SCREENING OF ANTIBIOTIC-PRODUCING BACTERIA FROM SOILS AT
UNIVERSITI MALAYSIA SARAWAK

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APPROVAL SHEET
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Screening of Antibiotic-Producing Bacteria from Soils at Universiti Malaysia Sarawak Campus

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ABSTRACT

A study was conducted on the isolation of soil bacteria to screen for antibacterial activity against Gram positive and Gram negative bacteria. Soil samples were taken two times: which the first samples were isolated with six soil bacteria and the second samples were isolated with 50 soil bacteria. Dilution of the soil samples were made and were spread onto nutrient agar. Bacteria colonies with inhibition zone around them were isolated, subcultured and further screened for their antibacterial properties. Overall screenings consist of cross-streak test, perpendicular-streak test, disc diffusion test, and agar gel diffusion test. After that, Gram staining and morphological characterizations of antibiotic-producing bacteria were made. Two soil bacteria isolates: A5.1 and A5.7 were observed with antibacterial activity against Enterobacter aerogenes and Bacillus cereus. Both bacteria have bacilliform and are Gram negative. These findings suggest that soil in UNIMAS may contain antibiotic-producing bacteria that could have potential of being newly isolated bacteria or having newly isolated antimicrobial compound.

Key words: antibiotic-producing bacteria, soil bacteria.

ABSTRAK


Kata kunci: Bakteria penghasil antibiotik, bakteria tanah
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1.0 Introduction

The first discovery of antibiotics was in 1928 by Alexander Flemming. He discovered penicillin produced by *Penicillium notatum* that have antibacterial properties. However, in 1942 a first penicillin resistance reported where strains of *Staphylococcus aureus* were detected to have resistance to this antibiotic. After that, many resistance of bacteria to antibiotic were reported such as methicillin-resistant *Staphylococcus aureus* (MRSA) in 1961, bacteria resistant to Linezolid in 2001, and full vancomycin-resistant strains of *Staphylococcus aureus* in 2002 (Lubelchek, 2008). Some bacteria possess multidrug-resistant properties whereby it can withstand the inhibitory action of a number of antibiotics. It has been common phenomena that each time after a new antibiotic is approved for use, resistant of bacteria to this antibiotic will be reported after a few years. The overuse of antibiotics in medicine and agriculture promote emergence of resistance (Lubelchek, 2008).

Antibiotics can be produced by bacteria, actinomycetes and fungi (Kobayashi, 1990). These microorganisms can be found in almost every area in environment such as in soils, lake, sea, river, and air. They normally produce different antibiotics for example, genus of *Streptomyces* which is a group of bacteria that can be found in soils and freshwater sediments produced streptomycin, tetracyclines, and erythromycin (Kobayashi, 1990). The microorganisms can be isolated from all area in environment either from air, soils or water. However, many antibiotic-producing bacteria and actinomycetes are isolated from soils from different country with different climate.
Without effective antibiotics, severe infections by bacteria can cause fatal disease that cannot be treated. The major strains of bacteria that show resistant to many antibiotics are *Staphylococcus aureus*. The bacteria can cause pneumonia, and bloodstream and surgical infection.

Therefore, continuous research to discover new antibiotics is essential. There have been discoveries of antibiotics from soils in Malaysia and Borneo. One of the antibiotics is vancomycin which was derived from a soil bacterium isolated from forest of Borneo (Lubelchek, 2008). Many studies have been carried out by local universities and Forest Research Institute Malaysia (FRIM) to isolate antibiotics from soils in Malaysia. In 2006 FRIM and Nimura Genetic Solution, which is a company from Japan made a research to search for new antibacterial compound in Malaysia (Numata & Nimura, 2003). In 2006, they discovered a new antibiotic compound derived from microorganism in Malaysia (Nimura Genetic Solutions Co. Ltd., 2006). There is high possibility that new antibiotics can be discovered from Malaysia’s environment especially soils because tropical rain forests of Malaysia rich with high diversity of flora and fauna.

In this study, isolation and characterization of antibiotic-producing bacteria and actinomycetes were made. The objective of this study is:

i. To isolate antibiotic-producing bacteria and from soil of UNIMAS Campus.

ii. To identify the soil bacteria to genus level and its antibiotic.

iii. To determine antibiotic-producing ability of the isolated bacteria.
2.0 Literature Review

2.1 Antibiotics

According to Waksman and Lechevalier (1962), antibiotic is a chemical solution that is produced by the microorganism that has the ability to kill or inhibit the growth of other microorganism or bacteria. Meanwhile, Burman & Olsson-Liljequist (2001) define antibiotics as the chemical compounds that are synthesized or, commonly, derived from natural sources and further modified, and which exert a certain action on the growth of bacteria. Normally, antibiotics are used to treat diseases that are caused by infection from microorganism. The infection can occur in animals, plants or human.

Penicillin was the first antibiotics discovered. It was discovered by Waksman and Woodruff in 1940 from a streptomycete (Queener & Day, 1986). The discovery started in 1928 when Alexander Flemming accidently discovered the inhibition of Staphylococcus sp. by diffusion products from Penicillium notatum that contaminated his culture of the Staphylococcus. Since then, many antibiotics have been isolated from various microorganisms, of which actinomycetes and streptomyces are the most important (Nicolaou, 2008).

Antibiotics can mainly be group into two groups which are antibiotics with bactericidal activity and antibiotics with bacteriostatic activity (Kobayashi, 1990). Antibiotics with bactericidal activity are able to kill susceptible microorganisms without
the intercession of humoral or cellular immune defense. Meanwhile, antibiotics with bacteriostatic activity can inhibit essential metabolic processes, and the metabolism can take place again after their level become subinhibitory.

The main classes of antibiotics are Penicillins, Cephalosporins, Macrolides, Fluoroquinolones, Sulfonamides, Tetracyclines, and Aminoglycosides. Each antibiotic can treat for only certain types of infections and act specifically. One of the examples is Penicillin which works by inhibiting the formation of the bacterial cell wall (APUA, 1999). It blocks the cross-linking of the bacteria cell wall structure.

2.2 Antibiotic-Resistant Bacteria

Bacteria that are not affected at all by antibiotics or only affected in lesser extent are referred to antibiotic-resistant bacteria (Burman & Olsson-Liljequist, 2001). The selection of multidrug-resistant bacteria in humans has been attributed to the uncontrolled use of antibiotic in medicine (Bradford et al., 1999; Davies et al., 1999).

Resistance of bacteria can be innate in all members in a species. In innate resistance the bacteria can be resistant because of inability of the antibiotic to enter into the bacteria through its cell envelope; absence of the specific target site of the antibiotics; ability of the bacteria to bypass a blocked metabolic pathway, and the production of an enzyme that inactive the antibiotics. Besides, the resistance can be acquired by bacteria because of mutation or derived from another microorganism through genetic exchange (Sherris & Plorde, 1990).
Staphylococcus aureus is one of the bacteria that have shown resistance to antibiotics. Initially it was reported to resist on penicillin (Lubelchek, 2008). The resistant did not occur when penicillin was first discovered, it only showed resistance a few years after that. It was caused by natural mutation. After that Staphylococcus aureus was reported to resist on many newly approved antibiotics such as methicillin, vancomycin, linezolid, and daptomycin. During 2008, over 90 percent of Staphylococcus aureus strains were reported to be immune to penicillin (Lubelchek, 2008).

Due to the difficulties in controlling the development of new strains with multi-drug resistance, new natural or synthetic antibiotics must be discovered. One such approach is to screen for new antibiotics from bacteria in the soils.

2.3 Soil Bacteria

Most of the bacteria can produce antibiotics that have the potential to be used in human medicine. Some of the bacteria are able to produce antibiotics under anaerobic conditions of growth or produce more than one antibiotic substance (Sturgen & Casida, 1961).

Soil has a higher number of bacterial diversity compared to other natural habitats. It contains about $10^7$ to $10^{10}$ cells per gram dry soil of bacteria (Elsas et al., 2006). Most of the bacteria are found in the top layer of soil which is at the top 2-3 centimeters (The University of Western Australia, 2004).
According to Lavelle and Spain (2001), bacteria are aquatic organisms that live in the water-filled pore spaces in soil. The bacteria mostly accumulate inside the soil aggregates. Clayey soils often have more bacteria than sandy soils because many small pores present in the clay than the sandy soils (The University of Western Australia, 2004). It offers better protection for the bacteria. Most of the bacteria are unable to move and are normally found on the surfaces of mineral or organic particles or assembles around particles of decaying plant and animal remains (Lavelle and Spain, 2001).

### 2.4 Discovery of Antibiotic-Producing Bacteria in Malaysia

One of the bacteria that produce antibiotic that are commercially available in market is *Nocardia orientalis*. In 1956, this bacteria strain was discovered to produce vancomycin. It was isolated from soil sample taken from jungle in Borneo (Nicolaou and Montagnon, 2008). This antibiotic is useful to treat disease that cause by methicillin-resistant *Staphylococcus aureus* infection. It is glycopeptide antibiotic that binds with peptides and inhibit peptidoglycan synthetase structurally. This action will suppress the cell wall synthesis.
2.5 Actinomycetes

In the article by MedicineNet.com (2003), it was described that actinomycete is a group of gram-positive bacteria in order Actinomycetales that produce various bioactive agents including antibiotics, enzymes, and vitamins (2003). The American Heritage® Medical Dictionary (2004) defines actinomycete as any various filamentous or rod-shaped, often pathogenic microorganisms of the genus *Actinomyces*, that is a member of the family Actinomycetaceae and a member of the order Actinomycetales. Apart from that, Saunders Comprehensive Veterinary Dictionary (2007) defines actinomycete as a mold-like bacterium in the Order Actinomycetales that occurred as elongated, frequently filamentous cells, with a branching tendency.

This group of bacteria produces spores and is highly distributed in soils (Todar, 2008). The bacteria are widespread in dry soils and not grow well in wet soils (Wellington et al., 1994). Most antibiotics are produce by actinomycetes (Miyadoh, 1993).

Streptomyces are bacteria that in Order of Actinomycetes and Family of Streptomycetaceae. They are fungus-like bacteria with aerial mycelia and chains of asexual spores (Kobayashi, 1990). They are able to produce bioactive molecules, including enzymatic inhibitors with antibiotic activity and many commercially valuable enzymes (Ravel, Wellington and Hill, 2000). In nature, the bacteria produce secondary metabolites to inhibit other competing cells, so that the bacteria can utilize more nutrients for survival (Kumari, Ponmurugan, and Kannan, 2006). Many secondary
metabolites possess the antibacterial activities (Sanglier, Haag, Huck and Fehr, 1993). They normally have the odor of freshly turned over soil (Kobayashi, 1990).

3.0 Material and Method

3.1 Source of Materials

3.1.1 Test bacteria

This study used five different test bacteria in the overall screening process. These bacteria are Gram positive and Gram negative bacteria. *Staphylococcus aureus, Salmonella typhi, Enterobacter aerogenes, Escherichia coli,* and *Bacillus cereus* were used as test bacteria. *Staphylococcus aureus* and *Bacillus cereus* are Gram positive bacteria while *Escherichia coli, Salmonella typhi,* and *Enterobacter aerogenes* are Gram negative bacteria. They were obtained from FSTS stock culture, stores in 80% glycerol at -4°C. The bacteria were subcultured on NA to get pure bacteria colony.

3.1.2 Soil samples

Soil samples used were taken from UNIMAS surroundings. They were taken using sterile garden scoop at depth of about 10–15 cm after removing approximately 10 cm of the soil surface. A total of two soil samples from reserved forest in UNIMAS were
taken in first sampling and a total of 11 soil samples were taken from different area around UNIMAS in second sampling. They were taken at area with many trees and shrubs (Figure 3.1). They were put into sterile plastic bags. Then the plastic bags were sealed and stored in refrigerator at 4°C until use.

![Figure 3.1: One of the area where soil samples were taken.](image)

### 3.2 Isolation of Antibiotic-producing Bacteria

#### 3.2.1 Preparation of the samples

Approximately 1 g of soil samples were aseptically added to 9 ml sterile PBS buffer and shaken using vortex machine. Then they were diluted to $10^{-2}$ and $10^{-3}$ in sterile PBS buffer. Aliquots of 100 µl of each sample dilution were pipetted onto Nutrient agar (NA) with volume approximately 20 ml, and then spread throughout the agar surface. The plates were dried for 10 minutes prior to inoculation. The inoculated plates were incubated at 28°C for 7 days.
3.2.2 Isolation of bacteria with antibacterial properties

After 7 days of incubation, the plates were observed for bacteria colonies with halo or inhibition zone around them (Figure 3.2). Each of the bacterial colonies were isolated and streaked onto NA separately. They were labeled specifically. The NA plates were incubated at 28°C for 24 hours. Then, any bacteria colony that grew normally and produced single colony growth were streaked again onto NA to subculture it and to get pure colony. The plates were incubated at 28°C for 24 hours.

3.3 Initial Screening of Positive-Antibiotic-Producing Isolates

3.3.1 Cross-streak test

Isolates from first soil samples were initially screened using cross-streak test. Positive-antibiotic-producing isolates were streaked in a straight line on NA. After 3 days incubation at 28°C, test bacteria were streaked across the isolates colony (Figure 3.3). Then, the plates were incubated at 37°C for 24 hours. *Enterobacter aerogenes, Staphylococcus aureus, Bacillus cereus, Escherichia coli and Salmonella typhi* were used as test bacteria.
3.3.2 Perpendicular-streak test

Isolates from second soil samples were initially screened using perpendicular-streak test (Figure 3.4). Each positive-antibiotic-producing isolates was streaked straight on NA. Then, they were incubated for 3 days at 28°C. After that, each test bacteria were streaked perpendicularly to the sample bacteria. They were incubated at 28°C for 24 hours. The test bacteria growths were observed after the incubation period for any inhibition of their growth. *Enterobacter aerogenes*, *Staphylococcus aureus*, and *Salmonella typhi* were used as the test bacteria.

3.4 Secondary Screening of Antibiotic-Producing Bacteria

Only isolates from second soil samples were further screened for their antibacterial properties. In secondary screening, bacteria samples with positive result in primary screening were incubated in 3.0 ml nutrient broth (NB) inside Bijou bottles. Bottles
were then incubated at room temperature for 7 days. Subsequently, 1.5 ml of the broth was centrifuged at 7,000 rpm for 15 minutes. The resulting supernatant was then used for subsequent antibacterial screening test.

3.4.1 Disc diffusion test

In disc diffusion test (Figure 3.5), firstly NA plates were swabbed with test bacteria broth using sterile cotton stick. Then sterile filter paper discs with diameter of 6 mm were cut and put on the NA at suitable position and not too near to each other. One NA plate was arranged with a maximum number of nine discs. Aliquot of 10μl of each supernatant was pipette onto different disc. They were tested with Enterobacter aerogenes, Escherichia coli, and Bacillus cereus. The plates were incubated at 37°C for 24 hours. After that, they were observed for any inhibition zone produced around each disc.
3.4.2 Agar gel diffusion test

This test was used to further verify the presence of antibacterial substance in the supematant obtained from the isolates. In this test, NA plates were swabbed with test bacteria using sterile cotton stick. *Enterobacter aerogenes*, *Escherichia coli*, *Bacillus cereus* and *Staphylococcus aureus* were used as the test bacteria. After the plates were dried out for 5 minutes, wells with 8.0 mm diameter were bore in the agar plates (Figure 3.6). They were made using sterile altered pipette tips. The holes depths were same as the agar thickness. A maximum of seven holes were made at each agar and they were made at positions which were not too close to each other. Aliquots of 30 µl of each supematant were pipette into each hole. The plates were incubated at 37°C for 24 hours. Then, they were observed for any inhibition zone produced around each well.

![Figure 3.6: Agar gel diffusion test](image)
3.5 Analysis of Antibiotic Activity

The antibacterial properties of supernatants with positive result in previous test were further test using different volumes. Aliquots 30 µl and 50 µl of each supernatant with antibacterial properties were tested using agar gel diffusion test. The size of each inhibition zone was observed and measured. In this test, Enterobacter aerogenes, Escherichia coli, Bacillus cereus and Staphylococcus aureus were used as the test bacteria.

3.6 Morphological Characterization

Gram staining was made to isolates with antibacterial properties. Their morphological properties were observed under light microscope.

4.0 Result

4.1 Isolation of bacteria from soils

A total of six bacteria with antibacterial activity were isolated from first soil samples. These isolates were code-designated according to place where the sample had been taken.
In a second sampling a total of fifty bacteria with antibacterial activity were isolated from second soil samples. These isolates were named specifically and according to place where the sample had been taken. They were isolated from nine soil samples taken from different places. The bacteria were isolated from soil sample number 1, 2, 3, 4, 5, 6, 7, 8, and 10. Soil sample number 9 and 11 did not have any bacterial colony with antibacterial activity.

4.2 Initial Screening of Positive-Antibiotic-Producing Isolates

The isolates in the first sampling were screened for their antibacterial activity using cross-streak test. In this test the antibacterial activity of the isolates could not be observed and analyzed clearly. All the tested isolates, namely F1.3, F1.6, F2.4, F2.5, F2.7 and F2.9 showed negative result against the test bacteria, which were Bacillus cereus, Escherichia coli, Salmonella typhi, Enterobacter aerogenes and Staphylococcus aureus.

Of the fifty isolates screened in initial screening of the screened sampling, 22 showed activity against Enterobacter aerogenes, 6 against Salmonella typhi, and 4 against Staphylococcus aureus. Among them, three isolates showed activity against all three test bacteria, two against Staphylococcus aureus and Enterobacter aerogenes, and four against Enterobacter aerogenes and Salmonella typhi (Table 4.2). There were isolates that showed activity only to one test bacteria while other isolates did not showed any activity against test bacteria.