

EVALUATION OF ANTIBACTERIAL COMPOUND PRODUCED BY
STREPTOMYCETES CULTURED IN OPTIMIZED CONDITION



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การประเมินฤทธิ์ของสารต้านจุลชีพที่ผลิตโดยเชื้อสเตรปโตมัยซีท
ที่เพาะเลี้ยงในสภาวะที่เหมาะสม



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วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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วรรณิกา ปะนา : การประเมินฤทธิ์ของสารต้านจุลชีพที่ผลิตโดยเชื้อสเตรปโตมัยซีที่เพาะเลี้ยงในสภาวะที่เหมาะสม (EVALUATION OF ANTIBACTERIAL COMPOUND PRODUCED BY STREPTOMYCETES CULTURED IN OPTIMIZED CONDITION) อาจารย์ที่ปรึกษา : ผู้ช่วยศาสตราจารย์ ดร.นวรรตน์ นันทพงษ์, 67 หน้า.

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การพัฒนาของเชื้อแบคทีเรียดื้อยาเป็นปัญหาสำคัญที่เกี่ยวข้องกับการใช้ยาปฏิชีวนะซึ่งเกิดจากการที่เชื้อแบคทีเรียสามารถเรียนรู้ที่จะทนต่อยาปฏิชีวนะที่จะทำลายหรือยับยั้งการเจริญเติบโตของมันได้ ดังนั้นการเกิดโรคติดเชื้ออุบัติใหม่และการดื้อยาที่เพิ่มมากขึ้น เป็นแรงบันดาลใจให้นักวิจัยศึกษาชนิดใหม่จากเชื้อจุลินทรีย์ ซึ่งเป็นที่รู้กันดีว่าประมาณสองในสามของยาปฏิชีวนะผลิตได้จากเชื้อสเตรปโตมัยซี ดังนั้นการศึกษาในครั้งนี้มีวัตถุประสงค์ คือการแยกและระบุเชื้อสเตรปโตมัยซีในดินที่สามารถผลิตยาปฏิชีวนะได้ เชื้อสเตรปโตมัยซีทั้งหมด 44 ตัว ประกอบด้วย WPN1-WPN44 ที่ได้จากการคัดแยกและเก็บรวบรวมดินจากสวนพฤกษศาสตร์ มหาวิทยาลัยเทคโนโลยีสุรนารี ได้ถูกทดสอบหาฤทธิ์ต้านเชื้อจุลชีพ ประกอบด้วย เชื้อแบคทีเรียแกรมบวก 5 สายพันธุ์ และเชื้อแบคทีเรียแกรมลบ 8 สายพันธุ์ พบว่าการคัดกรองปฐมภูมิโดยใช้เทคนิคการขีดเชื้อแบบ Perpendicular พบว่า 9% มีขอบเขตในการออกฤทธิ์แคบกับเชื้อแบคทีเรียแกรมบวกเท่านั้น และอีก 1% พบว่ามีขอบเขตในการออกฤทธิ์กว้าง สามารถออกฤทธิ์ต้านเชื้อแบคทีเรียแกรมบวกและเชื้อแบคทีเรียแกรมลบ และอีก 90% ไม่แสดงฤทธิ์ต้านเชื้อจุลชีพ ในการศึกษาครั้งนี้ WPN32 ได้รับการคัดเลือกมาเพื่อศึกษาการประเมินฤทธิ์และลักษณะของสารต้านจุลชีพ สายพันธุ์ WPN32 แสดงความคล้ายคลึง 99.45% กับ *Streptomyces cyaneochromogenes* ซึ่งเป็นเชื้อสเตรปโตมัยซีที่แยกได้จากดินที่ปนเปื้อนแมงกานีสในประเทศจีน การศึกษาการผลิตสารต้านเชื้อจุลชีพของสายพันธุ์ WPN32 ถูกศึกษาในอาหาร อุณหภูมิ และระยะพักตัวที่แตกต่างกัน ส่วนของน้ำเลี้ยงเชื้อที่มีสารต้านจุลชีพจะถูกประเมินสารออกฤทธิ์โดยใช้วิธี agar-well diffusion ผลการศึกษาพบว่าการผลิตสารต้านจุลชีพสูงสุดของสายพันธุ์ WPN32 สามารถเก็บได้มาจากอาหารเหลว SC ที่อุณหภูมิ 30 องศาเซลเซียส และบ่มเป็นระยะเวลา 5 วัน สารประกอบของสารต้านจุลชีพที่ผลิตโดย WPN32 จะถูกสกัดด้วย ethyl acetate โดยสารออกฤทธิ์จะถูกทำให้บริสุทธิ์โดยวิธี thin-layer chromatography (TLC) column chromatography (CC) และศึกษาลักษณะของสารประกอบโดย LC-MS จากผลการศึกษาสารออกฤทธิ์ของเชื้อสายพันธุ์ WPN32 สามารถแยกออกเป็นสารประกอบหลัก 2 ชนิด โดยประกอบแรกถูกระบุว่าเป็น Anisomycin ในขณะที่สารประกอบที่ 2 ถูกระบุว่าเป็น Actinomycin D

ซึ่งในการศึกษาครั้งนี้ ถือว่าเป็นการศึกษาแรกของการศึกษาสภาวะการเลี้ยงเชื้อที่เหมาะสม และ
ศึกษาลักษณะของสารออกฤทธิ์ที่แยกได้จากเชื้อสายพันธุ์ *Streptomyces cyaneochromogenes*



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ลายมือชื่อนักศึกษา
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สมานันท์ ปะเทศ

WANNIKA PANA : EVALUATION OF ANTIBACTERIAL COMPOUND PRODUCED BY
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The development of drug-resistant bacteria is a major problem related to antibiotic usage. Microorganisms evolve the ability to tolerate antibiotics made to kill or inhibit them. Therefore, the emergence of new infectious diseases and drug resistance inspired the researcher to study a new drug derived from microorganisms. Streptomyces are well known for producing around two-thirds of all antibiotics. The aim of this study was to isolate and identify the antibiotic-producing soil streptomyces. A total of 44 streptomyces strains (WPN1-WPN44) were isolated from botanical garden soil at Suranaree University of Technology. They were tested for antibacterial activity against 5 Gram-positive and 8 Gram-negative bacteria. From the primary screening using perpendicular streak method, 9% of the isolates showed narrow spectrum against only Gram-positive bacteria, 1% showed broad spectrum against both Gram-positive and Gram-negative bacteria, and 90% showed no antibacterial activity. In this study, the strain WPN32 was chosen to evaluate and characterize its antimicrobial compounds. The strain WPN32 showed 99.45% similarity to *Streptomyces cyaneochromogenes*, a novel streptomyces isolated from manganese-contaminated soil in China. The antibacterial production of strain WPN32 was studied on different medium, temperatures, and incubation periods. The supernatant contained antibacterial metabolites were evaluated for antibacterial activity using agar-well diffusion method. The results showed that the highest antibacterial compound of WPN32 was obtained with SC broth at 30°C for 5 days of incubation. The antibacterial compounds produced by WPN32 were extracted with ethyl acetate. The active compounds were partially purified by using thin-layer chromatography (TLC), column chromatography (CC) and characterized by using liquid chromatography and mass spectrum (LC-MS). The result showed that active compounds of WPN32 could be separated to 2 major compounds. The first compound

was identified as anisomycin, while the second was actinomycin D. To date, this is the first study of optimization of culture conditions and characterization of active compounds derived from *Streptomyces cyaneochromogenes*.



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CONTENTS

	Page
ABSTRACT IN THAI.....	I
ABSTRACT IN ENGLISH.....	III
ACKNOWLEDGEMENTS.....	V
CONTENTS.....	VI
LIST OF TABLES.....	IX
LIST OF FIGURES.....	X
LIST OF ABBREVIATIONS.....	XI
CHAPTER	
I INTRODUCTION.....	1
1.1 Background / Problem.....	1
1.2 Research objectives.....	2
1.3 Research hypothesis.....	3
1.4 Scope and limitation of study.....	3
II LITERATURE REVIEWS.....	4
2.1 The Streptomyces.....	4
2.2 Antibiotics.....	6
2.3 Antibacterial resistance.....	8
2.4 Situation in Thailand.....	10
III MATEERIALS AND METHODS.....	14
3.1 Materials.....	14
3.1.1 Test pathogens.....	14
3.1.2 Culture media.....	14
3.1.3 Buffers and solutions.....	15
3.1.4 Primers used for PCR amplification.....	15
3.1.5 Research kits.....	15
3.1.6 Miscellaneous material.....	15

CONTENTS (Continued)

	Page
3.1.7 Equipments.....	16
3.2 Methods.....	16
3.2.1 Sample collection.....	16
3.2.2 Screening and isolation of streptomycetes.....	16
3.2.3 Primary screening of antibacterial-producing soil isolates using perpendicular-streak plate.....	17
3.2.4 Identification of antibacterial-producing soil isolate by 16S rRNA Gene sequencing.....	17
3.2.4.1 Cultural and morphological characteristics.....	17
3.2.4.2 Preparation of DNA template.....	18
3.2.4.3 Amplification of the 16S rRNA gene by PCR.....	18
3.2.4.3 The DNA sequencing.....	19
3.2.5 Phylogenetic tree construction.....	19
3.2.6 Optimization of antibacterial compounds production.....	19
3.2.6.1 Effect of culture medium.....	19
3.2.6.2 Effect of Temperature.....	20
3.2.6.3 Optimum incubation period.....	20
3.2.6.4 Agar-well diffusion method.....	20
3.2.7 Crude extract preparation.....	20
3.2.8 Purification of antibacterial compounds.....	21
3.2.8.1 Purification of bioactive compound by thin-layer chromatography.....	21
3.2.8.2 Detection of bioactive compound by contact Bioautography.....	21
3.2.9 Characterization of antibacterial compounds.....	22
IV RESULTS.....	23
4.1 Isolation of streptomycetes.....	23

CONTENTS (Continued)

	Page
4.2 Primary screening of antibacterial producing soil isolates.....	25
4.3 Identification of antibacterial-producing soil isolates.....	27
4.4 Optimization of antibacterial compounds production.....	35
4.4.1 Effect of culture medium.....	35
4.4.2 Effect of temperature.....	37
4.4.3 Optimum incubation period.....	37
4.5 The preparation of crude extract from WPN32.....	40
4.6 Purification of antibacterial compounds.....	40
4.6.1 Purification of bioactive compound by thin-layer chromatography...40	
4.7 Characterization of purified antibacterial compounds.....	41
V DISCUSSION AND CONCLUSION.....	45
REFERENCES.....	51
APPENDICES.....	59
APPENDIX A SEQUENCE OF ANTIBACTERIAL PRODUCING STREPTOMYCETES SOIL ISOLATE.....	60
APPENDIX B LIST OF CLOSET STRAINS FROM EZBIOCLOUD.....	65
CURRICULUM VITAE.....	67

LIST OF TABLES

Table	Page
2.1 CDC's antibiotic resistance threats in 2013.....	9
2.2 WHO global priority pathogens list in 2017.....	10
3.1 List of primers used in this study.....	15
3.2 List of instruments used in this study.....	16
3.3 Mixture of PCR reactions	18
4.1 Primary antibacterial activity screening of soil isolates.....	26
4.2 Colony morphology of five antibacterial-producing soil isolates.....	28
4.3 Closely relative strains of antibacterial producing streptomycetes from EzBioCloud database.....	31
4.4 Effect of incubation period on antibacterial activity of WPN32.....	38
4.5 The closely related compounds match to Massbank library of WPN32 compound.....	42

LIST OF FIGURES

Figure	Page
2.1 The life cycle of streptomycetes.....	5
2.2 Timeline of antibiotic drug.....	7
2.3 The targets and mechanism of action of antibiotics.....	8
2.4 Indication of antibiotic use.....	12
2.5 Imipenem-resistant <i>K. pneumoniae</i>	13
2.6 Antibacterial resistance rates of <i>Acinetobacter</i> spp.	13
3.1 The perpendicular-streak method.....	17
3.2 Conditions for PCR amplification of the 16S rRNA gene.....	19
3.3 Contact bioautography on agar.....	22
4.1 Map of the sampling site.....	23
4.2 Morphological characteristics of streptomycetes isolates in ISP2 agar.....	24
4.3 Primary screening using perpendicular streak method.....	25
4.4. Agarose gel electrophoresis.....	30
4.5 Phylogenetic tree using neighbor-joining method of 5 strains.....	32
4.6 Phylogenetic tree using neighbor-joining method of WPN32.....	34
4.7 The antibacterial activity by using agar-well diffusion method.....	35
4.8 The effect of culture medium of WPN32.....	36
4.9 The effect of temperature of WPN32.....	37
4.10 The effect of incubation period of WPN32 with yield.....	39
4.11 The effect of incubation period of WPN32.....	39
4.12 The ethyl acetate extract of WPN32.....	40
4.13 TLC-based bioautography of crude ethyl acetate of WPN32.....	41
4.14 LC-MS chromatogram from WPN32 active compounds.....	42
4.15 LC-MS analysis of WPN32 in negative ion mode of peaks 8.....	43
4.16 LC-MS analysis of WPN32 in negative ion mode of peaks 9.....	44

LIST OF FIGURES (Continued)

Figure	Page
5.1 Chemical structure of Actinomycin D.....	49
5.1 Chemical structure of Actinomycin D.....	49



LIST OF ABBREVIATIONS

g	Gram
h	Hour
L	Liter
μl	Microliter
mg/g	MilliGram per Gram
ml	Milliliter
mm	Millimeter
mM	Millimolar
M	Molar
nm	Nanometer
PCR	Polymerase chain reaction
p.s.i	Pound per square inch
rpm	Round per minute
rRNA	Ribosomal ribonucleic acid
TBE	Tris-borate-EDTA
v/v	Volume per volume
w/v	Weight per volume

CHAPTER I

INTRODUCTION

1.1 Background / Problem

Infectious diseases are a major cause of death in hospitals. Pathogenic bacteria can infect and destroy human tissues by themselves or their toxins to damage the host cell (Clardy et al., 2009; Kirmusaoğlu et al., 2019). Normally, infectious diseases from microbial infections can be treated by inhibiting or killing the pathogens using antibacterials (Kapoor et al., 2017; Kirmusaoğlu et al., 2019). Antibacterial agents target the production of biological metabolic components as well as the synthesis of the cell wall, cell membrane, and proteins and nucleic acids (Kirmusaoğlu et al., 2019). However, bacterial pathogens have developed molecular defence mechanisms to protect themselves from antibiotics (Sköld, 2011). Besides pathogen evolution, the overuse of antibiotics in clinical practice has led to bacterial antibacterial resistance (Kapoor et al., 2017). Antibacterial resistance has been recognized as a key problem in the treatment of infectious diseases (Hasan and Al-Harmoosh, 2020; Kapoor et al., 2017).

In 1928, Alexander Fleming found the first antibiotic, penicillin. In the 1940s, penicillin was used to treat infections and was successful in preventing pathogen infections during World War II (De Lima Procopio et al., 2012; Ventola, 2015). In the late 1930s, the discovery of penicillin motivated Selman Waksman to study microorganisms as producers of antibacterial compounds (Hutchings et al., 2019). Waksman defined the term “antibiotic” to describe any small substance produced by a microbe that affects the growth of other microbes (Clardy et al., 2009). Waksman discovered various antibiotics produced by actinomycetes, including the first Tuberculosis drug, neomycin and streptomycin. Waksman’s research started the Golden Age of antibiotic discovery between the 1940s and 1960s. Many of these antibiotics are still used in medical settings, but the emergence of antibacterial resistance has reduced their efficacy

(Hutchings et al., 2019). The emergence of new infectious diseases and pathogens resistant to old antibiotic motivated the development of a new drug derived from bacteria, fungi, and actinomycetes (Ambarwati et al., 2012).

Nowadays, researchers have focused on the study of streptomycetes, which is the group of actinobacteria that produces the greatest number of antibiotics. (Ambarwati et al., 2012). Streptomycetes is the largest actinobacterium in the Streptomycetaceae family. They are widespread in the soil environment, and they play an important role in the organic material turnover (Seipke et al., 2012). They are chemoorganotrophic, Gram-positive filamentous bacteria with a high GC content in their DNA (Baghdadi and Alblady, 2019; Dharmaraj, 2010; Tarkka and Hampp, 2008).

According to the Antibiotic Literature Database (ABL), 45.6% of the 8000 biological antibacterial products are produced by streptomycetes, 21.5% by fungi, 16.9% by other bacteria, and 16% by strains of rare genera of actinomycetes (Lazzarini et al., 2000). Streptomycetes produced around two-thirds of well-known antibiotics and approximately 75% of commercially and clinically useful antibiotics (Khattab et al., 2016).

Thus, this study aims to isolate antibacterial producing streptomycetes from soil. The antibacterial compounds produced by soil isolate will be evaluated, purified, and characterized. The culture condition of streptomycetes that induce the production of antibacterial agents will also be optimized.

1.2 Research objectives

1.2.1 To isolate an antibacterial producing streptomycetes from soil.

1.2.2 To identify the antibacterial producing soil isolates by morphological characteristics and 16S rRNA gene sequencing.

1.2.3 To improve the production yield of antibacterial compounds by optimizing streptomycetes culture conditions.

1.2.4 To evaluate the antibacterial activity of crude extract produced by soil isolate streptomycetes against test pathogens.

1.3 Research hypothesis

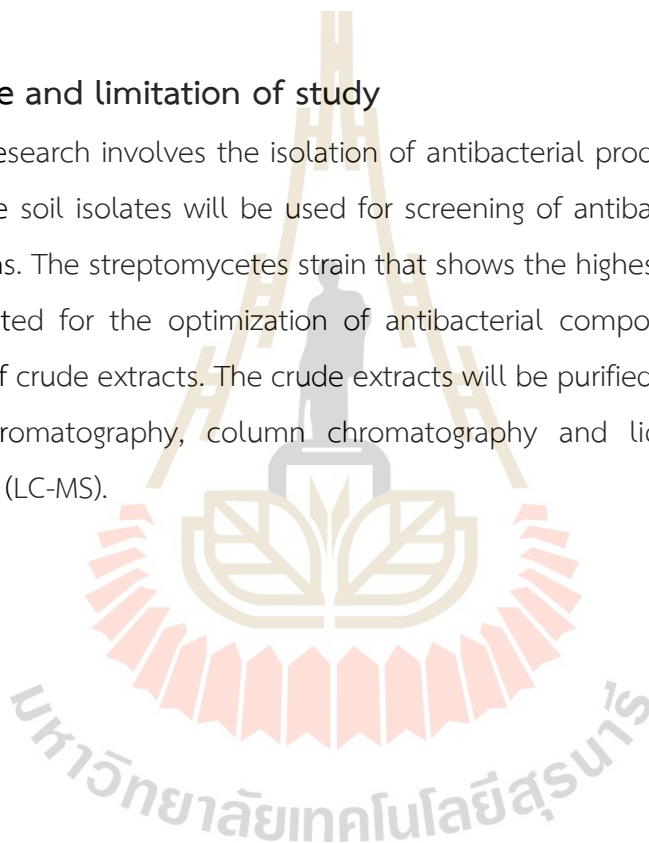
1.3.1 Soil isolate streptomycetes are able to produce antibacterial agents against test pathogens.

1.3.2 The active compounds of soil isolate streptomycetes are able to purify by chromatography techniques.

1.3.3 The purified active compounds of streptomycetes are active against test pathogens.

1.4 Scope and limitation of study

This research involves the isolation of antibacterial producing streptomycetes from soil. The soil isolates will be used for screening of antibacterial activity against test pathogens. The streptomycetes strain that shows the highest antibacterial activity will be selected for the optimization of antibacterial compounds production and preparation of crude extracts. The crude extracts will be purified and characterized by thin-layer chromatography, column chromatography and liquid chromatography spectrometry (LC-MS).



CHAPTER II

LITERATURE REVIEW

2.1 The Streptomyces

Streptomyces is the most abundant group in the Streptomycetaceae family, which is member of the Actinomycetales order and Actinobacteria phylum. They are Gram-positive aerobic filamentous chemoorganotrophic bacteria with a high G+C content in their DNA (De Lima Procopio et al., 2012; Hasani et al., 2014). *Streptomyces* are nonmotile, degrade starch, gelatin, casein, adenine, esculin, hypoxanthine, and L-tyrosine, and reduce nitrates to nitrites (Baghdadi and Alblady, 2019).

Streptomyces are normally found in the sea, plants, and mostly in soil (Cheng et al., 2015). Soil is the most important streptomyces habitat, accounting for 40% of soil microorganisms. The filamentous structure of streptomyces strengthens the soil surface and protects it from wind and rain erosion (Baghdadi and Alblady, 2019). In addition to the common environment, they may be found in extreme habitats such as deserts, deep seas, volcanic conditions, and extremely low cold or cryoenvironments et al., 2019). Moreover, rather than living as free-living bacteria, many streptomyces have adapted to living in symbiosis with plants, insects, fungi, and sponges (Van Der Meij, 2017).

Streptomyces colonies grow slowly and commonly have a soil-like smell because of the synthesis of volatile metabolites and geosmin. (Hasani et al., 2014). Streptomyces cultured on solid media involve two different structures: a substrate or vegetative mycelium and an aerial or reproductive mycelium (Manteca et al., 2005). Substrate mycelium grows on and into the culture medium (Figure 2.1) (Mendez et al., 1985). The important role of substrate mycelium is nutrient absorption via multiple hyphae by tip extension and branching (Seipke et al., 2012). Under the microscope, substrate mycelium is slender, translucent, and more branching than aerial hyphae. However, aerial mycelium is developed from substrate mycelium and grows into the

air (Li et al., 2016). Initially, colonies have a smooth surface, but later, the aerial mycelium develops into a powdery, granular, floccose, or velvety surface (Holt, Krieg, Sneath, Stanley, and Williams, 1994).

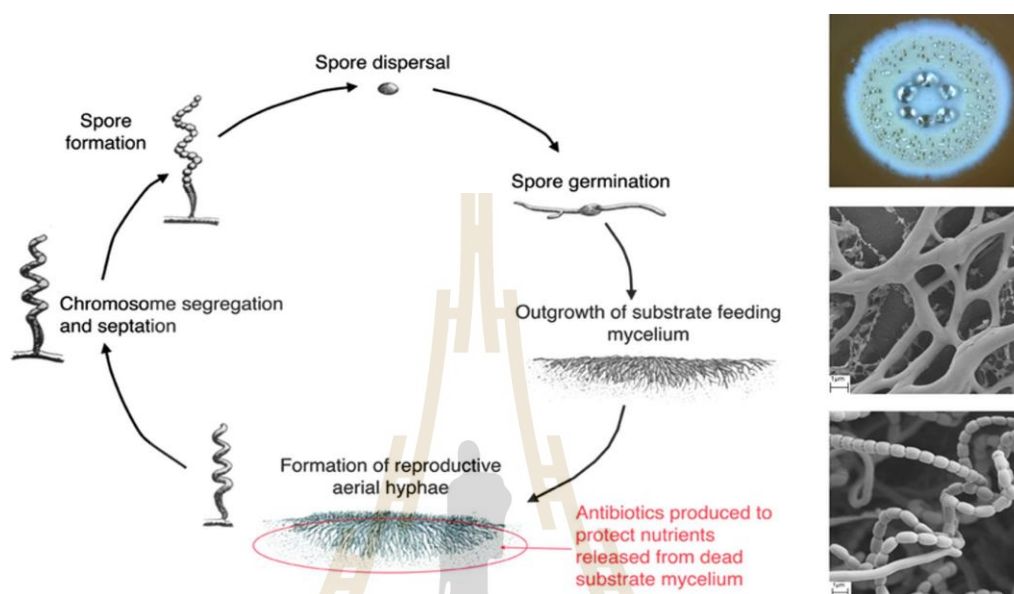


Figure 2.1 The life cycle of streptomycetes (Seipke et al., 2012).

Pigments observed in substrate mycelia include yellow, green, white, red, blue, orange, and others. Some types can develop non-water soluble or water-soluble pigments. Water-soluble pigments can leak into culture media, resulting in a medium and colony of the same color. The non-water-soluble pigment is only found in the colony. The significance of color or the presence of soluble pigments is utilized as a reference to identify new species (Holt et al., 1994; Li et al., 2016).

The natural substances of streptomycetes are important in various industries such as pulp and paper, agriculture, textiles, and waste management industries (Mukhtar et al., 2017). Streptomycetes produce a wide range of primary and secondary substances that can be utilized to fight pathogenic microbes, promote growth, and degrade organic polymers such as chitin and cellulose (Seipke et al., 2012; Van Der Meij et al., 2017; Wanbanjob, 2008). Primary metabolites have a major role in the organism, supporting a physiological function and related to reproduction,

development, and optimal growth (Hasani et al., 2014). Streptomycetes are responsible for half of all secondary metabolites discovered, including two-thirds all discovered antibiotics (Dharmaraj, 2010; Van Der Meij et al., 2017). The ability of Streptomycetes to synthesize bioactive substances with antibacterial, antitumoral, antiviral, and antifungal activities is their most important characteristic (Hasani et al., 2014; Van Der Meij et al., 2017). Secondary metabolites do not have a direct role in normal growth, development, or reproduction, they develop before the formation of aerial hyphae, when sporulation starts, and the rate of growth of bacterial filaments has slowed (Hasani et al., 2014; Tarkka and Hampp, 2008). Furthermore, streptomycetes' secondary metabolites can be produced because of their symbiosis with the plant. The antibiotic protects the plant against infections, whereas plant exudates help streptomycetes to develop (De Lima Procopio et al., 2012).

2.2 Antibiotics

The discovery of antibiotics started in 1928 with Alexander Fleming's discovery of penicillin (Figure 2.2). Penicillin played a key role in treating bacterial diseases in soldiers during World War II. However, the resistance to penicillin rapidly became a serious therapeutic problem (Ventola, 2015).

The history of antibiotics derived from streptomycetes began with the discovery of streptothricin (from *S. griseus*) in 1944. Later, the researchers intensified the search for antibiotics within the streptomycetes (Baghdadi and Alblady, 2019; De Lima Procopio et al., 2012).

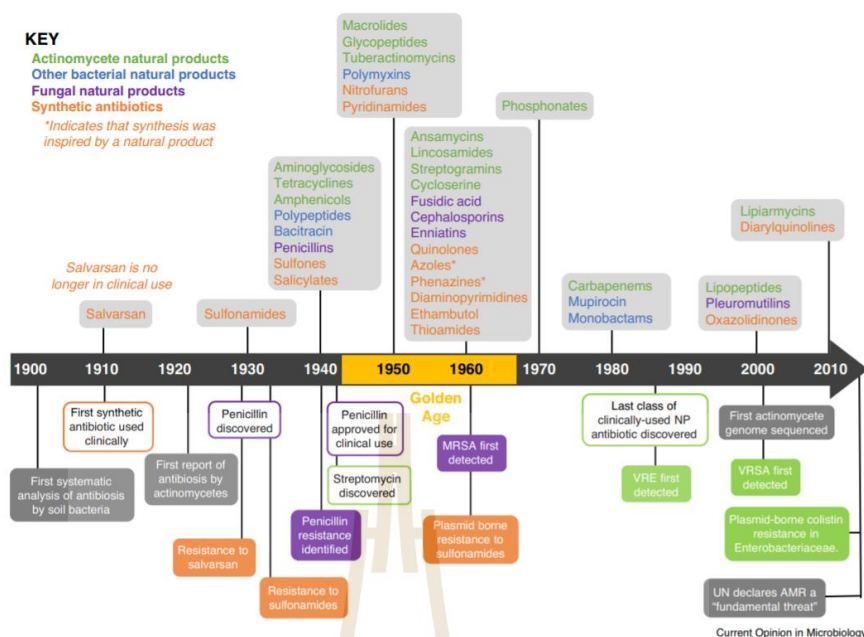


Figure 2.2 Timeline of antibiotic drug (Hutchings et al., 2019).

Since the discovery of penicillin, over 23,000 antibiotics have been reported, the largest number of which are made by bacteria, especially by the Actinomycetaceae family (Katz and Baltz, 2016). About 80 percent of antibiotics are derived from streptomycetes. They are the most well-known and widespread genus in the Actinomycetaceae family, which contains over 600 species (Berdy, 2005; De Lima Procopio et al., 2012; Van Der Meij et al., 2017). Streptomycetes compounds have been found in a wide range of antibiotic families, including macrolides, aminoglycosides, beta-lactams, and tetracyclines (Katz and Baltz, 2016).

The target of action of antibiotics is classified by the interaction of antibiotics and cellular functions in bacterial cells (Figure 2.3). (1) The mechanism of disruption of cell wall synthesis, (2) injury to the cell membrane, (3) inhibition of metabolic reactions, (4) inhibition of protein synthesis and (5) inhibition of DNA synthesis (De Lima Procopio et al., 2012; Kirmusaoğlu et al., 2019).

The main mechanisms of antibiotic resistance are (1) modifications of drug-receptor and location targets, (2) enzymatic inactivation of antibiotics, (3) decreased drug uptake into the cell, and (4) increased active pump system (Hasan and Al-Harmoosh, 2020).

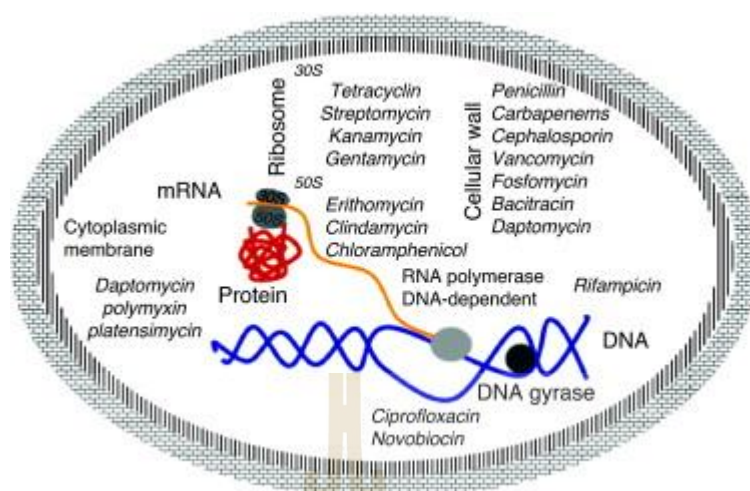


Figure 2.3 The targets and mechanism of action of antibiotics (De Lima Procopio et al., 2012).

2.3 Antibacterial resistance

The use of antibiotics has been related to the emergence and development of antibiotic-resistant bacteria. Drug-sensitive competitors are killed by antibiotics, allowing resistant microorganisms to reproduce and allowing antibiotic resistance genes to transfer from one species to another (Ventola, 2015). In 1946, Alexander Fleming warned that “the public will demand the drug and then will begin an era of abuses”. Unfortunately, Fleming's warning was ignored, and antibiotics are now endangered because of the rapid development of antibiotic resistance and the extremely low discovery of new antibiotics (Bartlett et al., 2013).

Many public health institutions have denoted the rapid evolution of resistant bacteria a “crisis” or “nightmare scenario” with “catastrophic consequences” (Ventola, 2015). The Centers for Disease Control and Prevention (CDC) said in 2013 that humankind has entered the “post-antibiotic age.” Moreover, the World Health Organization (WHO) identified antibiotic resistance as one of the three most serious health issues of the 21st century (Munita and Arias, 2016; Ventola, 2015).

The CDC evaluated antibiotic-resistant diseases based on seven criteria: clinical impact, economic impact, incidence, 10-year prediction of incidence, transmissibility,

availability of effective medications, and preventative hurdles. Each bacteria's threat level was then categorized as “urgent,” “serious,” and “concerning” (Table 2.1) (Ventola, 2015).

Table 2.1 CDC’s antibiotic resistance threats in 2013 (Ventola, 2015).

Threats level	Pathogens
Urgent	<i>Clostridium difficile</i> Carbapenem-resistant Enterobacteriaceae (CRE)
Serious	Drug-resistant <i>Neisseria gonorrhoeae</i> Multidrug-resistant <i>Acinetobacter</i> Drug-resistant <i>Campylobacter</i> Fluconazole-resistant <i>Candida</i> Extended spectrum beta-lactamase-producing Enterobacteriaceae (ESBLs) Vancomycin-resistant Enterococci (VRE) Multidrug-resistant <i>Pseudomonas aeruginosa</i> Drug-resistant nontyphoidal <i>Salmonella</i> Drug-resistant <i>Salmonella Typhimurium</i> Drug-resistant <i>Shigella</i> Methicillin-resistant <i>Staphylococcus aureus</i> (MRSA) Drug-resistant <i>Streptococcus pneumoniae</i> Drug-resistant tuberculosis
Concerning	Vancomycin-resistant <i>Staphylococcus aureus</i> (VRSA) Erythromycin-resistant Group A <i>Streptococcus</i> Clindamycin-resistant Group B <i>Streptococcus</i>

In 2017, the WHO published a list of global priority pathogens (GPP), which included 12 bacteria species with critical, high, or medium antibiotic resistance. Nine of the 12 GPPs were Gram-negative showed in Table 2.2 (Asokan et al., 2019).

Table 2.2 WHO global priority pathogens list in 2017 (Asokan et al., 2019).

Priority category	Pathogens
Critical	<i>Acinetobacter baumannii</i>
	<i>Pseudomonas aeruginosa</i>
	Enterobacteriaceae
High	<i>Enterococcus faecium</i>
	<i>Staphylococcus aureus</i>
	<i>Helicobacter pylori</i>
	<i>Campylobacter</i> spp.
	<i>Salmonella</i> spp.
	<i>Neisseria gonorrhoeae</i>
Medium	<i>Streptococcus pneumoniae</i>
	<i>Haemophilus influenzae</i>
	<i>Shigella</i> spp.

2.4 Situation in Thailand

Thai people are able to easily access antibiotics from private drug stores or pharmacies in Thailand. They can also self-medicate with antibiotics without a prescription (Sommanustweechai et al., 2018). Furthermore, antibiotics may be found in all grocery stores around 20–70%. Tetracycline, penicillin, aminopenicillins, and fluoroquinolones were the most common types of antibiotics found in pharmacies (Pumtong et al., 2020; Siltrakool et al., 2021). The capability to easily access antibiotics from several sources is a serious medical problem that puts humanity at risk of antibacterial resistance (AMR) (Pumtong et al., 2020).

According to the Thai National Statistics Office (NSO), In 2013, households in Thailand spent 0.9% of their income on purchasing self-medication, including antibiotics, and the percentage increased to 1.4% in 2016 (Siltrakool et al., 2021). The previous study showed that self-medication was commonly found among the working-age population at around 88.2% in Thailand's urban regions (Chautrakarn et al., 2021).

Previous studies reported that the significant factors in antibiotic misuse among Thais, particularly those located in rural locations, are lacking knowledge and misunderstandings about antibiotics (Chanvatik et al., 2019; Puntong et al., 2020). And most Thai people are also referring “antibiotics” as “anti-inflammatory drugs” (ya kae ak-seb), a word that has been in use for a long time. Thais believe antibiotics are magical drugs that can cure any disease caused by "inflammation" of various organs and systems in the body. The misconception of these abilities has spread to the treatment of diseases and unusual symptoms found in livestock and agriculture, animals and plants (Puntong et al., 2020).

Antibacterial resistance infections in Thailand result in 3.24 million increased hospitalization days and 38,481 deaths per year. Thailand spends USD 84.6–202.8 million USD per year on antibiotics and has a productivity loss of at least USD 1.3 billion as a result of morbidity and death (Boonyasiri et al., 2014; Siltrakool et al., 2021).

According to Chanvatik et al., identified the self-reported clinical indications for antibiotic usage into three categories. The majority of antibiotics (64.5%) were used to cure symptoms including fever (19.2%), sore throat (16.8%), headache (12.2%), cough (11.3%), and diarrhea (5.2 %). Antibiotics were also used to treat illnesses such as the flu, skin infections, pharyngitis, and unspecified symptoms and disorders (Figure 2.4). Most of the antibiotics (70.3%) were provided by public or private healthcare facilities, while 26.7 % were from pharmacies (Chanvatik et al., 2019). Antibiotics are usually used for bacterial infections, whereas some viral infection diseases might be prescribed by community pharmacists resulting in the misuse of antibiotics. This circumstance is one of the causes that lead to antibacterial resistance infections. (Intahphuak et al., 2021; Siltrakool et al., 2021)

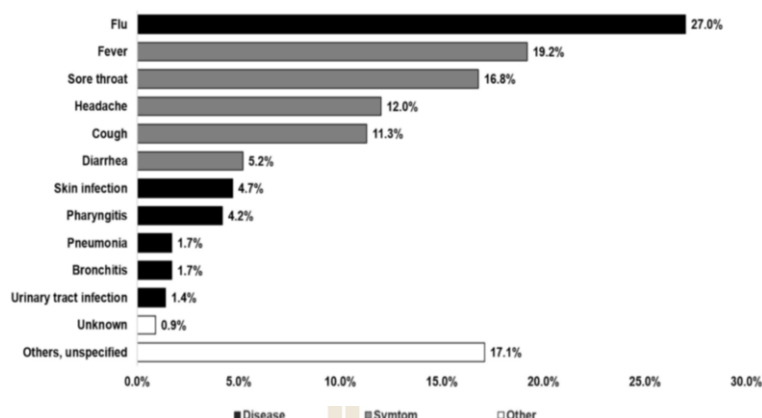


Figure 2.4 Indication of antibiotic use (Chanvatik et al., 2019).

Resistant Gram-negative bacteria cause nosocomial infections, which are a major problem in Thailand. Infections with *Acinetobacter* spp. and *Pseudomonas* spp., for example, are major causes of hospital mortality. The resistant *Escherichia coli* (*E. coli*), *Klebsiella* spp., and *Neisseria gonorrhoeae* (*N. gonorrhoeae*) are major public health concerns. *E. coli*, *Campylobacter* spp., and *Salmonella* spp. are major resistant bacteria in livestock and food production (WHO, 2017).

Moreover, the National Antibacterial Resistance Surveillance, Thailand: NARST, Department of Medical Sciences, reported that the crucial drug-resistant bacteria in Thailand are *Acinetobacter* spp., *P. aeruginosa* and Enterobacteriaceae which are resistant to Carbapenem, were commonly found in the hospital. Extended-spectrum beta-lactamase (ESBL)-producing Enterobacteriaceae, such as ESBL-producing *K. pneumoniae* and ESBL-producing *E. coli* are drug-resistant strains found in humans, animals and the environment (NARST, 2022).

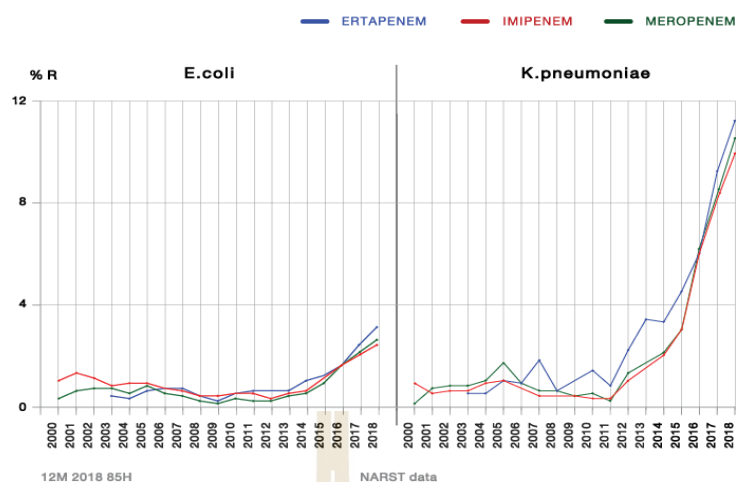


Figure 2.5 Imipenem-resistant *K. pneumoniae* (NARST, 2022).

As shown in figure 2.5, Imipenem-resistant *K. pneumoniae* increased from 0.7% (2000- 2010) to 8.4% (2010-2017), and the increase of this resistant bacteria was expected to continue rapidly in the future (NARST, 2022).

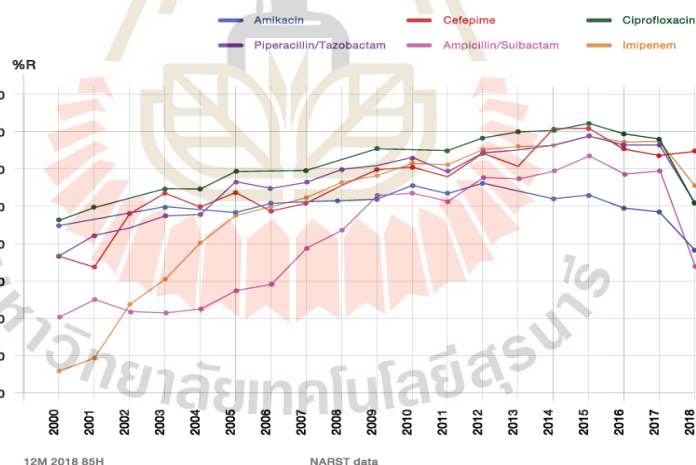


Figure 2.6 Antibacterial resistance rates of *Acinetobacter* spp. by year (51 hospitals) (NARST, 2022).

According to figure 2.6, in 2000 to 2011, the percentage of imipenem resistance in *Acinetobacter* spp., significantly increased from 14.4% to 63.4%, and between 2012 to 2017, the rate of resistance remained high and relatively stable at about 66.3-67.4% (NARST, 2022).

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

3.1.1 Test pathogens

In this study, the test pathogens were obtained from Thailand Institute of Scientific and Technological Research (TISTR) and American Type Culture Collection (ATCC). Test pathogens were *Bacillus cereus* TISTR687, *Staphylococcus aureus* ATCC29213, *Staphylococcus aureus* DMST20654 (MRSA 20654), methicillin-resistant *Staphylococcus aureus* DMST20651 (MRSA 20651), methicillin-resistant *Staphylococcus epidermidis*, *Escherichia coli* 8465, *Proteus mirabilis* TISTR100, *Proteus vulgaris*, *Salmonella* Typhimurium, *Enterobacter aerogenes* TISTR1540, *Pseudomonas aeruginosa* TISTR1287, *Pseudomonas aeruginosa* TISTR781, *Pseudomonas aeruginosa* N90Ps, *Klebsiella pneumoniae* TISTR1617, *Acinetobacter baumannii*, and *Serratia marcescens* TISTR1354.

3.1.2 Culture media

The International Streptomyces Project 2 (ISP2) medium included 10 g malt extract, 4 g yeast extract, and 4 g D-glucose per 1 L. The final pH was adjusted at 7.2.

The Luria-Bertani (LB) medium consisted of 10 g of peptone, 10 g of NaCl and 5 g of yeast extract per 1 L. The final pH was adjusted at 7.0.

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

The Starch Casein (SC) medium contained (per 1 L) 10 g of soluble starch, 0.3 g of casein acid hydrolysate, 2 g of KNO₃, 2 g of NaCl, 2 g of K₂HPO₄, 0.05 g of MgSO₄, 0.02 g of CaCO₃ and 0.01 g of FeSO₄. The final pH was adjusted to 7.0.

The Yeast Extract Glucose (YEG) medium contained (per 1 L) 10 g of D-glucose, 1 g of yeast extract, 0.5 g of KNO₃ and 0.1 g of K₂HPO₄. The final pH was adjusted to 7.0.

All solid media were added 15 g of agar per 1 L of medium. Sterilization of all media was prepared by autoclaving at 121°C, 15 p.s.i. for 15 min.

3.1.3 Buffers and solutions

3.1.3.1 Buffer for agarose gel electrophoresis

- Lysis buffer composed of 400 mM Tris-HCl pH 8.0, 60 mM EDTA pH 8.0, 150 mM NaCl and 1% w/v of sodium dodecyl sulfate.
- 10X TBE buffer contained (per 1 L) 108 g of Tris-HCl, 55 g of boric acid and 40 ml of 0.5 M EDTA and pH 8.0

3.1.4 Primers used for PCR amplification

The sequences and names of the oligonucleotides are listed in Table 3.1.

Table 3.1 List of primers used in this study.

Primers	Primer's sequences (5'-3')	References
27F	AGAGTTTGATCCTGGCTCAG	(Lane, 1991)
1525R	AAGGAGGTGTCCARCC	(Lane, 1991)

3.1.5 Research kits

FavorPrep™ GEL/PCR Purification Mini kit purchased from Favogen, Taiwan.

3.1.6 Miscellaneous material

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

One hundred bp DNA Ladder. The marker was purchased from HiMedia Laboratories, India.

3.1.7 Equipments

The instruments used in this investigation are all listed in Table 3.2.

Table 3.2 List of instruments used in this study.

Name	Source
Autoclave	Tomy, USA
Centrifuge machine	Hettich, Germany
Fume hood	S.K. POWERABLE, Thailand
Hot air oven	Memmert, Schwabach
Incubator shaker	N-biotek, Germany
Laminar flow	Esco, Singapore
pH meter	METTLER-TOLEDO, USA
Rotary evaporator	Buchi, Switzerland
Spectrophotometer	PG Instrument, UK
Thermal cycle	Thermo Fisher Scientific, US
Vortex mixer	FINEPCR, Korea

3.2 Methods

3.2.1 Sample collection

The study location was collected at botanical garden soil in Suranaree university of technology. The soil samples were selected at random and placed in a sterile bag.

3.2.2 Screening and isolation of streptomycetes

One Gram of the soil samples was suspended in 99 ml of sterile water in 250 ml Erlenmeyer flask. The suspension of soil samples was serially diluted to 10^{-3} , 10^{-4} and 10^{-5} using sterile water. Diluted samples were inoculated on an agar plate from the International Streptomyces Project no.2 (ISP2). All plates were incubated for 5-10 days at 37 °C or until the bacterial colonies appeared. The streptomycetes colony was selected and purified by a new ISP2 agar.

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

The antibacterial activity of soil isolates was tested using the perpendicular streak technique. All strains were cultured on the corner side of Muller Hilton agar (MHA). The plates were incubated for 5 days at 37 °C to allow the streptomycetes to produce antibiotic compounds and release into an agar media. The plates were inoculated with test pathogens, including opportunistic pathogens and resistant strains, by streaking perpendicular to the soil isolate colony. After 24 h of incubation, the zone of inhibition against test pathogens of each isolate was detected. (Figure 3.1).

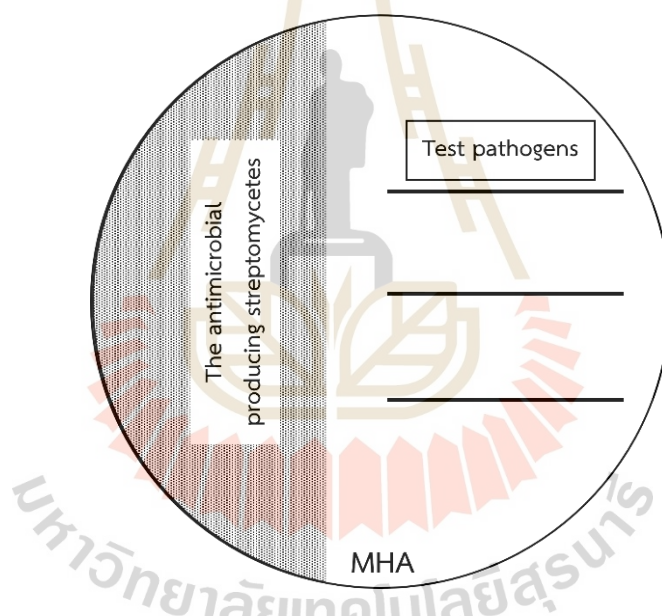


Figure 3.1 The perpendicular-streak method.

3.2.4 Identification of antibacterial-producing soil isolate by 16S rRNA gene sequencing

3.2.4.1 Cultural and morphological characteristics

The cultural morphology of antibacterial-producing bacterial soil isolate was determined on ISP2 medium for 14 days. The color of aerial mycelium, substrate mycelium, reverse color of colony, diffusible pigments, and texture of colony were observed and recorded.

3.2.4.2 Preparation of DNA template

The genomic DNA of soil isolates were grown on ISP2 medium for 3 days. The cell pellets were obtained after centrifugation at 13,000 rpm for 5 min. DNA extraction started with placing the cell pellets in a mortar with 500 μ l of lysis buffer. The suspensions were transferred to microcentrifuge tube and added 165 μ l of 5 M NaCl. The suspensions were mixed and centrifuged for 10 min at 13,000 rpm. The supernatants were transferred to a new tube and added with 800 μ l of chloroform: isoamyl alcohol (1:1). After mixing the suspensions until the solution became milky, the mixture was centrifuged for 10 min at 13,000 rpm. The aqueous layer was removed and added with an equal volume of chloroform. The mixture was centrifuged for 5 min at 13,000 rpm. The genomic DNA was precipitated with two volumes of absolute alcohol and centrifuged for 5 min at 10,000 rpm and washed with 800 μ l of 70% cold ethanol. The genomic DNA was dissolved in nuclease-free water and used as a DNA template for 16S rDNA amplification.

3.2.4.3 Amplification of the 16S rDNA gene by PCR

The genomic DNA of soil isolates were used as the template for PCR amplification of 16S rDNA gene using universal primer 27F and 1525R (Table 3.1). The reaction was carried out in a thermal cycler under the following conditions: 5 min of initial denaturation at 95 °C, denaturation for 60 s at 95 °C, annealing for 30 s at 55 °C, extension for 90 s at 72 °C, and final extension for 7 min at 72 °C (Figure 3.2). The amplification products were examined with 0.7% agarose gel and purified using FavorPrep™ GEL/PCR Purification Mini kit.

Table 3.3 Mixture of PCR reactions.

Reaction mixture	Volume (μ l)
Forward primer (10 μ M)	1
Reverse primer (10 μ M)	1
Green master mixed	12.5
Genomic DNA template	5
Nuclease free water	5.5
Final volume	25

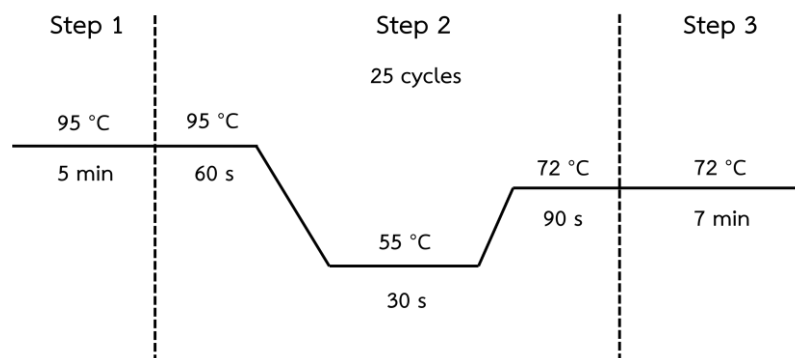


Figure 3.2 Conditions for PCR amplification of the 16S rDNA gene.

3.2.4.4 The DNA sequencing

The purified DNA was used for sequencing analysis at ACTG Co., Ltd, Thailand. The DNA sequencing containing 16S rDNA gene was compared against the EzBiocloud database using BLAST proGram.

3.2.5 Phylogenetic tree construction

The 16S rDNA gene sequences from soil isolates were matched to known sequences in the EzBiocloud database. The sequences using CLUSTAL W were aligned with closely related strains. The constructed phylogenetic tree was performed using MEGA software version X, and the confidence level was evaluated using bootstrap analysis (1,000 replications).

3.2.6 Optimization of antibacterial compounds production

3.2.6.1 Effect of culture medium

The antibacterial producing streptomycetes were grown on ISP2 medium for 3 days. Four milliliters of 3 days culture was transferred to 100 ml Erlenmeyer flasks containing 36 ml of 4 different culture medium which were ISP2, SC, MH, and LB broth. The flasks were incubated at 30 °C for 5 days. Cell suspensions were centrifuged at 13,000 rpm for separated cell pellets and cell-free supernatants. The antibacterial activity of cell supernatants was determined using the agar-well diffusion technique. The diameter (mm) of the inhibition zone was measured and recorded.

3.2.6.2 Effect of Temperature

The antibacterial producing streptomycetes were grown on ISP2 medium for 3 days. Four milliliters of cell inoculated was transferred to 100 ml Erlenmeyer flasks containing 36 ml of different temperature between 30 °C and 37 °C. Cell suspensions were centrifuged at 13,000 rpm for separated cell pellets and cell-free supernatants. The antibacterial activity of cell supernatants was determined using the agar-well diffusion technique. The diameter (mm) of the inhibition zone was measured and recorded.

3.2.6.3 Optimum incubation period

The antibacterial producing streptomycetes were grown on ISP2 medium for 3 days. After incubation, four milliliters of cell inoculated was transferred to 100 ml Erlenmeyer flasks containing 36 ml of SC medium and incubated at 30 °C. The cell inoculated was collected every 24 h for 10 days. Cell suspensions were centrifuged for separated cell-free supernatants and cell pellets at 13,000 rpm. The antibacterial activity of cell supernatants was determined using the agar-well diffusion technique. The diameter (mm) of the inhibition zone was measured and recorded.

3.2.6.4 Agar-well diffusion method

The antibacterial activity of cell-free supernatants was determined using the agar-well diffusion technique. The test pathogens were prepared to the turbidity standard of 0.5 McFarland. MH medium was swabbed by the cotton swab of test pathogens. A 6 mm diameter hole was punched with a sterilized cork-borer and the agar plug was removed by using a sterile toothpick. Fifty microliters of cell-free supernatants were loaded into the well. The plates were stood for 1 h to allow the supernatants to diffuse into the agar medium before incubated at 37 °C for 24 h. After incubation, the diameter (mm) of the inhibition zone was measured and recorded.

3.2.7 Crude extract preparation

The crude extract was prepared by submerged state fermentation. A soil isolate was grown on suitable conditions. The fermented broth was centrifuged at 9000 rpm for 5 min after incubation. The fermented broth was filtrated through Whatman No.1 filter paper (Whatman™, GE Healthcare, UK) to remove bacterial cells. The cell-

free supernatant containing antibacterial compounds was extracted with ethyl acetate in the ratio of 1:1 (v/v) and shaken for 1 h at 250 rpm. The organic solvent phase was collected using separatory funnel and the organic solvent was evaporated until dry under reduced pressure. The crude extract was used for antibacterial activity test. Cell dry weight was used for calculating the yield of crude compounds.

3.2.8 Purification of antibacterial compounds

3.2.8.1 Purification of bioactive compound by thin-layer chromatography

A mixture compounds were separated and seen using thin layer chromatography (TLC). Crude compound was spotted on the TLC plates. The spot was left to dry. The TLC plates were placed in a developing chamber containing organic solvent using chloroform: methanol (9.2: 0.8) as a mobile phase system. The TLC plate was removed when the solvent reached 0.5 cm from the top of the plate. After that, the TLC plate was left to dry before being visualized under UV light at 254 nm. The bands of chromatograms retention factor R_f value were calculated. The TLC plates were tested for antibacterial activity using contact bioautography to determine bioactive metabolites among different bands of silica plate. The bioactive bands were recovered for further study.

Column chromatography is a technique for purifying individual chemicals in mixtures of compounds. Silica gel (60-120 mesh) slurry was packed into a glass column. After that, the crude compound was subjected to column chromatography using silica gel as a stationary phase and pre-eluted with n-hexane followed by step-wise solvent with mixtures of chloroform: methanol. All purified fractions were collected and observed by using TLC. The purified bioactive compound was evaluated for its antibacterial activities by contact bioautography. The fractions showing antibacterial activity against test pathogen were collected for future study.

3.2.8.2 Detection of bioactive compound by contact bioautography

The antibacterial activity of bioactive compounds was determined by the contact bioautography method. The TLC plate was sterile by exposure under UV for 30 min and placed on the agar medium seeded with test pathogens (0.5 McFarland). In order to allow the substances to diffuse into the agar

medium, the TLC plate was left for 30 min. Then, the TLC plate was removed from the agar plates. The agar plates were incubated at 37 °C for 24 h. After incubation, the inhibition zone was observed around the band that contains bioactive compounds (Figure 7.2).

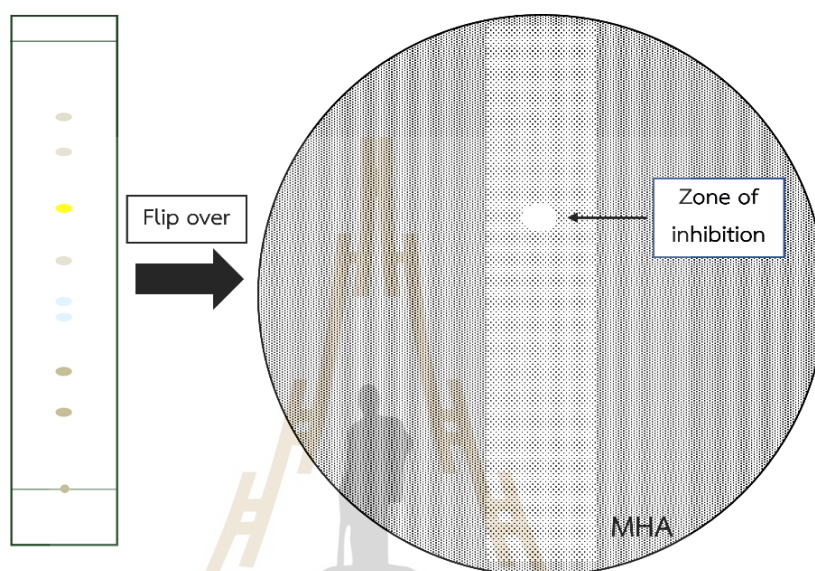


Figure 3.3 Contact bioautography on agar.

3.2.9 Characterization of antibacterial compounds

Liquid chromatography-mass spectrometry (LC-MS)

The mass spectrum of the antibacterial compounds was assessed using liquid chromatography and mass spectrometry (LC-MS). The antibacterial compounds solution was subjected to the Q-TOF spectroscopy. The mass spectrometer was operated in negative and positive full scan modes. The peak was detected and examined by comparing the mass spectra to the mass bank database.

CHAPTER IIV

RESULTS

4.1 Isolation of streptomycetes

The soil samples were taken from botanical garden at Suranaree University of Technology, Nakhon Ratchasima (14.8712° N, 102.0218° E). Soil samples were aseptically transferred to the laboratory in sterile bags.



Figure 4.1 Map of the sampling site. Botanical garden is situated in Suranaree University of Technology, Nakhon Ratchasima. The latitude of Botanical garden is 14.8712 and longitude is 102.0218.

The streptomycetes strains were isolated based on their unique colony morphology on ISP2 agar. They were screened and purified after 5-7 days of incubation. A total of 44 strains were given the name WPN1-WPN44. Most of the strains showed morphological characteristics such as a powdery to winkle texture and a white-colored

colony and some colonies produced diffusible pigments. Some of the morphological appearance of these isolates on ISP2 medium are shown in Figure 4.2

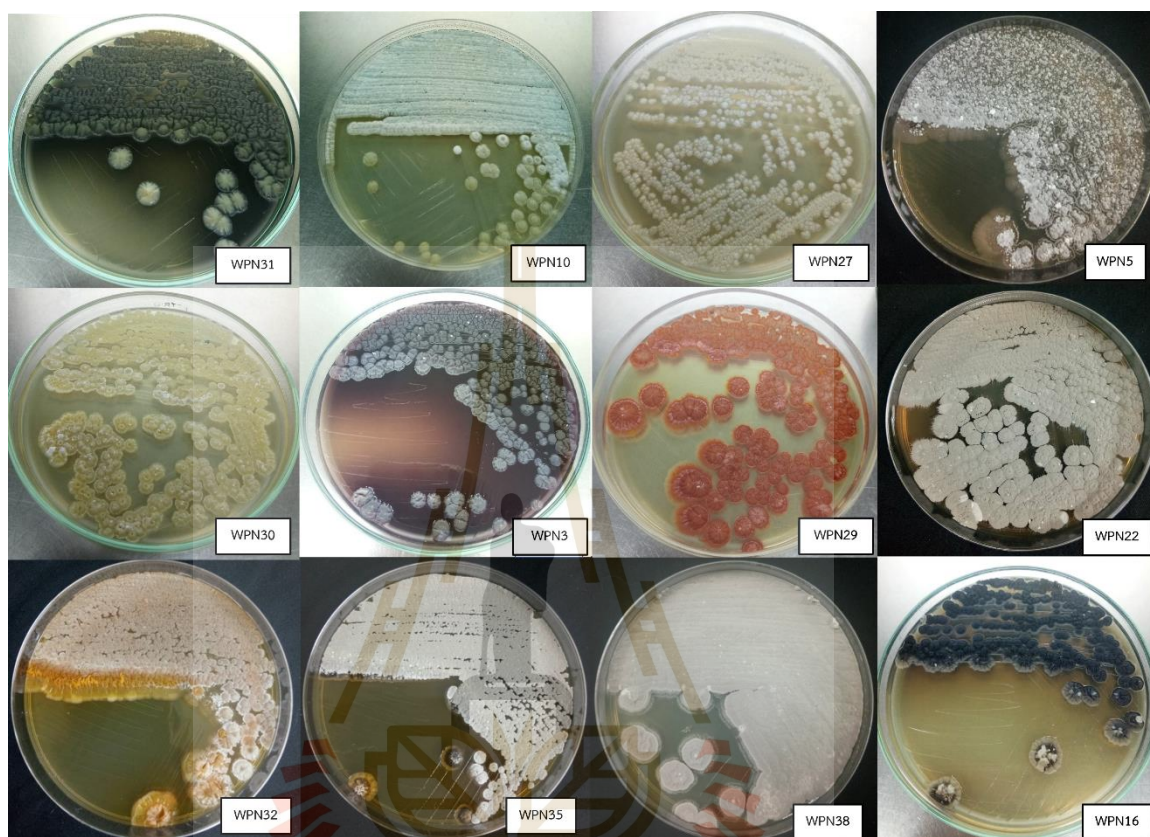


Figure 4.2 Morphological characteristics of streptomycetes strains in ISP2 agar

4.2 Primary screening of antibacterial producing soil isolates

The perpendicular streak method was used for the primary screening of antibacterial producing soil isolates using MH medium. (Figure 4.1).

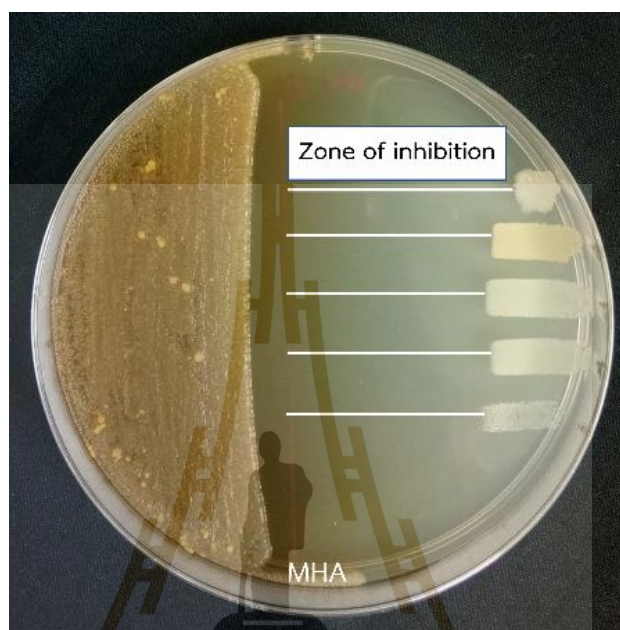


Figure 4.3 Primary screening using perpendicular streak method for antibacterial activity of the soil isolate.

A total of 44 soil isolates were tested for their ability to produce antibacterial metabolites against *Bacillus cereus* TISTR687, *Staphylococcus aureus* ATCC29213, *Staphylococcus aureus* DMST20654 (MRSA 20654), methicillin-resistant *Staphylococcus aureus* DMST20651 (MRSA 20651), methicillin-resistant *Staphylococcus epidermidis* (MRSE), *Escherichia coli* 8465, *Proteus mirabilis* TISTR100, *Proteus vulgaris*, *Salmonella* Typhimurium, *Enterobacter aerogenes* TISTR1540, *Pseudomonas aeruginosa* TISTR1287, *Pseudomonas aeruginosa* TISTR781, *Pseudomonas aeruginosa* N90Ps, *Klebsiella pneumoniae* TISTR1617, *Acinetobacter baumannii*, and *Serratia marcescens* TISTR1354. Out of 44 samples, 11% of isolates exhibited antimicrobial activity against Gram-positive and Gram-negative bacteria. The results are shown in Table 4.1.

Table 4.1 Primary antibacterial activity screening of soil isolates.

Test pathogens	Zone of inhibition (mm)				
	WPN5	WPN22	WPN32	WPN35	WPN38
<i>B. cereus</i>	5±0	11±2	29±1	35±3	22±2
<i>S. aureus</i>	10±1	16±1	21±1	30±2	17±0
MRSA 20651	11±1	16±1	22±2	30±2	13±1
MRSA 20654	12±1	18±2	21±1	31±1	13±0
MRSE	11±0	15±1	18±1	29±1	19±0
<i>E. coli</i>	-	-	-	-	9±0
<i>P. mirabilis</i>	-	-	-	-	14±2
<i>P. Vulgaris</i>	-	-	-	-	14±2
<i>S. Typhimurium</i>	-	-	-	-	9±0
<i>E. aerogenes</i>	-	-	-	-	11±0
<i>K. pneumoniae</i> 1617	-	-	-	-	12±0
<i>A. baumannii</i>	-	-	-	-	11±1
<i>S. marcescense</i>	-	-	-	-	8±1

-: no activity

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

Among them, WPN5, WPN22, WPN32 and WPN35 showed narrow spectrum of antibacterial activity against only Gram-positive bacteria, *B. cereus*, *S. aureus*, MRSA 20651 MRSA 20654, and MRSE.

On the other hand, WPN38 exhibited broad antibacterial spectrum against both Gram-positive and Gram-negative bacteria. WPN38 was active against *B. cereus*, *S. aureus*, MRSA 20651 MRSA 20654, MRSE, *E. coli*, *P. mirabilis*, *P. vulgaris*, *S. typhimurium*, *E. aerogenes*, *K. pneumoniae*, *A. baumannii*, and *S. marcescens*.

4.3 Identification of antibacterial producing soil isolates

The antibacterial-producing soil isolates were confirmed by their morphological and phylogenetic characteristics.

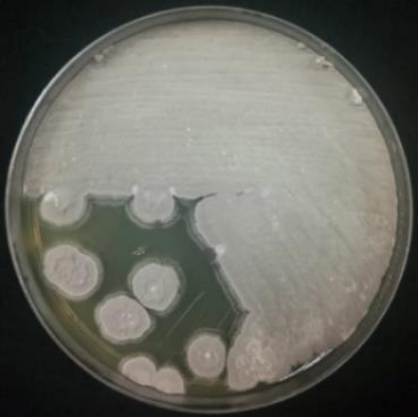
Table 4.2 shows the cultural and morphological characteristics of 5 antibacterial-producing soil isolates. The development of microbial growth, colony, aerial mycelium, and pigment production were observed after 14 days of growth on ISP2 medium. Five isolates were gray, white, and yellow of mycelium and colony pigmented. Strains WPN32 and WPN38 produced yellow-colored diffusible pigment on ISP2 agar. The results are shown in Table 4.2.



Table 4.2 Colony morphology of five antibacterial-producing soil isolates.

Characteristics	Isolates No.		
	WPN5	WPN22	WPN32
Macroscopic views			
Texture of colony	Powdery and smooth surface	Powdery and wrinkle surface	Hard and wrinkle surface
Colony color	White	White	Yellow
Aerial mycelium color	Grey and white	Grey	White
Reverse color of colony	Yellow	Yellow	Yellow
Diffusible pigment	-	-	Yellow

Table 4.2 Colony morphology of five antibacterial-producing soil isolates (Continued).

Characteristics	Isolates No.	
	WPN35	WPN38
Macroscopic views		
Texture of colony	Hard and wrinkle surface	Powdery and wrinkle surface
Colony color	Yellow	White
Aerial mycelium color	Grey	Grey
Reverse color of colony	Yellow	Yellow
Diffusile pigment	Yellow	-

The antibacterial producing streptomycetes isolates were identified at the species level using 16S rRNA gene sequencing. To amplify the 16S rRNA, universal primers were used, and the PCR products were approximately 1,500 bp (Figure 4.4). All sequences were compared with nucleotide sequences of *Streptomyces* species from the EzBioCloud database (<https://www.ezbiocloud.net/>).

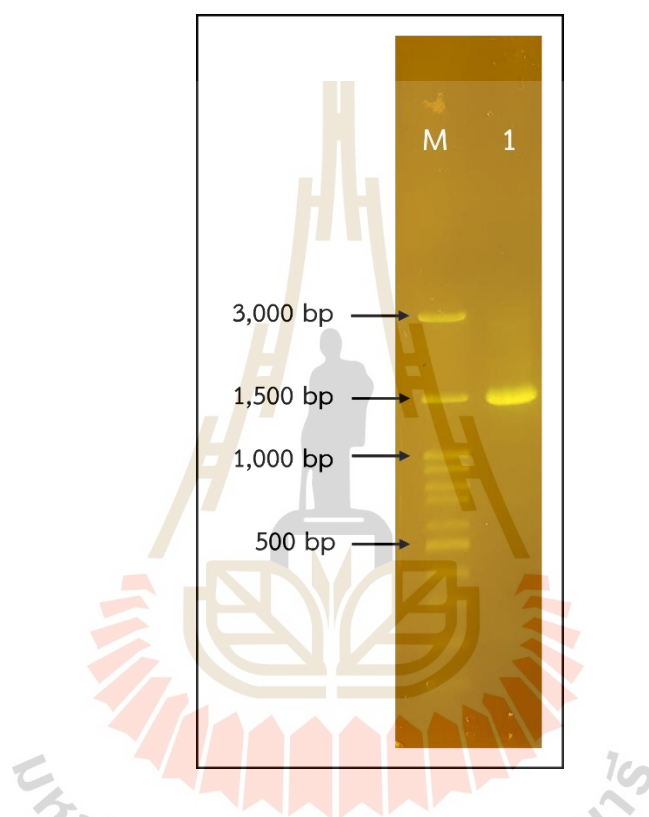


Figure 4.4 Agarose gel electrophoresis (0.7%) of PCR amplification product of 16S rRNA gene. Lane M: 100 bp DNA ladder, Lane 1: PCR product of WPN32.

The result revealed that all selected streptomycete isolates belonged to the genus *Streptomyces*. WPN5 and WPN22 shared 100% similarity to *S. pseudogriseolus*. WPN32 showed the highest similarity of 99.45% to *S. cyaneochromogenes* with an overlapping of 1462 bp. The sequences of WPN35 and WPN38 were closest to *S. antibioticus* and *S. rochei*, respectively. The result of closely related strains is summarized in Table 4.3.

Table 4.3 Closely relative strains of antibacterial producing streptomycetes from EzBioCloud database.

Isolates	Length (bp)	Identification (nearest match)	Top-hit strain	Similarity (%)
WPN5	1446	<i>Streptomyces pseudogriseolus</i>	NRRL B-3288(T)	100
WPN22	1448	<i>Streptomyces pseudogriseolus</i>	NRRL B-3288(T)	100
WPN32	1462	<i>Streptomyces cyaneochromogenes</i>	MK-45(T)	99.45
WPN35	1456	<i>Streptomyces antibioticus</i>	NBRC 12838(T)	100
WPN38	1445	<i>Streptomyces rochei</i>	NRRL B-2410(T)	100

Furthermore, the phylogenetic tree between soil isolates and reference *Streptomyces* was constructed with neighbor-joining method. The phylogenetic analysis of WPN5, WPN22, WPN32, WPN35 and WPN38 isolates showed a distant relationship with reference species from EzBioCloud database. The strains WPN5, WPN22, WPN35 and WPN38 formed a monophyletic clade with their closest type strains. On the other hand, strain WPN32 formed a monophyletic clade with *Streptomyces aquilus* at bootstrap value of 96% and out of clade with closet strains (Figure 4.5).

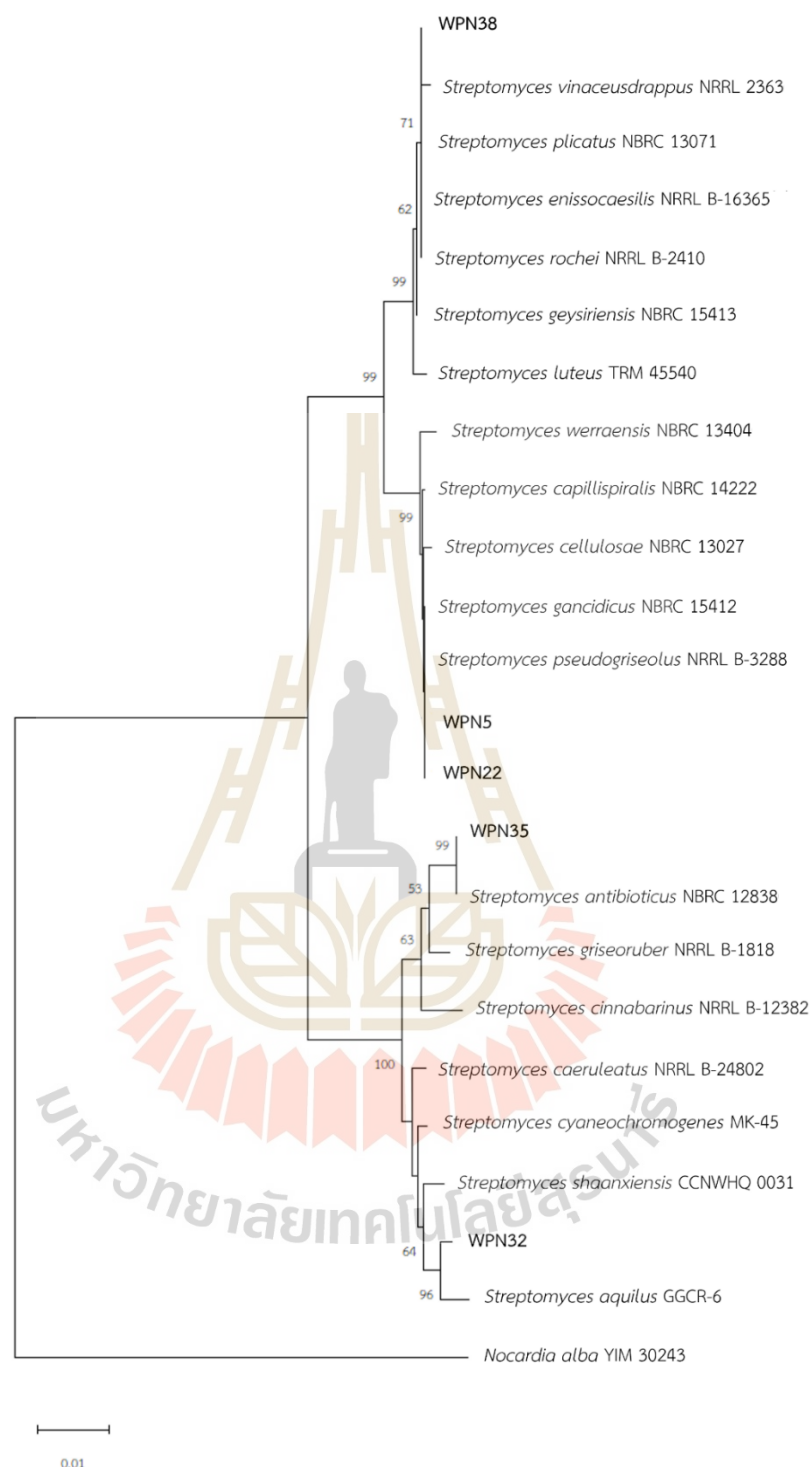
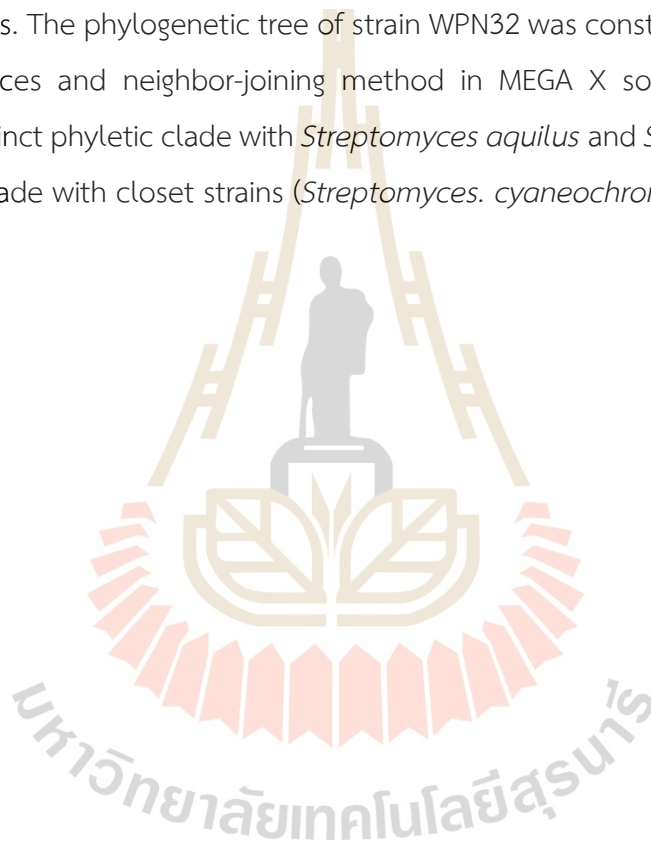


Figure 4.5 Phylogenetic tree using neighbor-joining method based on 16S rRNA gene sequences of strains WPN5, WPN22, WPN32, WPN35 and WPN38. *Nocardia alba* YIM 30243 was used as an outgroup. The bootstrap values show the number on the nodes (1000 replications), only values more than 50% are shown. The scale bar indicated 0.10 nucleotide substitution per nucleotide.

Strains WPN5, WPN22, WPN32, WPN35 and WPN38 were identified to be a genus *Streptomyces* based on cultural, morphological, and phylogenetic characteristics.

Since WPN32 showed a narrow spectrum against Gram-positive bacteria and the 16S rRNA sequences showed the highest similarity to *Streptomyces cyaneochromogenes*. The species *S. cyaneochromogenes*, was first identified and reported in 2019 (Tang et al., 2019). To date, the antibacterial compounds of *S. cyaneochromogenes* remain uninvestigated. Therefore, strain WPN32 was chosen for further studies. The phylogenetic tree of strain WPN32 was constructed using 16S rRNA gene sequences and neighbor-joining method in MEGA X software. Strain WPN32 formed a distinct phyletic clade with *Streptomyces aquilus* and *Streptomyces cyaneus* and out of clade with closet strains (*Streptomyces. cyaneochromogenes*) (Figure 4.6).



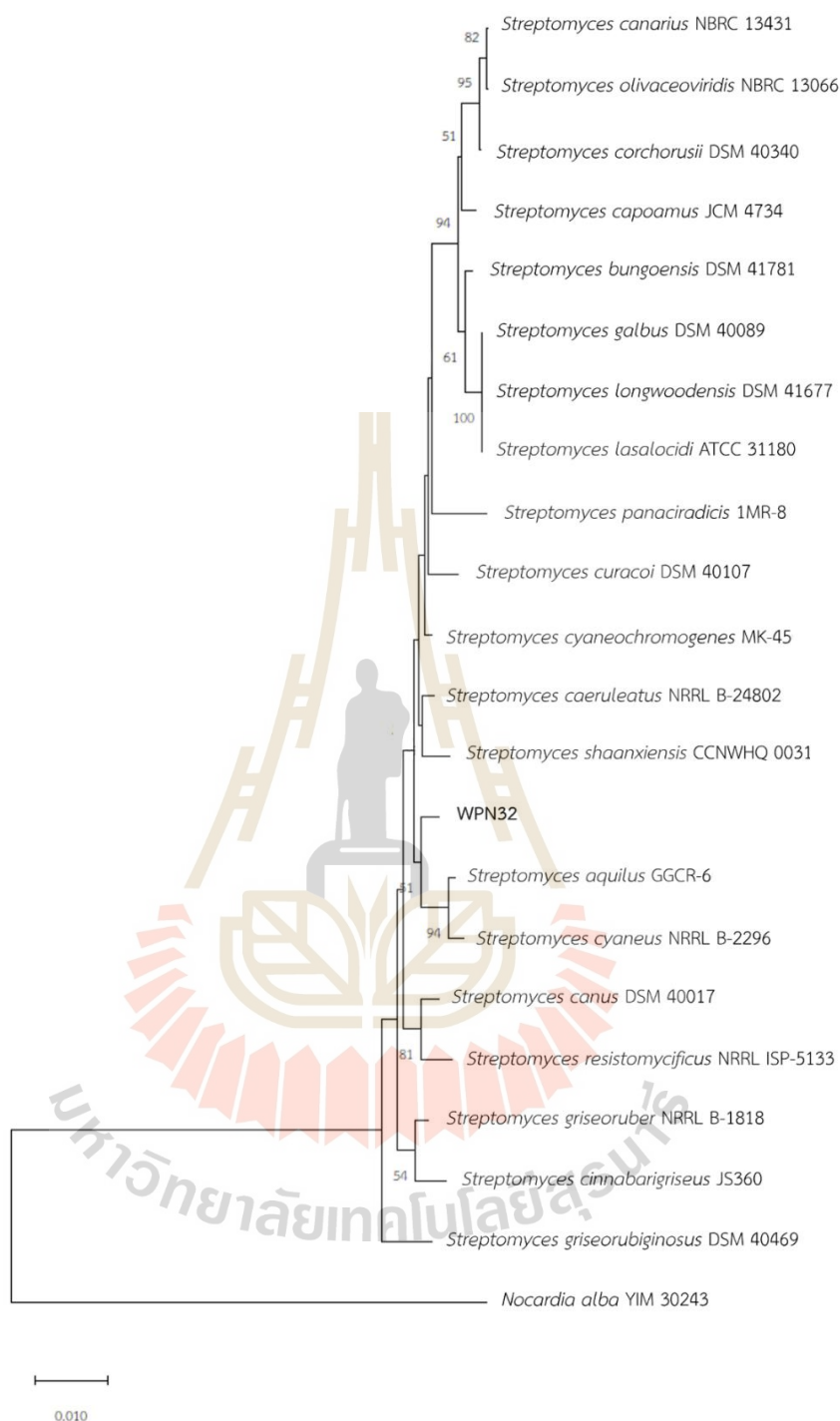


Figure 4.6 Phylogenetic tree using neighbor-joining method based on 16S rRNA gene sequences of strain WPN32. *Nocardia alba* YIM 30243 was used as an outgroup. The bootstrap values show the number on the nodes (1000 replications); only values more than 50% are shown. The scale bar indicated 0.01 nucleotide substitution per nucleotide.

4.4 Optimization of antibacterial compounds production

4.4.1 Effect of culture media

The examination of antibacterial compound productions were determined at different media, temperatures, and incubation periods. In this study, the effect of culture media on the growth of WPN32 was investigated on four different media, MH, LB, SC and ISP2 broth. The supernatant containing antibacterial compounds was collected and evaluated their antibacterial activity using agar well diffusion method (Figure 4.7).

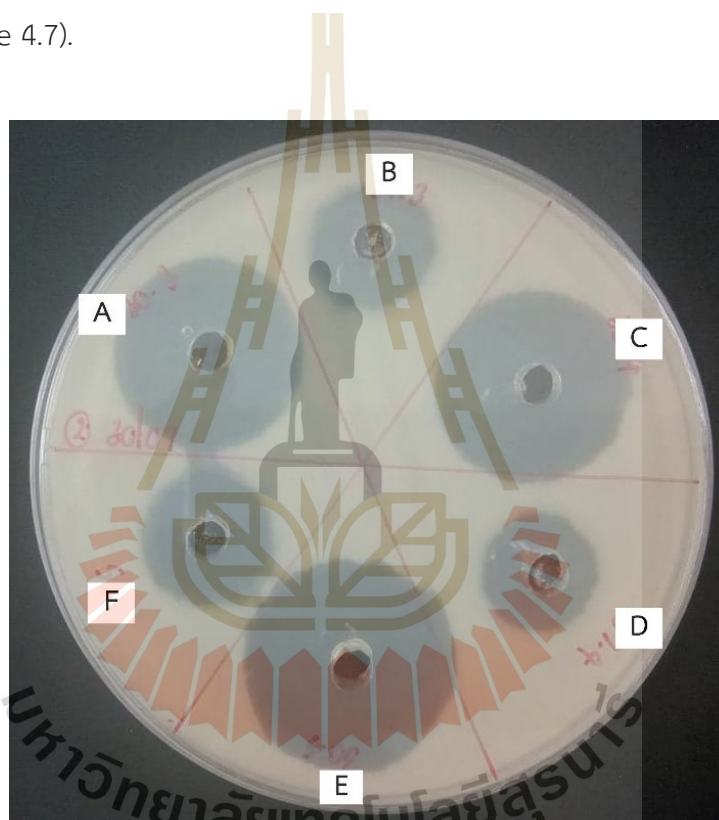


Figure 4.7 The antibacterial activity against test pathogens by using agar-well diffusion method. The inhibition zone of A: 3 days at 37 °C, B: 3 days at 30 °C, C: 4 days at 37 °C, D: 4 days at 30 °C, E: 5 days at 37 °C, F: 5 days at 30 °C of incubation.

The results of Figure 4.8 revealed that the highest antibacterial activity against test microorganisms of WPN32 was observed on SC broth (cell dry weight 1.73 mg/ml). The Maximum activity was measured against *B. cereus* (23.3 mm), followed by MRSA 20651 and MRSA 20654 (21 mm), *S. aureus* (19.7 mm), and MRSE (19.3 mm)

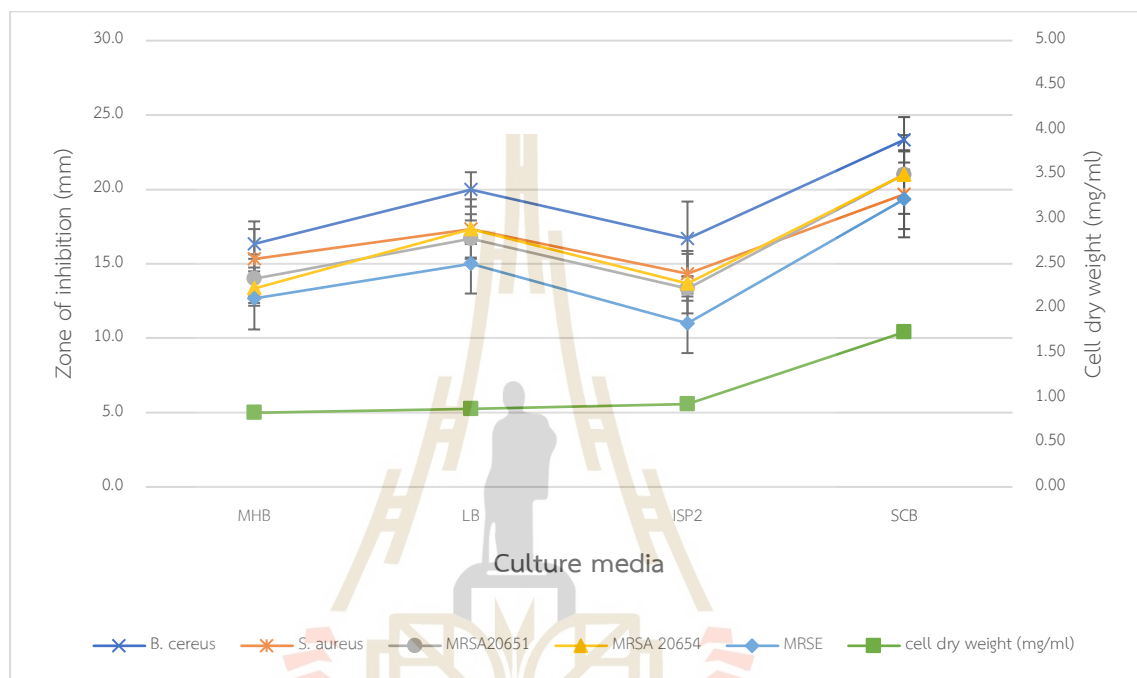


Figure 4.8 The effect of culture medium of WPN32 cultured with ISP2, LB, MH and SC medium and cell dry weight.

4.4.2 Effect of temperature

The fermented broth of WPN32 cultured at 30 °C and 37 °C were collected and tested for antibacterial activity. The highest antibacterial activity was obtained at 30 °C (cell dry weight 2.16 mg/ml) (Figure 4.9). The temperature increases to 37 °C showed a decrease in antibacterial activity and cell dry weight (1.79 mg/ml).

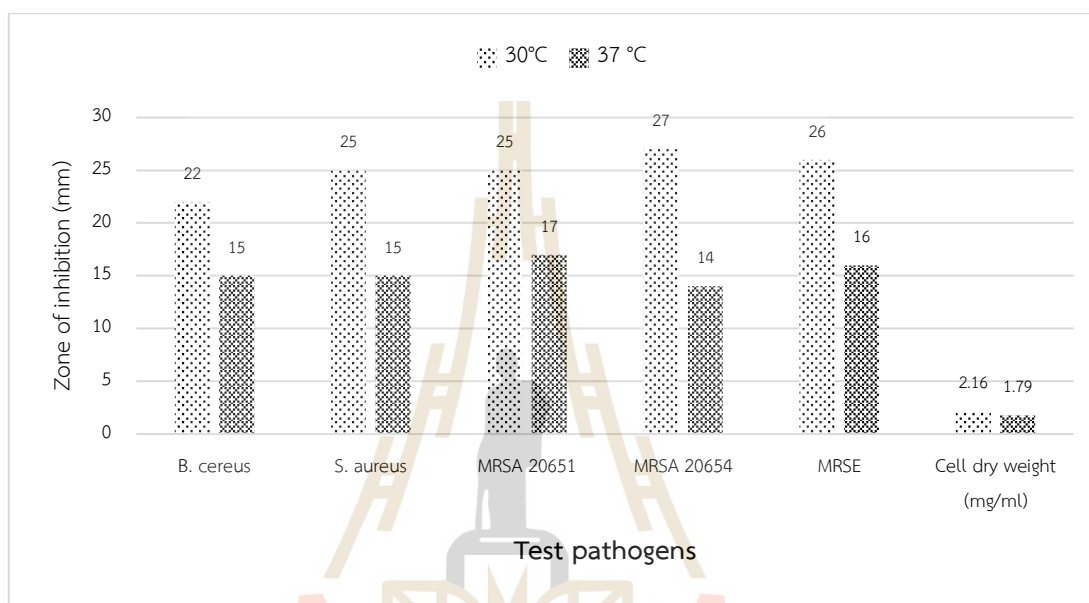


Figure 4.9 The effect of temperature of WPN32 cultured with SC medium and incubated at 30 °C and 37 °C.

4.4.3 Optimum incubation period

Strain WPN32 was cultured in SC medium at 30 °C. The fermented broth was collected every 24 h for 10 days. The agar-well diffusion method was used to evaluate the antibacterial activity of WPN32 fermented broth. The antibacterial activity of WPN32 against *B. cereus*, MRSA 20651, and MRSA 20654 was observed from day 1 of incubation, while *S. aureus* and MRSE showed from day 2 of incubation (Table 4.5). The activity against *B. cereus*, MRSA 20651, MRSA 20654 and MRSE were stable from day 2 to day 10, while *S. aureus* was unchanged from day 3 to day 10.

Table 4.4 Effect of incubation period on antibacterial activity of WPN32.

Days	Zone of inhibition				
	<i>B. cereus</i>	<i>S. aureus</i>	MRSA 20651	MRSA 20654	MRSE
1	15.3±0	0	16.7±1.2	16.3±2.1	0
2	21.5±1.2	19±3	20.3±2.5	20.3±2.4	19±1
3	22.5±1.2	19±3	20.3±2.1	20.7±1.9	20±2
4	22±1.9	18±2	19.7±1.9	21.3±3.1	18.5±1.5
5	23.5±1.4	21±2	20.7±2.5	21±2.2	21±1
6	22.5±1.2	19±4	20.7±2.9	21.3±2.5	20±3
7	23.5±1.2	20.5±2.5	21.3±1.9	22.3±1.7	21±1
8	24.5±1.4	20.5±0.5	20.7±0.5	22.3±1.2	19.5±1.5
9	24±0.8	20.5±0.5	20.3±0.9	22±1.4	21±1
10	24±0.8	20.5±0.5	20.3±0.9	22±0.8	21.5±0.5

Data are means (n=3).

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

The antibiotic production and cell dry weight were collected every 24 h for 10 days (Figure 4.10). The result showed that the highest antibiotic production was recorded at 5 days (72.5 ug/ml) with 1.19 mg/ml of cell dry weight. However, the maximum yield was obtained at 4 days (9.31 g/g) of incubation with cell dry weight 1.17 mg/ml (Figure 4.8).

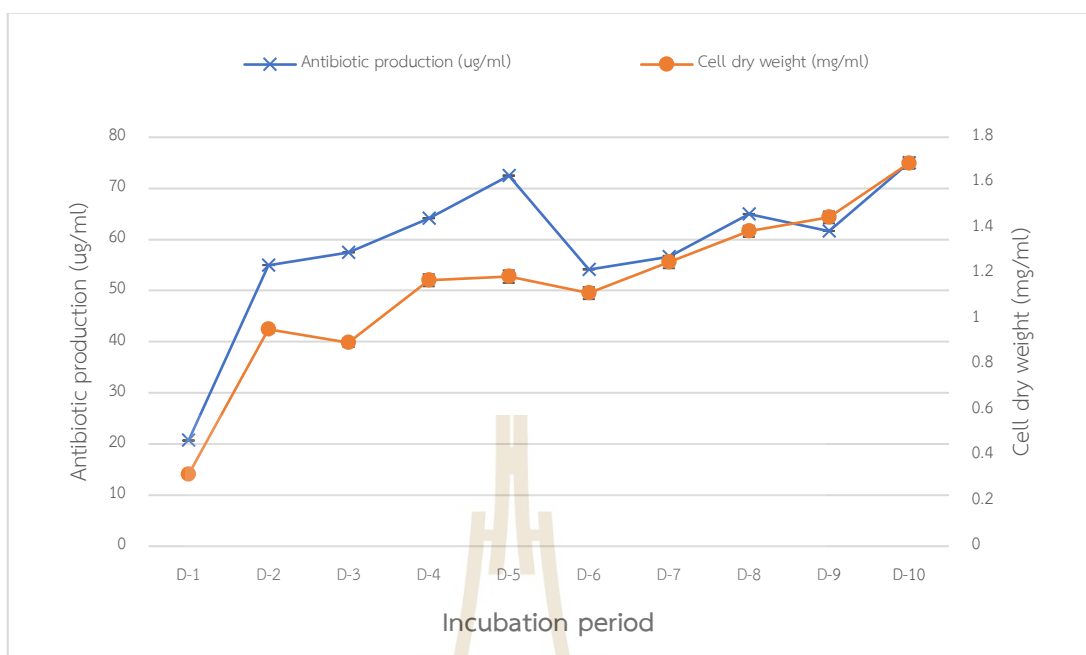


Figure 4.10 The Effect of incubation period of WPN32 cultured with SC medium. Incubation period with antibacterial production and cell dry weight collect every 24 hrs.

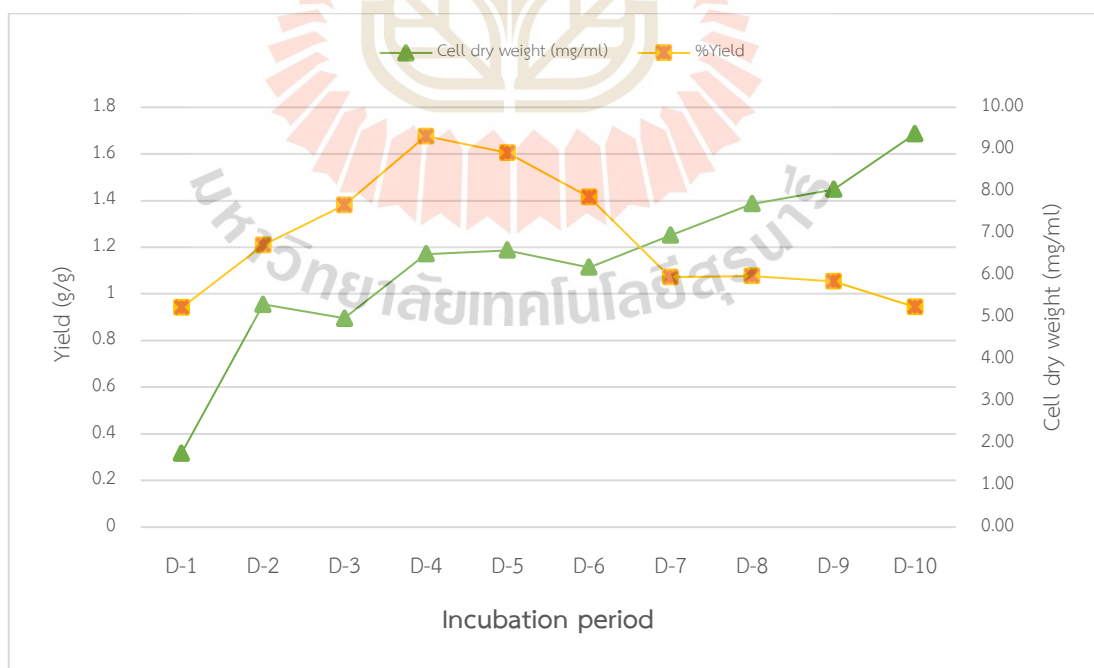


Figure 4.11 The Effect of incubation period of WPN32 cultured with SC medium. Incubation period with yield and cell dry weight collect every 24 hrs.

4.5 The preparation of crude extract from WPN32

The strain WPN32 was cultured in SC medium for 5 days at 30 °C. After incubation, the cultured was centrifuged and filtrated to separate the cell pellet. The cell-free supernatant was extracted with ethyl acetate (1:1), and the organic phase was evaporated under reduced pressure using a rotary evaporator. The ethyl acetate extract of WPN32 showed an orange color (Figure 4.12).



Figure 4.12 The ethyl acetate extracted of WPN32.

4.6 Purification of antibacterial compounds

4.6.1 Purification of bioactive compound by thin-layer chromatography

The crude ethyl acetate extracts were redissolved in ethyl acetate. Separation of crude compounds was performed on a silica gel plate using chloroform and methanol (9.2:0.8) as a mobile phase system. The bioactive compounds were tested against test microorganisms using contact-bioautography method. Figure 4.13 revealed that the yellow-coloured fraction with R_f value 0.73 showed antibacterial activity against MRSA 20654. The bioactive band was scraped out from TLC plates and separately purified until one single band was observed. The single band on TLC plates was tested for antibacterial activity. The partially purified compound was used for column chromatography.

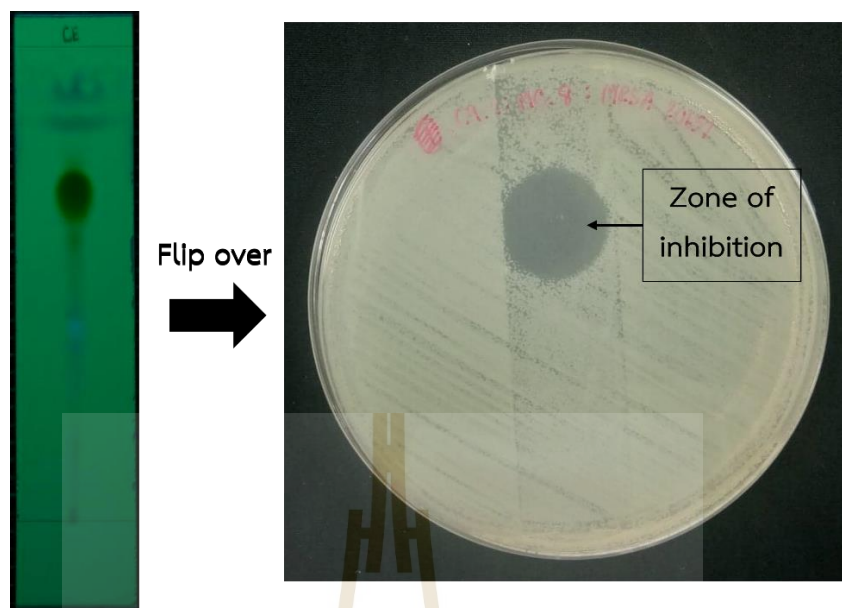


Figure 4.13 TLC-based bioautography of crude ethyl acetate of WPN32 exhibited antibacterial activity against MRSA 20654.

Active compounds obtained from TLC were further purified using silica gel column chromatography technique. The active compound was subjected to silica gel (60-120 mesh) with the gradient of chloroform: methanol. The polarity of methanol gradually decreased using the chloroform up to 39.9:0.1 v/v of chloroform: methanol. All fractions were tested using contact bioautography method for their antibacterial activity. Active band with R_f value 0.71 showed 12 mm of inhibition zone against MRSA 20654.

4.7 Characterization of purified antibacterial compounds

The bioactive compounds were identified using LC-MS based on their mass spectrum. The MS peaks were analyzed and identified by matching the mass spectra with the MassBank of North America (MoNA) (<https://mona.fiehnlab.ucdavis.edu/>). Figure 4.14 showed that the purified compounds of WPN32 showed 8 mixtures (8 peaks) of organic compounds (Figure 4.14). The summarized molecular weight retention time and the closely related compounds of partially purified WPN32 are shown in Table 4.5.

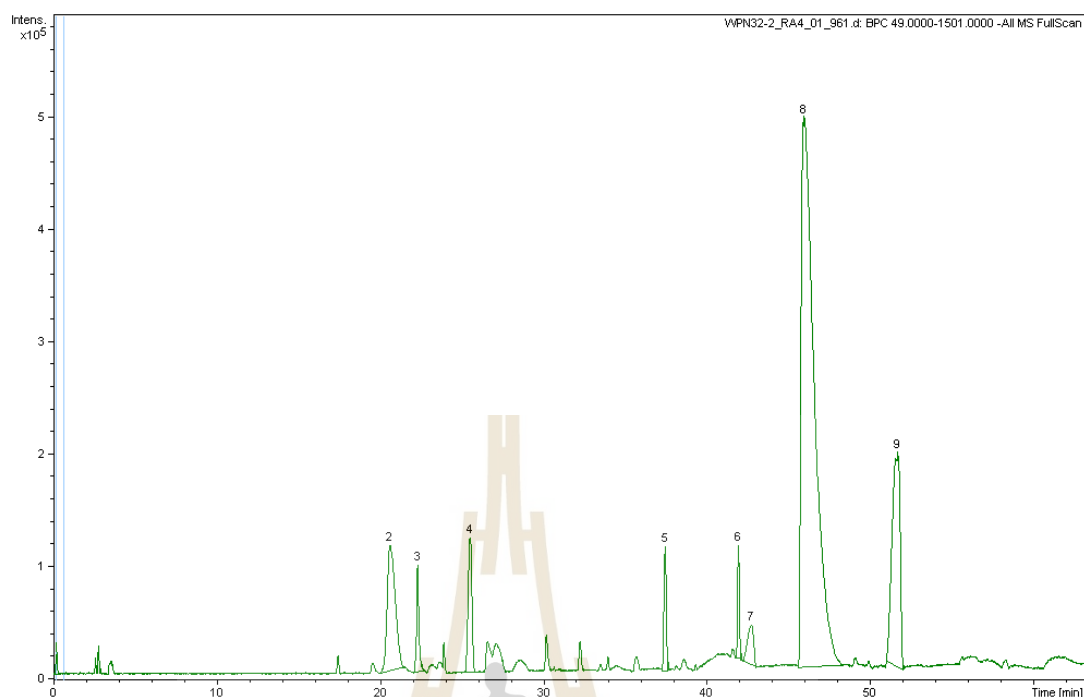


Figure 4.14 LC-MS chromatogram from WPN32 active compounds.

Table 4.5 The closely related compounds match to Massbank library of WPN32 compound.

RT (min)	Name of the Compound	Molecular formular	Molecular weight (g/mol)
20.5	Biflavonoid	C ₃₁ H ₂₀ O ₁₀	552.1056
22.2	Phosphatidylethanolamine alkenyl	C ₄₁ H ₇₄ NO ₇ P	723.5203
25.4	Quercetin	C ₂₃ H ₂₂ O ₁₃	506.106
37.4	Rimsulfuron	C ₁₄ H ₁₇ N ₅ O ₇ S ₂	431.0569
41.9	Cerivastatin	C ₂₆ H ₃₄ FNO ₅	459.2421
42.7	Phosphatidylcholine	C ₄₂ H ₇₄ NO ₈ P	751.5152
45.9	Anisomycin	C ₁₄ H ₁₉ NO ₄	265.1314
51.6	Actinomycin D	C ₆₂ H ₈₆ N ₁₂ O ₁₆	1254.629

Based on LC-MS analysis showed the two highest peaks at the retention time of 45.9 and 51.6 min. The first peak was found with molecular ion peak at m/z 265. $[M+H]^+$ related to anisomycin (Figure 4.12). The peak at retention time 51.6 min with molecular ions M^- at m/z 1253 was matched to actinomycin D or dactomycin (Figure 4.13).

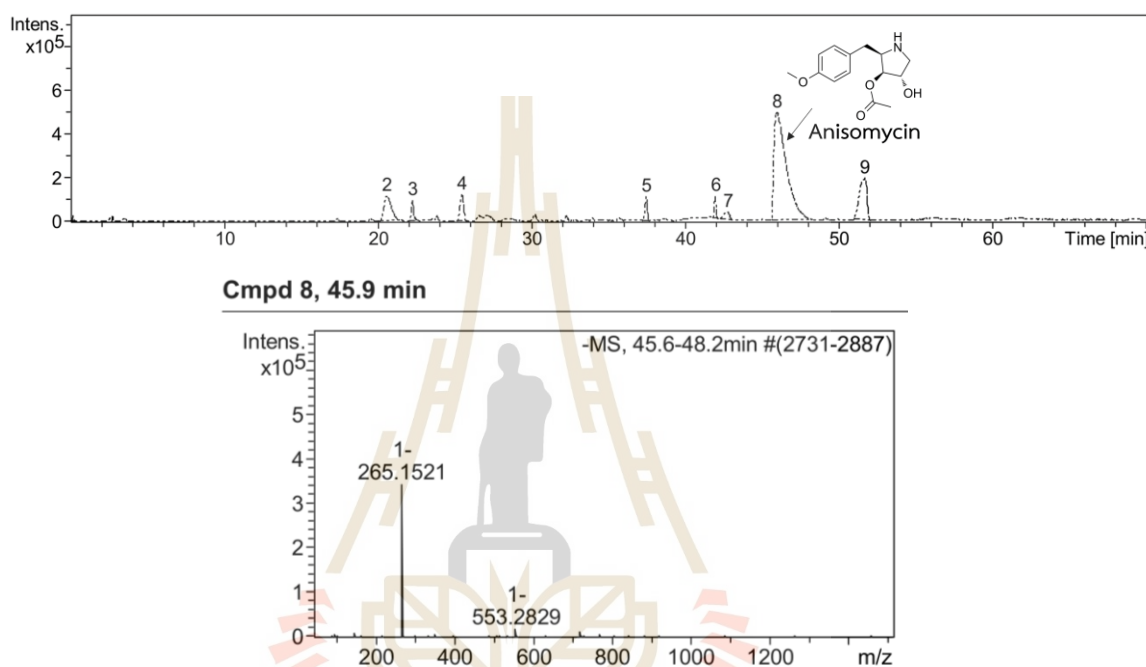


Figure 4.15 LC-MS analysis of WPN32 in negative ion mode. (a) LC-MS chromatogram and (b) MS spectrum of peaks 8 with retention time 45.9 min at m/z 256.1521 related to anisomycin.

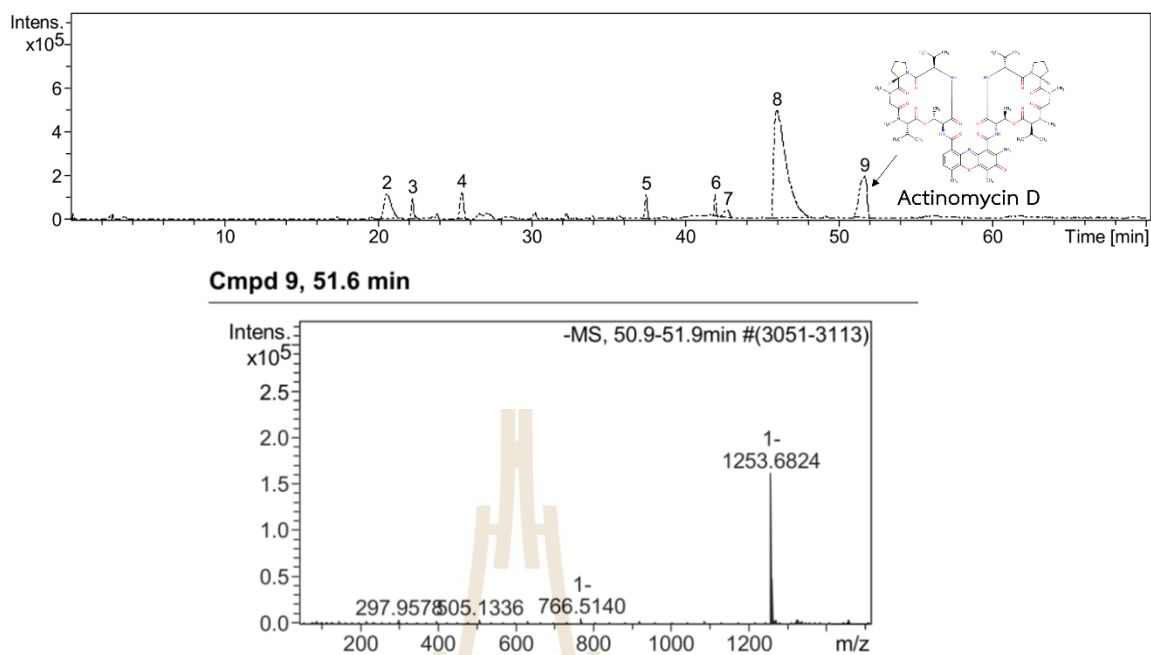


Figure 4.16 LC-MS analysis of WPN32 in negative ion mode. (a) LC-MS chromatogram and (b) MS spectrum of peak 9 with retention time 51.9 min at m/z 1253.6824 related to actinomycin D

CHAPTER V

DISCUSSION AND CONCLUSION

Streptomycete isolates that produce antibacterial activity were screened from soil samples. In this research, forty-four streptomycetes were isolated and purified using ISP2 medium. Streptomycetes colonies are easily separated by their opaque, non-spreading structure, rough, and normally embedded into the agar medium. The substrate and aerial mycelia appear in a variety of colors, and the earthy odor in most colonies are important characteristic of streptomycetes (Intra et al., 2011). Among 44 isolates, they consisted of yellow, pink, red, dark purple, black, gray, and white of colony color. Gray and white mycelial pigmentation were mainly found in this study.

For screening study of antibacterial activity using the perpendicular steak method, 5 Gram-positive and 8 Gram-negative bacteria were selected as test microorganisms. The results showed that 90% of the 44 streptomycetes exhibited no antibacterial activity. Ten percentage showed positive antibacterial activity as suggested by the zone of inhibition. Nine percentage of all strains showed narrow spectrum against only Gram-positive bacteria and 1% showed broad spectrum against Gram-positive as well as Gram-negative microorganisms. These results revealed that the finding of streptomycetes, which possess antimicrobial activity against Gram-negative bacteria, was difficult.

The composition and structure of bacterial cell walls cause the differences between Gram-positive and Gram-negative bacteria (Kurnianto et al., 2020). Lipopolysaccharide (LPS) is a major structural component of Gram-negative bacterial cell walls (Singh et al, 2014). It forms a barrier to protect hydrophilic and hydrophobic compounds with various molecular weights. Choi and Lee reported that several antibacterial compounds with small hydrophilic molecule (<600Da) can diffuse via porins to permeate the outer membrane. (Choi and Lee, 2019). Gram-positive bacteria are more sensitive to antibiotic substances because they lack LPS in the cell walls. They only have a peptidoglycan layer, which is ineffective as a permeability barrier

(Gebreyohannes et al., 2013; Singh et al., 2014) Antibacterial producing streptomycetes were selected for further studied. Strains WPN5, WPN22, WPN32, WPN35 and WPN38 were studied of morphological and phylogenetic characteristics. All of strains grown on ISP2 medium for 14 days were observed based on morphological characteristic. The findings indicated that the majority of the isolates produced grey and white colored of aerial mycelium and white to yellow colored of colony. Two stains, WPN32 and WPN35, produced yellow pigment that changed the color of the medium. Whereas pigments color is not produced by WPN5, WPN22, and WPN38.

Five antibacterial producing streptomycetes were identified at the species level based on molecular characteristics. The 16S rRNA sequencing of strains WPN5, WPN22, WPN32, WPN35, and WPN38 confirmed that these stains belonged to the *Streptomyces* genus. Stains WPN5 and WPN22 shared 100% similarity to *Streptomyces pseudogriseolus*. Strain WPN32 showed a highest similarity of 99.45% to *Streptomyces cyaneochromogenes*. Moreover, strains WPN35 and WPN38 were identified as *Streptomyces antibioticus* and *Streptomyces rochei* with 100% similarity, respectively. Zhi et al. demonstrated that the percentage of similarity with closet stains was less than 98% suggesting the possibility of different species (Zhi et al., 2009). According to Hagstrom et al., strains with more than 97% sequence homology can represent the same species. The percentage ranged between 93% and 97% for the same genus but differed at the species level. The percentage of sequence homology was less than 93% indicated the new of the genus level (Hagstrom et al., 2000). Furthermore, the phylogenetic analysis of strains WPN5, WPN22, WPN35 and WPN38 were formed a monophyletic clade with their closet strains. Strain WPN32 formed a distinct phyletic clade with *S. aquilus* and *S. cyaneus* and showed out of clade from their closet group (*S. cyaneochromogenes*). These results indicated strain WPN32 may represent a novel strain. Kurnianto and Semêdo described that these strains may show the possibility to belong in a different strain or new strains with new characteristics (Kurnianto et al., 2020; Semêdo et al., 2001).

The 16S rRNA gene sequencing showed that the strain WPN32 were classified as strain *S. cyaneochromogenes* MK-45. In 2019, Tang reported a novel *Streptomyces* species, *S. cyaneochromogenes* MK-45, could be isolated from manganese-

contaminated soil. The morphological, physiological, and biochemical characters of *S. cyaneochromogenes* MK-45 were described (Tang et al., 2019). As a result, WPN32 was chosen for further investigation in this research.

Streptomycetes can either gain or entirely lose their capacity to produce antibacterial compounds depending on the environmental and dietary factors they are exposed to. This is because antibiotic production is a microorganism-specific property that is affected by culture conditions (Bundale et al., 2015). The important factors in antibiotic production are the nutrients, pH, temperature, and incubation time (Usha Kiranmayi et al., 2011). Thus, the study of culture media, temperature, and incubation period were investigated in order to achieve the maximal antibacterial production.

The agar-well diffusion technique was used to examine the zone of inhibition against test microorganisms. The production of antibacterial compounds was highest on SC broth. SC medium contains starch and casein acid hydrolysate as carbon and nitrogen sources (Bundale et al., 2015). Several studies have found that starch is the best carbon source to produce antibacterial compounds. It has been shown that streptomycete isolates produced the most bioactive compounds in medium containing starch and cellobiose (Bundale et al., 2015; Singh et al., 2014). According to El-Naggar, starch-containing medium yielded the maximum antibacterial production in *Streptomyces violates* (El-Naggar et al., 2003). Another study by Singh et al. and Ansari et al. also reported that the composition of cultured medium containing starch increased the production of bioactive compounds (Al-Ansari et al., 2020; Singh et al., 2014).

In this study, strain WPN32 showed the highest zone of inhibition when cultivated at 30 °C. This finding is consistent with Khattab's research, which found that 30 °C is the suitable temperature for maximum production of antibacterial compounds (Khattab et al., 2016). The highest antibacterial compounds production was also observed at 30 °C with *Streptomyces* sp. KGG32 and *Streptomyces violates* (Hassan and Al-Harmoosh, 2004 and Oskay, 2016).

The incubation period study's antibacterial activity against *B. cereus*, MRSA 20651, and MRSA 20654 started after day 1 of incubation. MRSE and *S. aureus* started to appear on day 2 of incubation. The maximum level of antibacterial compounds was

obtained on day 5 of incubation. Moreover, the optimum antibacterial activity and % yield of antibacterial compound was found between days 4 and 5 of incubation period. According to Kathiresan, 5 days of incubation of actinomycetes strains produced the highest value of inhibition zone (Kathiresan et al., 2005). In contrast, the research by Singh found that after 7 days of incubation showed the highest antibacterial activity (Singh et al., 2014). Other studies of incubation time for optimized bioactive metabolites have also been reported. The study by Kurnianto and Sharma obtained the best yield after 3 to 10 days of incubation (Kurnianto et al., 2020; Sharma and Manhas, 2019). Srinivasan described that the maximum production of antibacterial compounds was obtained after the exponential phase reaches the stationary phase (Srinivasan et al., 1991). According to Sejiny and Kandula, antibacterial compound synthesis occurs during the late exponential and stationary phases. The initial phase is high growth rate with biomass production, followed by slow growth with increased antibacterial production (Kandula and Terli, 2013; Sejiny, 1991).

The fermentation of strain WPN32 was performed after incubation for 5 days in SC broth at 30 °C. After incubation, the fermented broth was extracted by using ethyl acetate as an organic solvent. Crude compounds were partially purified by thin-layer chromatography (TLC) and column chromatography (CC). LC-MS analysis of WPN32 active compounds showed a major peak with molecular ion peaks m/z at 265 and 1253. The MassBank of North America (MoNA) revealed that the WPN32 produced 2 major compounds, including anisomycin and actinomycin D.

Anisomycin is a pyrrolidine antibiotic used as an antiprotozoal drug (Sun et al., 2013). The chemical structure is shown in Figure 5.1. In 1954, the anisomycin antibiotic was first discovered from *S. griseolus* and *S. roseochromogenes* by Sobin and Tanner (Jiménez and Vázquez, 1979; Sobin and Tanner, 1954). The mode of action of anisomycin inhibits the eukaryotic protein synthesis and DNA synthesis. Anisomycin was also utilized as a topical anticandidal and antiamoebic treatment in humans (Xing et al., 2008).

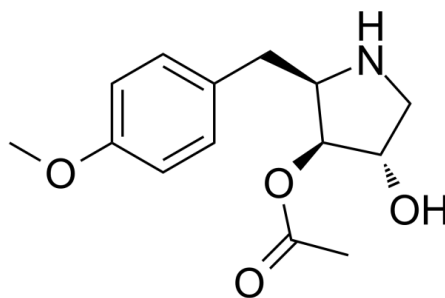


Figure 5.1 Chemical structure of Anisomycin.

Actinomycin D is the antibiotic discovered by Waksman and Woodruff in 1940 (Figure 5.2). According to Kurosawa, at least 23 species of *Streptomyces* can produce actinomycin. Examples of *Streptomyces* strains include *S. antibioticus*, *S. flavus*, *S. galbus* (Frommer, 1959), *S. nasri*, *S. plicatus*, and *S. violascens* (El-Naggar, 1998; Hirata and Nakanishi, 1949; Kurosawa et al., 2006; Lam et al., 2002; Praveen et al., 2008; Roos and Loane, 2004; Waksman and Woodruff, 1940). Actinomycin D is a transcription inhibitor, by binding to DNA duplexes in the transcription initiation complex, RNA polymerase elongation is blocked (Singh et al., 2010)

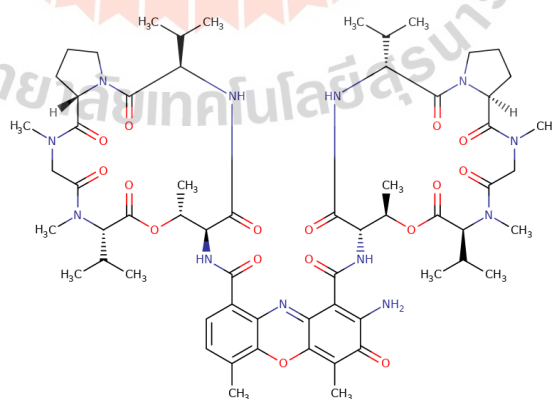
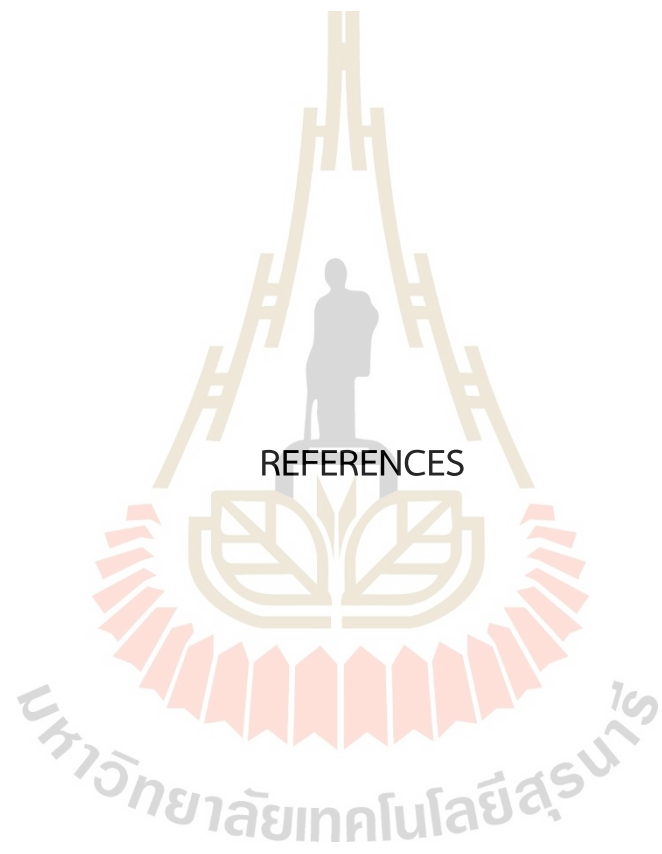


Figure 5.2 Chemical structure of Actinomycin D.

To date, this is the first report to study the optimized culture condition and purified antibacterial compound produced by *S. cyaneochromogenes*. Furthermore, this work demonstrates the first study of anisomycin and actinomycin D produced from *S. cyaneochromogenes*. The production of anisomycin and actinomycin D from WPN32 may have an important function in the pharmaceutical industry. These medicines are important for treating patients with anticancer and antiprotozoal drug in their communities. For the future studies of WPN32, the characterization of these active compounds using NMR and FT-IR should be further investigated.



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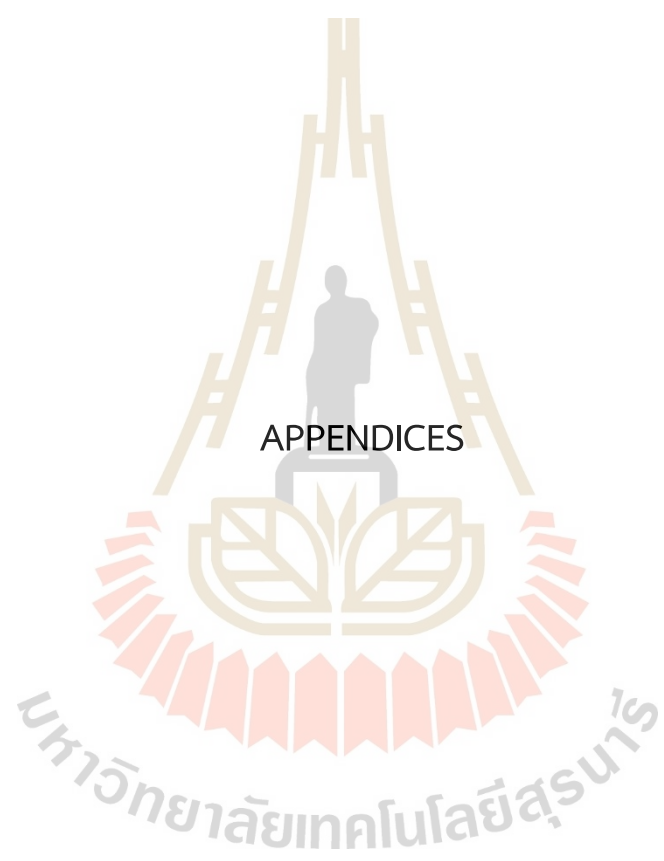
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APPENDIX A
SEQUENCE OF ANTIBACTERIAL PRODUCING STREPTOMYCETES
SOIL ISOLATE

WPN5

TCAGGACGAACGCTGGCGGCGTGCTTAAACACATGCAAGTCGAACGATGAACCACTTCGGTGG
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GGTGCAGGATGAGCCCGCGGCCTATCAGCTTGTTGGTGAGGTAATGGCTCACCAAGGCGACGA
CGGGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTAC
GGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAG
GGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAAGCGAAAGTGACGGTACCTGC
AGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCGAGCGTTGTC
CGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGCTTGTCGCGTCGGTTGTGAAAGCCCGGGGC
TTAACCCCGGGTCTGCAGTCGATACGGGCAGGCTAGAGTTCGGTAGGGGAGATCGGAATTCCT
GGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGATCTCTGGGC
CGATACTGACGCTGAGGAGCGAAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCC
ACGCCGTAAACGGTGGGCACTAGGTGTGGGCGACATTCCACGTCGTCCGTGCCGCAGCTAACG
CATTAAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACCTCAAAGGAATTGACGGGGGC
CCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGA
CATACACCGGAAAACCTTGGAGACAGGGTCCCCCTTGTTGGTCGGTGACAGGTGGTGATGGCT
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TTGCCAGCAGGCCCTTGTTGGTGCTGGGGACTCACGGGAGACCGCCGGGGTCAACTCGGAGGAA
GGTGGGGACGACGTCAAGTCATCATGCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCC
GGTACAATGAGCTGCGATACCGCGAGGTGGAGCGAATCTCAAAAAGCCGGTCTCAGTTCGGATT
GGGGTCTGCAACTCGACCCCATGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCATTGCTGCGG
TGAATACGTTCCCGGGCCTTGTAACACCGCCCGTCACGTCACGAAAGTCGGTAACACCCGAAG
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WPN22

TGGCTCAGGACGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAACGATGAACCACTTCGGT
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 CTGGAAACGGGGTCTAATACCGGATACTGATCATCTTGGGCATCCTTGGTGATCGAAAGCTCCG
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 GACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCT
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 GCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCGAGCGTTG
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 CCGGTACAATGAGCTGCGATACCGCGAGGTGGAGCGAATCTCAAAAAGCCGGTCTCAGTTCGG
 ATTGGGGTCTGCAACTCGACCCCATGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCATTGCTG
 CGGTGAATACGTTCCCGGGCCTTGACACACCGCCCGTCACGTCACGAAAGTCGGTAACACCCG
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WPN32

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 TACAATGAGCTGCGAAACCGTGAGGTGGAGCGAATCTCAAAAAGCCTGTCTCAGTTCGGATTGG
 GGTCTGCAACTCGACCCCATGAAGTCGGAGTTGCTAGTAATCGCAGATCAGCATTGCTGCGGTG
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WPN35

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 GGGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAGACACGGCCAGACTCCTACG
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WPN38

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APPENDIX B

LIST OF CLOSET STRAINS FROM EZBIOCLOUD

A1 List of closet strains from EzBioCloud using 16S rRNA gene database of soil isolate WPN32

Hit taxon name	Hit strain name	Similarity (%)	Completeness (%)
<i>Streptomyces cyaneochromogenes</i>	MK-45	99.45	100
<i>Streptomyces aquilus</i>	GGCR-6	99.43	96.6
<i>Streptomyces shaanxiensis</i>	CCNWHQ 0031	99.3	98.1
<i>Streptomyces caeruleatus</i>	NRRL B-24802	99.24	100
<i>Streptomyces griseoruber</i>	NRRL B-1818	99.17	100
<i>Streptomyces curacoi</i>	DSM 40107	99.03	100
<i>Streptomyces capoamus</i>	JCM 4734	98.96	100
<i>Streptomyces canus</i>	DSM 40017	98.96	100
<i>Streptomyces cyaneus</i>	NRRL B-2296	98.96	100
<i>Streptomyces bungoensis</i>	DSM 41781	98.9	100
<i>Streptomyces lasalocidi</i>	ATCC 31180	98.83	100
<i>Streptomyces longwoodensis</i>	DSM 41677	98.83	100
<i>Streptomyces galbus</i>	DSM 40089	98.83	100
<i>Streptomyces corchorusii</i>	DSM 40340	98.76	100
<i>Streptomyces cinnabarinus</i>	JS360	98.69	100
<i>Streptomyces resistomycificus</i>	NRRL ISP-5133	98.69	100

A1 List of closet strains from EzBioCloud using 16S rRNA gene database of soil isolate WPN32 (Continued)

Hit taxon name	Hit strain name	Similarity (%)	Completeness (%)
<i>Streptomyces canarius</i>	NBRC 13431	98.69	99.9
<i>Streptomyces olivaceoviridis</i>	NBRC 13066	98.62	99.9
<i>Streptomyces panaciradicis</i>	1MR-8	98.55	100



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