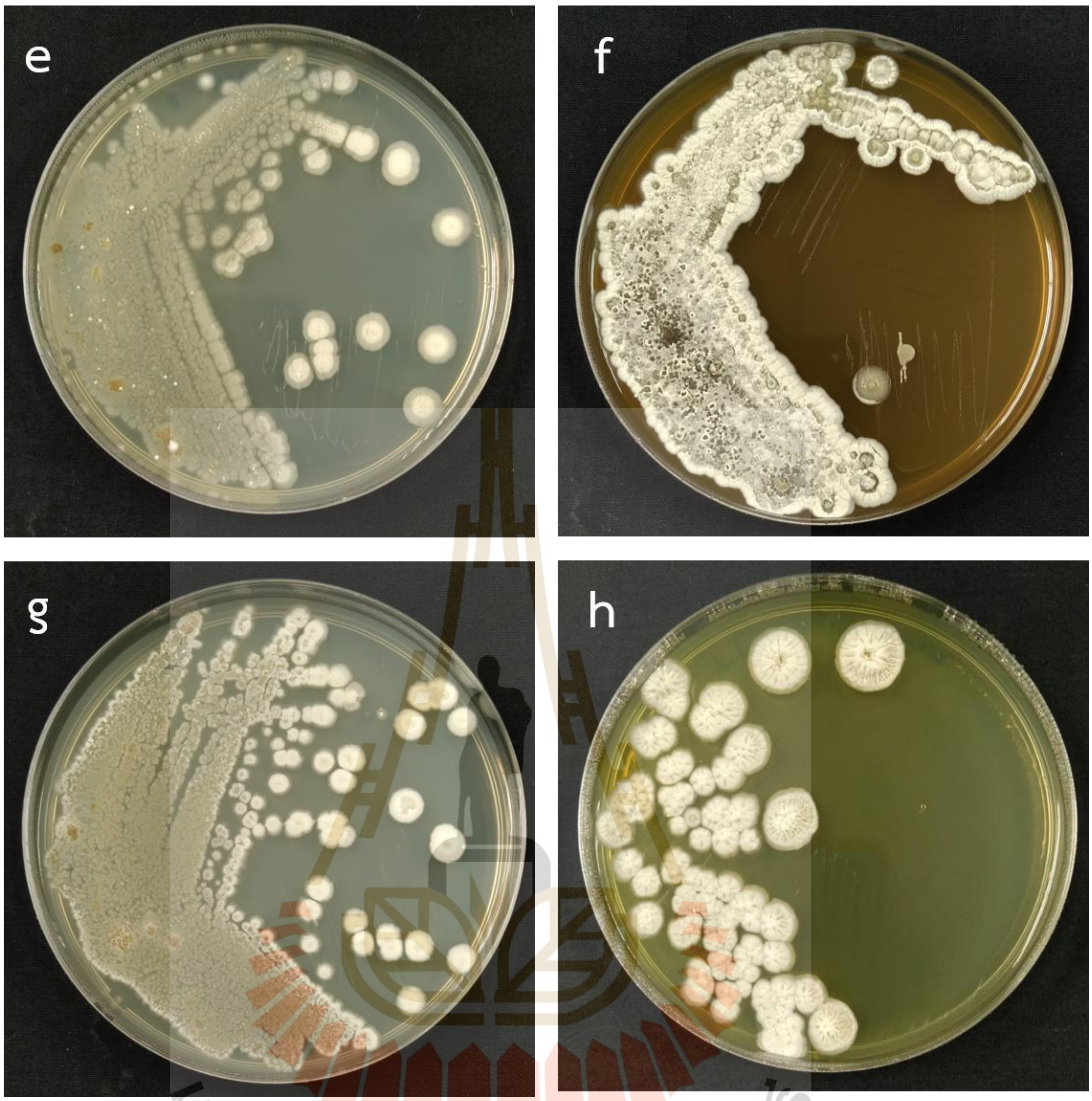


Figure 4.3 Colony morphology of PKA51 on various agar media, a: International *Streptomyces* Project no. 1 (ISP-1), b: ISP-2, c: ISP-3, d: ISP-4, e: ISP-5, f: ISP-6, g: ISP-7, h: ISP-8, i: ISP-9, j: Starch casein agar SCA), k: Czapek's agar, and l: Bennet's agar (Continued).

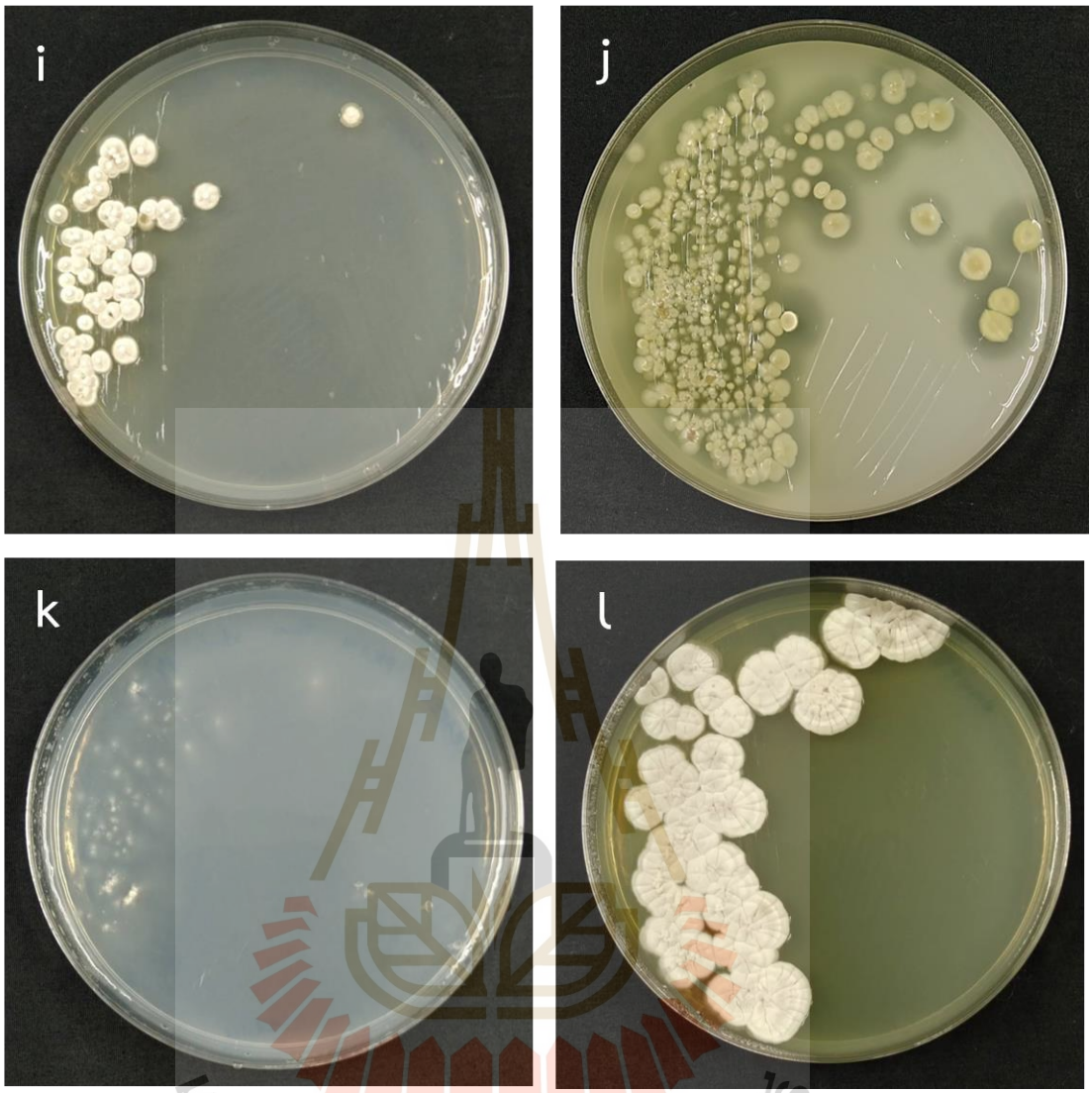


Figure 4.3 Colony morphology of PKA51 on various agar media, a: International *Streptomyces* Project no. 1 (ISP-1), b: ISP-2, c: ISP-3, d: ISP-4, e: ISP-5, f: ISP-6, g: ISP-7, h: ISP-8, i: ISP-9, j: Starch casein agar SCA), k: Czapek's agar, and l: Bennet's agar (Continued).

Table 4.2 Cultural characteristics of PKA51 on different media.

Medium	Growth	Colony morphology	Diffusible pigment
ISP-1	Abundant	Circular, Convex, White colonies	Strong yellow
ISP-2	Abundant	Irregular, Convex, White colonies	Strong yellow
ISP-3	Abundant	Circular, Flat, Grey colonies	Yellow
ISP-4	Abundant	Circular, Raised, White colonies	Yellow
ISP-5	Abundant	Circular, Raised, Pale grey colonies	Light yellow
ISP-6	Abundant	Irregular, Convex, White colonies	Black
ISP-7	Abundant	Circular, Raised, Pale grey colonies	Light yellow
ISP-8	Abundant	Circular, Convex, White colonies	Strong yellow
ISP-9	Moderate	Circular, Umbonate, White colonies	Light yellow
SCA	Moderate	Circular, Flat, Pale grey colonies	Yellow
Czapek's agar	Slight	Irregular, Flat, White colonies	-
Bennet's agar	Abundant	Circular, Convex, White colonies	Strong yellow

-: Non-diffusible pigment produced by colonies.

The species-level of strain PKA51 was identified by 16s rRNA gene sequence analysis. The 16s rRNA gene was amplified by using universal primers 27F and 1525 R. The amplified fragments (1481 bp) (Figure 4.4) were then cloned into a pTG19-T cloning vector and sequenced. A full-length 16s rRNA sequence was sequenced by using M13F and M13R primers. The nucleotide sequence of the 16S rRNA gene of PKA51 was deposited into the NCBI database with the accession number ID MZ497354. Molecular identification based on 16S rRNA gene analysis was performed by comparing to the

reference sequences of the EzTaxon database (https://www.ezbiocloud.net). The results revealed that the 16S rRNA gene of the strain PKA51 was classified as a member of the genus *Streptomyces.* The sequence of the 16S rRNA gene was blasted and aligned with top hit strains from the EzBioCloud database. List of 30 hits taxon of PKA51 from the EzBioCloud 16S rDNA database is shown in Table 4.3.

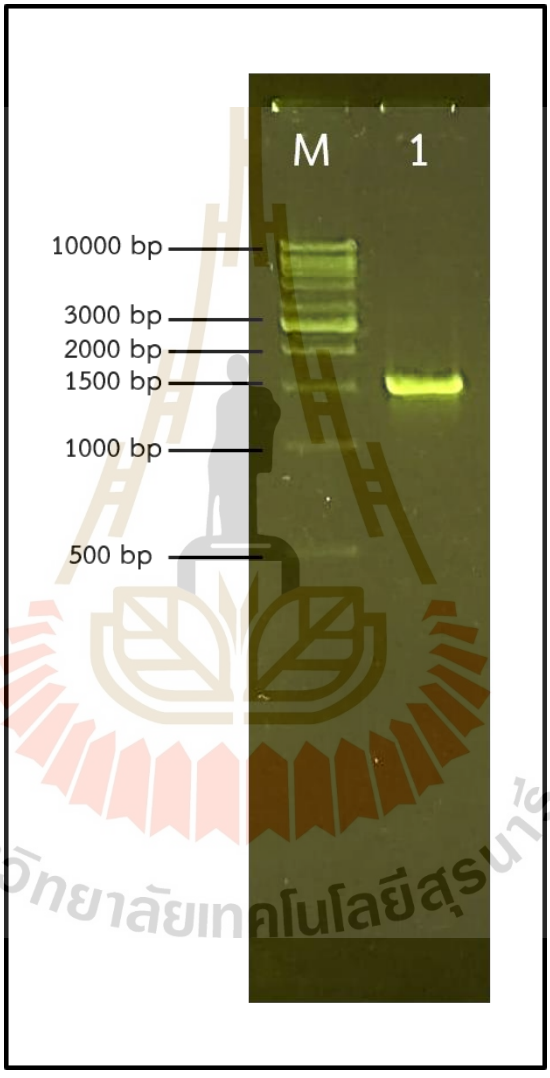


Figure 4.4 PCR products of 16s rRNA gene on 0.7% (w/v) agarose gel, lane M: 1 kb DNA ladder (GeneDirex, Taiwan), lane 1: purified PCR product of PKA51 (1481 bp).

Table 4.3 List of hits taxon of PKA51 from EzBioCloud 16S rDNA database.

No.	Top hit taxon	Accession No.	% Similarity
1	*Streptomyces luteosporeus* NBRC 14657^T	AB184607	99.93
2	*Streptomyces alboverticillatus*	AB249978	98.62
3	*Streptomyces abikoensis* NBRC 13860^T	AB184537	98.61
4	*Streptomyces hiroshimensis* NBRC 3839^T	AB184802	98.54
5	Streptomyces morookaense LMG 20074^T	AJ781349	98.41
6	*Streptomyces griseocarneus* JCM 4580^T	MT760576	98.39
7	*Streptomyces lilacinus* NRRL B-1968^T	JNXU01001020	98.34
8	*Streptomyces pseudoechinosporeus* NBRC 12518^T	AB184100	98.27
9	*Streptomyces thioluteus* LMG 20253^T	AJ781360	98.27
10	*Streptomyces cinnamoneus* NBRC 12852^T	AB184850	98.27
11	*Streptomyces mobaraensis* NBRC 13819^T	AORZ01000256	98.20
12	*Streptomyces tirandamycinicus* HNM0039^T	CP029188	98.19
13	*Streptomyces mashuensis* DSM 40221^T	X79323	98.13
14	*Streptomyces roseifaciens* MBT76^T	MG947385	98.13
15	*Streptomyces lacticiproducens* GIMN4.001^T	GQ184344	98.03

Table 4.3 List of hits taxon of PKA51 from EzBioCloud 16S rDNA database (Continued).

No.	Top hit taxon	Accession No.	% Similarity
16	*Streptomyces wuyuanensis* CGMCC 4.7042^T	jgi.1085054	97.99
17	*Streptomyces palmae* CMU-AB204^T	LC073309	97.93
18	*Streptomyces blastmyceticus* NRRL B-5480^T	AY999802	97.92
19	*Streptomyces caatingaensis* CMAA 1322^T	LFXA01000004	97.86
20	*Streptomyces orinoci* NBRC 13466^T	AB184866	97.85
21	*Streptomyces ardus* NBRC 13430^T	AB184864	97.84
22	*Streptomyces olivoverticillatus* NBRC 15273^T	AB184636	97.84
23	*Streptomyces ochraceiscleroticus* NRRL ISP-5594^T	JOAX01000062	97.79
24	*Streptomyces angustmyceticus* NRRL B-2347^T	MUAY01000275	97.79
25	*Streptomyces luteireticuli* NBRC 13422^T	AB249969	97.79
26	*Streptomyces varsoviensis* NRRL ISP-5346^T	JOBF01000056	97.72
27	*Streptomyces sioyaensis* NRRL B-5408^T	DQ026654	97.65
28	*Streptomyces catenulae* NRRL B-2342^T	JODY01000075	97.65
29	*Streptomyces violens* NRRL ISP-5597^T	JOBH01000039	97.65
30	*Streptomyces sparsogenes* ATCC 25498^T	MAXF01000077	97.65

According to 30 top hit taxa from the EzBioCloud 16s rDNA database exhibited that the strain PKA51 belongs to the genus *Streptomyces*. The strain PKA51 exhibited the highest sequence similarity to *Streptomyces luteosporeus* NBRC 14657^{T} (99.93 % similarity). Therefore, PKA51 can be classified as *S. luteosporeus*.

Furthermore, the phylogenetic relationship between the isolate PKA51 and other closely related species was studied in the EzTaxon database (Figure 4.5). *Nocardia alba* YIM 30243 was used as an outgroup. The nodes are indicated to construct a majority phylogenetic tree. The phylogenetic tree of strain PKA51 based on the neighbor-joining algorithm formed a distinct monophyletic clade with *S. luteosporeus* NBRC14657^{T}. The phylogenetic tree's confidence was validated using the bootstrap value (1,000 replications of analysis). The strain PKA51 shares a common ancestor with *S. luteosporeus* NBRC14657^{T} and can be identified as *S. luteosporeus* PKA51.

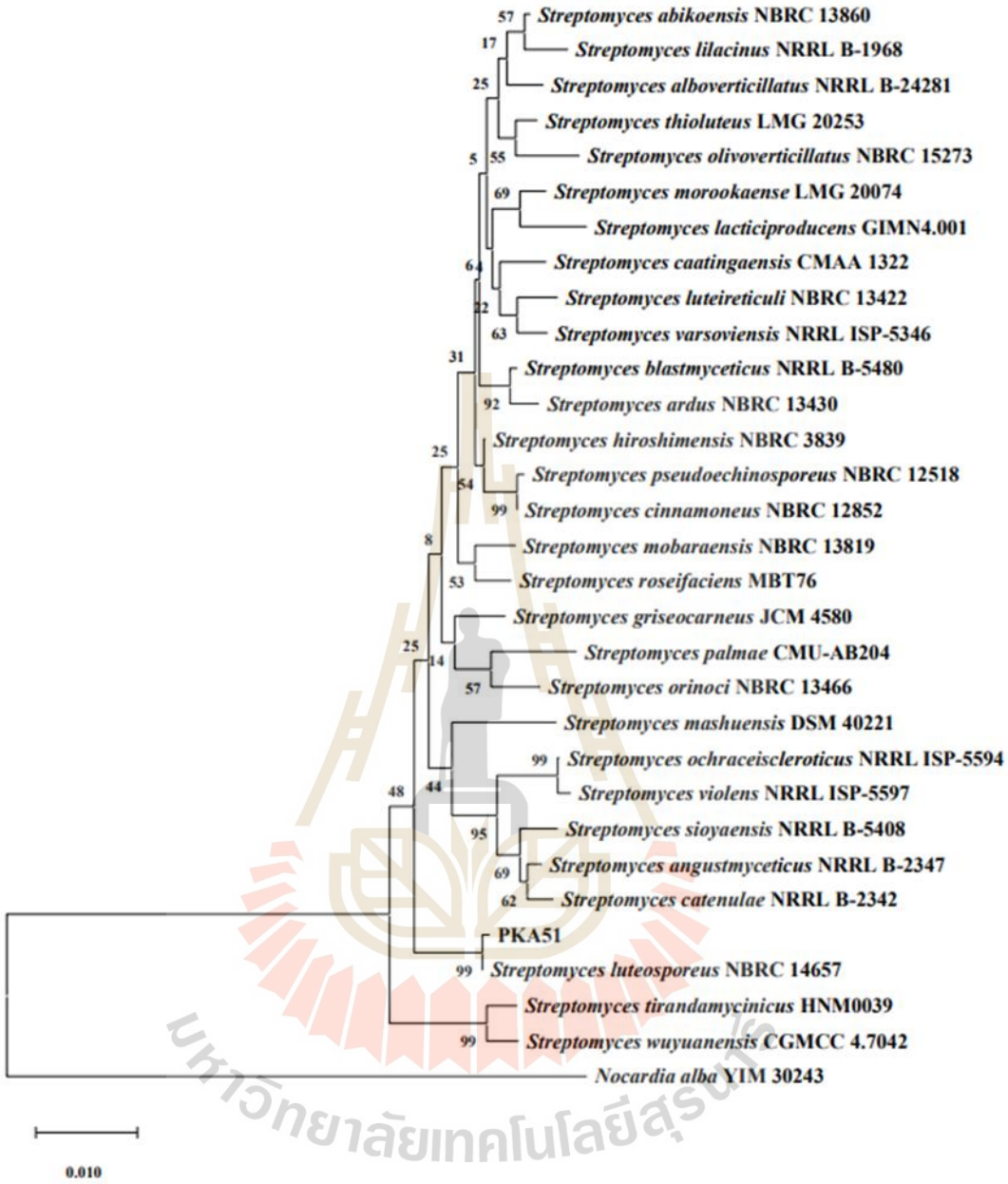


Figure 4.5 Neighbor-joining phylogenetic tree constructed using 16S rRNA gene sequences showing the relationships between *Streptomyces* sp. PKA51 and other closely related species. The numbers at the nodes represent bootstrap values of the tree topology's confidence (1,000 repetitions of analysis). The scale bar indicates 0.010 substitutions per nucleotide position.

4.3 Crude compounds extraction and antimicrobial activity of a crude compounds

It has been reported that the optimum culture condition for antimicrobial production of the strain PKA51 is at 28 °C, 200 rpm for 3 days in MHB medium (Khowangklang, 2017). As a result, the condition used in this work was based on Khowangklang's earlier published condition.

The strain PKA51 was inoculated on ISP-2 broth for 3 days. Then, 40 ml of PKA51 inoculum was transferred and cultivated in 1,000 ml Erlenmeyer flasks containing 400 ml of MHB medium. Following the incubation, the cell-free supernatant was collected, and used for the extraction of a crude compound with ethyl acetate. Cell pellets and mycelium were harvested and dried at 65 °C for 3 days to obtain cell-dry weight. The characteristics of the crude compound of PKA51 were semi-solid with yellow-colored (Figure 4.6). The ratio of crude extract to cell dry weight ($mg \cdot g^{-1}$) was used to calculate the yield. The following formula was used to calculate the yield of the crude compound:

Yield = Crude compound (mg) / The dry weight of cell pellets and mycelium (g)

The crude compound yield was 36.25 ± 8.04 $mg \cdot g^{-1}$.

Figure 4.6 Ethyl acetate crude extract of *Streptomyces* sp. PKA51.

An evaluation of the antimicrobial activity of crude extract PKA51 was performed by the agar well diffusion method (Table 4.4). Although PKA51 crude extract displayed broad-spectrum antimicrobial activity against Gram-positive, Gram-negative, and antibiotic-resistant bacteria, its antibacterial activity against Gram-positive bacteria was the most potent. The maximum inhibition zone was observed against clinical isolate MRSE (35.00 ± 1.00 mm), *B. subtilis* TISTR008 (34.00 ± 0.00 mm), *B. cereus* TISTR684 (32.00 ± 1.73 mm), *S. epidermidis* TISTR518 (30.67 ± 1.52 mm), *S. aureus* ATCC1466 (28.67 ± 2.89 mm), MRSA DMST20654 (28.43 ± 0.58 mm), and *E.coli* TISTR780 (28.00 ± 1.83 mm) While moderate to weak inhibition activity was observed from *E. coli* TISTR780, *S.* Typhimurium TISTR292, *E. aerogenes* TISTR1540, clinical isolate *K. pneumoniae* 1617, clinical isolate *E. coli* 2026, and *S. marcescens* TISTR1354 (inhibition zone range of 13.86 ± 0.11 to 9.67 ± 0.58 mm). These results revealed that Gram-positive bacteria were more susceptible to PKA51 crude compounds than Gram-negative bacteria.

Table 4.4 Antimicrobial activity of PKA51 crude extract.

	Test pathogens	Inhibition zone (mm)
Gram-Positive bacteria	*B. subtilis* TISTR008	34.00 ± 0.00
	B. cereus TISTR684	32.00 ± 1.73
	S. epidermidis TISTR518	30.67 ± 1.52
	S. aureus ATCC1466	28.67 ± 2.89
Gram-Negative bacteria	*E. coli* TISTR780	28.00 ± 1.73
	S. Typhimurium TISTR292	13.86 ± 0.11
	E. aerogenes TISTR1540	12.67 ± 0.58
	S. marcescens TISTR1354	9.67 ± 0.58
	P. mirabilis TISTR100	33.67 ± 1.15
Antibiotic-resistant bacteria	*S. aureus* DMST20654 (MRSA)	28.34 ± 0.58
	Clinical isolate MRSE	35.00 ± 1.00
	Clinical isolate *K. pneumoniae* 1617	11.67 ± 0.58
	Clinical isolate *E. coli* 2026	10.00 ± 0.00

Data are means ± standard deviation (n = 3)

4.4 Purification of bioactive compounds of PKA51

The partial purification of bioactive compounds presenting in the crude extract was conducted by thin-layer chromatography (TLC). The crude compound was applied to the bottom edge of TLC plates. Among various solvent systems, chloroform and methanol were given the best separation on chromatograms. Thus, the TLC plates were immersed and developed in a developing chamber by using chloroform and methanol (9.9: 0.1, v/v) as a solvent system. Then, the chromatogram was left to dry and visualized under UV light ($\lambda_{254\ nm}$) (Figure 4.7).

Then, contact bioautography was used to locate bioactive compounds using MRSA DMST20654 as a tested pathogen. The results demonstrated an inhibition zone of crude PKA51 in the R_f values ranging from 0.35 to 0.40 against MRSA DMST20654 (Figure 4.8). In order to identify bioactive compounds, the active zone was scraped and redissolved in ethyl acetate before column chromatography purification. Partially purified bioactive compounds were subjected to silica column chromatography and eluted with chloroform: n-hexane as an eluent.

All fractions were collected from column chromatography. The fractions were tested for their antibacterial activities against MRSA DMST20654 by contact bioautography. The active fraction was collected and defined as active fraction 1 and active fraction 2. The result indicated that R_f values of active fractions 1 and 2 were 0.40 and 0.36, respectively (Figure 4.9). On the other hand, the physical properties of the bioactive compounds were yellowish and needle-like crystals.

Moreover, the chemical structure of bioactive compounds which presents in the active fractions 1 and 2 were further characterized and identified by spectral analysis including UV-VIS spectroscopy, LC-MS, and NMR spectroscopy.

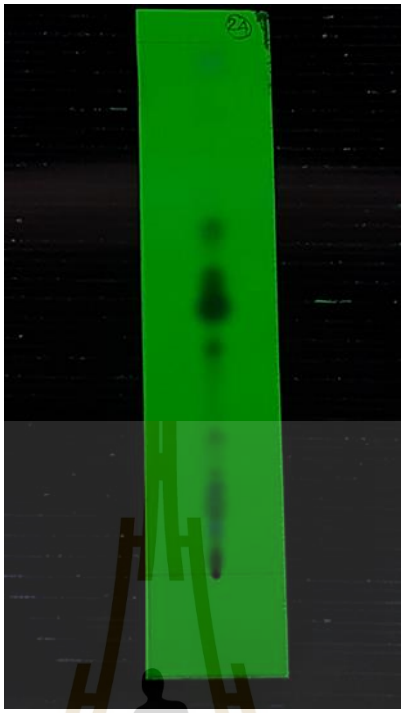


Figure 4.7 The TLC chromatogram of PKA51 crude extract using chloroform and methanol (9.9: 0.1, v/v) as a mobile phase.

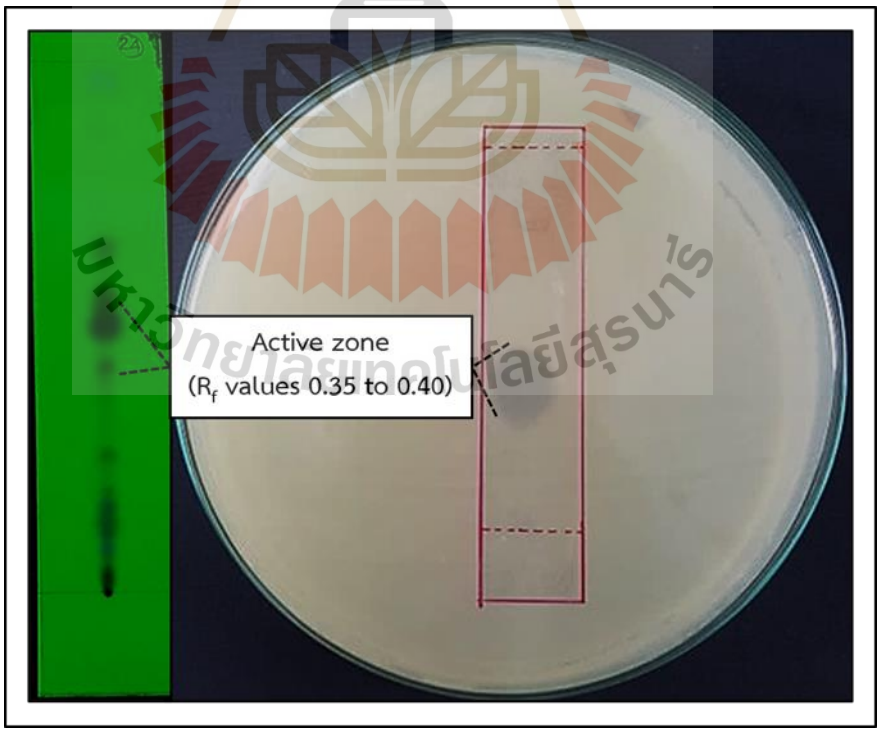


Figure 4.8 Contact bioautography of crude extract PKA51 on petri dish seeded with MRSA DMST20654.

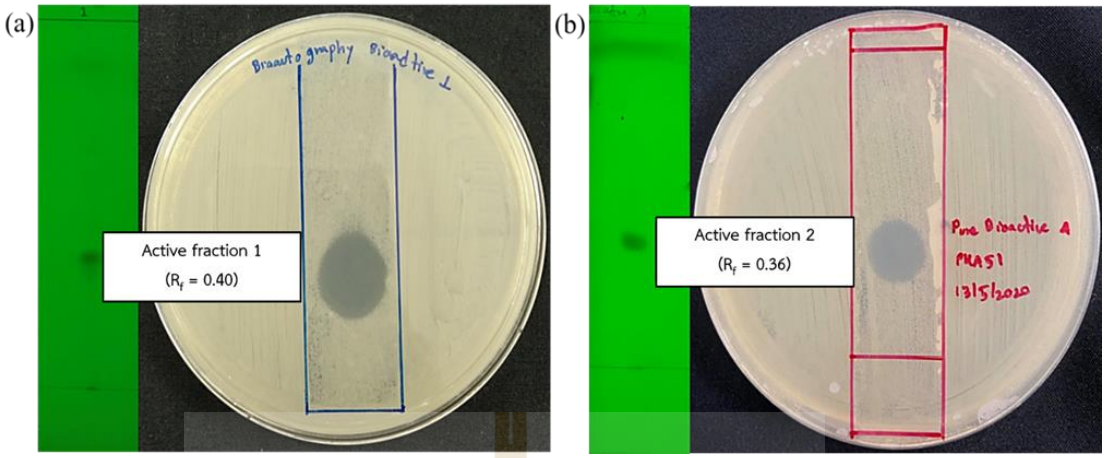


Figure 4.9 Contact bioautography of (a) purified active fraction 1 and (b) purified active fraction 2 against MRSA DMST20654.

4.5 Characterization of bioactive compounds

4.5.1 Bioactive compound 1

UV-VIS spectroscopy

UV-Vis absorption spectra of the bioactive compound present in the active fraction 1 were recorded by a UV-Visible spectrophotometer (T80+, Shimadzu). The UV-visible absorption spectra of purified bioactive compound 1 were scanned (200-800 nm). Ethyl acetate was used as a blank control. The UV absorption spectra of compound 1 showed maximum peaks at 311 and 391 nm indicating the presence of the pyrrothine core, a core scaffold of dithiolopyrrolone (DTP) compounds (Figure 4.10). It has been reported that pyrrothine derivatives are classified by their different *N-acyl* groups (Li et al., 2014; Merrouche et al., 2019).

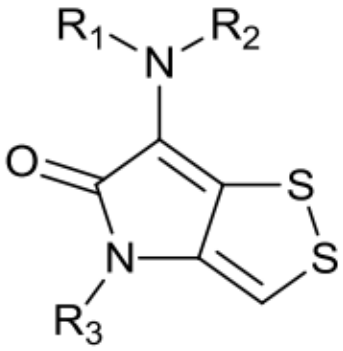


Figure 4.10 General chemical structure of pyrrothine core. Various pyrrothine derivatives are characterized by their different *N*-acyl groups (R_1, R_2, and R_3 groups).

Liquid chromatography-mass spectrometry (LC-MS) analysis

The mass spectrum of the purified bioactive compound 1 was determined by LC-MS analysis. The mass spectrum peak was identified by matching mass spectra with the MassBank database (https://massbank.eu/MassBank and https://mona.fiehnlab.ucdavis.edu). According to the HPLC-MS chromatogram, purified bioactive compound 1 exhibited one major peak at the retention time of 11.6 min. The MS chromatogram also showed five minor peaks at the retention time of 14.9, 18.9, 19.6, 38.6, and 41.5 min (Figure 4.11a). These minor peaks show the low intensity and could be identified as fragmentations of impurities. Therefore, purified bioactive compound 1 was detected at a retention time of 11.6 min and had an exact mass of 226.9965 *m/z* at negative mode (Figure 4.11b).

On the basis of exact mass, the smart formula search manual software was used for predicting molecular formulas from mass spectrometry. The chemical constituents of carbon (C), hydrogen (H) oxygen (O), and nitrogen (N) were calculated by atomic mass fundamentally. Seven molecular formulas of exact mass 266.9965 *m/z* $[M-H]^-$ were $C_5H_3N_6O_3S$, $C_4H_7N_2O_2O_7S$, $C_4H_3N_8S_2$, $C_8H_7N_2O_2S_2$, $C_{16}H_3S$, $C_5H_{11}N_2O_2S_3$, and $C_{13}H_7S_2$. The smart formula search result of an MS peak at 226.9965 *m/z* is shown in Appendix E. Additionally, NMR analysis was used to confirm the chemical structure of purified bioactive molecule 1.

Nuclear magnetic resonance (NMR) analysis

The NMR spectroscopic technique is a sophisticated characterization method for confirming the chemical structures of a substance. The purified bioactive compound 1 had been tested ^{1}H and ^{13}C NMR. The ^{1}H NMR spectrum of purified bioactive compound 1 was 7.32 (1H, s at position H-3), 3.25 (3H, s at position N (4)-CH_3), 9.95 (1H, s at position C (6)-NH), and 2.02 (3H, s at position CO-CH_3) (Figure 4.12a). The ^{13}C NMR spectrum of purified bioactive compound 1 was 110.85 ppm, 132.40 ppm, 27.51 ppm, 166.16 ppm, 114.79 ppm, 135.98 ppm, 168.83 ppm, and 22.35 ppm (Figure 4.12b). The ^{1}H and ^{13}C NMR spectrum was compared with published data described by Lamari et al. (2002). Therefore, bioactive compound 1 produced by PKA51 might be identified as thiolutin. The comparison of ^{1}H and ^{13}C NMR spectra of compound 1 and thiolutin are summarized in Tables 4.5-4.6. The results showed that they were consistent, thus compound 1 was identified as thiolutin ($C_8H_8N_2O_2S_2$). The chemical structure of thiolutin was shown in Figure 4.15a.

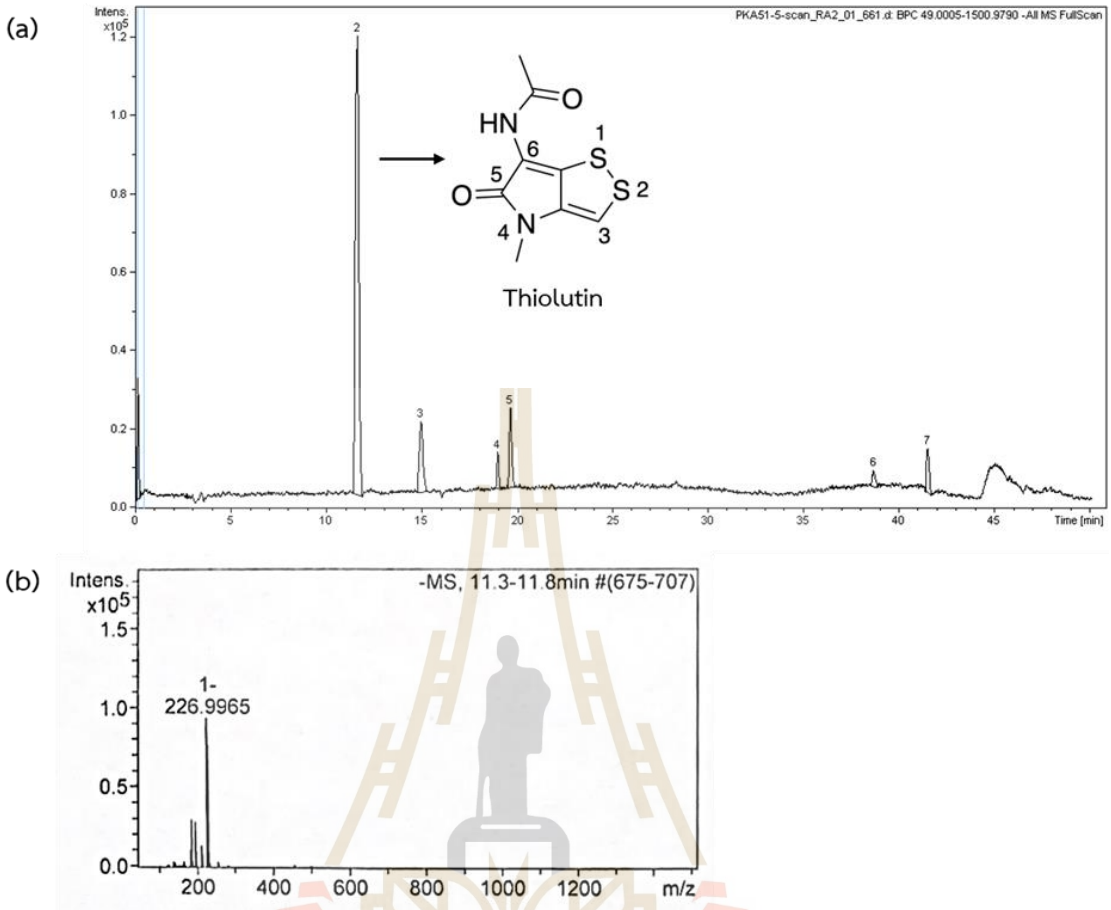


Figure 4.11 Chromatogram for liquid chromatography-mass spectrometry (LC-MS) of purified bioactive compound 1 in negative ion polarity mode $[M-H]^-$. (a) HPLC-MS chromatogram of purified bioactive compound 1. (b) Mass spectrum of peak at a retention time of 11.6 min from purified bioactive compound 1 (226.9965 *m/z* at negative mode)

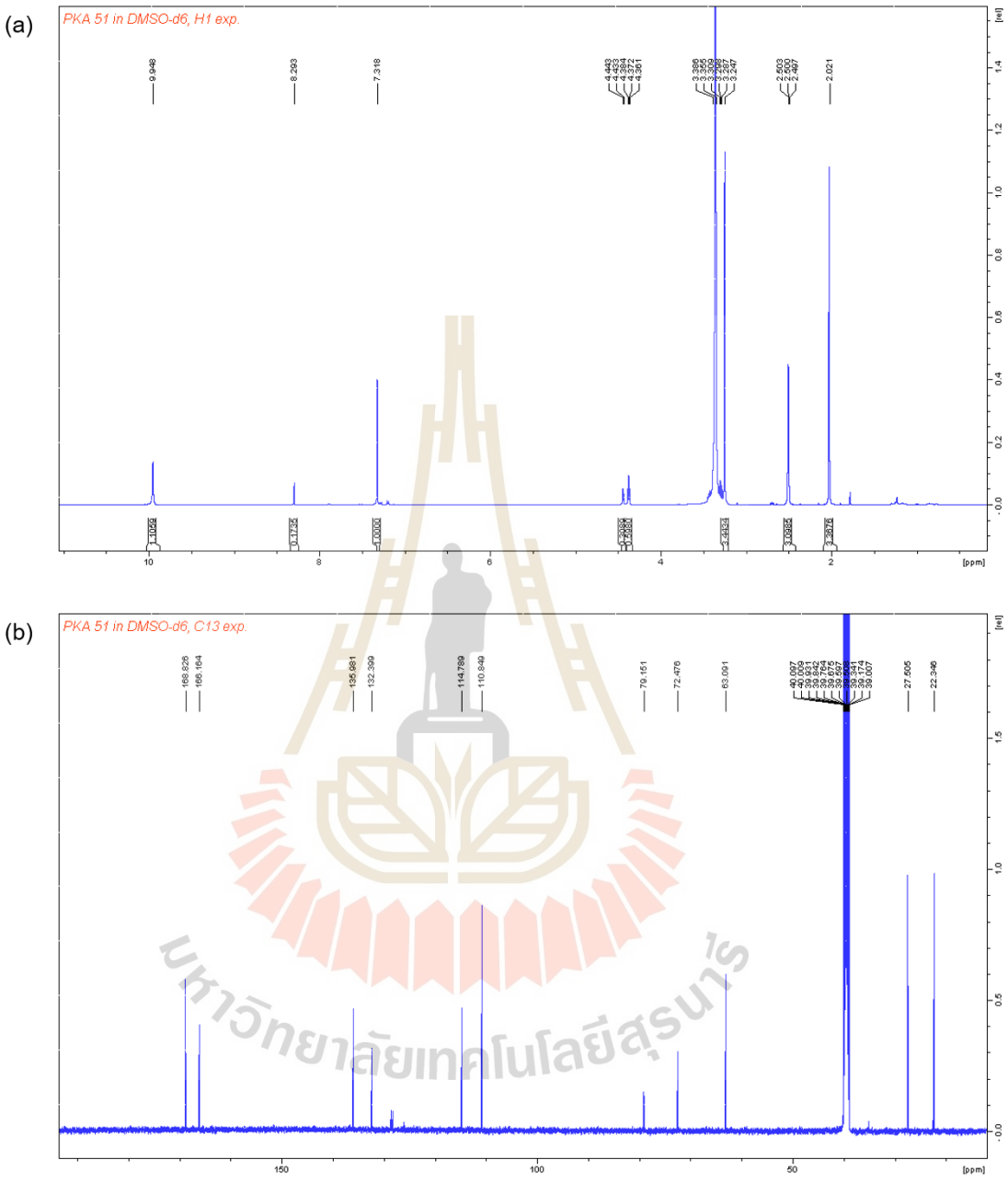


Figure 4.12 Nuclear magnetic resonance (NMR) chromatogram of compound 1 extract from *S. luteosporeus* PKA51. (a) ^{1}H NMR spectrum, (b) ^{13}C NMR spectrum.

Table 4.5 ^{1}H NMR spectra of compound 1 and thiolutin.

Position	Compound 1	Thiolutin
	δH (ppm)	δH (ppm)
H-3	7.32 (1H, s)	7.30 (1H, s)
N (4)-CH_3	3.25 (3H, s)	3.20 (3H, s)
C (6)-NH	9.95 (1H, s)	9.95 (1H, s)
CO-CH_3	2.02 (3H, s)	2.50 (3H, s)

Table 4.6 ^{13}C NMR spectra of compound 1 and thiolutin.

Position	Compound 1	Thiolutin
	δC (ppm)	δC (ppm)
3	110.85	112.1
3a	132.40	133.6
N (4)-CH_3	27.51	27.8
5	166.16	167.3
6	114.79	115.9
6a	135.98	137.1
Amino moiety	168.83	170.0
	22.35	23.5

4.5.2 Bioactive compound 2

UV-VIS spectroscopy

UV-Vis absorption spectra of the bioactive compound present in the active fraction 2 were recorded by determining maximum absorbance wavelengths. The bioactive compound 2 of PKA51 exhibited maximum UV absorption peaks at 313 and 385 nm, which was the characteristic of the pyrrothine core. The second antimicrobial compound also showed a UV-Vis spectrum similar to compound 1 at 311 and 391nm indicating the presence of the pyrrothine core (Li et al., 2014; Merrouche et al., 2019).

Liquid chromatography-mass spectrometry (LC-MS) analysis

Purified bioactive compound 2 derived from *S. luteosporeus* PKA51 was subjected to LC-MS analysis for chemical structure elucidation. The exact mass (*m/z*) and fragmentation peaks of MS spectrum peaks were used to identify purified bioactive compound 2. According to the HPLC-MS chromatogram. Purified bioactive compound 2 exhibited 1 major peak at the retention time of 13.3 min. The MS chromatogram also showed 2 minor peaks at the retention time of 26.6 and 29.8 min (Figure 4.13a). Therefore, purified bioactive compound 2 was detected at a retention time of 13.3 min and had an exact mass of 258.9848 *m/z* at negative ion mode (Figure 4.13b).

The elemental composition of C, H, O, N, and S of purified bioactive compound 2 was predicted using the smart formula search manual software based on their accurate mass. Nine molecular formulas of exact mass 258.9848 *m/z* $[M-H]^-$ were $C_4H_3N_8O_2S_2$, $C_8H_7N_2O_4S_2$, $C_4H_7N_2O_9S$, $C_{11}H_3N_2O_4S$, $C_9H_3N_6S_2$, $C_3H_7N_4O_6S_2$, $C_5H_{11}N_2O_4S_3$, $C_{16}H_3O_2S$, and $C_4H_{11}N_4OS_4$. The table of smart formula search manual software of an MS peak at 258.9848 *m/z* is shown in Appendix E. Furthermore, NMR analysis was used to confirm the chemical structure of purified bioactive compound 2.

Nuclear magnetic resonance (NMR) analysis

The purified bioactive compound 2 was tested ^{1}H and ^{13}C NMR. The ^{1}H NMR spectrum of compound 2 was 7.54 (1H, s at position H-3), 3.10 (3H, s at position N (4)-CH_3), 10.66 (1H, s at position C (6)-NH), and 2.09 (3H, s at position CO-CH_3) (Figure 4.14a). The ^{13}C NMR spectrum of purified bioactive compound 2 was 109.63 ppm, 145.57 ppm, 27.93 ppm, 165.35 ppm, 123.09 ppm, 114.12 ppm, 170.59 ppm, and 22.60 ppm (Figure 4.14b). The ^{1}H and ^{13}C NMR spectrum was compared with the published patent no. EP1372640B1 described by Claudia et al., (2002). Therefore, bioactive compound 2 produced by PKA51 might be identified as thiolutin dioxide. The comparison of ^{1}H and ^{13}C NMR spectra of compound 2 and thiolutin dioxide are listed in Tables 4.7-4.8. The results showed that they were consistent and confirmed bioactive compound 2 as thiolutin dioxide ($C_8H_8N_2O_4S_2$). The chemical structure of thiolutin dioxide was shown in Figure 4.15b.

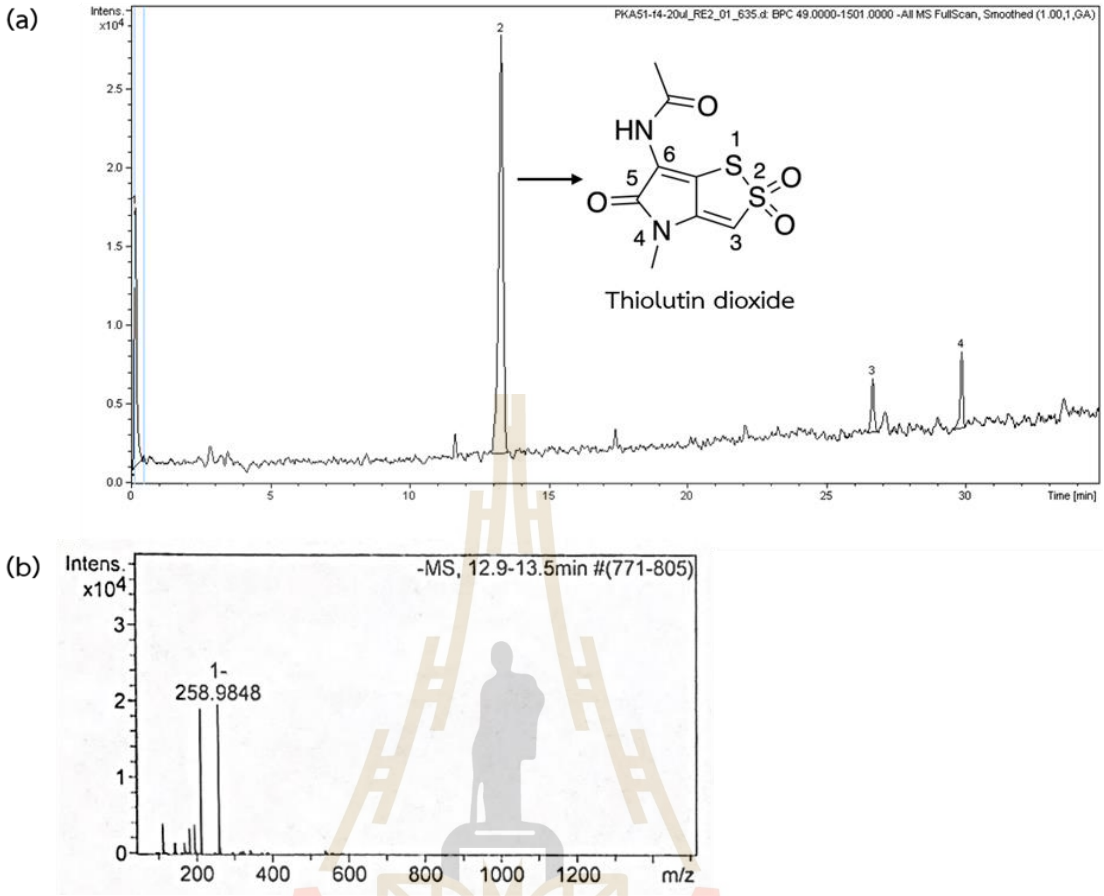


Figure 4.13 Chromatogram for liquid chromatography-mass spectrometry (LC-MS) of purified bioactive compound 2 in negative ion polarity mode $[M-H]^-$. (a) HPLC-MS chromatogram of purified bioactive compound 2. (b) Mass spectrum of peak at a retention time of 13.3 min from purified bioactive compound 2 (258.9848 *m/z* at negative mode.

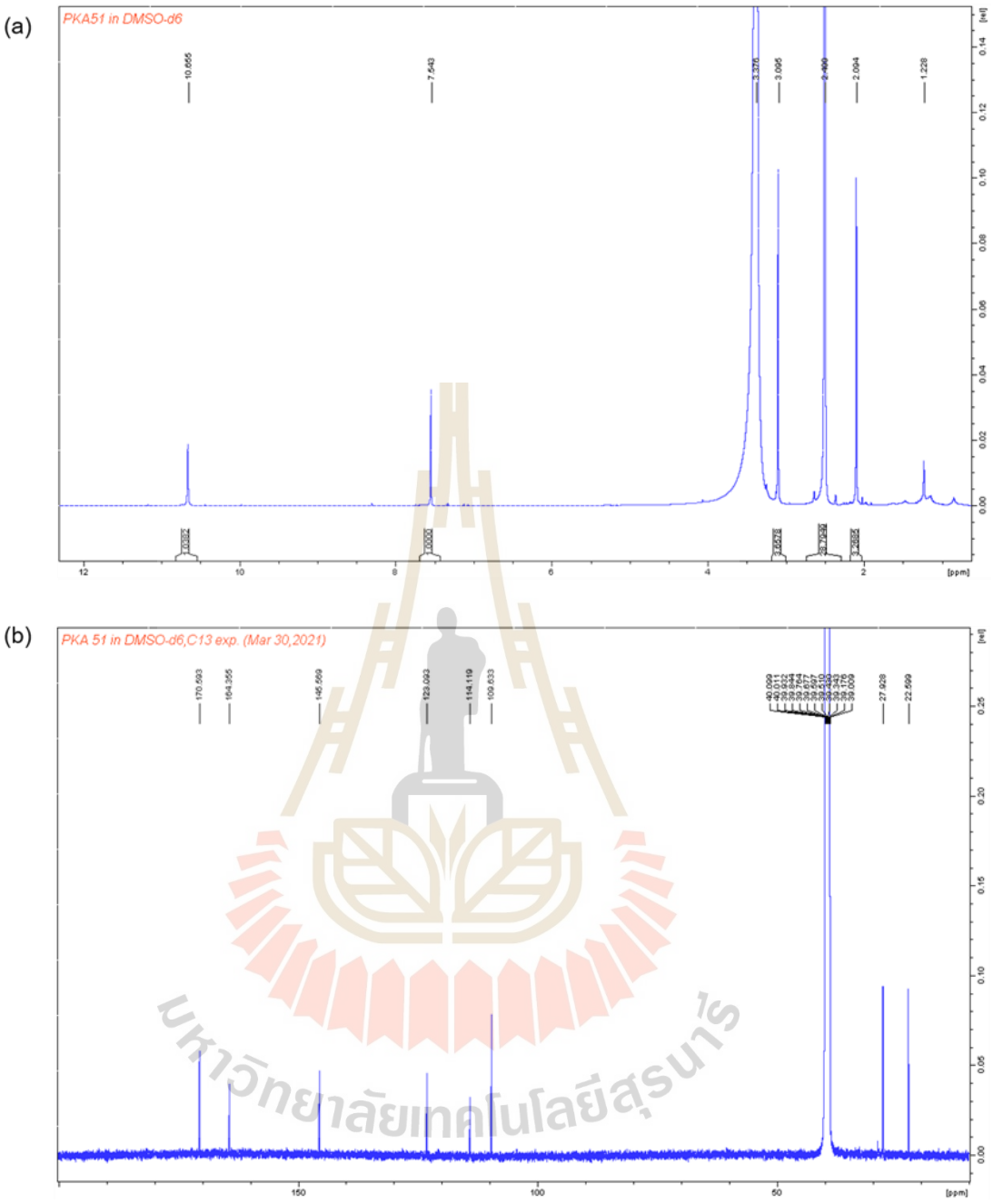


Figure 4.14 Nuclear magnetic resonance (NMR) chromatogram of compound 2 extract from *S. luteosporeus* PKA51. (a) ^{1}H NMR spectrum, (b) ^{13}C NMR spectrum.

Table 4.7 ^{1}H NMR spectra of compound 2 and thiolutin dioxide.

Position	Compound 2	Thiolutin dioxide
	δH (ppm)	δH (ppm)
H-3	7.54 (1H, s)	7.55
N (4)-CH_3	3.10 (3H, s)	3.10
C (6)-NH	10.66 (1H, s)	10.65
CO-CH_3	2.09 (3H, s)	2.10

Table 4.8 ^{13}C NMR spectra of compound 2 and thiolutin dioxide.

Position	Compound 2	Thiolutin dioxide
	δC (ppm)	δC (ppm)
3	109.63	109.58
3a	145.57	145.48
N (4)-CH_3	27.93	27.85
5	165.35	164.27
6	123.09	123.01
6a	114.12	114.05
Amino moiety	170.59	170.46
	22.60	22.51

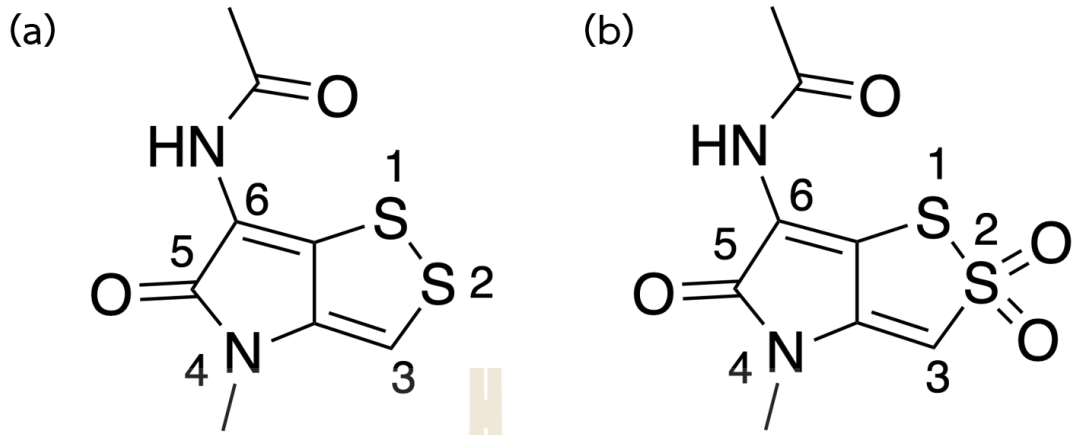


Figure 4.15 Chemical structure of (a) thiolutin, and (b) thiolutin dioxide produced by *S. luteosporeus* PKA51.

4.6 Antimicrobial activity of purified bioactive compounds

The MIC values of purified bioactive compounds were determined by the broth microdilution method using 96-well plates against antibiotic-resistant bacteria. Compound 1 (thiolutin) was active against MRSA, MRSE, clinical isolate *E. coli,* and clinical isolate *K. pneumoniae* with MIC values 2, 4, 32, and 128 μg·ml^{-1}, respectively. MIC values of compound 2 (thiolutin dioxide) against MRSA, MRSE, clinical isolate *E. coli*, and clinical isolate *K. pneumoniae* were 8, 4, 32, and 64 μg·ml^{-1}, respectively. Furthermore, MBC of compound 1 and compound 2 were examined by plating aliquot assay (Nithya et al., 2018; Rajivgandhi et al., 2018). The MBC values of compound 1 and compound 2 against MRSE were 8 and 4 μg·ml^{-1}. While the MBC values of compound 1 and compound 2 were >256 μg·ml^{-1} for MRSA, clinical isolate *E. coli*, and clinical isolate *K. pneumoniae*, respectively. It should be noted that MIC of compound 2 against MRSE, clinical isolate *E. coli*, and clinical isolate *K. pneumoniae* were lower than standard drugs (vancomycin, and tetracycline). The antimicrobial activity of compound 1 and compound 2 were summarized in Table 4.9.

Table 4.9 MIC and MBC of compound 1 and compound 2 extracted from *Streptomyces* sp. PKA51 against antibiotic-resistant bacteria.

Antibiotic-resistant bacteria	Minimum inhibitory concentration (μg·ml^{-1}) (MIC)				Minimum bactericidal concentration (μg·ml^{-1}) (MBC)			
	VAN	TC	Thiolutin	Thiolutin dioxide	VAN	TC	Thiolutin	Thiolutin dioxide
MRSA DMST20654	2	ND	2	8	2	ND	>256	>256
Clinical isolate MRSE	4	ND	4	4	8	ND	8	4
Clinical isolate *E. coli* 2026	256	64	32	32	256	>256	>256	256
Clinical isolate *K. pneumoniae* 1617	256	256	128	64	>256	>256	>256	>256

Each value is a mean of n = 3 experiments., ND = Not determined, VAN = Vancomycin, TC = Tetracycline

CHAPTER V
DISCUSSION AND CONCLUSION

The genus *Streptomyces* from terrestrial soil is a potential biological source of bioactive metabolites. They are known to produce a wide variety of substances such as antimicrobial compounds, antifungals, antivirals, herbicides, pesticides, and antitumor agents. (Al-Dhabi et al., 2016; Bérdy, 2005; Dahal and Chaudhary, 2018; Procópio et al., 2012; and Quinn et al., 2020). The diversity of bioactive metabolites from the genus *Streptomyces* is driven by evolution and natural selection (Hibbing et al., 2010). In bacteria communities, the genus *Streptomyces* has evolved various strategies to disrupt and kill other microorganisms including the production of antimicrobial agents and protein toxins. They compete for resources and space within their communities by defending and attacking other bacteria. (Meij et al., 2017; Hussain et al., 2018). This phenomenon resulted in the synthesis of various metabolites. Several *Streptomyces* sp. are frequently distributed in various habitats such as the Tharban hot spring, Mount Everest, Himalayan region, Egyptian desert, Saudi Arabia desert, Indian saline desert, volcanic cave, the lake of Ghana, and mangrove soil. The *Streptomyces* spp. can produce a variety of bioactive compounds and contribute to the development of novel antimicrobial agents (Al-Dhabi et al., 2016; Gurung et al., 2009; Hozzein et al., 2011; Ser et al., 2015; Nithya et al., 2018; Radhakrishnan et al., 2010; Selim et al., 2021; Tawiah et al., 2012; Thumar et al., 2010).

In 2017, Khowangklang (2017) isolated 77 strains of actinobacteria from dry dipterocarp soil in Suranaree University of Technology, Nakhon Ratchasima province. The antimicrobial test has shown that 6 soil isolates (PKA2, PKA20, PKA45, PKA50, PKA51, and PKA62) had antimicrobial activity against test pathogens (*S. aureus* TISTR2466, MRSA DMST20654, *S. epidermidis* TISTR518, *B. subtilis* TISTR008, *B. cereus* TISTR687, *S.* Typhimurium TISTR292, and *P. mirabilis* TISTR100) (Khowangklang, 2017).

Strain PKA51 was picked for this study since the strain showed high-antimicrobial activity against Gram-positive and Gram-negative bacteria. The colony morphology of PKA51 is rough-surfaced and covered by a web of aerial mycelium. They could produce earthy smell and soil odors. These earthy odors are caused by the production of geosmin and siderophore, which are mostly generated by the genus *Streptomyces* (Flärdh and Buttner, 2009; Hasani et al., 2014). Based on the results of 16S rDNA and phylogenetic tree analysis, PKA51 exhibited the highest sequence similarity with *Streptomyces luteosporeus* NBRC 14657^{T} (99.93 % similarity). Therefore, PKA51 can be classified as *S. luteosporeus* (Basonym: *Streptoverticillium album*).

S. album was first discovered by Locci and co-workers in 1969. It was isolated from plant rhizosphere soil in Kuala Lumpur, Malaysia (Locci et al., 1969; Ting et al., 2009). In 1990, Witt and Stackebrandt studied the taxonomic relationship between *Streptoverticillium album* and *Streptomyces luteosporeus*. These two species shared chemotaxonomic and 16S rRNA gene sequence. Therefore, the *Streptoverticillium album* was reclassified as *Streptomyces luteosporeus* (Euzéby, 1997; Witt and Stackebrandt, 1990).

It has been reported that the antibacterial activity of *S. luteosporeus*, *S. luteosporeus* was active against Gram-positive bacteria (*S. aureus*) while antibacterial activity against Gram-negative bacteria (*K. pneumoniae*, *E. coli*) was weak (Gayathri et al., 2011; Ting et al., 2009). In this study, the antimicrobial activity of *S. luteosporeus* PKA51 was evaluated against Gram-positive bacteria and Gram-negative bacteria. PKA51 exhibited broad-spectrum antimicrobials against Gram-positive bacteria (*B. subtilis* TISTR008, *B. cereus* TISTR684, *S. epidermidis* TISTR518, *S. aureus* ATCC1466, MRSA DMST20654, and clinical isolate MRSE) and Gram-negative bacteria (*E. coli* TISTR780, *S.* Typhimurium TISTR292, *E. aerogenes* TISTR1540, *S. marcescens* TISTR1354, *P. mirabilis* TISTR100, clinical isolate *K. pneumoniae* 1617, and clinical isolate *E. coli* 2026). *S. luteosporeus* is known as a producer of thiolutin (acetopyrrothine), quinaldopeptin, and indolmycin antibiotics (BacDive, 2019; Toda et al., 1990; Celmer and Solomons, 1955).

In the present study, bioactive compounds from *Streptomyces* sp. PKA51 were purified and characterized by using LC-MS and NMR spectroscopy. The results revealed that PKA51 produced two bioactive compounds which were designated as compound 1 and compound 2. Both compounds exhibited a maximum absorbance in a range of 385 - 391 nm indicating the presence of pyrrothine core, a nucleus aromatic in bioactive compounds (Li et al., 2014; Merrouche et al., 2018; Qin et al., 2014). Based on HPLC-MS, compound 1 was detected at a retention time of 11.6 min and had an exact mass of 226.9965 *m/z* $[M-H]^-$ which matched $C_8H_7N_2O_2S_2$. Compound 2 was detected at a retention time of 13.3 min and its exact mass was 258.9848 *m/z* $[M-H]^-$ and matched $C_8H_7N_2O_4S_2$. The chemical structure of bioactive compounds was confirmed by NMR spectroscopy analysis. The results revealed that compound 1 was thiolutin which was similar to the previous report by Celmer and Solomons, (1955); Lamari et al. (2002). While compound 2 was identified as N-(4-methyl-2,2-dioxido-5-oxo-4,5-dihydro- [1,2] dithiolo[4,3-b] pyrrol-6-yl) acetamide), a thiolutin dioxide derivative.

Thiolutin and thiolutin dioxide have been characterized as antibiotics belonging to the dithiolopyrrolone (DTP) group. The chemical structure of DTP was characterized by a distinct bridged bicyclic, which contains a disulfide between two-end thiols (Figure 5.1). The conjugation of two end-thiols and the presence of the pyrrothine core in these molecules are responsible for the yellow color and maximum absorbance at 388 nm (λ_{388}). DTPs are grouped into 3 subfamilies including thiolutin type (*N*-methyl, *N*-acylpyrrothine derivatives), holomycin type (*N*-acylpyrrothine derivatives), and thiomarinol type. Many DTP members have been isolated from several microorganisms, such as *Streptomyces* sp., *Xenorhabdus bovienii*, *Alteromonas rava*, and *Saccharothrix* sp. (Celmer and Solomons., 1955; Li et al., 2014; Qin et al., 2013; Zhai et al., 2016).

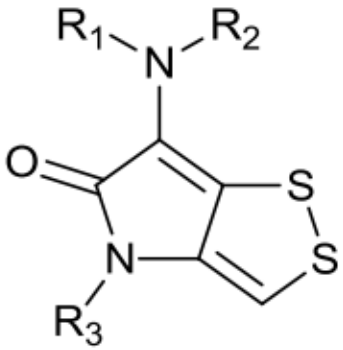


Figure 5.1 Chemical structure of dithiolopyrrolone compounds. The R_1, R_2, and R_3 groups for each pyrrothine core will differ in structure.

It has been shown that mode of action of thiolutin is to block DNA-dependent RNA-polymerase, resulting in the inhibition of RNA synthesis (Oliva et al., 2001). Thiolutin acts as a direct RNA synthesis inhibitor in the process of RNA elongation in bacteria and yeast (*Saccharomyces cerevisiae*) (Khanchatourians and Tipper, 1974; Oliva et al., 2001; Villain-Guillot et al., 2007). In the current study, MIC values of thiolutin derived from PKA51 against drug-resistant pathogens were determined by using the broth microdilution method. Thiolutin produced by PKA51 exhibited a strong inhibitory effect against MRSA DMST20654, clinical isolate MRSE, and clinical isolate *E. coli* 2026 with MIC values 2, 4, 32 $\mu g \cdot ml^{-1}$, respectively. While the MIC value of thiolutin against clinical isolate *K. pneumoniae* 1617 was 128 $\mu g \cdot ml^{-1}$.

It has been reported the antibacterial activity of commercial thiolutin isolated from *Streptomyces albus* is active against *S. epidermidis*. The MIC and MBC values of commercial thiolutin against *S. epidermidis* were 6.25 and 12.5 $\mu g \cdot ml^{-1}$, respectively. (Villain-Guillot et al., 2007). It should be noted that the MIC and MBC values of thiolutin from *S. luteosporeus* PKA51 against *S. epidermidis* were lower than commercial thiolutin (*S. albus*). Moreover, it has been discovered that thiolutin has anti-angiogenic properties by inhibiting a focal adhesion kinase involved in tumor malignancy. Thiolutin has strong anti-angiogenesis on human cancer cell lines, lung-H460 ($IC_{50} \approx 190$ nM), and breast-LCC6 ($IC_{50} \approx 220$ nM). Therefore, thiolutin was defined as an antitumor with

moderates cytotoxic to noncancerous cells (Fu and Macmillan, 2015; Li et al., 2007; Minamiguchi et al., 2001; Toda et al., 1990).

Thiolutin dioxide is a known substance. The semi-synthesis process of thiolutin dioxide was described by Schachtner in 1999. Thiolutin dioxide was synthesized by oxidizing thiolutin with meta-chlorperbenzoic acid (mCPBA) (Schachtner et al., 1999). However, the semi-synthetic compound had several disadvantages, including byproduct formation, chemical hazards, and toxic substances. Compared to the semi-synthetic process, the biological synthesis of thiolutin dioxide from PKA51 is naturally occurring, environmentally safe, and chemical-free (Usman et al., 2018). In 2002, it has been shown that thiolutin dioxide can be isolated from *Nocardiopsis* sp. ST100692 (DSM13834) (Claudia et al., 2002). In 2014, it was reported that there are around 27 naturally occurring DTP compounds including aureothricin, thiolutin, holomycin, isobutyropyrrothine, butanolypyrrothine, senecioylpyrrothine, tigloylpyrrothine, propanoylholothine, VD-844, xenorhabdin no. 1 - 13, and thiomarinol A-G (Li et al., 2014; Qin et al., 2013). Since 2014, there have been no reports of thiolutin dioxide derived from microorganisms (Li et al., 2014). However, in 2020 Zhou and co-workers reported the hypothesis of thiolutin dioxide's biosynthesis route from thiolutin. This hypothesis has been proposed by the conversion of holomycin to thiolutin. They suggested that the oxidation reaction of thiosulphate (-RSO_2SR'-) to thiolutin results in the conversion of thiolutin to thiolutin dioxide (Li and Walsh, 2010; Zhou et al., 2020). As a result, thiolutin dioxide was synthesized from thiolutin and rarely produced by microorganisms.

In this study, the ratio of bioactive compound to cell dry weight ($mg \cdot g^{-1}$) was used to calculate the yield. The obtained yield of thiolutin and thiolutin dioxide from PKA51 were 2.98 and 1.36 $mg \cdot g^{-1}$, respectively. Thus, PKA51 is a potential candidate for the production of thiolutin dioxide due to its shorter fermentation period.

To date, there has been no report on the antibacterial activity of thiolutin dioxide against drug-resistant pathogens. In this study, the antibacterial activity of thiolutin dioxide was first evaluated. The results showed that thiolutin dioxide was the most effective against clinical isolate MRSE (MIC = 4 $\mu g \cdot ml^{-1}$). While the highest MIC value was observed against clinical isolate *K. pneumonia* 1617 (MIC = 64 $\mu g \cdot ml^{-1}$). In

addition, the results showed Gram-positive bacteria (MRSA and MRSE) have more susceptible to thiolutin dioxide than Gram-negative bacteria (*E. coli* and *K. pneumoniae*).

To best knowledge, there has only been one report of naturally-occurring thiolutin dioxide from *Norcardiopsis* sp. ST100692 (Claudia et al., 2002). This is the first report on isolation and characterization of thiolutin dioxide from *S. luteosporeus*. Thiolutin dioxide showed strong antibacterial activity against antibiotic-resistant pathogens. Therefore, thiolutin dioxide from *S. luteosporeus* PKA51 may be a promising antibacterial drug candidate for pharmaceutical applications.

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APPENDICES

APPENDIX A

PREPARATION OF CULTURE MEDIUM

1. Tryptone yeast extract agar (ISP medium no.1)

Ingredients	$g \cdot l^{-1}$
Tryptone	5.0
Yeast extract	3.0
Agar	15.0
Final pH (25 °C)	7.0 ± 0.2

2. Yeast malt agar (ISP medium no. 2)

Ingredients	$g \cdot l^{-1}$
Peptone	5.0
Yeast extract	3.0
Malt extract	3.0
Dextrose	10.0
Agar	15.0
Final pH (25 °C)	6.2 ± 0.2

3. Oat meal agar (ISP medium no. 3)

Ingredients	$g \cdot l^{-1}$
Oat meal	20
Agar	18

Trace salts	-
$FeSO_4 \times 7H_2O$	0.001
$MnCl_2 \times 4H_2O$	0.001
$ZnSO_4 \times 7H_2O$	0.001
DI water	1,000 ml
Final pH (25 °C)	7.3 ± 0.2

4. Inorganic salt starch agar (ISP medium no. 4)

ingredients	$g{\cdot}l^{-1}$
Starch soluble	10.0
K_2HPO_4	1.0
$MgSO_4 \times 7H_2O$	1.0
NaCl	1.0
$(NH_4)_2SO_4$	2.0
$CaCO_3$	2.0
$FeSO_4 \times 7H_2O$	0.001
$MnCl_2 \times 4H_2O$	0.001
$ZnSO_4 \times 7H_2O$	0.001
Agar	20
DI water	1,000 ml
Final pH (25 °C)	7.2 ± 0.2

5. Glycerol asparagine agar base (ISP medium no. 5)

Ingredients	$g{\cdot}l^{-1}$
L-Asparagine	1.0

K_2HPO_4	1.0
Trace salt solution (ml)	1.0
Agar	20.0
1 ml of Trace salt solution contains	-
$FeSO_4 \times 7H_2O$	0.001
$MnCl_2 \times 4H_2O$	0.001
$ZnSO_4 \times 7H_2O$	0.001
Glycerol	10 ml
DI water	1,000 ml
Final pH (25 °C)	7.4 ± 0.2

6. Peptone yeast extract iron agar (ISP medium no.6)

Ingredients	$g \cdot l^{-1}$
Peptone	15.0
Proteose peptone	5.0
Yeast extract	1.0
$C_6H_8FeNO_7$	0.5
K_2HPO_4	1.0
$Na_2O_3S_2$	0.08
Agar	15.0
DI water	1,000 ml
Final pH (25 °C)	6.7 ± 0.2

7. Tyrosine agar (ISP medium no.7)

Ingredients	$g \cdot l^{-1}$
L-Asparagine	1.0
L-Tyrosine	0.5
K_2HPO_4	0.5
$MgSO_4 \times 7H_2O$	0.5
NaCl	0.5
Trace salt solution (ml)	1.0
Agar	20.0
Trace salt solution containing	-
$FeSO_4 \times 7H_2O$	1.360 mg
$CuCl_2 \times 2H_2O$	0.027 mg
$CoCl_2 \times 6H_2O$	0.040 mg
$Na_2MoO_4 \times 2H_2O$	0.025 mg
$ZnCl_2$	0.020 mg
H_3BO_3	2.850 mg
$MnCl_2 \times 4H_2O$	1.800 mg
$Na_2C_4H_4O_6$	1.770 mg
Glycerol	15 ml
DI water	1,000 ml
Final pH (25 °C)	7.3 ± 0.1

8. Nitrate agar (ISP medium no.8)

Ingredients	$g \cdot l^{-1}$
Peptone	5.0
HM peptone B (Meat extract B)	3.0
KNO_3	1.0
Agar	12.0
DI water	1,000 ml
Final pH (25 °C)	6.8 ± 0.2

9. Carbon utilizing agar (ISP medium no.9)

Ingredients	$g \cdot l^{-1}$
$(NH_2)_2SO_4$	2.64
KH2PO4	2.38
$K_2HO_4P \times 3H_2O$	5.65
$MgSO_4 \times 7H_2O$	1.0
$CuSO_4 \times 5H_2O$	0.0065
$FeSO_4 \times 7H_2O$	0.0011
MnCl2 x 4H2O	0.0079
$ZnSO_4 \times 7H_2O$	0.0015
Agar	15
Starch soluble (10%, w/v)	100 ml
DI water	1,000 ml
Final pH (25 °C)	7.0 ± 0.2

10. Bennet's agar

Ingredients	$g \cdot l^{-1}$
Yeast extract	1.0
Beef extract	1.0
Casein enzymatic hydrolysate	2.0
Dextrose	10.0
Agar	15.0
DI water	1,000 ml
Final pH (25 °C)	7.3 ± 0.2

11. Czapek's agar

Ingredients	$g \cdot l^{-1}$
$C_{12}H_{22}O_{11}$	30.0
$NaNO_2$	2.0
K_2HPO_4	1.0
$MgSO_4$	0.5
Kcl	0.5
$FeSO_4$	0.01
Agar	15.0
DI water	1,000 ml
Final pH (25 °C)	7.3 ± 0.2

12. Starch casein agar (SCA)

Ingredients	$g \cdot l^{-1}$
Starch soluble	10.0
Casein acid hydrolysate	0.3
KNO_3	2.0
$MgSO_4 \times 7H_2O$	0.05
K_2HPO_4	2.0
NaCl	2.0
$CaCO_3$	0.02
$FeSO_4 \times 7H_2O$	0.01
Agar	15.0
DI water	1,000 ml
Final pH (25 °C)	7.3 ± 0.2

13. Mueller Hinton broth (MHB)

Ingredients	$g \cdot l^{-1}$
Beef extract	300.0
Casein acid hydrolysate	17.5
Starch	1.5
DI water	1,000 ml
Final pH (25 °C)	7.3 ± 0.1

Mueller Hinton broth was purchased from Himedia®, India.

14. Luria Bertani (LB) broth

Ingredients	$g \cdot l^{-1}$
Tryptone	10.0
Yeast extract	5.0
nAcL	10.0
DI water	1,000 ml
Final pH (25 °C)	7.5 ± 0.2

LB agar was prepared by adding 15.0 g of agar into 1000 ml of LB broth. The sterilization of all-culture media was sterilized by autoclaving at 121 °C, 15 lbs. pressure for 15 min.

APPENDIX B

INFORMATION OF CLINICAL ISOLATES

Strains	Source	Susceptible	Drug resistant
K. pneumoniae 1617	Sputum	Gentamycin	Ampicillin
			Amikacin
			Amocxicillin-Clavulanate
			Piperacillin-Tazabactam
			Cefoxitin
			Cefazolin
			Ceftriaxone
			Ceftazidime
			Cefepime
			Sulfamethoxazole
			Ciprofloxacin
			Levofloxacin
			Imipenem
			Ertapenem
			Meropenem
E. coli 2026	Urine	Gentamycin	Ampicillin
		Amikacin	Cefazolin
		Amocxicillin-Clavulanate	Ceftriaxone
		Piperacillin-Tazabactam	Ciprofloxacin
		Cefoxitin	Levofloxacin
		Imipenem	Cefepime
		Ertapenem	
		Meropenem	

APPENDIX C

16s rRNA NUCLEOTIDE SEQUENCE

Streptomyces sp. PKA51 (1481 bp)

GACGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAACGATGAACCTCCTTCGGGAGGGGA
TTAGTGGCGAACGGGTGAGTAACACGTGGGCAATCTGCCCTGCACTCTGGGACAAGCCCTGGA
AACGGGGTCTAATACCGGATATGACCTTCGAAGGCATCTTTGAAGGTGGAAAGCTCCGGCGGTG
CAGGATGAGCCCGCGGCCTATCAGCTGGTTGGTGGGGTGATGGCCTACCAAGGCGACGACGGG
TAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGA
GGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGAT
GACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAAGCGAGAGTGACGGTACCTGCAGA
AGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTGTCCGG
AATTATTGGGCGTAAAGAGCTCGTAGGCGGCTTGTCGCGTCGGATGTGAAAGCCCGGGGCTTAA
CCCCGGGTCTGCATTCGATACGGGCAGGCTAGAGTTCGGTAGGGGAGATCGGAATTCCTGGTG
TAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGATCTCTGGGCCGAT
ACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCC
GTAAACGTTGGGCACTAGGTGTGGGCCACATTCCACGTGGTCCGTGCCGCAGCTAACGCATTAA
GTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCAC
AAGCAGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACA
CCGGAAAGCATCAGAGATGGTGCCCCCCTTGTGGTCGGTGTACAGGTGGTGCATGGCTGTCGTC
AGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCTGTGTTGCCA
GCATGCCCTTCGGGGTGATGGGGACTCACAGGAGACTGCCGGGGTCAACTCGGAGGAAGGTGG
GGACGACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTAC
AATGAGCTGCGATACCGCGAGGTGGAGCGAATCTCAAAAAGCCGGTCTCAGTTCGGATTGGGGT
CTGCAACTCGACCCCATGAAGTTGGAGTTGCTAGTAATCGCAGATCGGCATTGCTGCGGTGAAT
ACGTTCCCGGGCCTTGTACACACCGCCCGTCACGTCACGAAAGTCGGTAACACCCGAAGCCGGT
GGCCCAACCCTTGTGGAGGGAGCCGTCGAAGGTGGGACTGGCGATTGGGACGAAGTCGTAACA
AGGTAGCCGTACCGGAAGGTGC

APPENDIX D

MOBILE PHASE USED IN THIN-LAYER CHROMATOGRAPHY

Table 1D List of various solvent systems tried for TLC analysis of crude extract PKA51.

No.	Solvent systems	Composition (v/v)
1	Hexane: Chloroform	40: 60
2	Hexane: Chloroform	50: 50
3	Hexane: Chloroform	60: 40
4	Butanol: Acetic acid: Water	70: 20: 10
5	Butanol: Acetic acid: Water	60: 30: 10
6	Chloroform: Methanol	60: 40
7	Chloroform: Methanol	50: 50
8	Chloroform: Methanol	70: 30
9	Chloroform: Methanol	30: 70
10	Chloroform: Methanol	80: 20
11	Chloroform: Methanol	20: 80
12	Chloroform: Methanol	10: 90
13	Chloroform: Methanol	90: 10
14	Chloroform: Methanol	75: 25
15	Chloroform: Methanol	95: 5
16	Methanol: Hexane	90: 10
17	Chloroform: Hexane	90: 10
18	Chloroform: Hexane	80: 20
19	Chloroform: Methanol	98: 2
20	Chloroform: Hexane	95: 5
21	Chloroform: Hexane	98: 2
22	Chloroform: Hexane: Methanol	90: 5: 5
23	Chloroform: Hexane: Methanol	95: 2.5: 2.5
24	Chloroform: Methanol	99: 1

Table 1D List of various solvent systems tried for TLC analysis of crude extract PKA51 (Continued).

No.	Solvent systems	Composition (v/v)
25	Chloroform	100
26	Chloroform: Methanol	40: 60
27	Methanol	100
28	Chloroform: Methanol	96: 4
29	Methanol: Water	70: 30
30	Methanol: Water	60: 40
31	Methanol: Water	50: 50
32	Butanol: Methanol: Water	60: 10: 30
33	Butanol: Methanol: Water	60: 20: 20
34	Butanol: Methanol: Water	60: 30: 10
35	Hexane: Ethyl acetate	80: 20
36	Hexane: Ethyl acetate	50: 50
37	Hexane: Ethyl acetate	25: 75
38	Butanol: Acetic acid: Water	50: 25: 25
39	Butanol: Acetic acid: Water	40: 30: 30
40	Butanol: Acetic acid: Water	40: 10: 50
41	Methanol: Hexane	60: 40
42	Methanol: Hexane	50: 50
43	Methanol: Hexane	40: 60
44	Chloroform: Dichloromethane: Water	33: 33: 33
45	Hexane: Chloroform: Water	40: 30: 30
46	Hexane: Chloroform: Water	65: 25: 10
47	Hexane: Isopropanol	80: 20
48	Hexane: Isopropanol	90: 10
49	Hexane: Isopropanol	70: 30
50	Hexane: Isopropanol	60: 40
51	Benzene: Acetic acid: Water	40: 10: 50
52	Acetonitrile: Water	92.5: 7.5

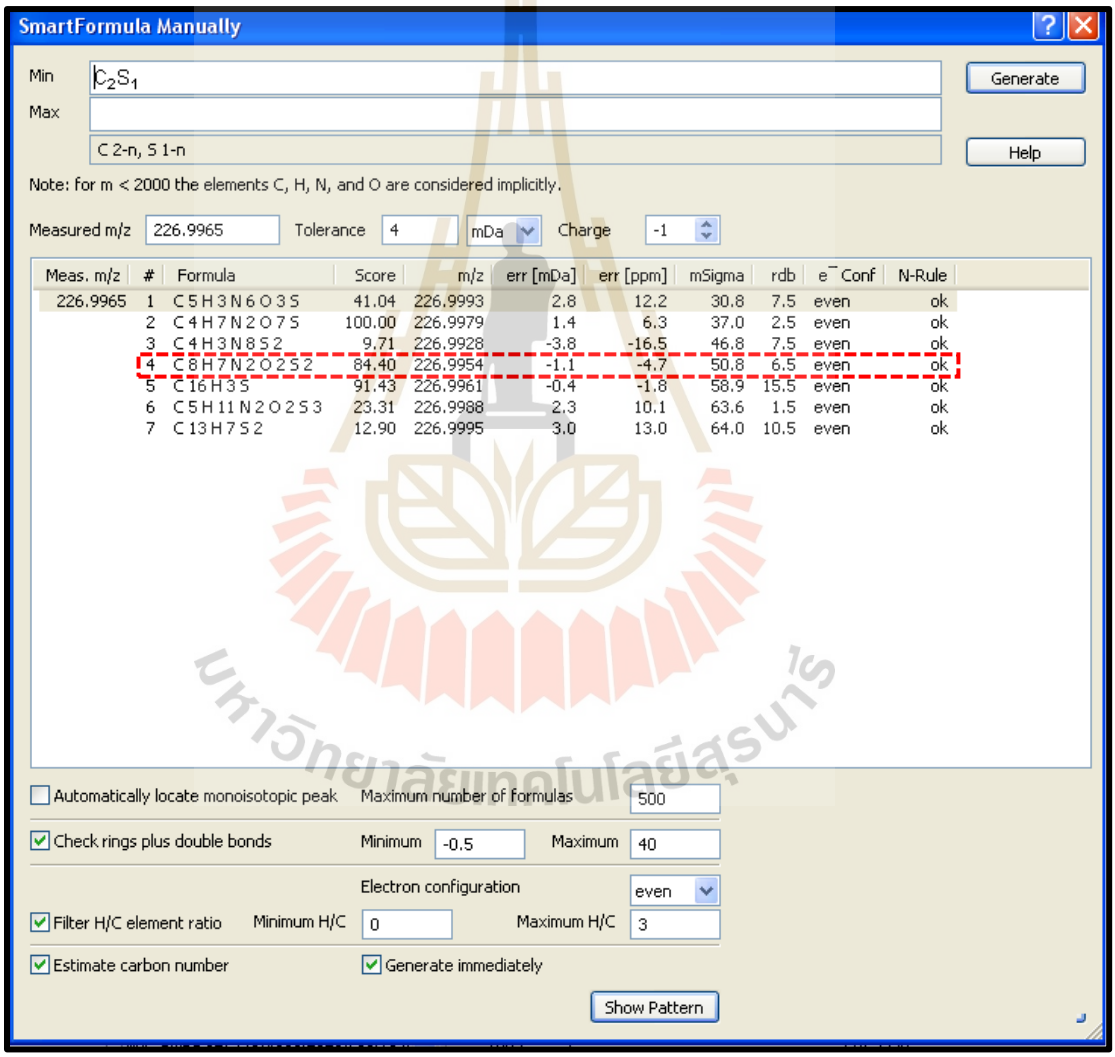
Table 1D List of various solvent systems tried for TLC analysis of crude extract PKA51 (Continued).

No.	Solvent systems	Composition
53	Acetonitrile: Water	60: 40
54	Acetonitrile: Water	50: 50
55	Acetonitrile: Water	40: 60
56	Ethyl acetate: Methanol	83.33: 16.67
57	Hexane: Acetone	70: 30
58	Hexane: Acetone	50: 50

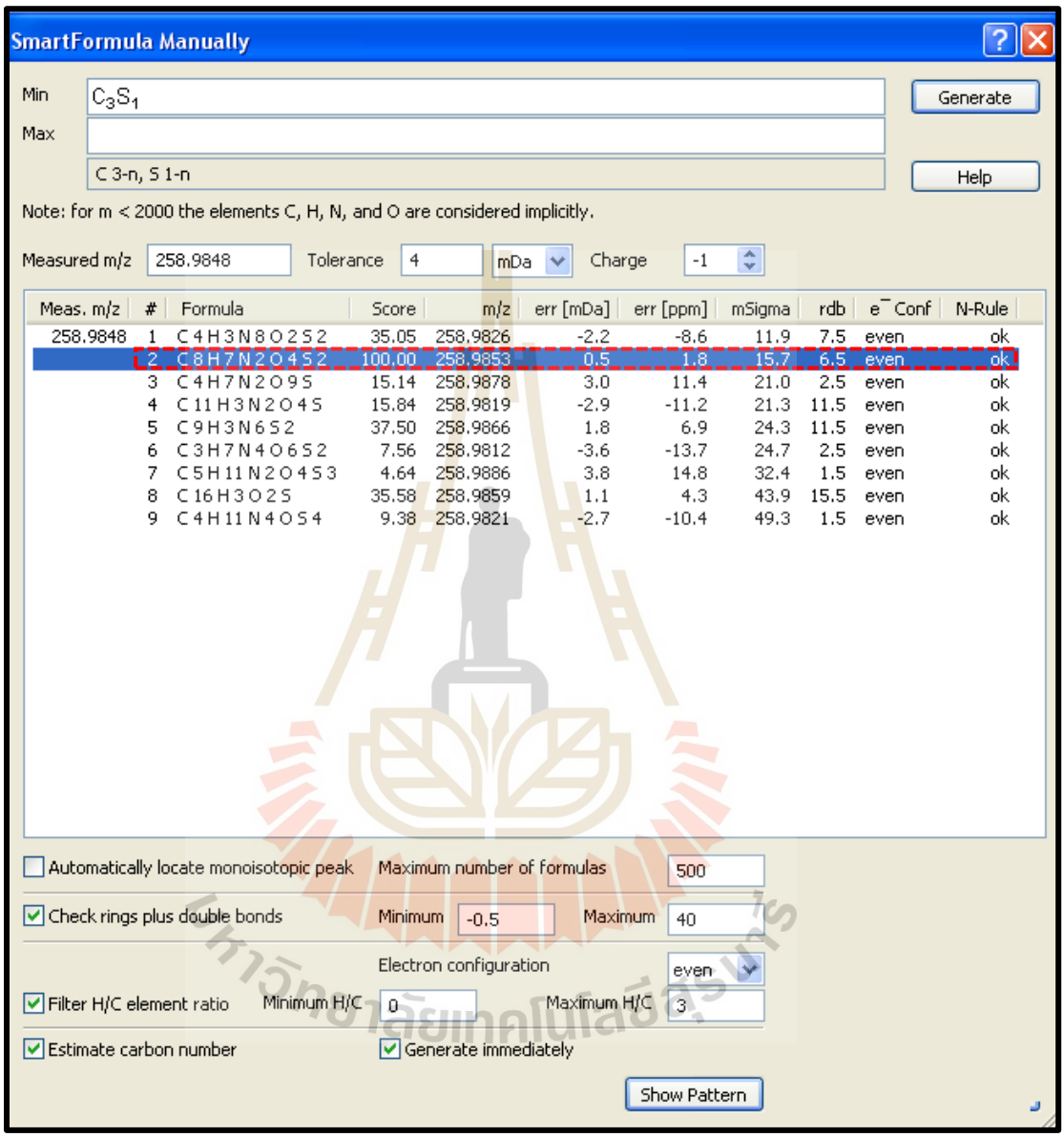
APPENDIX E

CHARACTERIZATION OF BIOACTIVE COMPOUNDS

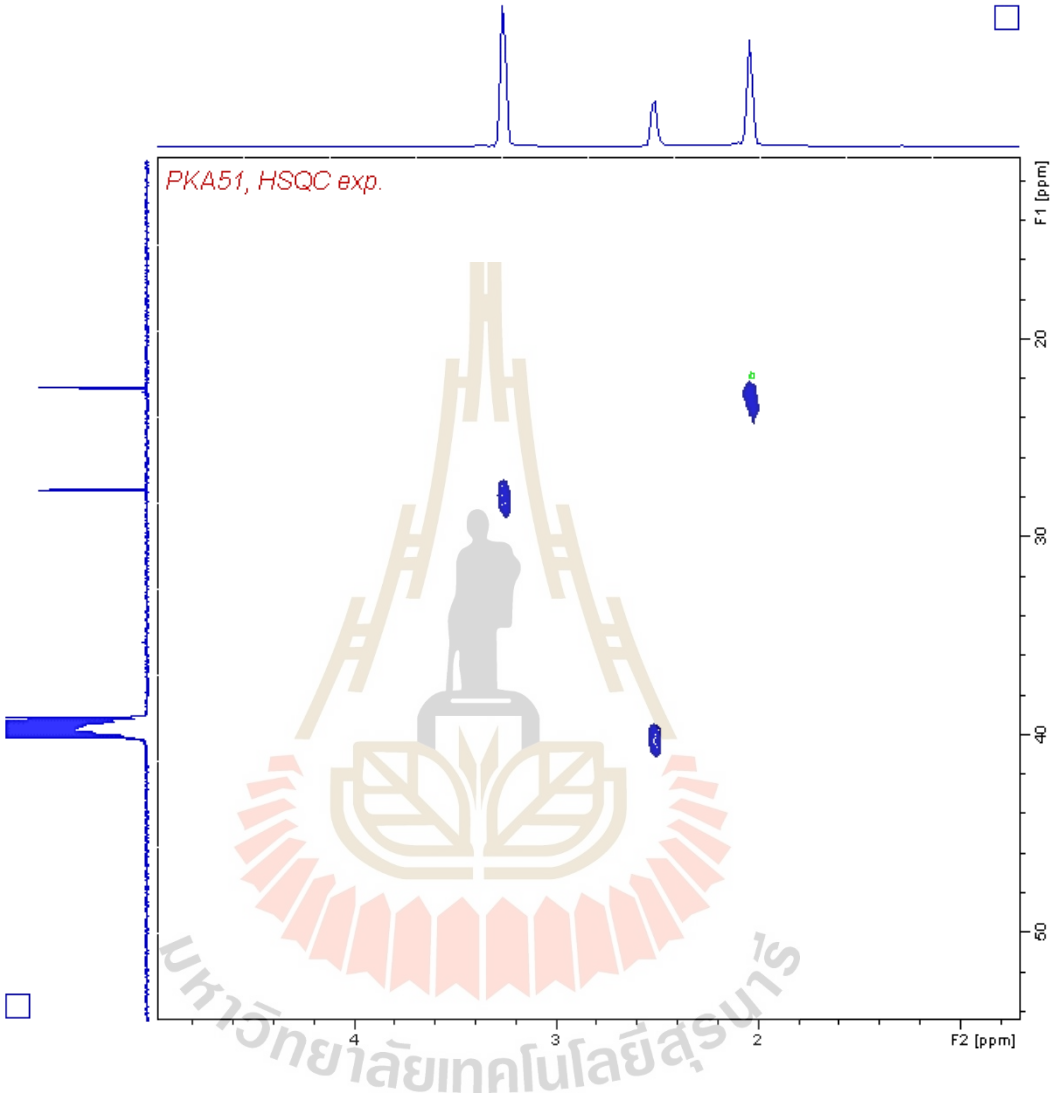
Smart formula search manually software: the prediction of molecular formula in mass spectra peak 226.9965 *m/z* (negative ion mode).



Smart formula search manually software: the prediction of molecular formula in mass spectra peak 258.9848 *m/z* (negative ion mode).



Two-dimensional NMR spectroscopy (Heteronuclear Single Quantum Coherence; HSQC) of purified bioactive compound 2 (thiolutin dioxide).



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