BIOASSAY-GUIDED ISOLATION OF ANTIBIOTICS FROM SELECTED MARINE BACTERIA

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Bioassay-guided isolation of antibiotics from selected marine bacteria

Jenny Choo Cheng Yi (21119)

A final project report submitted in partial fulfillment of the Final Year Project II (STF 3015) course

Supervisor: Prof. Dr. Ismail bin Ahmad

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I declare that this thesis entitled “Bioassay-guided isolation of antibiotics from selected marine bacteria” is the result of my own research paper except as cited in the references. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.

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List of Abbreviations:

HTS – High Throughput Screening

NA – Nutrient Agar

NB – Nutrient Broth

NDM-1 – New Delhi metallo-β-lactamase 1

MHA – Mueller-Hinton Agar

MHB – Mueller-Hinton Broth

MIC – Minimum Inhibitory Concentration

MRSA – Methicillin-resistant Staphylococcus aureus

TLC – Thin-layer Chromatography
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Bioassay-guided isolation of antibiotics from selected marine bacteria

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ABSTRACT

As a response to the widespread emergence of antibiotic resistant bacteria, a study was devised to isolate selected marine bacteria to test for its antibacterial activity. A total of six bacterial isolates which were P5.1.2, 3.1.2, B2.4A, B.24C, B2.6A, and S1.2.3 were subjected to preliminary screening via agar-well diffusion assay against Gram-positive (Staphylococcus aureus) and Gram negative (Escherichia coli, Enterobacter aerogenes, and Salmonella typhi) test bacteria. Bacterial isolate P5.1.2 which was tentatively identified as Klebsiella sp. was only found active against Gram positive test bacteria while others showed no activity. Antibiotic extraction from this isolate P5.1.2 was done from culture growing it on solid media. Dried cultures were then subjected to subsequent extraction using six solvents which were hexane, chloroform, dichloromethane, ethyl acetate, methanol, and water. A time-course experiment was conducted to determine the optimum age of harvesting. All six extracts from the selected bacterial isolate were subjected to antibacterial screening against the test bacteria via agar-well diffusion method and the presence of halo around the wells was examined. Methanol, chloroform, and ethyl acetate crude extracts showed antibacterial activities and the minimum inhibitory concentration (MIC) values were 1 mg/ml, 0.25 mg/ml and 0.125 mg/ml respectively. These results supports that marine environment is capable of producing new antibiotic substances.

Keywords: Antibacterial activity, marine bacteria, extracts, minimum inhibitory concentration

ABSTRAK

Dalam respon bagi kemunculan bakteria rintang antibiotik, satu kajian terhadap bakteria yang dipencilkan dari persekitaran laut telah dijalankan untuk mengkaji aktiviti antibakteria. Sejumlah enam pencilan bakteria iaitu P5.1.2, 3.1.2, B2.4A, B2.4C, B2.6A, and S1.2.3 telah melalui penyaringan pertama terhadap bakteria ujian Gram-positif (Staphylococcus aureus) dan Gram-negative (Escherichia coli, Enterobacter aerogenes, dan Salmonella typhi) melalui kaedah resapan telaga agar. Pencilan bakteria P5.1.2 yang dikenalpasti sebagai Klebsiella sp. didapati menunjukkan aktiviti antibakteria terhadap bakteria ujian Gram-positif manakala tiada aktiviti didapati daripada pencilan bakteria lain. Pengekstratan antibiotik dari pencilan bakteria P5.1.2 telah dilakukan dengan menggunakan media pepejal. Kultur kering dikenakan pengekstratan seterusnya dengan menggunakan enam pelarut iaitu heksana, kloroform, dikerometana, etil asetat, metanol, dan air. Experiment jangka masa dilakukan untuk mengenalpasti masa optimum pengekstratan kultur. Penyaringan antibakteria untuk enam ekstrak tersebut telah dilakukan terhadap empat bakteria ujian melalui kaedah resapan telaga agar dan kemunculan halo di sekitar telaga agar telah diperiksa. Ekstrak metanol, kloroform, dan etil asetat menunjukkan aktiviti antibakteria dan konsentrasi perencatan minimum yang dicatatkan adalah 1 mg/ml, 0.25 mg/ml, dan 0.125 mg/ml masing-masing. Keputusan yang didapati dapat membuktikan bahawa persekitaran laut mempunyai potensi yang tinggi untuk pencarian antibiotik baru.

Kata kunci: aktiviti antibakteria, bakteria laut, ekstrak, konsentrasi perencatan minimum
1.0 Introduction
Antibiotics are naturally occurring antimicrobial drugs that are produced by many different bacteria and fungi, which have been successfully used to inhibit and kill disease-causing microorganisms (Madigan et al., 2009). Since the discovery of Penicillin and other major antibiotic classes between 1929 and 1962, infectious diseases have generally been conquered and the mortality rates have decreased (Coates & Hu, 2007). However, the success of antibiotics in treating infectious diseases throughout the past decades could not change the fact that bacterial infectious diseases are still among the leading causes of death worldwide (Schmidt, 2004). Thus, this has lead to the increase demand of discovering new antibiotic-producing bacteria.

The inability of antibiotic treatment to cope with disease is generally attributed to the emergence of antibiotic resistant bacteria. This phenomena has since been considered as a major health problem and it also has been identified as a global threat (World Health Organization, 2000). The increasing numbers of drug resistant Gram-positive and Gram-negative infections have been continuously posing healthcare problems (Grayson & Eliopoulos, 1990). However, the greater concern than resistance to single antibiotics is inevitably the development of bacterial resistance towards multiple antibiotic types (Tan et al., 2000). The rapid emergence of multiple drug resistant bacteria such as the methicillin-resistant *S. aureus* (MRSA) has posed a major problem worldwide and this leads to the increased use of vancomycin (Neu, 1992). Although vancomycin is often used to treat infections with multi-drug resistant bacteria, vancomycin-resistant *S. aureus* has been identified in 1997 (Levy, 1998). Recently, the rapid emergence of New Delhi metallo-β-lactamase 1 (NDM-1) in multidrug-resistant Enterobacteriaceae in India, Pakistan, and the United Kingdom has also posed a serious threat to the health problem worldwide (Kumarasmy et al., 2010). In addition, healthcare facilities also continue to face major
health threat due to the recent emergence of superbug carbapenem-resistant *Klebsiella pneumonia* which is reported to be resistant to almost all antibiotics (Cool, 2011). Thus, the driving force to search and to screen for new antibiotics substances is the evolution and spread of the antibiotic resistance bacteria (Silver & Bostian, 1993). Therefore, the urgent need for the rapid screening for new antibiotics is imminent. It is perhaps the only feasible way to keep pace with the changing face of bacterial antibiotic susceptibility and to combat the widespread emergence of antibiotic resistant bacteria.

The discovery and screening of new antibiotics can be made from natural resources such as soil bacteria, extract from plant and fungi, and even marine microorganisms. Since the discovery of Penicillin in 1928, soil derived microorganism has been studied extensively and research has shown that microorganisms are a potential source of producing novel compounds (Fenical, 1993). Although soil microorganism has shown to produce secondary metabolites such as penicillin and streptomycin, it is found that the discovery of new compounds has been decreasing due to the exhaustive studies of soil microorganism which produce redundancy of previously described compounds (Fusetani, 2000). Marine environment has reported to offer potential source of discovering antibiotic-producing bacteria since the discovery of bacterium *Pseudomonas bromoutilis* which produce antibiotic pentabromopseudiline (Burkholder et al., 1966). Hence, researchers need to look for alternative resources such as the marine environment.

Recently, Liu Yu Choi (2010) and Nur Izzah Fateha (2010) had described the screening for antimicrobial activities in microorganisms isolated from the marine environment. Several bacteria had been successfully identified and were reported to be active against the test bacteria. This current study selects bacterial which they have isolated and were subjected to further studies in order to isolate and characterize the antibiotics produced by the selected bacterial isolates. Therefore, antibiotic screening among
microorganism is crucial in searching for new novel compounds to replace the old antibiotics that are losing its efficiency against the infectious diseases.

Objectives:

1. To screen for the presence of antibacterial activities in isolated bacteria.
2. To characterize and identify bacteria isolates with antibacterial activities.
3. To extract the antibiotics from bacterial isolates with the most potential of producing antibiotics.
4. To determine the minimum inhibitory concentration (MIC) value of the extracted antibiotics.
2.0 Literature Review

2.1 Antibiotic

Antibiotic is derived from the Greek word, which means “against life”. In 1889, antibiotic was coined by French researcher Paul Vuillemin to describe a substance he isolated some years earlier from *Pseudomonas aeruginosa*. The substance called pyocyanin, which inhibited the growth of other bacteria in test tubes, but was said to be too toxic to be used in disease therapy. Vuillemin’s term has survived to the current era (Alcamo, 1997). Refinement of the definition of antibiotic was made by Selman Waksman, who in 1940 had described antibiotic as a “chemical substance produced by microorganism which has the ability to inhibit the growth of bacteria and even destroy bacteria and other microorganism in dilute solution” (Black, 2005). The discovery of antibiotics can be said to mark the beginning of modern medicine as it has tremendously serve to fight against infectious diseases since its discovery. Thus, antibiotics have traditionally been known as miracle drugs.

Antibiotics exert their effects mainly by inhibiting the cell wall synthesis, disruption of cell membranes, interference with protein synthesis or with nucleic acid synthesis (Hogg, 2005). For the past years, several microorganisms have been isolated which are served as a source of antibiotics. Antibiotic penicillin has been found from *Penicillium notatum* and *Penicillium chrysogenum*; streptomycin from *Streptomyces griseus*; and tyrocidin from *Bacillus brevis*. However, according to Madigan et al., (2009), less than 1% of the thousands of known antibiotics are clinically useful, often because of toxicity or lack of capable of host cells. It is also said that 50% of all antibiotics made are used in animal agriculture applications, and even said to be extensively used in aquaculture and fruit production.
2.2 Antibiotic resistant bacteria

The emergence of antibiotic resistant bacteria is a natural consequence of overuse and misuse of antimicrobial drugs, which kills the sensitive organisms and eventually the resistant ones are left to survive and multiply (Holloway, 2000). The drug resistant Gram-positive infections is increasingly reported among *Staphylococcus aureus*, coagulase-negative staphylococci, corynebacteria, and enterococci, while drug resistant in Gram-negative organisms such as *Pseudomonas*, *Serratia*, and *Acinetobacter* species continue to pose problems (Grayson & Eliopoulos, 1990). The emergence of antibiotic resistant bacteria seems inevitable and has been said to pose major health problems due to the increase level of resistance that decreases in the efficacy of the drug in human population. According to Black (2005), the resistance of microorganism to an antibiotic means that the microorganism is no longer effective to the formerly susceptibility action of the antibiotic.

Bacteria can become resistance towards antibiotic through chromosomal mutation or expression of a latent gene (Neu, 1992). Neu (1992) also believes that bacteria can confer resistance through exchange of genetic material via transformation, transduction, or conjugations by plasmids. Apart from that, it is also believed that transposons, the so-called jumping genes also play its role by conferring antibiotic resistance to the bacteria by having the ability to enter transmissible plasmids or chromosomes. Besides that, Williams (2000) also shares the same view with Neu (1992) whereby mutation or acquisition from other bacteria of resistance genes can cause bacteria to develop resistance towards antibiotics. Thus, this clearly explains the rapid spread of resistant strains among microbes and eventually replaces a previously drug-susceptible population of bacteria. Williams (2000) also believes that when antibiotic resistant bacteria occurs, it is the microbe that is resistant and not the drug nor the patient.
2.3 Marine environment

The ocean, which constitutes approximately 70% of the earth’s surface, is the habitat of the vast diversity of unique marine microorganisms (Fenical, 1993). According to Hagstrom et al. (2002), the marine environment of the oceans constitutes approximately $10^6$ bacterial cells per ml, and hence making it undeniably the habitat of rich diverse microorganisms. Since the mid 1970s, the marine environment has begun intensively investigated for its sources of novel secondary metabolites. Among the many phyla found in the oceans, bacteria, fungi, certain group of algae, sponges, coelenterates, sea hares, bryozoans, tunicates, and nudibranchs were the most studied organisms (AL-Zereini, 2006).

One of the earliest isolations of antibiotic-producing marine microorganisms was the isolation of the fungus *Cephalosporium acremonium* which produces cephalosporin in 1948. Apart from that, the isolation of the first marine metabolite from bacterium *Pseudomonas bromoutilis* which produce antibiotic pentabromopseudiline also contributed to the discovery of novel compounds from the marine environment (Burkholder et al., 1966).

According to Schwartsmann et al. (2001), the secondary metabolites produced by marine microorganisms have more novel and unique structures, and the bioactive are much stronger compared to its terrestrial counterparts. This is due to the complex living circumstance and the diversity of the species living in the marine environment. Furthermore, Armstrong et al. (2001) also stated that the production of many natural products processing medical and industrial values is mainly due to the competition among microorganisms for space and nutrient in the marine environment. The result from the study done by Penesyan et al. (2010) is consistent with the studies of Armstrong et al. (2001). In Penesyan et al.’s studies, it is also reported that microbes such as the marine surface-associated microorganisms are required to evolve allelochemicals which are
capable of protecting the producer against the competition between the microorganisms on the surface of marine eukaryotes. Hence, the marine environment is proven to be a rich source of novel compounds due to the competition among the microorganism for survival and protection.

2.4 Marine biofilm

Marine biofilms as described by Hall-Stoodley et al. (2004) are surfaces being concentrated with a population of microorganisms through an exo-polymeric matrix. It has been reported that the matrix attributed to the formation of the biofilm and also serves as a protection for the microorganisms (Davey et al., 2000; Gilbert et al., 1997; Flemming, 1993). According to a study by Corterston et al. (1999), it has been found that almost all microbial populations live and attached to microbial communities such as biofilm. The study was supported by Satpathy et al. (1999) in which biofilm has been described as world’s largest colonialism as biofilm can be found almost everything, including highly irradiated area of nuclear power plant. With the abundance population of microbial being attached to the biofilm, the potential of discovery antibiotic-producing bacteria is high. Apart from that, from the study of Lemos et al. (1986), it has been reported that bacteria in biofilms attached to surface of marine microorganism are recorded to have greater potential of discovering antibiotic producing bacteria compared to other marine environment. Thus, the marine biofilm can serve as a great source for the discovery of new antibacterial drugs.

2.5 Antimicrobial screening assays

According to Madigan et al. (2009), new antibiotics are discovered by laboratory screening programs. Through this approach, a large amount of potentially antibiotic-producing microorganisms are obtained from nature in pure culture. Its antibiotic production is tested
by assaying for diffusible materials that are inhibitory to the growth of test bacteria. It is reported that since the discovery of the first antibiotics which are brought into clinical used in the 1930s and 1940s, standard methods for isolating more new antibiotics have been developed by researchers. Candidate drugs were routinely isolated from natural sources such as *Streptomyces* or *Penicillium* cultures and systematically screened for antimicrobial activity using standard MIC (Minimum Inhibitory Concentration) or agar diffusion method to find new antimicrobial compounds.

2.5.1 Spot Inoculation method

The spot inoculation method is frequently being conducted using the agar-overlay technique for screening the antimicrobial activity of the microorganisms (Fleming *et al.*, 1975). The isolated microbes are spotted on the agar plates in an appropriate distance from each other. The agar-overlay technique uses the principle of having the lower base of the agar which is seeded with the isolated microbes to be overlaid by a soft agar layer that is seeded with the test bacteria. The isolated microbes are then being transferred onto the soft agar layer seeded with the test bacteria via diffusion process (Marston and Hostettmann, 1999).

According to Mesbah and Wiegel (2006), the soft agar plates should be incubated right side up for the first 12-20 hours in order for the top layer of soft agar to adhere tightly to the base layer of the agar. The plates are then inverted for the remaining incubation period in order to minimize the smearing of colonies by water. The agar-overlay technique is applicable to a broad spectrum of microorganisms as it produces well-defined zone of inhibition and it is not sensitive to contamination (Marston and Hostettmann, 1999).
2.5.2 Disc diffusion method

According to Prescott et al. (1996), the disc diffusion test most often used is the Kirby-Bauer method, which was developed in the early 1960s at the University of Washington Medical School by William Kirby, A. W. Bauer, and their colleagues. This method operates on the principle that the antibiotics will diffuse from a paper disk or small cylinder into an agar medium containing test organism.

When the Mueller-Hinton agar plate which has been inoculated with the test organism is placed with an antibiotic impregnated disk, the disk eventually picks up moisture and the antibiotic diffused outward through the agar. Thus, an antibiotic concentration gradient is produced. High concentration of antibiotic is present near the disk and it affects minimally susceptible microorganisms. The antibiotic concentration drops when the distance from the disk increases and allowing only the more susceptible pathogens are being harmed. After incubation, a clear zone or ring, known as the zone of inhibition, is present around an antibiotic disk when there is inhibition of bacterial growth by the antibiotics. The wider the zone surrounding a disk, the more susceptible the pathogen is towards the antibiotics.

2.5.3 Agar-well diffusion method

The agar-well diffusion method is an alternatively approach on testing the antimicrobial activity of the isolate microbes (Teo & Hai, 2006). The agar-well diffusion method also uses the principle of diffusion process in which the antibiotic extract is diffused onto the agar plate seeded with the test bacteria. In the agar-well diffusion method, the wells are aseptically created by using a hole-borer in the solidified agar medium and the wells are being filled with the antibiotic extracts. Similarly with the disk-diffusion method, the
antimicrobial activity is determined by the clear zone of inhibition, which is present around
the well in this method.

2.6 Recent development of drug discovery technologies

The discovery of antibiotic substances using assays that measure growth inhibition or
killing of a microbial pathogen tend to be disappointing slow (Walsh, 2003). High
throughput screening (HTS) is one of the rapid practical method that has been established
in order to improve the cost effective screening for large numbers of synthetic compounds
in vitro assays cost effectively (Pereira & Williams, 2007). This has also leads to the
increase acceptance of high throughput screening in the Pharmaceutical and Biotechnology
industry.

The application of HTS started as early in 1984 where the natural products
screening automation was designed to process and assay 10,000 fermentation broths per
week, and was completely implemented in Nagoya, Japan in 1990 (Pereira & Williams,
2007). Apart from that, HTS had also been conducted by Moy et al. (2009) to screen for
novel antimicrobials compound using Caenorhabditis elegans. In Moy et al.’s research,
37,200 compounds and natural products extracts had been subjected to automated high
throughput screening to determine the extracts that enhance the survival of C. elegans
infected with E. faecalis. Thus, the application of HTS has permitted the process to be
automated, which leads to the increase of speed and screening capacity for the
antimicrobial compound (Pereira & Williams, 2007).

Another recent development of drug discovery technologies is the applications of
metagenomics (Schmeisser et al., 2007). Metagenomics is a new field of research that has
been established over the past decade due to the increase of biotechnological demands for
biomolecules and novel enzymes. The application of metagenomics allows to elucidate the
genomes of non-cultured microbes as the not-yet cultivated microbes offer a vast resource for the development of novel chemical compounds, genes, and enzymes (Schmeisser et al., 2007).

The metagenomic libraries have been successfully used in detecting a range of novel antibiotics (Gillespie et al. 2002; Brady and Clardy 2003; Brady et al. 2004). Based on the studies by MacNeil et al. (2001) and Lim et al. (2005), soil metagenomic libraries for indirubin, a microbial product used in the treatment of leukaemia, has been reported to be successfully screened. Similarly, an antibiotic palmitoylputrescine has been extracted from bromeliad using metagenomic DNA technique (Brady and Clardy, 2004a). Thus, metagenomics has driven the discovery of novel drug and enzyme and contribute tremendously for the biotechnological use.
3.0 Materials and Methods

Marine bacteria which were isolated by Liu Yu Choi (2010) and Nur ‘Izzah Fateha (2010) were used for testing its antibacterial activities. The samples were originally collected at Satang Island, Sarawak by the crew from Aquatic Science Department. The bacterial isolates that were used are P.5.1.2, B2.4, B2.6, 3.1.2., and S.1.2.3. These bacterial isolates were chosen based on their strong antibacterial activities against the test bacteria. Gram positive *S. aureus* and Gram negative *Eschericia coli, Enterobacter aerogenes,* and *Salmonella typhi* bacteria were used as the test bacteria.

3.1 Media Preparation

3.1.1 Preparation of Nutrient Agar

The preparation of Nutrient Agar (NA) supplemented with 0.35% (w/v) sodium chloride is considered to be equivalent to 10% (v/v) salinity of the ocean (Bergman, 2001). First, 2X concentration of 3.5% (w/v) sodium chloride stock solution had been prepared by dissolving 14g of sodium chloride in 200ml of distilled water. The stock solution was then kept in a sterilized media bottle. In preparing the NA, the media agar solution supplemented with sodium chloride was boiled using a stirring hotplate and then autoclaved at 121°C, 15 psi for 20 minutes. The media was then left to cool in a water bath at 50°C before poured onto the Petri dishes. The pouring of the media was done in a laminar flow hood to prevent contamination. The agar plates and the sodium chloride stock solution were stored at 4°C after the media had been solidified.

3.1.2 Preparation of Nutrient Broth

Nutrient broth (NB) had been prepared to grow the test bacteria by suspending the NB with 1 L distilled water. The solution were mixed using hotplate and 4ml of the NB had been
pipetted to each of the 5ml Bijour bottles. The Bijour bottles containing the broth had been autoclaved for 15 minutes at 121°C and were kept in the refrigerator for further use.

3.2 Revival and Storage of Microbial Isolates

The isolated bacteria from the stock cultures from Liu Yu Choi (2010) and Nur 'Izzah Fateha (2010) were revived by culturing it onto a fresh plate containing the same culture media from which it was derived. Bacteria were incubated for 1 to 2 days at room temperature (28°C). The bacterial isolates were subcultured at least twice using the streak plate method as described by Prescott et al. (2002). After pure cultures were obtained, the bacterial isolates were then inoculated onto slant agars using 20ml Universal bottle and subsequently incubated at similar temperature and length. The Universal bottle containing slant agars were then stored at 4°C as stock culture after incubation (Green, 2009).

3.3 Gram staining of bacterial isolates

The standard Gram staining method as described by Bruckner (2008) was conducted on the six bacterial isolates to differentiate those bacteria into Gram-positive and Gram-negative bacteria.

3.4 Preparation of Test Bacteria

Three Gram negative and one Gram positive bacteria were used as test organisms and the test bacteria were *E. coli*, *E. aerogenes*, *S. typhi* and *S. aureus*.

The test bacteria from the stock were streaked on NA medium and the plates were then incubated overnight at 37°C. The single colony from each bacteria obtained were then inoculated into 4ml of NB separately and incubated overnight at 37°C. After incubation, the bacteria cultures of log phase was measured using spectrophotometer and adjusted until it reached optical density value of 0.68 at wavelength 550nm.