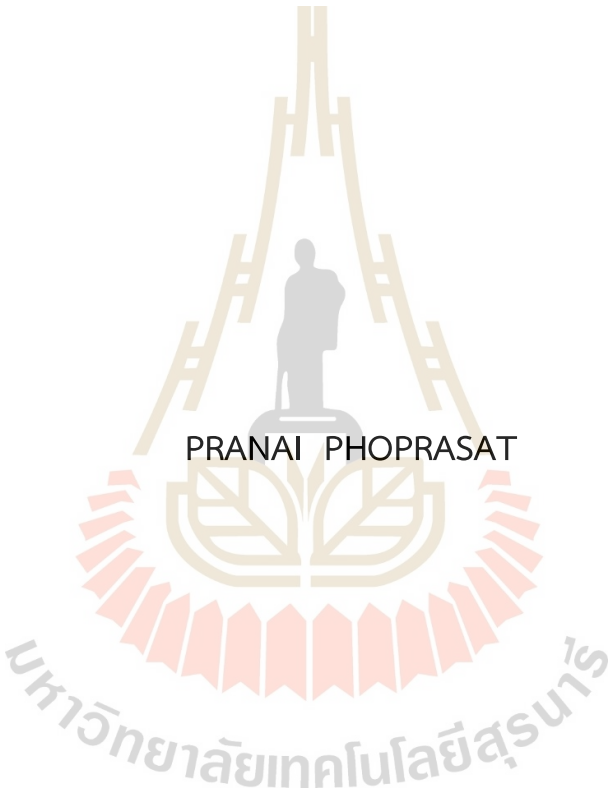
ISOLATION AND PURIFICATION OF ANTIBIOTIC FROM SOIL BACTERIA

PRANAI PHOPRASAT



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การแยก และการทำให้บริสุทธิ์ของยาปฏิชีวนะจากแบคทีเรียในดิน

นายประณัย โพธิ์ประสาท

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาชีวเคมี
มหาวิทยาลัยเทคโนโลยีสุรนารี
ปีการศึกษา 2565

ISOLATION AND PURIFICATION OF ANTIBIOTIC
FROM SOIL BACTERIA

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

Thesis Examining Committee

(Asst. Prof. Dr. Anyanee Kamkaew)
(Chairperson)

(Dr. Sakesit Chumnarnsilpa)
Member (Thesis advisor)

(Assoc. Prof. Dr. Jaruwan Siritapetawee)
Member

(Assoc. Prof. Dr. Panida Khunkaewla)
Member

(Asst. Prof. Dr. Nawarat Nantapong)
Member

(Assoc. Prof. Dr. Chatchai Jothityangkoon)
Vice Reactor for Academic Affairs
and Quality Assurance

(Prof. Dr. Santi Maensiri)
Dean of In Stitute of Science

ประณัย โพธิ์ประสาท : การแยก และการทำให้บริสุทธิ์ของยาปฏิชีวนะจากแบคทีเรียในดิน (Isolation and Purification of Antibiotic from Soil Bacteria) อาจารย์ที่ปรึกษา : ดร.เศกสิทธิ์ ชำนาญศิลป์, 63 หน้า

คำสำคัญ: แบคทีเรียดื้อยา/ การแยก/ การทำให้บริสุทธิ์/ การค้นหายาชนิดใหม่/ ยาปฏิชีวนะ

แบคทีเรียดื้อยาเป็นภัยคุกคามต่อสุขภาพของประชากร เนื่องจากมีความสามารถในการกลายพันธุ์ได้เร็วว่ายาปฏิชีวนะชนิดใหม่ที่มีการค้นพบ ดังนั้นเราจึงมีการพัฒนาเทคนิคหรือค้นหายาชนิดใหม่เพื่อมาต่อสู้กับเชื้อแบคทีเรียดื้อยาเหล่านี้

วิทยานิพนธ์นี้มีจุดมุ่งหมายเพื่อค้นหายาปฏิชีวนะชนิดใหม่จากดินที่เป็นแหล่งปนเปื้อนยาปฏิชีวนะ โดยเลือกใช้อาหารขนไก่เพื่อคัดแยกแบคทีเรียที่ผลิตยาปฏิชีวนะจากตัวอย่างดินที่เก็บจากโรงฆ่าสัตว์ สุกร โรงบำบัดน้ำเสียมูลสุกร และเครื่องกำเนิดก๊าซชีวภาพของฟาร์มสุกรทั่วจังหวัดนครราชสีมา แล้วนำแบคทีเรียที่มีความสามารถผลิตยาปฏิชีวนะมาแยก จำแนกลักษณะ และระบุโดยใช้ 16s rRNA

จากการแยกเชื้อแบคทีเรียและระบุสายพันธุ์ได้เป็น *Bacillus siamensis* ที่ได้จากบริเวณคอกหมู แล้วนำมาทดสอบการยับยั้งการเจริญของเชื้อด้วยวิธี plug diffusion method ซึ่งมีความสามารถยับยั้งการเจริญของเชื้อ *Shigella flexneri Pseudomonas aeruginosa Escherichia coli*

การวิเคราะห์โครมาโทกราฟีแบบชั้นบาง แสดงแถบของสารประกอบออกฤทธิ์ที่ยับยั้งได้โดยสารสกัดโดย 20% เอทิลอะซิเตตในเฮกเซน การทำให้บริสุทธิ์เพิ่มเติมโดยคอลัมน์โครมาโทกราฟีแสดงให้เห็นว่าสารประกอบที่ถูกชะด้วย 10% 40% 90% เอทิลอะซิเตทในเฮกเซนและเอทิลอะซิเตตในเฮกเซนและเอทิลอะซิเตท 100% ยับยั้งการเจริญเติบโตของแบคทีเรียที่ทดสอบซึ่งบ่งชี้ว่าสารสกัดประกอบด้วยสารออกฤทธิ์หลายชนิด สารประกอบ ผลลัพธ์ของงานนี้แสดงหลักฐานว่า *B. siamensis* สามารถผลิตสารต้านแบคทีเรียได้หลายชนิด ซึ่งต้องได้รับการตรวจสอบเพิ่มเติมในอนาคต

สาขาวิชาเคมี
ปีการศึกษา 2565

ลายมือชื่อนักศึกษา ประณัย โพธิ์ประสาท
ลายมือชื่ออาจารย์ที่ปรึกษา

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Keyword: MULTIDRUG-RESISTANT/ ISOLATION / PURIFICATION / DEVELOPING NEW DRUG / ANTIBIOTIC

Multidrug-resistant (MDR) bacteria are threats to public health because they mutate faster than development of new antibiotics. Therefore, new techniques or drugs must be developed to combat them.

This thesis aims to screen for the new antibiotic from antibiotics contaminated soil. The selective media chicken feathers (CF) were used to screen antibiotic producing bacteria from the soil samples collected from a slaughterhouse, a pigsty, a pig manure wastewater treatment plant, and a biogas generator of the pig farm around Nakhon Ratchasima province, Thailand. The antibiotic producing bacteria were isolated, characterized and identified by 16s rRNA sequencing.

The antibiotic producing bacterium, *Bacillus siamensis* wad isolated from the pigsty of the pig farm. The plug diffusion method showed that *B. siamensis* was able to inhibit growth of *Shigella flexneri, Pseudomonas aeruginosa and Escherichia coli.*

Thin-layer chromatography analysis showed the band of active compounds in the inhibition zone extracted by 20% ethyl acetate in hexane. The further purification by column chromatography showed that the compounds eluted by 10%, 40%, 90% ethyl acetate in hexane and ethyl acetate in Hexane and 100 % ethyl acetate inhibited growth of the tested bacteria, suggesting that the extract comprised several kinds of active compounds. These results of this work provide us evidence that *B. siamensis* is able to produce several kinds of antibacterial growth substances that must be further investigated.

School of Chemistry
Academic Year 2022

Student's signature_____
Advisor's signature_____

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Pranai Phoprasat

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LIST OF ABBREVIATIONS

CF media	Selective media feather
cm	Centimeters
DI water	Distilled water
DMSO	Dimethyl sulfoxide
DNA gyrase	Topoisomerase II
g	Grams or gram
hr	hour
LB	Luria-Bertani medium
LB agar	Luria-Bertani medium agar
LAB	Lactic acid bacterium
LPS	Lipopolysaccharides
MDR bacteria or MDR	Multidrug-resistant bacteria
µL	Microliter
mL	Milliliter
mm	Millimeter
NaCl	Sodium choline
PBP	Penicillin-binding proteins
TLC	Thin-layer chromatography
rpm	Revolutions per minute

CHAPTER I
INTRODUCTION

The bacteria that resist to more than one type of antibiotic are referred to as multidrug-resistant bacteria (MDR bacteria) (Magiorakos et al., 2012). MDR bacteria seriously threaten public health because, they develop must faster than discovery of new antibiotics (Duin and Paterson, 2016). The primary cause of MDR bacteria is the improper and excessive use of antibiotics. Dadgostar, 2019 andDuin and Paterson, 2016 say that multidrug resistance can lead to treatment failure, higher rates of illness and death, and higher healthcare costs for patients.

There is an urgent need to find strategies to fight against MDR bacteria. Options to treat MRD bacterial infections include phage treatment, antibiotic synergy, and new types of antibacterial substances. Even though the rate at which MDR bacteria change is much faster than the rate at antibiotics development, screening for new antibiotic remain an important strategy. The recent discovery of new classes of antibiotics from bacteria creates a new chance to speed up the discovery of antibiotics (Azam et al., 2015; Brives and Pourraz, 2020; Tacconelli et al., 2018). The main idea of this research is to screen the antibiotics producing bacteria from the antibiotics contaminated soil from animal farms.

1.1 Antibiotic

An antibiotic is generally a microorganism's secondary metabolite that is able to inhibit bacterial growth. Antibiotics are generally used to treat bacterial infections. Antimicrobial drugs, as opposed to antibiotics, are artificial or natural chemicals that include a broader spectrum of agents that act on microbes. The term "microbe" refers to various species, including bacteria, fungi, viruses, and protozoa. Only a few antibiotics also have antiprotozoal properties. Therefore, antibiotics are useless against viruses like the flu or the common cold (Cycon et al., 2019). However,

"antibiotics" could also mean drugs made from semi-natural materials. Alexander Fleming discovered penicillin, the first antibiotic, in 1928. (Figure 1.1). Since 1961, it has been widely employed to treat bacterial infections. There are currently 13 groups of antibiotics based on their structural characteristics. as displayed in Figure 1.1.

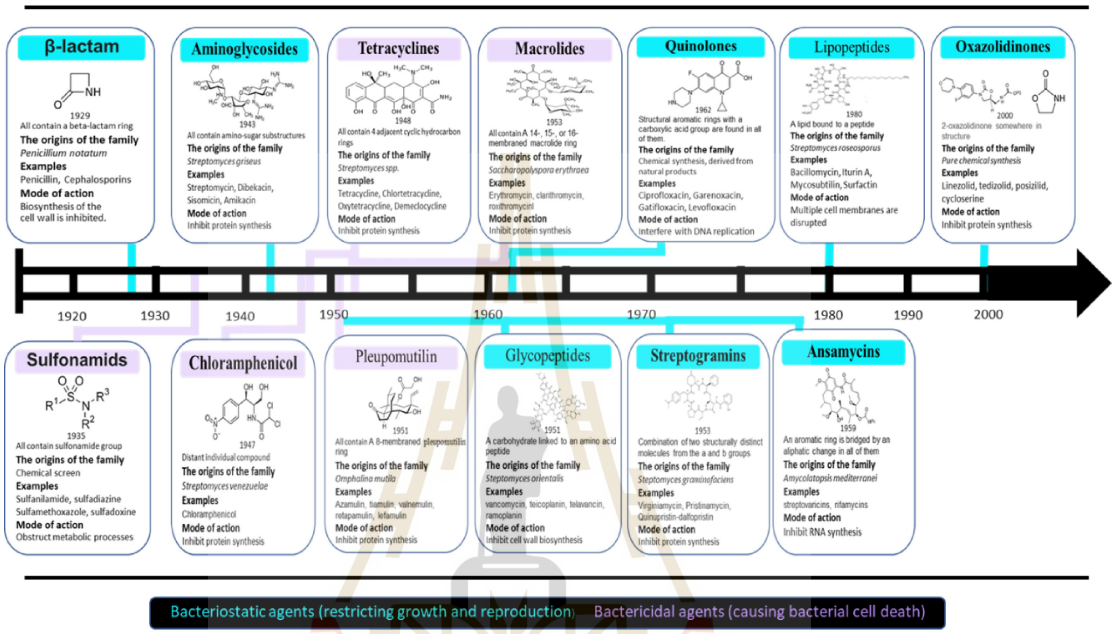


Figure 1.1 *Overview of Classes of Antibiotics (modified from Farrell et al., 2018).*

1.2 Mechanisms of Antibiotic Action and Resistance

1.2.1 Mechanisms of antibiotic action against bacterial cells

Antibiotics work against bacteria through five main mechanisms: 1) block the formation of cell walls, 2) inhibit protein synthesis, 3) interfere the cell membranes integrity, 4) disrupt the making of nucleic acids, and 5) prevent the action of antimetabolites. (Figure 1.2).

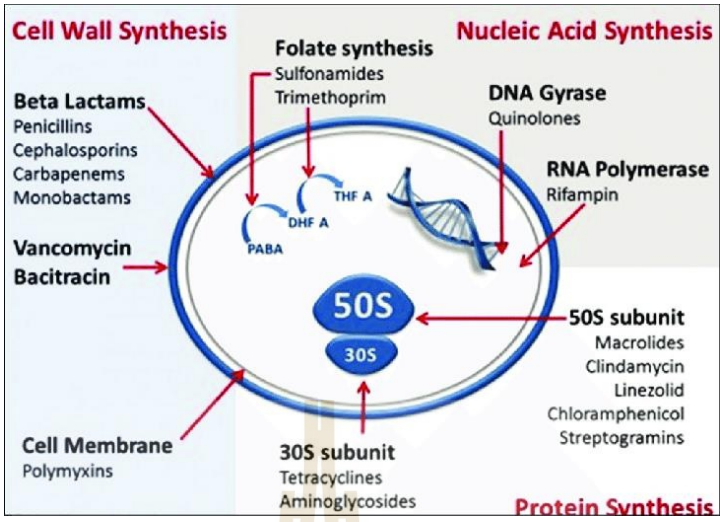


Figure 1.2 *The mechanisms of Antibiotic Action (Kapoor et al., 2017).*

1.2.2 Mechanisms of antibiotic resistance

Bacteria have created several ways to counteract the effects of antibiotics. Three main ways exist through which bacteria might develop resistance to an antibiotic's effects (Pal, 2017; Sanz-García et al., 2021; Seukep et al., 2020). These mechanisms include blocking the antibiotic from reaching its target in adequate quantities, altering or bypassing the drug's target, and obtaining genetic material from other bacteria. (Figure 1.3).

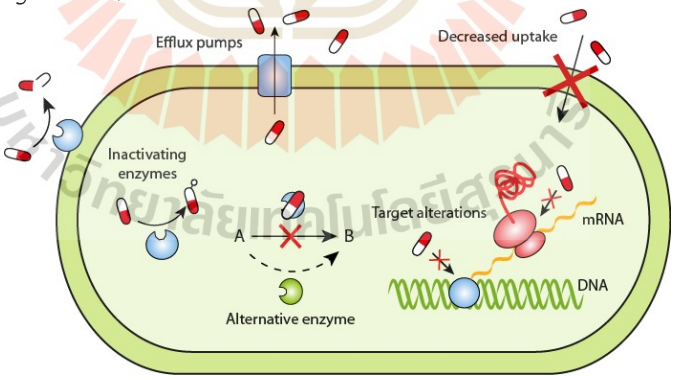


Figure 1.3 *Bacterial mechanisms have a way of avoiding the effects of antibiotics. For example, they can stop them from getting to where they should work, change, or prevent them. (Pal, 2017).*

1.2.2.1 Preventing the antibiotic from reaching its target in sufficient quantity

Efflux pumps; Pumps that the bacteria have created may be found in the cell wall or membrane of the bacterium. Efflux pumps are membrane proteins that bacteria use to move antibiotics out of the cell. Rarely, modifications to the bacteria's DNA may cause them to produce more of a particular pump, which raises their resistance level (Pal, 2017; Sanz-García et al., 2021; Seukep et al., 2020).

Diminish the membrane's permeability; make the membrane surrounding the bacterial cell less permeable. Because of the changes to the bacterial barrier and the tightening of the outer membrane, the porin-mediated route for antibiotics to get into the cell will work less well, and the bacteria will absorb much less of the antibiotic. (Delcour, 2009; Pal, 2017).

Remove or alter the structure of antibiotics; bacterial enzymes can render antibiotics useless. One of these enzymes, beta-lactamase, degrades the active component of penicillin, the beta-lactam ring, and may also produce enzymes that can add other chemical groups to antibiotics. In addition, it prevents the antibiotic from binding to the bacterial cell's target (Pal, 2017; Wright, 2005).

1.2.1.2 Modifying or bypassing the drug's target

Conceal the location of the target; A mutation in the bacterial DNA may alter the target's structure by adding new chemical groups, protecting it from the antibiotic, which can prevent the antibiotic from interacting with the target (Pal, 2017; Sanz-García et al., 2021; Seukep et al., 2020).

Binding with other proteins; Bacteria may make new proteins to replace inactive ones due to the antibiotic. For instance, Staphylococcus aureus may develop a novel penicillin-binding protein by acquiring the resistance gene mecA.

Change target site. Occasionally; bacteria can develop a unique structure variation that they need. For instance, bacteria that are resistant to vancomycin produce a cell wall that is distinct from those bacteria that are vulnerable to the antibiotic. The antibiotic does not interact with this form of the cell wall as effectively as other types (Pal, 2017; Sanz-García et al., 2021; Seukep et al., 2020).

1.2.1.3 Acquiring genetic

They are obtaining genetic material from other microorganisms. Bacteria utilize three primary strategies to exchange or spread resistance genes horizontally (Figure 1.4). During transformation, bacteria ingest a piece of DNA floating in their environment. During conjugation, DNA is transported between bacteria through a tube between cells (transduction) (Porter and Dorman, 2006; Modi et al., 2014; Bello-López et al., 2019).

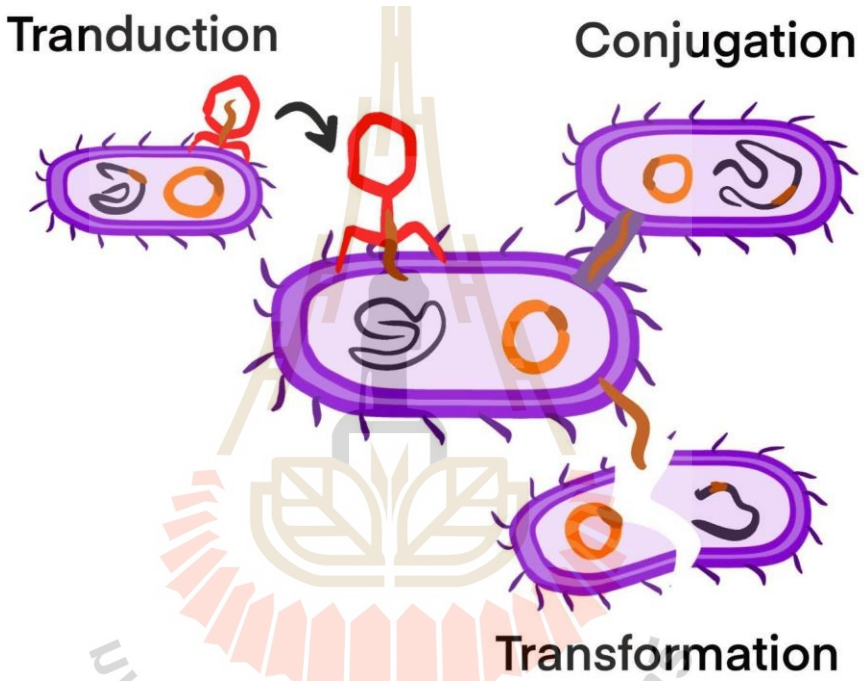


Figure 1.4 *The bacteria acquire genetic material from other organisms for drug mutation.*

1.2.3 Action and resistance mechanisms of antibiotics on target of bacteria

1.2.3.1 Inhibitors the formation of cell walls

Antibiotics of the beta-lactam and glycopeptide families suppress bacterial cell wall production. Beta-lactam antibiotics have a β-lactam nucleus in their molecules and stop the formation of cell walls (Kapoor et al., 2017; Srinivasan et al., 2020). Penicillin derivatives (called "penams"), cephalosporins (called "cephems"), monobactams, and carbapenems are all in this group (Wright, 2005). Antibiotics containing glycopeptides consist of glycosylated cyclic or polycyclic non-ribosomal peptides. Vancomycin, teicoplanin, telavancin, bleomycin, ramoplanin,

and decaplanin are effective glycopeptide antibiotics (Zeng et al., 2016) and glycopeptide antibiotics like vancomycin are essential by inhibiting the production of peptidoglycan, this group of medicines stops weak microorganisms from making cell walls. The two kinds of antimicrobial drugs stop or mess with the cell wall formation of the bacteria they are meant to kill. Since animal cells do not have cell walls, antibiotics often stop bacteria from making peptidoglycan-filled cell walls. The peptidoglycan layer is important to the structure of the cell wall because it is the most abundant and outermost part of the cell wall. Penicillin and cephalosporin are beta-lactam antibiotics. They inhibit peptidoglycan crosslinking, the final stage in forming bacterial cells. Because the structure of β-lactams is similar to that of peptidoglycan subunits, they can covalently bind to and inhibit the enzymatic activity of D-alanyl-alanine transpeptidase or DD-transpeptidase (a type of penicillin-binding proteins, PBP) (Lobanovska and Pilla, 2017). (Figure 1.6). A lack of peptidoglycan crosslinking causes osmotic lysis by weakening the cell wall. Even though there have been significant efforts in medicinal chemistry to change beta-lactam antibiotics, some bacterial strains have been able to resist every antibiotic used in clinical settings.

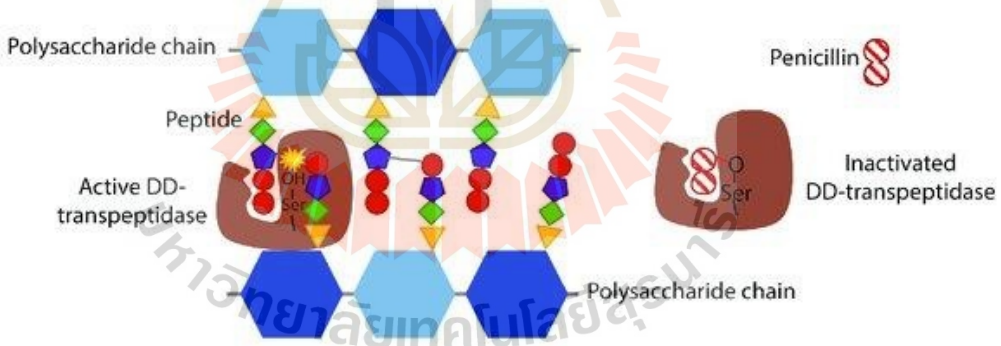


Figure 1.5 *Inhibition activity of Penicillin with peptidoglycan (Lobanovska and Pilla, 2017).*

Most bacteria that make the enzyme -lactamase, which breaks down the β-lactam ring, are resistant to antibiotics with the -lactam ring. Serine -lactamases and metallo-lactamases (MBL) are the two types of -lactamases. Furthermore, second-generation cephalosporins and essential serine—lactamases Include extended-spectrum lactamases (ESBL), which break down cephalosporins and carbapenem antibiotics like Klebsiella pneumoniae carbapenem (KPC). MBLs are enzymes that need Zn (II) to work. Their active site will break down almost all -lactam

antibiotics, including carbapenems. Recent global dispersion of Gram-negative bacteria with plasmid-encoded MBLs, such as the New-Delhi Metallo—lactamase (NDM-1), has raised the clinical significance of this class of β-lactamases.

Vancomycin is a glycopeptide. Glycopeptides are the class of chemicals to which vancomycin belongs. It is antimicrobial and can inhibit cell wall production—binding to the D-Ala-D-Ala terminal of the expanding peptide chain during cell wall formation. Vancomycin stops the transpeptidase from working, which stops the peptidoglycan matrix from getting longer and more cross-linked. Because vancomycin is a large, complicated molecule that binds to the end of the peptide chain of cell wall precursors, its action does not directly stop penicillin-binding proteins that are not affected by β-lactams. As a result, vancomycin is lethal to gram-positive bacteria.

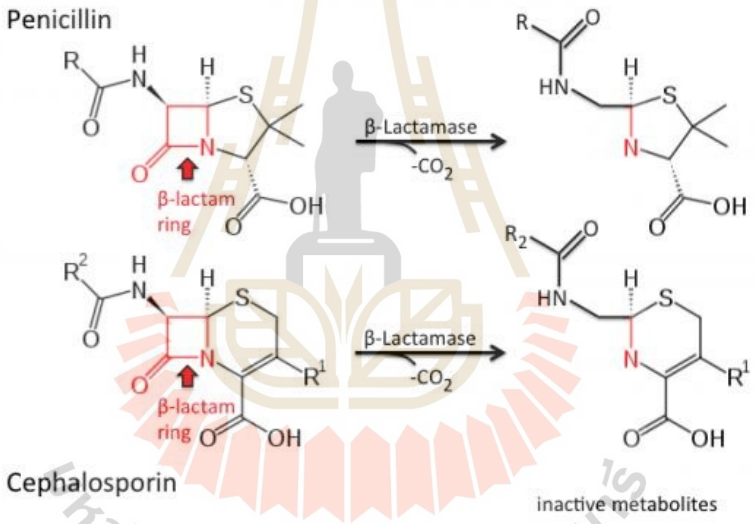


Figure 1.6 *Beta lactam core of penicillin, cephalosporin antibiotics and hydrolysis by beta-lactamas. Beta-lactam is the core component of penicillin, cephalosporin, and beta-lactamase-catalyzed antibiotics. Both penicillin and cephalosporins include a beta-lactam ring with four atoms. Certain gram-negative bacteria produce a family of enzymes called beta-lactamases. They make bacteria resistant to beta-lactam medicines by breaking down the ring, which stops the molecule from killing bacteria. There are four distinct classes of beta-lactamases with distinct substrate specificities. For example, clavulanic acid can inhibit some beta-lactamases while others remain unresponsive. (Zango and Abubakar Shawai, 2019).*

Vancomycin resistance is possibly developed by a peptidoglycan terminal different from the conventional D-Ala-D-lac instead of the usual D-Ala-D-Ala. Reducing vancomycin bind makes it unable to stop the creation of

cell walls. The production of erroneous peptides false binding sites that bind vancomycin and prevent. From attaching to its receptor or an increase in peptidoglycan that results in thickened cell walls are two possible mechanisms vancomycin-intermediate Staphylococcus aureus and glycopeptide-intermediate S. aureus can develop resistance. Additionally, S. pneumoniae exhibits a particular form of resistance due to a mutation in the sensor-response system that controls the autolysin activity necessary to kill specific bacteria. (Esmaeillou et al., 2017; Levine, 2006; Schäfer et al., 1996; Singh et al., 2018). (Figure 1.7).

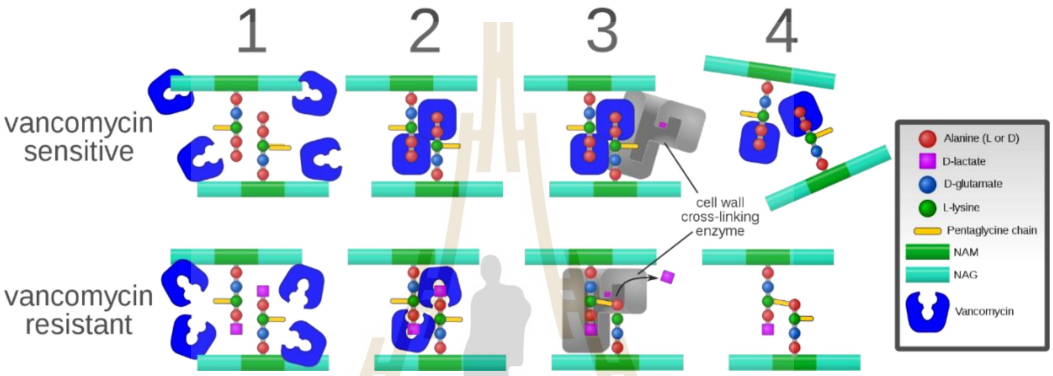


Figure 1.7 *Mechanisms of vancomycin action and resistance. 1) Vancomycin is added to the bacterial environment while it is trying to synthesize new cell wall. Here, the cell wall strands have been synthesized, but not yet cross-linked. 2) Vancomycin recognizes and binds to the two D-ala residues on the end of the peptide chains. However, in resistant bacteria, the last D-ala residue has been replaced by a D-lactate, so vancomycin cannot bind. 3) In resistant bacteria, cross-links are successfully formed. However, in the non-resistant bacteria, the vancomycin bound to the peptide chains prevents them from interacting properly with the cell wall cross-linking enzyme. 4) In the resistant bacteria, stable cross links are formed. In the sensitive bacteria, cross-links cannot be formed and the cell wall falls apart (Singh et al., 2018).*

Bacitracin is primarily bacteriostatic, but may have bactericidal activity depending upon the antibiotic concentration and the susceptibility of the bacteria. Bacitracin inhibits bacterial cell wall synthesis. This is achieved by preventing the final dephosphorylation step in the phospholipid carrier cycle, which interferes with the mucopeptide transfer to the growing cell wall (disrupts movement of peptidoglycan precursors). Bacitracin is active against many gram-positive and some gram-negative bacteria. Bacitracin resistance arises from a mutation of the bacitracin

permease and an active ABC-type efflux system resulting in losing control of antibiotic across the membrane (Choi et al., 2018; J. Ma et al., 2019).

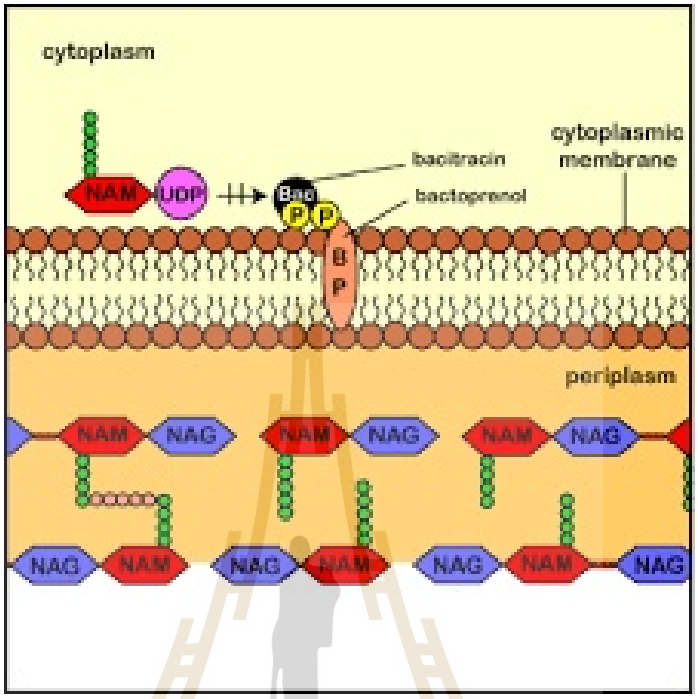


Figure 1.8 *Mode of Action Bacitracin (Kiran et al., 2021).*

1.2.3.2 Protein the making of protein

Interfering with the processes that directly create new proteins, a protein synthesis inhibitor slows or delays cell development or proliferation. Also, the look of ribosomes in animal cells (80S) differs from that in bacterial cells (70S). It makes protein synthesis a great selective target for antibiotics. Two types of inhibitors of protein synthesis exist. (Cocito et al., 1997, 1997; Damas et al., 2015).

Protein synthesis inhibitors that interact with the 30S subunit of bacterial ribosome

Aminoglycosides are antibacterial medicines that are large and highly polar compounds. These positively charged molecules require an energy-dependent active bacterial transport system, oxygen, and an active proton motive force, which lets the antibiotic enter the bacterium cell and bind to the 30S subunit of bacterial ribosomes. Aminoglycosides are effective broad-spectrum antibiotics, such as streptomycin, gentamicin, neomycin, and kanamycin. (Doi et al., 2016; Garneau-Tsodikova and Labby, 2016; Kulengowski, 2016). (Figure 1.9).

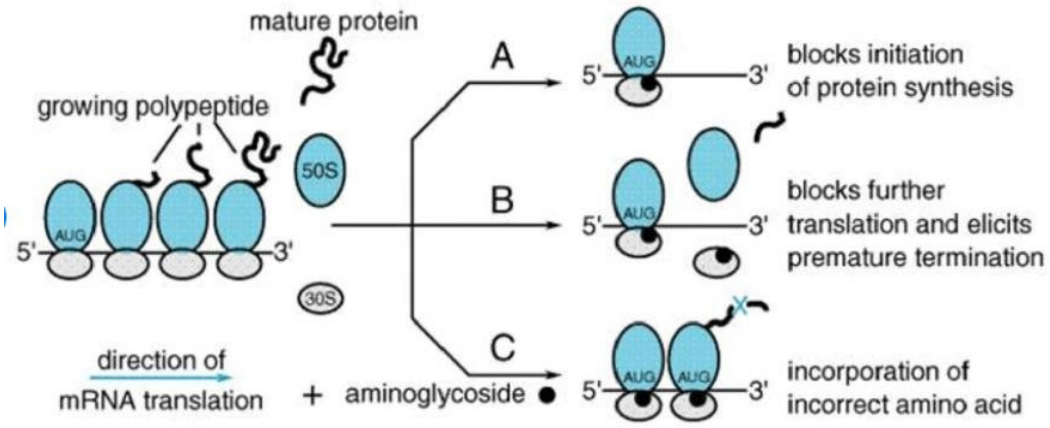


Figure 1.9 *Mechanism of action of aminoglycosides (Kulengowski, 2016).*

Two mechanisms to resistant to aminoglycosides are reduction in absorption and cellular permeability and the creation of enzymes that alter aminoglycosides.

Aminoglycoside resistant bacteria reduce cellular uptake or permeability due to a transport deficiency or membrane impermeability. Several strains of Pseudomonas aeruginosa and gram-negative bacilli resist to aminoglycosides. This process is likely chromosomally mediated and is responsible for cross-reactivity with all aminoglycosides. The observed amount of resistance is modest (i.e., intermediate susceptibility). Variations in Ribosome Binding Sites: Mutations at the aminoglycoside attachment site may inhibit ribosomal binding. This process can lead to streptomycin resistance because this drug binds to a single location on the 30S subunit of the ribosome. Since they can bind to many places on both ribosomal subunits and high-level resistance cannot be chosen in one step, this is a rare way for aminoglycosides to stop working (Mingeot-Leclercq et al., 1999; Doi et al., 2016).

The most prevalent form of aminoglycoside resistance is enzymatic modification. Over 50 distinct enzymes have been found. High-level resistance is caused by enzymatic alteration. The genes encoding aminoglycoside-modifying enzymes are usually found in plasmids and transposons. Multiple genes mediate the majority of gram-negative bacilli enzyme-mediated resistance. People think that the enzymes come from organisms that make aminoglycosides or from changes in the genes that code for the enzymes that help cells breathe(Mingeot-Leclercq et al., 1999; Doi et al., 2016; Garneau-Tsodikova and Labby, 2016).

Tetracyclines treat infections caused by susceptible bacteria, such as chlamydia, mycoplasma, protozoa, and rickettsia. Tetracyclines are different

from aminoglycosides. It stops bacteria from growing and prevents tRNAs from joining the ribosome during translation, which slows down protein production.

In a therapeutic setting, resistance to tetracycline is mostly caused by active efflux pumps and the production of ribosomal protection proteins (RPPs). (Figure 1.10). Along with enzymatic degradation, reduced drug permeability, and target mutation, enzymatic degradation is another way that resistance can develop. (Grossman, 2016; Nguyen et al., 2014; Sharma, 2021; Speer et al., 1992).

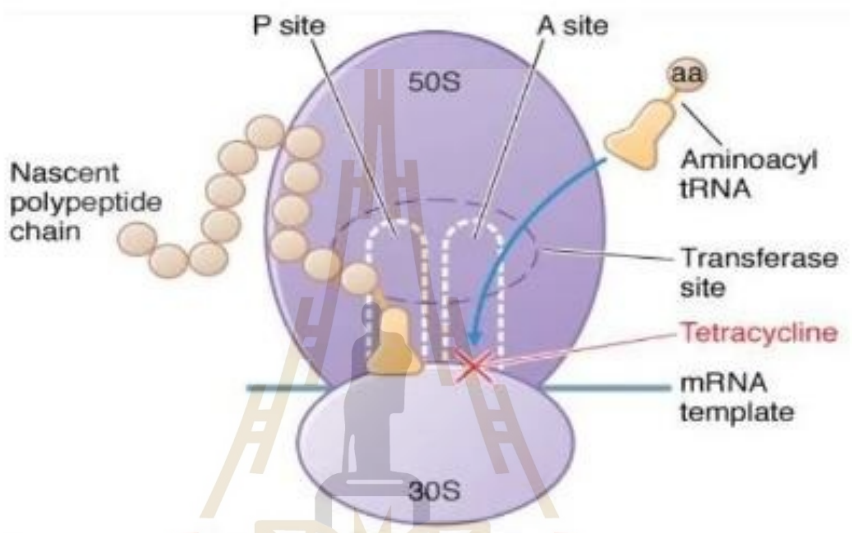


Figure 1.10 *Mechanisms of action and resistance to tetracyclines (Kiran et al., 2021; Sharma, 2021).*

Protein synthesis inhibitors that interact with the 50S subunit of bacterial ribosome

Macrolides are used to prevent and cure numerous bacterial diseases. Macrolides' ability to bind the peptidyl tRNA transfer from the A-site to the P-site is key to their mode of action. Moreover, partially block the bacterial 50S ribosomal subunit at the peptide escape tunnel (Figure 1.11). Therefore, macrolides have been viewed as tunnel plugs inhibiting and eliminating bacterial protein synthesis. It is frequently used to treat pneumonia, sinusitis, tonsillitis, and pharyngitis.

Bacteria can resist macrolide antibiotics in three ways: 1) by changing the target site through methylation or mutation, which stops the antibiotic from binding to its ribosomal target; 2) by getting rid of the antibiotic; and 3) by making the drug useless. These processes have been identified among macrolide and lincosamide manufacturers. They frequently employ multiple strategies to defend against the antimicrobials they produce. The incidence and clinical implications of

the three processes in pathogenic microorganisms are uneven. There is a contrast between efflux and inactivation, and modification of the ribosomal target confers broad-spectrum resistance to macrolides and lincosamides. (Fyfe et al., 2016; Leclercq, 2002; Schroeder and Stephens, 2016).

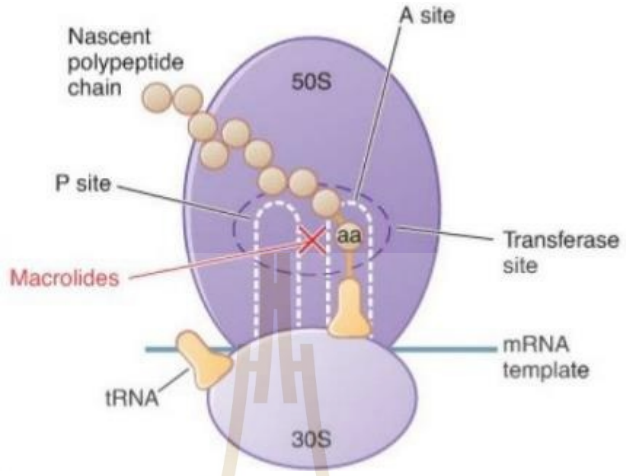


Figure 1.11 *Action mechanisms of macrolides (Kiran et al., 2021).*

1.2.3.3 Disrupt membrane integrity.

Cell membrane disrupting antibiotics are a subset of antibacterial agents that target the bacterial membranes. The majority of antibiotics in this class target at phospholipids in the cell membrane, influence the cell's physical features, such as its intrinsic curvature and fluidity.

Bacillus polymyxa was the first bacterium found producing polymyxins. The therapy of last resort is used to treat gram-negative bacterial infections. They contain features similar to detergents and are lipophilic. Polymyxins kill gram-negative bacteria due to an electrostatic interaction between the positively charged polymyxins and the negatively charged lipid A of the lipopolysaccharide. Given that Gram-positive bacteria lack an outer membrane containing Lipopolysaccharides (LPS), it is widely accepted that polymyxins are less effective against Gram-positive bacteria. Gram-positive bacteria create negatively charged teichoic acids, which may serve as polymyxin targets (X. Ma et al., 2018; O'Donnell et al., 2015; Satlin and Jenkins, 2017).

Developing tolerance to polymyxins has been linked to chromosomal alterations. This resistance happens when the LPS is changed by the pmrCAB operon, the phoPQ two-component system and its regulator mgrB, the pmrE gene, the pmrHFIJKLM operon, and the crrAB operon. This makes it impossible for the

LPS to get through the outer membrane of the bacteria. (Moffatt et al., 2019; Yu et al., 2015). (Figure 1.12).

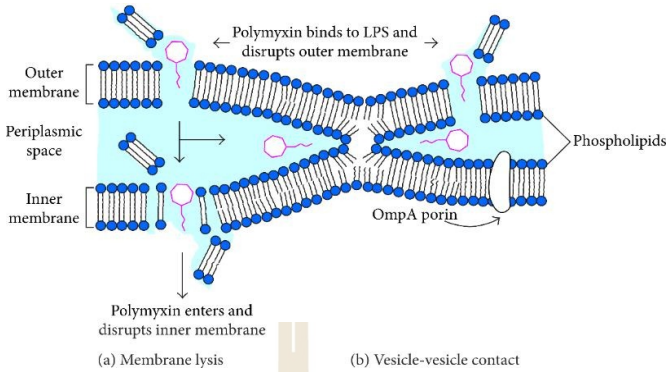


Figure 1.12 *The antibacterial properties of polymyxin. (a) the traditional process of membrane lysis, and (b) an alternate method of vesicle-vesicle interaction. (Yu et al., 2015).*

1.2.3.4 Stopping the making of nucleic acid

Antibacterial substances that inhibit the synthesis of nucleic acid are Classified in this class of antibiotic. Throughout a cell's existence, nucleic acids manage its metabolism, protein synthesis, enzyme production regulation, and genetic transmission.

Quinolones are antibiotics that inhibit topoisomerase, most often topoisomerase II (DNA gyrase), which is a key enzyme in DNA replication. DNA gyrase uses the energy from ATP hydrolysis to relax supercoiling DNA molecules. As a result, they make temporary breaks and fix phosphodiester links in super helical twists of closed-circuit DNA. DNA gyrase is an excellent target for quinolones because it is not found in eukaryotic cells, which are necessary for bacteria to grow. Now, we know that there are three different ways for quinolones to be resistant. These include mutations that change the drug targets (chromosomal changes in the genes that make the proteins), mutations that lower the amount of drug in the body, and plasmid-located genes associated with quinolone resistance. (Fàbrega et al., 2009; Hooper and Jacoby, 2015, 2016). (Figure 1.13).

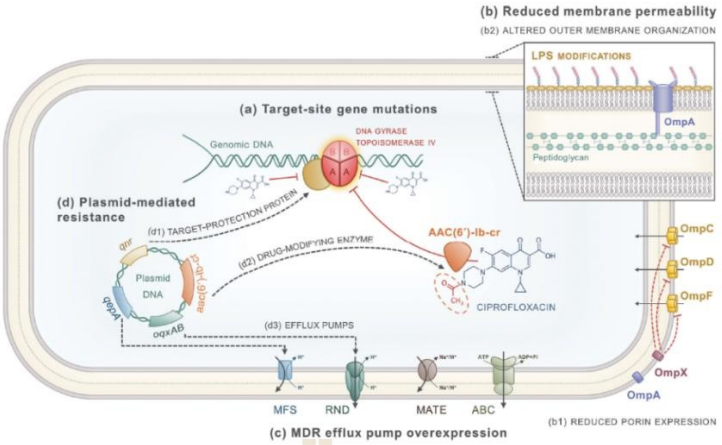


Figure 1.13 *Mechanisms of quinolone resistance. (Correia et al., 2017).*

Metronidazole is an antibiotic that suppresses anaerobic bacteria by cell membrane diffusion. The chemical structure of pyruvate-ferredoxin oxidoreductase is being altered. The decrease of metronidazole generates a concentration differential inside the cell that favors the absorption of additional medications and the generation of harmful free radicals. Then they interact with DNA, causing the loss of helical DNA structure and strand rupture. Therefore, it induces cell death in vulnerable species. Several processes may lead to metronidazole resistance, including lower absorption of the drug, higher clearance from the bacterial cell through efflux by decreasing the rate, and reduced metronidazole activation inside anaerobes (Dingsdag and Hunter, 2018, 2018) (Figure 1.14).

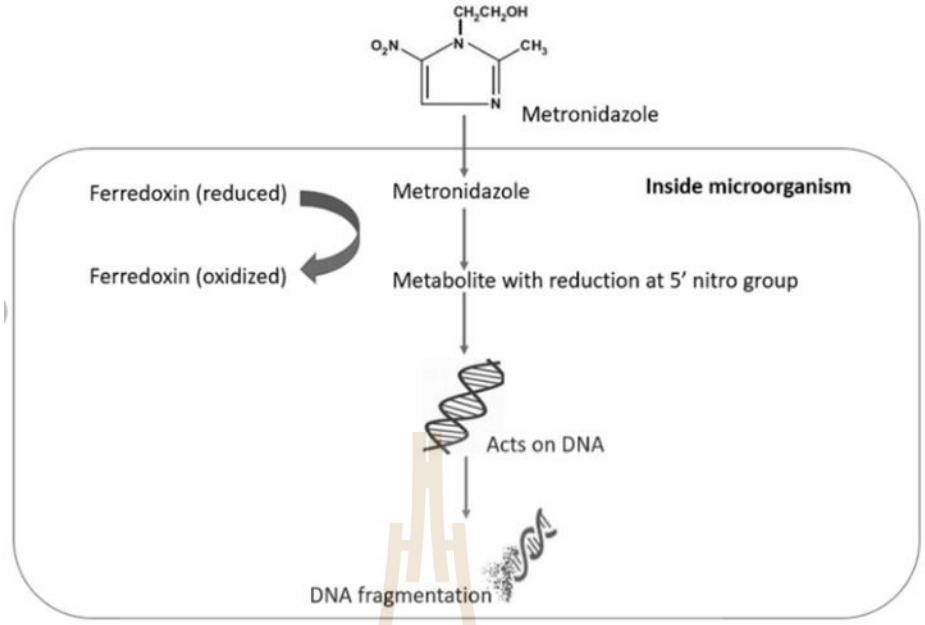


Figure 1.14 *Structure of metronidazole and its mechanism of action (Bhardwaj et al., 2009).*

RNA Synthesis Inhibitors

Rifampin is an antibiotic that can treat both mycobacterial and gram-positive bacterial infections. Also, some things stop bacterial DNA-dependent RNA polymerase from working. Rifampin occurs when a drug binds to the polymerase subunit deep inside the DNA and RNA channels, this stops RNA from being transcribed into a form that can be used to make proteins (Bliziotis et al., 2007; Portelli et al., 2020) Mutations that change the shape of the RNA polymerase beta subunit cause bacteria to be resistant to rifampin. Resistance to rifampin is not all or nothing. Scientists have found a wide range of RNA polymerases with different levels of sensitivity to rifampin (Wehrli, 1983; Goldstein, 2014; Cambau and Williams, 2015). (Figure 1.15).

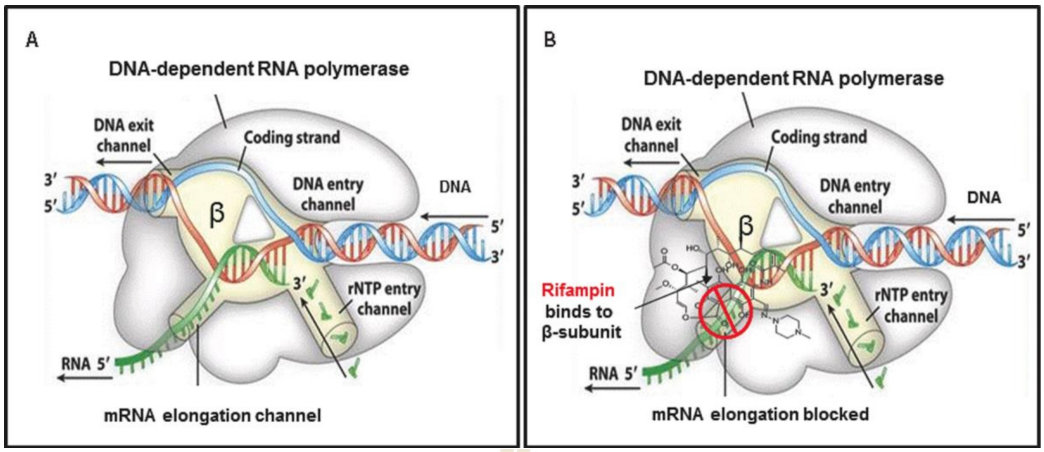


Figure 1.15 *Rifampin stops the DNA-dependent RNA polymerase from making RNA (Cambau and Williams, 2015).*

1.2.3.5 Inhibitors of metabolites.

Antimetabolites interfere by disguising themselves as metabolites. Blocking reduces its effectiveness by forming a non-covalent connection to active site of the particular enzyme. Sulfamide and trimethoprim are examples of drugs that limit DNA replication.

Sulfonamides are antimicrobials that inhibit bacterial development. Sulfonamides are structurally similar to para-aminobenzoic acid (PABA) and competitively inhibit dihydropteroate synthase. Sulfonamide resistance is caused by spreading exogenous folP or its parts from one pathogenic bacterial to another. Clinical resistance in gram-negative enteric bacteria is transferred by plasmids and affected by genes producing drug-resistant variants of DHPS enzymes. (Kim et al., 2019; Sköld, 2000; Wang et al., 2014). (Figure 1.16).

Trimethoprim hampered the conversion of tetrahydrofolate to dihydrofolate. Tetrahydrofolate is a crucial building block in the system that produces thymidine, and disruption of this mechanism prevents the production of bacterial DNA. Given that trimethoprim is regarded as bacteriostatic. It has bactericidal action when combined with sulfamethoxazole. Changes in cell permeability, loss of bacterial drug-binding ability, and overproduction of dihydrofolate reductase or mutations in dihydrofolate reductase can all lead to resistance to trimethoprim.(Kim et al., 2019; Sköld, 2000; Wang et al., 2014). (Figure 1.16).

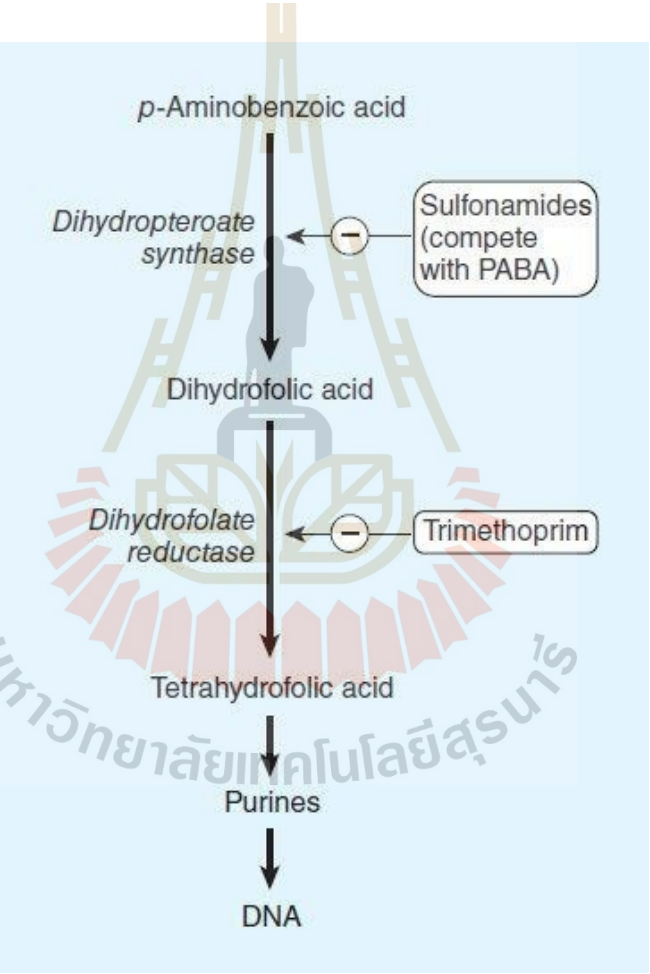


Figure 1.16 *Inhibition activity of sulfonamides and trimethoprim (Kim et al., 2019; Sköld, 2000; Wang et al., 2014).*

1.3 Multidrug resistance bacteria (MDR bacteria)

MDR bacteria were only found in hospitals, but now they can be found everywhere due to the globalization, are resulting of overuse of antibiotics in animal husbandry and aquaculture, the use of multiple broad-spectrum agents, and a lack of good antimicrobial stewardship. MDR bacteria are one of the most challenging things to deal with in the health system and pose a severe threat to public health. In the United States of America (USA), approximately 2.8 million antibiotic-resistant infections are reported annually. These infections cause over 35,000 deaths (Aslam et al., 2021; Pepi and Focardi, 2021). The Review on Antimicrobial Resistance says that by 2050, the antibiotic resistance crisis will severely threaten health worldwide and could lead to a pandemic. It will also be the leading cause of death (10 million deaths per year), more than cancer and HIV (Vivas et al., 2019).

ESKAPE is a group of bacteria that can evade commonly used antibiotics due to their increasing multi-drug resistance (MDR), is an acronym comprising: Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter spp. These bacteria are typical sources of hospital infections in severely ill and immunocompromised patients and healthy persons (Mulani et al., 2019a, 2019b; Rice, 2008). They are more resistant to antibiotics like penicillin, vancomycin, carbapenems, and others. Bacteria can develop antibiotic resistance by producing enzymes that attack the structure of the antibiotic (for example, β-lactamases, which prevent -lactam antibiotics from working), changing the antibiotic's target site so that it cannot bind properly, producing efflux pumps, and producing biofilm. Gram-negative bacteria have a part of their membrane called an efflux pump that constantly pumps out foreign substances, including antibiotics, so the inside of the cell never has a high enough drug concentration to have an effect. Biofilms are composed of different microbial communities and polymers that act as a physical barrier to keep antibiotics from killing the bacteria (Bennett, 2008; Lobanovska and Pilla, 2017; Pal, 2017; Sanz-García et al., 2021; Seukep et al., 2020).

1.4 Treatment of MDR Bacteria

Strategies to fight against MDR bacteria comprise developing of new drug, antibiotic synergy and phage therapy (Bayer et al., 1980; Brives and Pourraz, 2020; Hooper and Jacoby, 2015; Thakuria, 2013).

1.4.1 Developing of new drug

The process of identifying new antibiotics to combat MDR is called drug discovery. It may take years or decades to discover a new medication. The first antibiotic to be found was penicillin, which Alexander Fleming discovered in 1928 (Aminov, 2010; Hutchings et al., 2019). The discovery led to the creation of penicillin and other medicines. Their search process by the first step in drug discovery is finding an antibiotic from bacteria or fungus and can be done by looking for inhibits bacterial growth or killing bacteria. This can be done through various techniques, including screening, especially Soil-screening identify antibiotic-producing microorganisms (Cycon' et al., 2019b; Shetty et al., 2014; Suchada et al., 2008). Then Design and development drug are techniques used to find a new antibiotic to identify the specific antibiotic designed of molecules that relies on the knowledge of the three-dimensional structure (structure-based drug design), target to specific enzymes bacterial, cell walls synthesis, and other essential components, and target a wide range of bacteria by using antibiotics that are active against a broad spectrum of bacteria or drugs that have been designed to interfere with the synthesis of bacterial cell walls. Last test drugs analyze interactions with other antibiotics to ensure that they do not interfere with other treatments or become dangerous for humans (Chhibber et al., 2018; Choi et al., 2018; Gajdács, 2019; S.-F. Zhou and Zhong, 2017). (Figure 1.17).

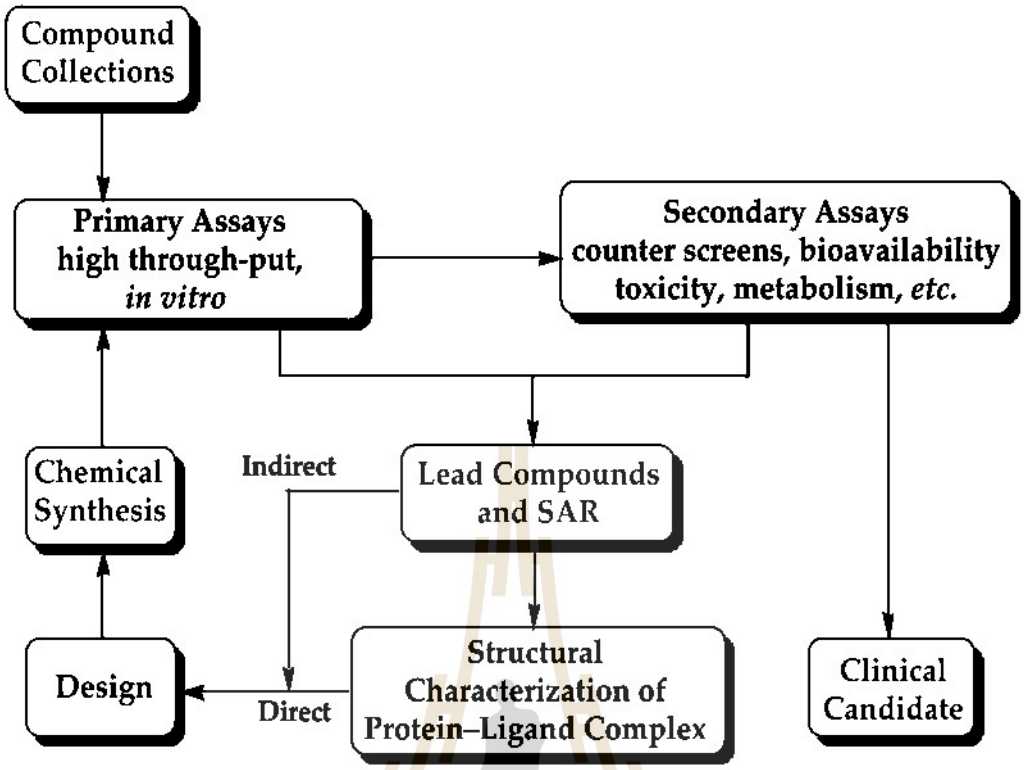


Figure 1.17 *Schematic diagram of the drug discovery and development process. (Shihab, 2020).*

1.4.2 Antibiotic synergy

Antibiotic synergy is when two or more antibiotics work together to have a more significant effect than if given separately. Compare the synergistic influence with the additive and antagonistic impacts. In the Bayer et al study from 1980, penicillin G worked better with streptomycin and gentamicin against 17 and 16 strains, respectively, while ampicillin-aminoglycoside combinations worked better with 12 and 15 pathogens. Similar to Magainin II exhibited synergistic effects with ceftriaxone, amoxicillin-clavulanate, ceftazidime, meropenem, piperacillin, and β-lactam antibiotics. (Y. Zhou and Peng, 2013). (Figure 1.18)

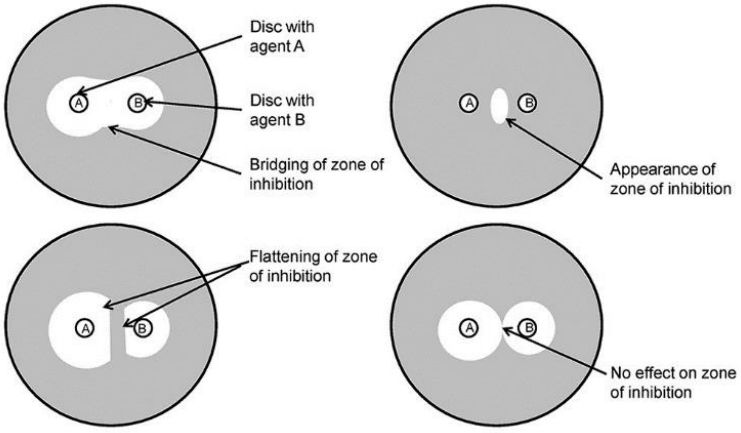


Figure 1.18 *Assessing synergy with the double-disk technique. (a) Antagonism (a widening of the zone of inhibition); (b) synergy (a narrowing of the zone of inhibition); (c) indifference/additive (no effect on the zone of inhibition); and (d) synergy (a new zone of inhibition appearing between agent A and B) are the four possible interactions. (Laishram et al., 2017).*

1.4.3 Phage therapy

Phage therapy is commonly referred to as viral phage therapy, treats bacterial illnesses. Viruses that infect bacteria are known as phages or bacteriophages. They solely target pathogenic bacterial infections; phages are non-toxic to humans, animals, and plants. Bacteriophages are bacteria's natural enemies. The term bacteriophage translates to "bacteria eater"; they are found in dirt, sewage, and other environments where bacteria thrive. Attaching to bacterial cells, the virus copies its DNA or RNA into them. By producing the viral genome, bacteria prevent the virus from functioning, ending the bacterial infection. The phage virus replicates itself within the bacteria. Virus cloning can produce up to one thousand trusted Sources of new viruses per bacteria. Finally, the virus penetrates the bacterial cell wall and releases new bacteriophages. Phages are only effective against specific bacterial strains; once all the bacteria have been lysed (killed), they will stop proliferating. Similar to other viruses, phages can hibernate until additional bacteria appear. This is a downside of phage therapy, as a phage can only kill a bacteria if it fits its specific strain (Brives and Pourraz, 2020; Liu et al., 2022; Wei et al., 2020).

1.5 Research objectives

The research aims to find new antibiotics for fighting against MDR by discovering bacteria producing.

1.5.1 To isolate and identify new antibiotic producing bacteria.

1.5.2 To extract, purify, and characterize of the active compound.

CHAPTER II
LITERATURE REVIEW

Competing for nitrogenous nutrients in soil is a key for bacterial survival. Several microorganisms have developed methods of suppressing their neighbors for the advantage of their own development. Fungi and bacteria are known to produce a vast array of antibiotics as a natural defensive mechanism and for nutrient competition. Most of news classes of antibiotic were discovered from bacteria. Because of huge biodiversity and high competition for nutrients of bacteria in soil, screening for new types of antibiotics in soil still stands a chance.

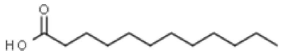
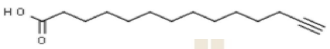
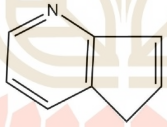
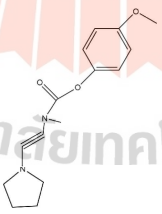
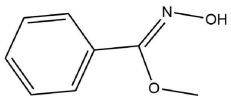
2.1 Antibiotics from bacteria

2.1.1 Gram-negative bacteria producing antibiotics

The most common gram-negative bacteria found to produce antibiotic is *Escherichia coli*.

E. coli is a gram-negative, facultatively anaerobic, rod-shaped bacterium. The cell is typically about 2.0 μm long and 0.25–1.0 μm in diameter. E. coli is commonly found in the lower intestine of warm-blooded organisms. Most E. coli strains are harmless, but some strains can cause serious food poisoning in the human gut and cause disease in their hosts. It lives on various substrates and uses mixed acid fermentation in anaerobic conditions, producing lactate, succinate, ethanol, acetate, and carbon dioxide. Research of Nadia Altaee (Nadia et al., 2016) have shown that E. coli produces several active compounds which have activity in anti-inflammatory, anti-diabetic, antioxidant, antibacterial. (Table 2.1)

Table 2.1 Bioactive chemical compounds identified in methanolic extract of *E. Coli.* (Nadia et al., 2016).

No.	Name	Structure	Molecular Weight	Pharmacological activity
1	Dodecanoic acid, 3-hydroxy		216.1725445	Anti-apoptotic and anti-inflammatory
2	13-Tetradecynoic acid, methyl ester		238.19328	Antitoxin and anti-inflammatory
3	12, 15-Ovtadecadiynoic acid		290.22458	Antioxidant, anti-inflammatory and antimicrobial
4	9-Tetradecen-1-ol		254.22458	Anti-inflammatory
5	1-Propornamine, 3 (methylthio)		105.06122	Anti-inflammatory and analgesic
6	Benzeneethanamine		121.0891495	Antimicrobial, Anti-inflammatory
7	5H-Pyrindine		117.078494	Anti-inflammatory, analgesic activates
8	4-Methoxyphenoxyformanide, methyl-N-[4-(pyrrolidinyl)-2		302.163042	Anti-inflammatory
9	Oxime-,methoxy-phenyl		151.063329	Antimicrobial

2.1.2 Gram-positive bacteria producing antibiotics

Actinomyces and Streptomyces, high G+C gram-positive bacteria, are the most common genera of the Actinobacteria class that are found to produce antibiotic. One of the most interesting genera in the class of Bacilli are Lactococcus and Bacillus, newly found to produce various active compounds.

2.1.2.1 Actinomyces

Actinomyces is a genus in the *Actinomycetia* subclass of Actinobacteria. All species in this genus are gram-positive, rod-shaped, and soil-dwelling. *Actinomyces spp*. exhibit facultative anaerobiosis (except *A. meyeri* and *A. israelii* are anaerobes). Some species generate endospores. The hyphae networks of Actinomyces colonies resemble those of fungi. *Actinomyces spp*. are widespread, appearing in soil and animal microbiomes, including the human microbiota. They are well-known for their crucial function in soil ecology; they generate a variety of enzymes that aid in the degradation of organic plant material, lignin, and chitin. Consequently, their existence is essential for the creation of compost. Actinomycetes are significant due to their ability to produce diverse classes of antitumor agents (e.g., doxorubicin and bleomycin), antifungal agents (e.g., amphotericin B and nystatin), immunosuppressive agents (e.g., FK-506 and rapamycin), insecticides (e.g., spinosyn A and avermectin B), and herbicides Current research indicates that *Actinomycetes spp*. are also a valuable resource for discovering novel natural antibiotics such as Bafilomycins, neomaclafungins, rosaramicins, spinosyns, tiacumicins, pikromycin, chartreusin, etc (De Simeis and Serra, 2021; Ezeobiora et al., 2022; Lo Grasso et al., 2016; Mast and Stegmann, 2019).

2.1.2.2 Streptomyces

Streptomyces is gram-positive, spore-forming bacteria with a filamentous shape resembling fungi. They can flourish in various habitats. *Streptomyces* produces aerial hyphae when resources are insufficient, resulting in sporulation to withstand harsh conditions to translocate to other locations or nutrient sources. Streptomyces is well known to produce number of complex secondary metabolites with various bioactive activities such as antifungals, antivirals, antitumoral, antihypertensives, and antibiotics. (Table 2.2). Almost all of *Streptomyces* bioactive substances are started at the same time with the aerial hyphal development. More than two-thirds of the clinically relevant natural antibiotics, including streptomycin, chloramphenicol, daptomycin, tetracycline, etc., are produced by *Streptomyces*. In addition, studies revealed that adding *Streptomyces* as probiotics in aquaculture by mixing them to feed might improve the

growth of aquatic creatures and shield fish and shrimp from infections (Procópio et al., 2012; Quinn et al., 2020; Rajan and Kannabiran, n.d.; Shetty et al., 2014).

2.1.2.3 Lactococcus

L. lactis is a non-motile, gram-positive, no spore forming coccus. It has oval shape with average length between 0.5 and 1.5 μm. Since ancient times, cheese, yogurt, and sauerkraut have been fermented using *L. lactis*, a lactic acid bacterium (LAB). Lactic bacteria are found in the commensal gut flora of both humans and animals. Antimicrobial compounds produced by LAB have a strong antagonistic effect on various pathogenic pathogens. The different metabolites seem to have a multifaceted role in the mechanisms underlying the LAB activity against infections. Earlier research revealed that the *Lactococcus* create several bactericidal substances. According to the evidence, some strains of *L. lactis* produce antibiotics called nisin (Figure 2.1) that has antimicrobial activity against pathogenic bacteria like *E. coli*, *Enterococcus feacalis*, *Pseudomonas aeruginosa, Staphylococcus aureus*, problematic pathogens in the ESKAPE group (Enan et al., 2013; Khemariya et al., 2013; Song et al., 2017; Soundharrajan et al., 2021).

Table 2.2 List of some antibiotics produced by *Streptomyces sp*. (Procópio et al., 2012; Quinn et al., 2020).

No.	Streptomyces sp.	Antibiotic	No.	Streptomyces sp.	Antibiotic
1	*S. orchidaccus*	Cycloserin	17	*S.ambofaciens*	Tetracycline
2	*S.oriantalis*	Vancomycin	18	*S.avermitilis*	Spiramycin
3	*S.fradiae*	Neomycin, Actinomycin, Fosfomycin, Dekamycin Amphotricin B	19	*S.alboniger*	Avermicin
4	*S.nodosus*	Nistatin	20	*S.niveus*	Puromycin
5	*S.noursei*	Rifampin	21	*S.platensis*	Novobicin
6	*S.mediterranei*	Streptomycin	22	*S.roseosporus*	Platenmycin
7	*S.griseus*	Kanamycin	23	*S.ribosidificus*	Daptomycin
8	*S.knanamyceticus*	Tobramycin	24	*S.garyphalus*	Ribostamycin Cycloserine
9	*S.tenebrarius*	Spectinomycin	25	*S.vinaceus*	Viomycin
10	*S.spectabilis*	Tetracycline	26	*S.clavuligerus*	Cephalosporin
11	*S.viridifaciens*	Lincomycin,	27	*Streptomyces spp.*	Oligomycin

Table 2.2 List of some antibiotics produced by *Streptomyces sp*. (Procópio et al., 2012; Quinn et al., 2020). (Continued)

No.	Streptomyces sp.	Antibiotic	No.	Streptomyces sp.	Antibiotic
12	*S.lincolensis*	Clindamycin	28	*Streptomyces spp.*	Pyrroles
13	*S.rimosus*	Oxytetracyclin	29	*S. lavendulae*	Mytomycin C
14	*S.erythraeus*	Antibiotic	30	*S. antibioticus*	Actinomycin D
15	*S.vensuella*	Erythromycin	31	*S. parvulus*	Actinomycin D
16	*S.aureofaciens*	Chloramphenicol Chlortetracycline, Dimethylchlor	32	*S. clavuligerus*	Clavulanic acid

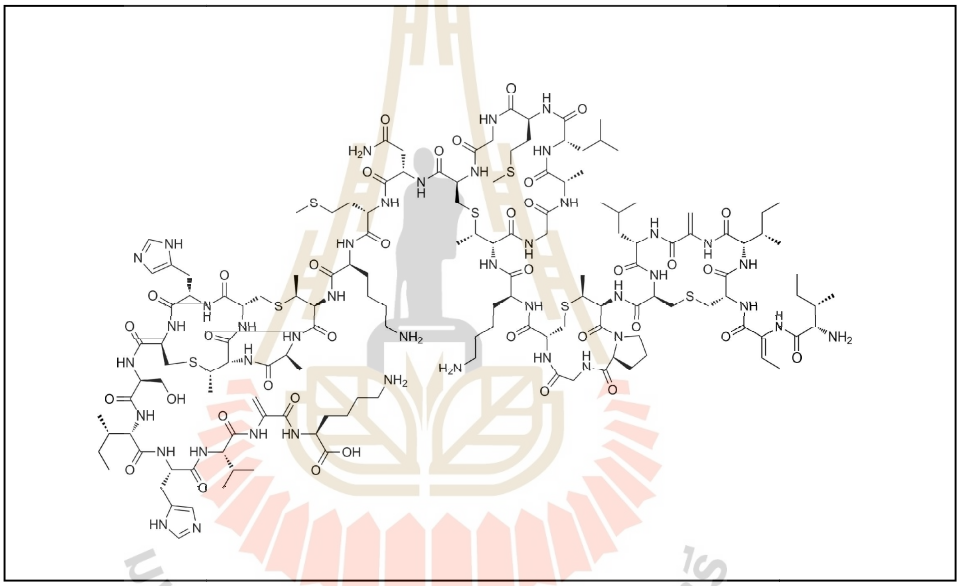


Figure 2.1 Antibacterial of nisin (Salmieri et al., 2014).

2.1.2.3 *Bacillus*

Bacillus is a genus of more than 300 species of spore forming gram-positive, rod-shaped bacteria. There are 1389 *Bacillus* strains have been found to have antimicrobial activity consist of 27 different species of metabolites. Their mechanisms of action have been characterized. Based on how they are made, peptide antibiotics from Bacillus species can be put into two groups. One of these subgroups consists of tiny microbial peptides created nonribosomally by large enzyme complexes, whereas the second group consists of ribosomally synthesized peptides (Caulier et al., 2019; Tran et al., 2022).

Their mechanisms of action have been characterized. Based on how they are made, peptide antibiotics from Bacillus species can be put into

two groups. One of these subgroups consists of tiny microbial peptides created nonribosomally by large enzyme complexes (gramicidin, tyrocidine, bacitracin, surfactin, iturins), whereas the second group consists of ribosomally synthesized peptides (glycocins, subtilisin, mersacidin). (Nakano and Zuber, 1990; Sumi et al., 2015)

2.2 Extraction and purification of antibiotics

Common techniques to extract bioactive compound from nature are solvent extraction, ultrasound, Soxhlet and microwave (Borges et al., 2020). Antibiotics from bacteria are active compounds that bacteria secrete out of the cells to inhibit their neighbor, most of the bacterial antibiotics dissolved in water and submerge culture is the most common to grow bacteria therefore solvent extraction is the most prevalent technique for the extraction. The procedure to extract and purify antibiotic from bacterial culture includes liquid-liquid extraction, concentration, chromatography, and crystallization (Idris and Mohd Nadzir, 2021; Skariyachan et al., 2014). The four steps in solvent extraction are 1) the solvent is introduced; 2) the solute dissolves in the solvents; 3) the solute is diffused out, and 4) the extracted solutes are gathered. Any component that increases diffusivity and solubility throughout the processes will aid the extraction. The extraction efficiency is affected by the characteristics of the extraction solvent, the particle size of the raw materials, the particle size, the solvent-to-ratio, polarity, and extraction time. The choice of solvent is a key of success in solvent extraction. According to the law of similarity and intervisibility (like dissolves like), solvents with a polarity value close to the solute's polarity are likely to perform better, and vice versa. Although alcohols are ubiquitous solvents for the solvent extraction, the solvent can be selected according to its properties (Alshammari et al., 2021; R. R. Kumar and Jadeja, 2018)

2.2.1 The criteria for the solvent selection.

The solvent for antibiotics extraction should have properties as described.

2.2.1.1 Immiscible pair of solvents.

A pair of immiscible solvents in the sample must be incompatible with the solvent extraction solution. For example, a water-based solution is typically extracted using an organic solvent. Therefore, organic solvents with strong polarities, like methanol, ethanol, and acetone, should be used to extract a sample. However, because they are miscible with water, they are unsuitable for liquid-liquid extraction; organic extracting solvents with low polarities, such as hexanes, toluene, dichloromethane, and diethyl ether, are typically used. (Castro and Alvarez-Sánchez, 2008; Kaczmarski et al., 2006; Kleiman et al., 2016). (Table 2.3).

Table 2.3 Polarity index of solvents. (Kaczmarski et al., 2006; Kleiman et al., 2016).

Solvent	Polarity index
Hexane	0.1
Isopropyl ether	1.83
Toluene	2.4
Benzene	2.7
Dichloromethane	3.1
Isopropanol	3.92
Ethyl Acetate	4.4
Methanol	5.1
Acetone	5.1
Ethanol	5.2
Acetonitrile	5.8
Dimethyl sulfoxide	7.2
Water	10.2

2.2.1.2 Select a solvent for the desired chemical.

The solute and solvent's physical and chemical characteristics are responsible for checking a structure's dissolve functions group. Common solutes will dissolve more effectively in similar solvents. For example, polar and nonpolar solutes dissolve more effectively in polar and nonpolar solvents. However, if this is problematic, larger molecules will be surrounded by dispersed molecules, and smaller molecules will result. (Kaczmarski et al., 2006; Kleiman et al., 2016; Sherwood, 2013).

CHAPTER III
MATERIALS AND METHODS

3.1 Materials

3.1.1 Equipment for screening of antibiotic-producing bacteria

Materials, media and chemicals used in Screening of antibiotic-producing bacteria and their sources are shown in Table 3.1

Table 3.1 Materials, media and chemicals used in for screening of antibiotic-producing bacteria.

Materials/Reagent/Chemicals	Company
Glove	commercial grade
Plastic bottle sterilization	commercial grade
Sterile Sampling Spoon	commercial grade
Plastic test tube 50 ml	Nuce
Distilled water (DI water)	Lab analysis
Sodium choline (NaCl)	commercial grade
Alcohol lamp	Lab analysis

3.1.2 Microbiological study method

3.1.2.1 Selective medium chicken feather (CF medium)

Weight 2 grams chicken feather, 0.27 grams NaCl with 18 ml, when we were preparing LB agar (Table 3.2). The sample CF medium was sterile at 15 psi, 121°C, for 15 to 20 minutes and we used the screening method.

3.1.2.2 Luria-Bertani medium agar (LB agar)/ Luria-Bertani medium broth (LB).

Weight 10 grams Peptone, 5 grams Yeast extract and 5 grams NaCl in with 1000 ml DI water (Table 3.1). (Add agar 15 grams, when we were preparing LB agar (Table 3.2). The sample agar or broth were sterile at 15 psi, 121°C, for 15 to 20 minutes.

3.1.3 Sequence of a pair of primers for the 16sr RNA sequencing

27F 5'-AGAGTTTGATCCTGGCTCAG-3'

1492R 5'-GGTTACCTTGTTACGACTT-3'

3.1.4 Microorganisms used in this work

Bacillus cereus

Bacillus subtilis.

Escherichia coli ATCC25422

Pseudomonas aeruginosa ATCC27853

Shigella flexneri.

Staphylococcus aureus

3.1.5 Equipment and chemical for extraction and purification

Materials and chemicals used in Equipment and chemical for extraction and purification are shown in Table 3.2.

Table 3.2 Materials, media and chemicals used in for screening of antibiotic-producing bacteria.

Materials/Chemicals	Company
Plastic test tube 50 ml	Nuce
Distilled water (DI water)	Lab analysis
UV lamp	Anatech
Sodium sulfate anhydrous crystal	carlo erba
Silica gel 60 (0.040-0.063 nm)	Merck
DMSO	
Hexane	commercial grade
Ethly acetate	commercial grade
Acetone	commercial grade
Thin layer chromatography (TLC)	Merck

3.2 Method

3.2.1 Screening of bacteria produce antibiotic from soil samples (colony with clear zone)

The soil samples were taken from a slaughterhouse, a pigsty, a pig manure wastewater treatment plant, and a biogas generator of the pig farm around Nakhon Ratchasima province, Thailand (Figure 3.1). An aseptic approach was used to take soil samples at a depth of 3 – 5 cm below the ground. A soil suspension was made by adding 10 g of the soil sample in 20 mL of 0.85% NaCl and filtering by Whatman filter paper No. 42. Two milliliters of each filtered sample were inoculated

into 18 mL of CF-medium and incubated at 30°C, shaking with 200 revolutions per minute for 15 days in shaker incubator.

Figure 3.1 The sampling sites at the pig farm A) slaughterhouse, B) a pigsty, C) pig manure wastewater treatment plant, and D) biogas generator.

The 4 samples of the 15 days bacterial culture from the first step were diluted with ten-fold serial dilution as shown in Figure 3.2. (Al-Dhabaan and Bakhali, 2017). The 10^{-7} to 10^{-9} were spread into LB agar plates, incubate at 30°C for 24 hr. The bacterial colony with the inhibition zone around was observed and isolated in the next step.

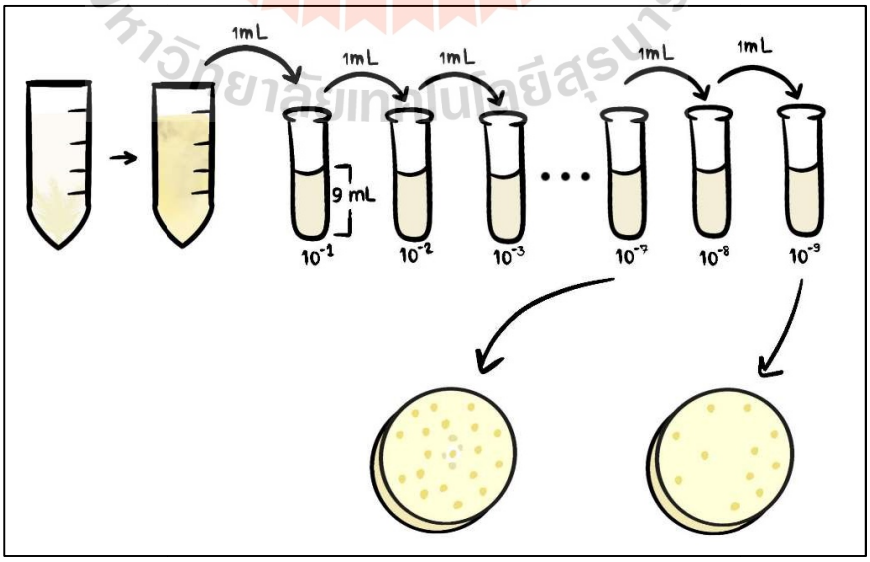


Figure 3.2 Serial dilutions method: 1 mL of the bacterial suspension added into 9 mL of diluent, 0.9 % NaCl (Modified from Cotton et al., 2019).

3.2.2 Isolation, characterization, and identification (16sr RNA sequencing).

The colonies with clear zone from the step 3.2.1 were isolated by streak plate method on LB agar plates (Figure 3.3). The LB agar plates were incubated at 30°C for 24 hr.

Morphology of isolated colony was observed by a stereo light microscope. Gram staining was used to investigate the type of cell of the isolated bacterial clone.

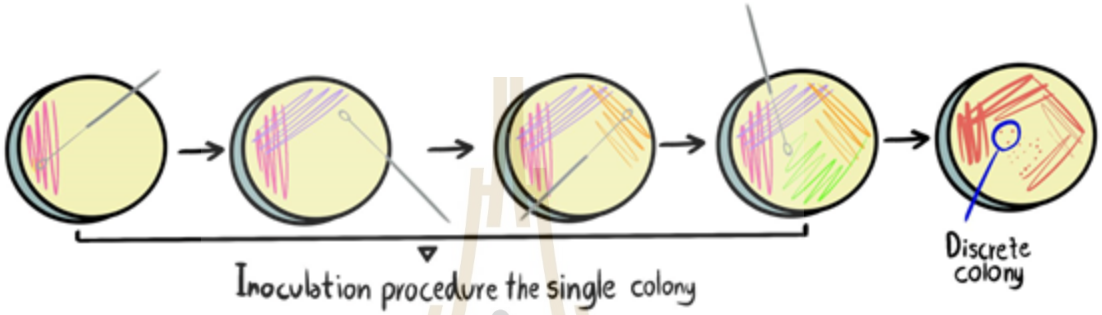


Figure 3.3 Cross streak plate method. (Modified from Zahrani et al., 2017).

The isolated colony was sent to Thailand Institute of Scientific and Technological Research (TISTR) for the 16S rRNA sequencing. The 16S rRNA sequences were analyzed by using the MEGA-X (S. Kumar et al., 2018) to compare with the database from the National Center for Biotechnology Information (NCBI) and the Ezbiocloud.

3.2.3 Antimicrobial activity of Bacillus siamensis

The plug diffusion technique (Balouiri et al., 2016) was performed to investigate inhibitory activity of B. siamensis. The standard 5 mm paper discs were soaked with 25 μL of B. siamensis liquid culture and placed on LB-agar plates containing test bacteria Bacillus cereus, Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa, and Shigella flexneri, Staphylococcus aureus. The plates were then incubated in an incubator at 30°C for 24 hr.

3.2.4 Extraction of the active compounds from the culture medium (agar and broth)

Initial extraction: The active compounds were extract by excised agar medium at the clear zone around the colony and soaked in ethyl acetate at 30°C for 24 hr. The extract solution was dry by vacuum-rotary evaporator, to remove the solvent. The dried extract was kept in microcentrifuge tube and stored at 4°C to be used as a control for thin-layer chromatography (TLC) analysis.

Liquid culture extraction: *B. siamensis* cultured in 1000 mL of LB broth at 30°C, shaking with 200 rpm for 3 days in shaker incubator. Then centrifuge the bacterial culture at 3500 rpm 25°C for 30 min to collect the supernatant for the extraction. The active compounds were extracted by 500 mL of Ethyl acetate, twice. The extract was dried by vacuum rotary evaporator. The dried extract was dissolved in 1 mL of ethyl acetate, transferred to an Eppendorf tube, and stored at 4°C. The presence of the active compounds was confirmed by thin-layer chromatography (TLC). Each 10 µL of the samples were loaded onto the TLC plate and used 20% ethyl acetate in hexane a mobile phase. The TLC band was visualized by 244 nm and 365 nm UV light. (Caulier et al., 2019; Kanwar, 2018; Sherwood, 2013)

3.2.5 Large scale preparation and Purification of active compounds by silica gel column chromatography

On a larger scale, the dry crude extract was prepared in the same way as describes in the Liquid culture extraction step of the 3.2.4. The dry crude extract was dissolved with 3 mL of ethyl acetate. The 3 mL sample solution was loaded in the silica gel column that was equilibrated with hexane. The compounds were stepwise eluted with each 300 mL of the various ratio of ethyl acetate: hexane, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, and 100% ethyl acetate. The chromatographic fractions were collected at every 100 mL (Ngo and Chua, 2019).

There were 33 fractions in total from the chromatographic step. Each fraction from the chromatography was dried by vacuum rotary evaporator and weighed by electronic digital balance. The dry sample of each fraction was dissolved by 50 µL of ethyl acetate. The dissolved samples were analyzed by stepwise thin-layer chromatography (TLC), using various ratio of ethyl acetate: hexane, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% and 100% hexane as mobile phases. The TLC band was visualized by 254 nm and 365 nm UV light. (Caulier et al., 2019; Kanwar, 2018; Sherwood, 2013)

3.2.6 Bacterial growth inhibition of the extracted compounds

The dissolved samples from the step 3.2.5 were freeze dried by speed vacuum and dissolved by 10 µL of DMOS (Balouiri et al., 2016). The bacterial growth inhibition activity of the dissolved samples from this step were investigated by disc diffusion method (Sherwood, 2013), using *S. flexneri* as a susceptible strain, on LB-agar plate, incubated at 30°C for 24 hr.

CHAPTER IV
RESULTS AND DISCUSSION

4.1 Results

4.1.1 Screening of bacteria produce antibiotic from soil samples (colony with clear zone)

The soil suspension collected from the 4 sites of the pig farm, slaughterhouse, pigsty, pig manure wastewater treatment plant, and biogas generator, as shown in Figure 4.1, were prepared as described in 3.2.1. The growth of bacteria in the CF-medium is shown in the Figure 4.2B in comparing with 4.2A. In the when we isolated antibiotic-producing bacteria from the CF medium (Figure 4.3B). The growth of bacteria were found in pigsty, suggestion that there were bacteria that can utilize chicken feather as nutrient.

The bacterial suspension from the pigsty was proceed to the screening as described in screening step of 3.2.1. Several colonies of bacteria with clear zone around the colonies were found as shown the Figure 4.2C. Antibiotic-making bacteria were found in the clear zone of 10^{-7} on the LB agar plate, which contained bacteria-producing antibiotics isolated from a pigsty. It was discovered to be a clear zone (Figure 4.3C). The result indicated that the bacteria can grow and try to compete for the limit nutrient by secreting active compounds and diffused through the agar to inhibit growth of other bacteria nearby. (Azam et al., 2015; Brives and Pourraz, 2020; Tacconelli et al., 2018).

Figure 4.1 Soil sample and washed by 0.85% NaCl and filtered in centrifuge tube 15 ml. 1) slaughterhouse, 2) pigsty, 3) pig manure wastewater treatment plant, and 4) biogas generator.

4.1.2 Isolation, characterization, and identification (16sr RNA sequencing)

The single colonies of bacterium inhibition zone from Figure 4.2C was able isolated from the screening plate, using the streak plate method, are shown in Figure 4.3A. The growth inhibition activity of the isolated clone was confirmed by producing clear zone in the LB-agar plate that had the *S. flexneri* as a susceptible strain (as described in 3.2.3).

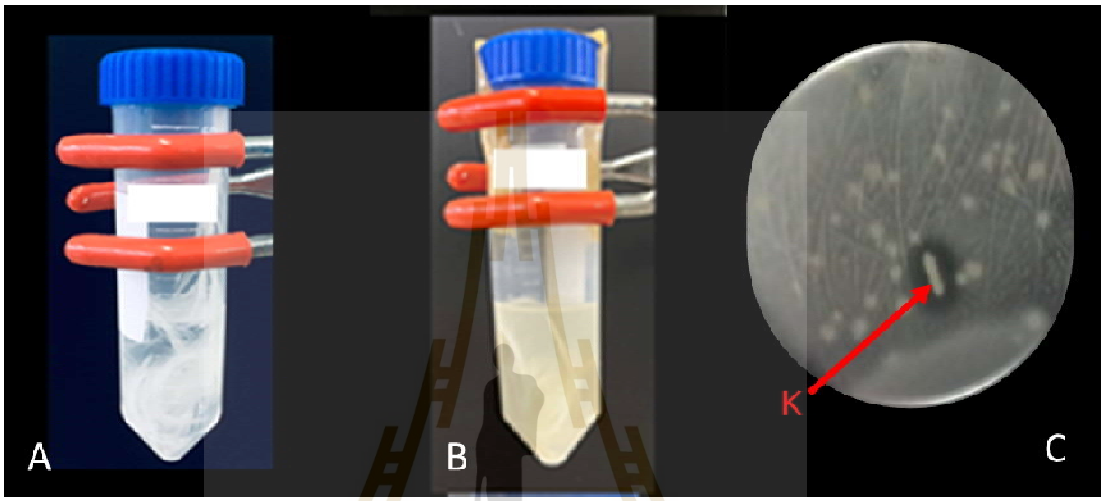


Figure 4.2 (A) Chicken feathers media before incubating, (B) chicken feathers after 15 days, (C) Example of bacteria colony with clear zone, named as K.

Stereo microscope (25X magnification) showed that the isolated K bacterium displayed a white colony with irregular, undulating, crater-shaped (Figure 4.3A). Gram-straining showed that the isolated bacterium is a spore forming gram positive bacilli. (Caulier et al., 2019, 2019; Landy et al., 1948; Tran et al., 2022). (Figure 4.3B). The results suggested that the bacterium is a Bacillus sp. which need to be further identify by 16S rRNA.

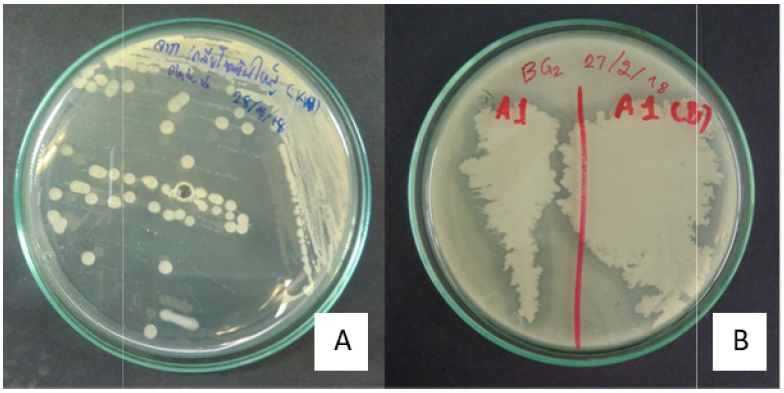


Figure 4.3 (A) Single colony by cross streak plate, (B) Test antibiotic activity by perpendicular streak method

The 16S rRNA sequencing method using a pair of universal primer as described in 3.2.3 showed that the RNA sequence of the K bacterium has 99.92% identity to *Bacillus siamensis* at 100.0 percent completeness, and 99.92% identity to *Bacillus velezensis* with 95.4 percent completeness (Table 4.1). The phylogenetic tree of the 16S rRNA is shown in (Figure 4.4). Regards to the 100% completeness the K bacterium is identified as *B. siamensis*.

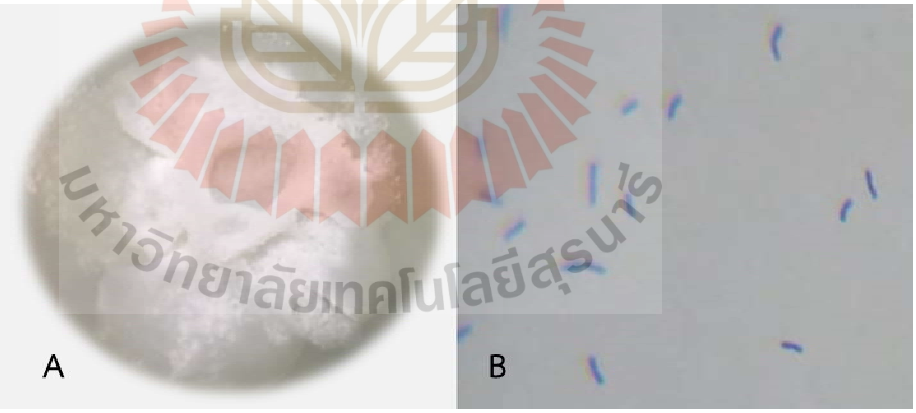


Figure 4.4 (A) The colony of the K bacteriuim, (B) Gram stain of the K bacterium.

Table 4.1 Nucleotide sequence relationship from gene of the k bacterium.

Isolate name	Nucleotide sequence relationship from gene		
	species	similar	completeness
K	*Bacillus siamensis*	99.92	100
	Bacillus velezensis	99.92	95.4

4.1.3 Antimicrobial activity (growth inhibition) of *Bacillus siamensis*

The antibacterial activity of *B. siamensis* was tested by the agar plug diffusion method. The detail of the method is described in the chapter 3.2.4. The results of the growth inhibition are shown in Table 4.1. *B. siamensis* was able to inhibit *S. flexneri*, *P. aeruginosa*, and *E. coli*. The diameter of the inhibition zone of about 0.68, 0.73 and 0.77 cm, respectively. *B. siamensis* could not inhibit *B. siamensis*, *B. cereus*, *B. subtilis*, *S. aureus*, *A. baumannii*.

Table 4.2 Antimicrobial activity of *B. siamensis*.

Bacteria	Inhibition zone		diameter of inhibition zone (average; centimeter)
	Positive	Negative	
Shigella flexneri	✓		0.68
Psudomonas aeruginosa,	✓		0.73
Escherichia coli	✓		0.77
Acinetobacter baumannii		✓	N/A
Bacillus cereus		✓	N/A
Bacillus subtilis		✓	N/A
Staphylococcus aureus		✓	N/A

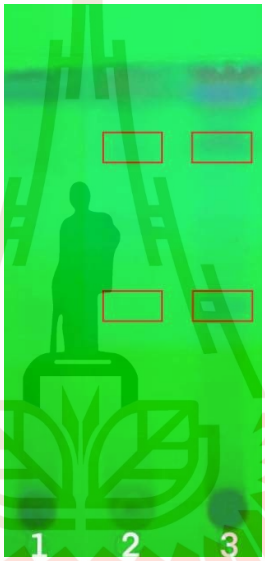


Figure 4.6 TLC plate using 20% ethyl acetate in hexane as a mobile phase: 1) The extract from LB broth (no bacterial culture), 2) the extract from the clear zone around the colony of *B. siamensis*, 3) the extract from the *B. siamensis* liquid culture. The red rectangular blocks show the presences of the active compounds.

4.1.4 Extraction and Isolation of active compound from the culture medium (LB agar and LB broth).

The active compounds extracted by the method described in the chapter 3.2.5 were analyzed by TLC. The results showed the presences of the compounds produced by *B. siamensis* in both the clear zone and the supernatant of the bacterial culture. (Figure 4.6). The TLC bands that presented in only the extract of the clear zone and the extract of the supernatant from the bacterial liquid culture, suggesting the presence of active compounds.

4.1.5 Large scale preparation and Purification of active compounds by silica gel column chromatography.

Three liters of *B. siamensis* culture were extracted by ethyl acetate and purified by column chromatography as described in the chapter 3.2.5. The stepwise TLC analysis showed several bands at the different mobile phases. The possible active compounds in compare with LB-broth were present in the faction 3, 5, 14, 16, 29, 30, 32, 33 which had proportion of ethyl acetate in hexane 5%, 10%, 40%, 50%, 90%, 90%, 100% and 100%, respectively.

4.1.6 Bacterial growth inhibition of the extracted compounds

The six extract samples from the purification step were dried by speed vacuum freeze dryer. Every sample was dissolved by 25 µL DMSO. The concentrate of each sample is shown in Table 4.2. The paper disc diffusion method using *Shigella flexneri* as a susceptible strain showed that fraction 5, 14, 30 and 33 had growth inhibition activity. (Figure 4.7)

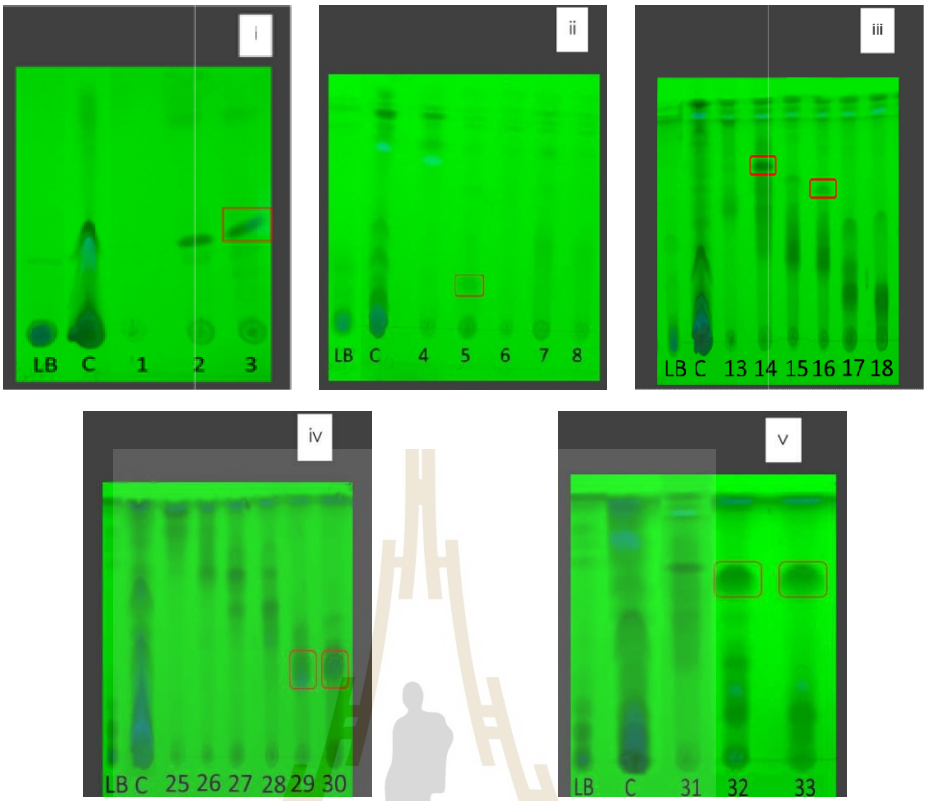


Figure 4.7 Showing suppose antibiotic purification of high-volume LB and extract difference solvent of ethyl acetate and hexane.

Figure 4.8 Disk diffusion method test inhibit *Shigella flexneri* of purification. D = DMSO, C = control and Sample faction (3, 5, 14, 16, 30, 33).

Table 4.3 The concentrate of each sample purification of the fraction 5, 14, 30 and 33.

Sample of fraction	The concentration of purification per volume DMSO (µg: 25µL)
3	100
5	50
14	50
16	200
30	100
33	100

4.2 Discussion

Screening for the antibiotic producing bacteria from the highly antibiotics contamination and scarce food conditions area provides high chance to discover new types of antibiotics. (Dadgostar, 2019; D'Costa et al., 2006, Hibbing et al., 2010). This research screened the antibiotic producing bacteria from a pig farm, a highly antibiotic contamination from misusing. The results found the bacterium that can produce the clear zone from the pigsty that is potentially highest dose of antibiotics contamination over the other sampling area because it is the nearest area that expose to the antibiotic usage. The antibiotic contamination was less in further sampling areas, possibly due to degradation from environmental factors.

Discovery of the bacterium produces an antibiotic zone, *B. siamensis* in this research provides an opportunity to find new antibiotic. Number of reports have shown that *Bacillus spp.* are able to produce various kind of antibiotics (Been et al., 2008). The newest class of antibiotics produced by *Bacillus spp.* is lipopeptide(Sumi et al., 2015). *B. siamensis* isolated from Sumpavapol et al. has been reported that it can be used as probiotic (Heo et al., 2021) and produces lipopeptide antibioctics However, the lipopeptide produced by *B. siamensis* has not been characterized (Xu et al., 2018). Five fractions from the column chromatography purification in this research showed growth inhibition activity suggesting that there were more than one actives compounds. This finding provide is the first evident to show that *B. siamensis* can produce more than one active compound that can inhibit bacterial growth, which is an important step to guide researcher to further investigate the active compound from *B. siamensis.*

CHAPTER V
CONCLUSION

This research discovered *B. siamensis*, a gram-positive bacterium from the pigtsy of the pig farm around Nakhonrasim. Plug diffusion method showed that *B. siamensis* was able to inhibit growth of *S. flexneri*, *P. aeruginosa*, and *E. coli* but *A. baumannii, B. cereus, B. subtilis, S. aureus.* The isolated *B. siamensis* produced at least 4 active compounds in LB-both after incubated at 30°C for 24 hr. The active compounds can be separated by column chromatography using various mobile phase, 10%, 40%, and 90% ethyl acetate in hexane, and 100% hexane. The four active compounds were able to inhibit the growth *Shigella flexneri*. However, the active compounds need to be further characterized.

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APPENDICES

APPENDIX A

THE FLOW CHART METHOD

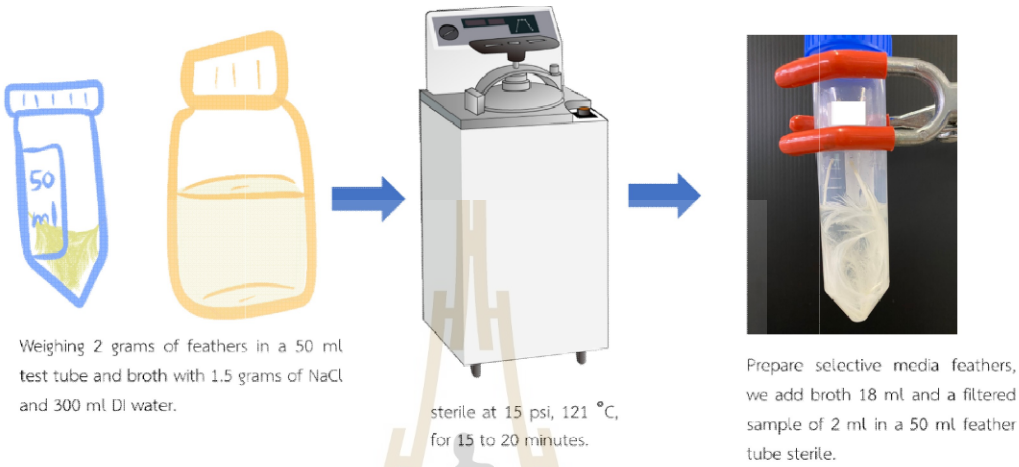

Figure A 1 Prepare selective media feather.

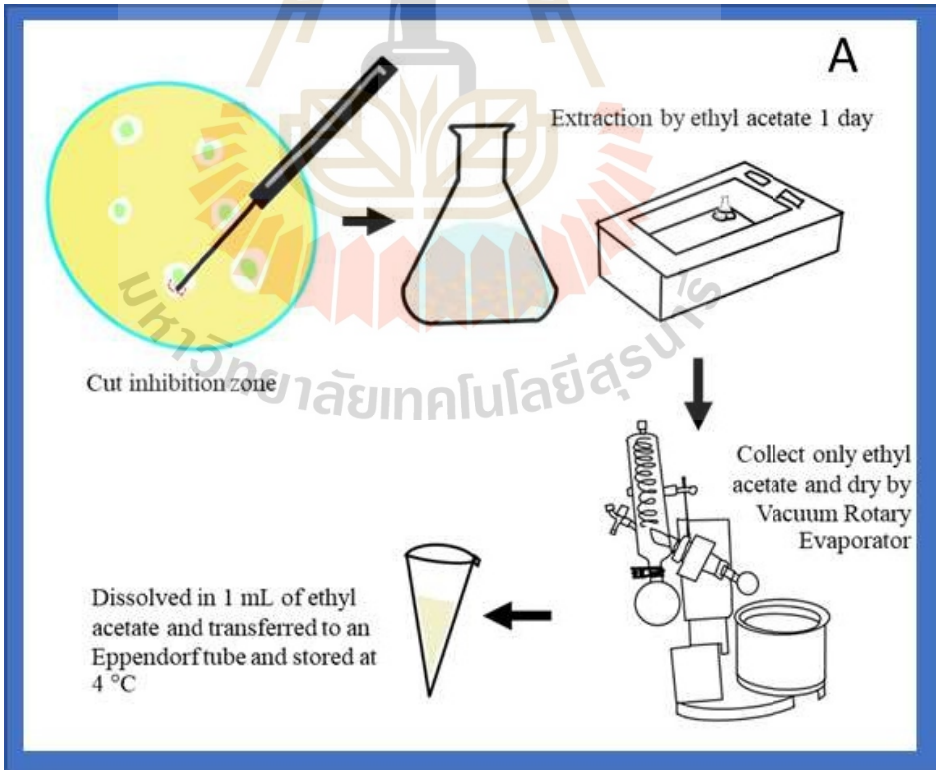

Figure A 2 Extraction and Isolation of antibiotic from LB agar.

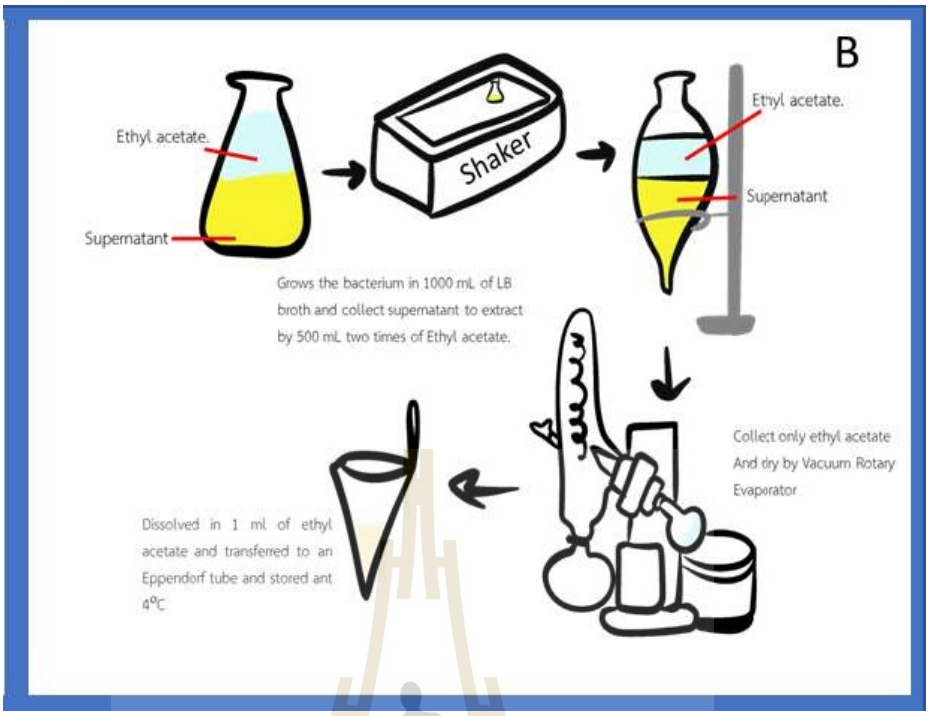

Figure A 3 Extraction and Isolation of antibiotic from LB broth.

APPENDIX B

16SrRNA SEQUNCEIN

16SrRNA sequnceing of K bacteria

GAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACC
GGATGGTTGTTTGAACCGCATGGTTCAGACATAAAAGGTGGCTTCGGCTACCACTTACAGATGG
ACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGAC
CTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAG
TAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTT
CGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTGCCGTTCAAATAGGGCGGCACCTTGACG
GTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAA
GCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGC
CCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGG
AATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTC
TCTGGTCTGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGG
TAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCT
AACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGG
GGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCT
TGACATCCTCTGACAATCCTAGAGATAGGACGTCCCCTTCGGGGGCAGAGTGACAGGTGGTGCA
TGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGAT
CTTAGTTGCCAGCATTCAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTG
GGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGACAGAA
CAAAGGGCAGCGAAACCGCGAGGTTAAGCCAATCCCACAAATCTGTTCTCAGTTCGGATCGCAG
TCTGCAACTCGACTGCGTGAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAAT
ACGTTCCCGGGCCTTGTACACACCGCCCGTCACAC

APPENDIX C

TEST ACTIVITY TOXIC OF DIMETHYL SULFOXIDE (DMSO) ON *Shigella flexneri* BY AGAR DISH DIFFUSION METHOD.

DMSO, which can be used to dissolve extracts for antibiotic assays without toxic effects on test Shigella flexneri. Different concentrations of 100%, 50%, 25%, and 12.5% DMSO with Distilled water (DI) and difference volume at 5 μl and 10 μl by disk diffusion method (Table 11).

Table A 1 Test of DMSO on toxic antibacterial activity.

Percent DMSO	Inhibition zone	
	Positive	Negative
1. 10 μl of 100% DMSO		✓
2. 5 μl of 100% DMSO		✓
3. 5 μl of 25% DMSO		✓
4. 10 μl of 25% DMSO		✓
5. 5 μl of 50% DMSO		✓
6. 10 μl of 50% DMSO		✓
7. 5 μl of 12.5% DMSO		✓
8. 10 μl of 12.5% DMSO		✓

The results indicated that bactericidal concentrations of DMSO hadn't a bactericidal effect on S. flexneri, So the exact maximum of DMSO that the tested bacteria can tolerate. The results of the experiment suggest the maximum of optimal. DMSO concentrations were used to dissolve antibiotic extraction assays to test anti-bacteria.

CURRICULUM VITAE

Name	Mr. Pranai Phoprasat
Date of Birth	September 21 1994
Place of Birth	Chonburi, Thailand
Education	
2018-2022	**Master** in School of Chemistry, Institute of Science, Suranaree University of Technology, Nakhon Ratchasima, Thailand
2014-2017	**Bachelor** of Post-harvest technology, Institute of Agriculture technology, Burapha University, Sakaeo Thailand and are or what all but what an