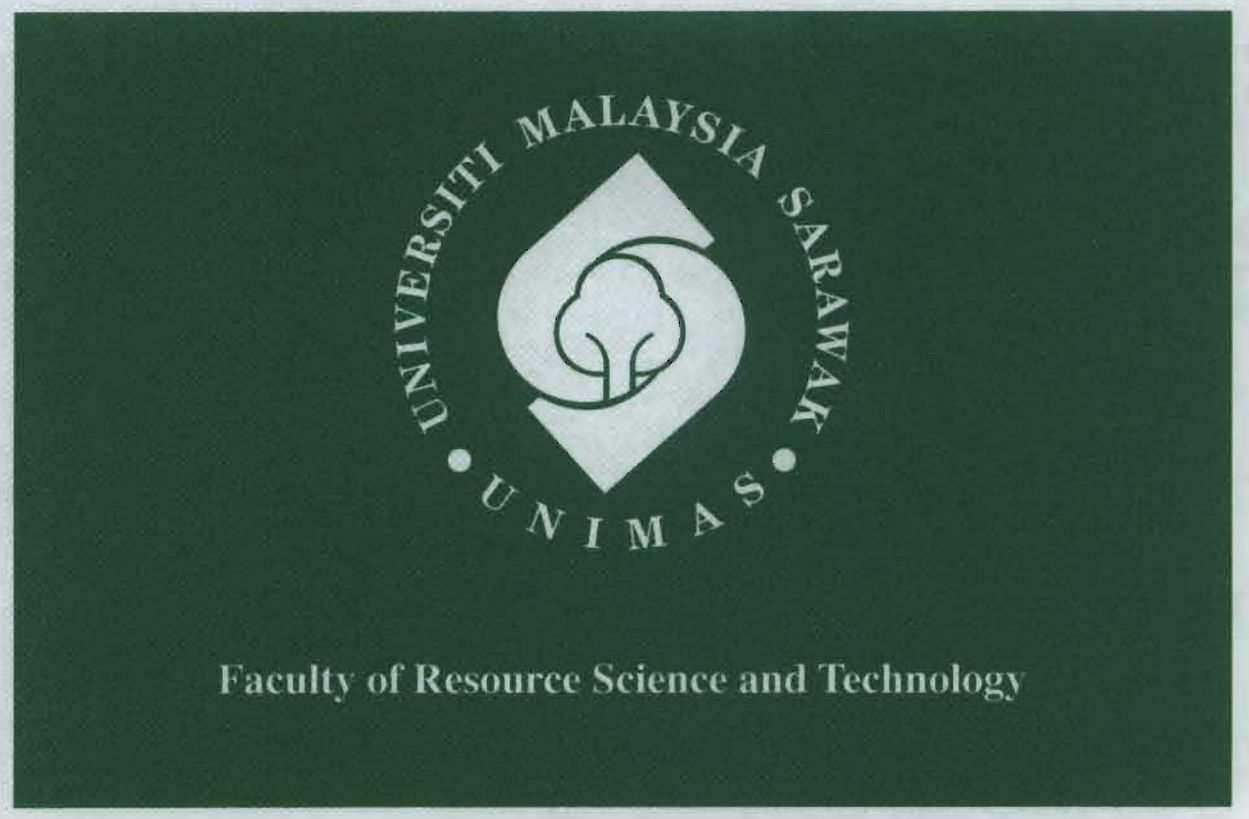

CHARACTERIZATION OF MARINE *PENICILLIUM* ISOLATES AND THEIR ANTIBIOTICS

Mohd. Noorzaifiqrudin Bin Khairol

Bachelor of Science with Honours
(Resource Biotechnology)
2012

CHARACTERIZATION OF MARINE *PENICILLIUM* ISOLATES AND THEIR ANTIBIOTICS

MOHD.NOORZAIFIQRUDIN BIN KHAIROL
24159

This project is submitted in the partial fulfilment of the requirements for the degree of Bachelor of Science with Honours

(Resource Biotechnology)

Department of Molecular Biology

Faculty of Resource Science and Technology

UNIVERSITI MALAYSIA SARAWAK

2012

Acknowledgement

First of all, I would like to express my deepest gratitude and thanks my supervisor, Professor Dr. Ismail bin Ahmad for his guidance, concerns and encouragement throughout this project. I am very grateful for being one of his FYP students.

I would like to take this opportunity to thank postgraduate students in Virology laboratory, Miss Anita, Miss Kathleen and Miss Felicia for their advice and guidance that helps me towards the completion of this project. Not to forget, Virology Laboratory Assistant, Mr Iskandarshah that has provided us with the materials we need for conducting the project.

Finally, thanks to my colleagues for their ideas, advices, information and cooperation throughout the project. I appreciate the valuable experience, knowledge and laboratory skills that I gained throughout this project.

DECLARATION

I hereby declared that this thesis entitled "Characterization of Marine *Penicillium* Isolates and their Antibiotics" submitted to Faculty of Resource Science and Technology, is a record of an original work done by me under the guidance of my supervisor, Prof. Dr. Ismail bin Ahmad. The findings embodied in this report have not been submitted to any other university or institute for any award.

Mohd. Noorzaifiqrudin bin Khairol

Faculty of Resource Science and Technology

Department of Molecular Biology

Universiti Malaysia Sarawak

TABLE OF CONTENTS

Acknowledgment	I
Declaration	II
Table of Contents	III
List of Abbreviations	V
List of Table	VI
List of Figures	VII
Abstract	VIII
1.0 Introduction	1
2.0 Literature Review	3
2.1Antibiotic-resistance microorganism	3
2.1 Antibiotic compounds	4
2.3 Marine fungi	5
2.4 Thin layer chromatography	6
3.0 Materials and Method	7
3.1 Preparation of culture media	7
3.2 Preparation of samples	7
3.3 Identification and characterization of fungi	8
3.3.1 Macroscopic examination	8
3.3.2 Microscopic examination	8
3.3.3 Molecular Identification	8
3.3.3.1DNA extraction of fungal isolates	8
3.3.3.2Polymerase Chain Reaction (PCR)	10
3.4 Antibacterial screening of fungal cultures	10
3.5 Antibiotics extraction	11
3.6 Antibiotic Assay of Crude Extracts	12
3.6.1 Test bacteria extraction	12
3.6.2 Disc diffusion assay	12
3.7 Thin layer chromatography	13

3.8 Bioautography assay of Crude Extracts 14

4.0 Results 15

4.1 Cultivation of Fungal Isolates 15

4.2 Identification and Characterization of Fungi 15
4.2.1 Macroscopic Examination 15
4.2.2 Microscopic Examination 15
4.2.3 Molecular Identification of Fungal Isolates 19

4.3 Preliminary test of Fungal Isolates 22

4.4 Disc Diffusion Assay of Crude Extracts 22

4.5 Thin Layer Chromatography 27

4.6 Bioautography Assay 28

5.0 Discussion 30

5.1 Cultivation of Fungal Isolates 30

5.2 Identification and Characterization of Fungal Isolates 30

5.3 Preliminary test of Fungal Isolates 32

5.4 Disc Diffusion Assay of Crude Extracts 33

5.5 Thin Layer Chromatography and Bioautography Assay of Crude Extracts 34

6.0 Conclusion 35

References 36

LIST OF ABBREVIATIONS

CIA	Chloroform: Isoamylalcohol
CTAB	Cetyl Trimethylammonium Bromide
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide-triphosphates
OD	Optical density
PCR	Polymerase Chain Reaction
RNA	Ribonucleic Acid
UV	ultraviolet
bp	base pair
Kbp	kilo base pair
ml	milliliter
mM	milimolar
ng	nanogram
nm	nanometer
rpm	revolutions per minute
µl	microliter
MIC	minimum inhibitory concentration
MRSA	methicillins-resistant *Staphylococcus aureus*
MTT	3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide

List of Tables

Table	Description	Page
Table 1	Thermal cycling profile	10
Table 2	Growth characteristics of fungal isolates	16
Table 3	Sequences of isolates SWS3 and SVM3 with their corresponding primer	20
Table 4	Results of ITS sequencing and Blast of fungal isolates	21
Table 5	The average antibacterial activities by the fungal isolates from the preliminary test	23
Table 6	Relative strength of extracts in five different concentrations against four test bacteria	25
Table 7	Thin layer chromatography (TLC) profiling of DCM extracts (6µl) of all extracts	27
Table 8	The presence of inhibition zone of the different spots against test bacteria	28

List of Figures

Figure	Description	Page
Figure 1	Fungal isolates; SVP3 (A), SWS3 (B), SVP4 (C), SVM3 (D), SVP3 (E) on PDA media.	17
Figure 2	Slide-culture of isolate SS3 (A), SWS3 (B), SVP4 (C), SVM3 (D), SVP3 (E).	18
Figure 3	Agarose gel profile of amplicon of PCR using DNA from fungal isolates, SVM3 (A), and SWS3 (B) as template.	19
Figure 4	Zone of inhibition as result of antibacterial activities of isolates SVM3 (D) and SVP3 (E) against S. aureus (1), S. typhi (2), E. coli (3) and E. aerogenes (4).	24
Figure 5	Disc diffusion for antibiotics screening of DCM extracts from isolates PDA S4 vortex (PDA) (A) and MEA S3 vortex (B) against S.aureus. Different dilution of extracted antibiotics: 1 mg/ml (1), 0.5 mg/ml (2), 0.25 mg/ml (3), 0.125 mg/ml (4), 0.0625 mg/ml (5), negative control: Sterile distilled water (6), positive control: 2.5x Penicillin-Sterptomycin (7).	26
Figure 6	Bioautography of DCM crude extracts of isolate: SVP4 (CDA) (A) and SVM3 (MEA) (B) against *S. aureus* and *E. aerogenes*, respectively.	29

Characterization of Marine *Penicillium* Isolates and their Antibiotics

Mohd.Noorzaifiqrudin Bin Khairol

Resource Biotechnology Programme
Faculty of Science and Technology
Universiti Malaysia Sarawak

ABSTRACT

The emergence of antibiotic resistant microorganisms has received growing concern on the problems they have brought to human and animal health. *Penicillium* sp. have been known as their potential of producing antimicrobial compounds. Recently, this species were discovered to produce more than one type of antimicrobial compounds. This discovery has been promising to encounter the ongoing emergence of antibiotic resistant microorganisms. In this study, a total of five pure fungal isolates were cultivated from marine environment. Out of five, two of them were putatively identified as *P. oxalicum* and *Ampelomyces* sp. From the preliminary test of fungi isolates, almost all the isolates were shown to have strong antibacterial activity against test bacteria, which were *S. aureus*, a Gram-positive bacteria as well as *S. typhi, E. coli* and *E. aerogenes*, Gram-negative bacteria. Contrary to this, only dichloromethane extracts of isolates SVM3 and SVP4 (PDA) were active against *S. aureus* observed in disc diffusion assay. The results were also inconsistent with the bioautography assay of the extracts, which showed only dichloromethane extracts of SVP4 (CDA) and SVM3 were active against *S. aureus* and *E. aerogenes*, respectively. The active compound in bioautography assay might be penicillin, as compared to penicillin-streptomycin as reference.

Keywords: *Penicillium oxalicum, Ampelomyces*, bioautography assay, disc diffusion assay

ABSTRAK

Kemunculan mikroorganisma yang tahan-antibiotik telah mendapat perhatian daripada ramai pihak mengenai masalah-masalah yang timbul disebabkan oleh mereka. Keupayaan *Penicillium* sp. untuk menghasilkan antibiotic telah diketahui. Baru-baru ini, spesies ini telah dijumpai untuk menhasilkan lebih daripada satu jenis antibiotic. Penemuan ini memberi harapan kepada kita untuk melawan kemunculan mikroorganisma tahan-antibiotik tersebut. Dalam kajian ini, sebanyak lima pencilan kulat telah dipencilkan dari persekitaran marin. Daripada lima pencilan kulat, dua daripadanya telah di kenal pasti sebagai *P. oxalicum* dan *Ampelomyces*. Ujian awal terhadap pencilan kulat menunjukkan hampir kesemua pencilan kulat menunjukkan kesan anti-bakteria yang kuat terhadap bakteria ujian, iaitu *S. aureus*, Gram-positif bakteria dan *S. typhi, E. coli* dan *E. aerogenes*, Gram-negatif bakteria. Sebaliknya, hanya *dichloromethane* ekstrak daripada pencilan SVM3 dan SVP4 (PDA) yang menunjukkan aktiviti anti-bakteria dalam *disc diffusion assay*. Manakala hasil daripada *bioautography assay* pula menunjukkan keputusan yang bercanggah, di mana ekstrak daripada SVP4 (CDA) aktif terhadap *S. aureus* dan ekstrak daripada SVM3 (MEA) aktif terhadap *E. aerogenes*. Bahan aktif dalam *bioautography assay* kemungkinan adalah penicillin, seperti dibandingkan dengan penicillin-streptomycin sebagai rujukan.

Kata kunci: *P. oxalicum, Ampelomyces, bioautography assay, disc diffusion assay*

1.0 Introduction

Antibiotics are antimicrobial drugs that are derived from microorganisms that can inhibit growth and kill bacteria (Walsh, 2003). Since the discovery of penicillin in 1929 by Alexander Fleming, antibiotics have been used extensively to treat varied types of infectious diseases. The development of penicillin and other major classes of antibiotic produced from soil such as streptomycin, chloramphenicol and tetracycline between 1945 and 1955 has mark the start of antibiotic era (Clardy *et al.*, 2009).

However, the widespread use and misuse of antibiotics has led to the emergence of pathogenic microorganisms that are resistant to various current antibiotics. The emergence of these antibiotic resistant microorganisms has led to numerous challenges in healthcare prospect. The problems that have arises from this emergence includes increased cost for drugs and disease control measures, prolonged duration of illnessess and increased patient mortality (British Columbia Centre for Disease Control, 2010). The report by British Columbia Centre for Disease Control (2010) showed that "the percent of *Staphylococcus aureus* isolates that are methicillin-resistant (MRSA) has increased since 2008". If the antibiotic resistant bacteria increases constantly through the years without applying any preventive measure, more major problems could arise worldwide. Subsequently, the era of antibiotic are threatened by the emergence of this antibiotic resistant bacteria. In some areas of United States, up to 30% of *Streptococcus pneumonie* is no longer susceptible to penicillin (Centers for Disease Control and Prevention, 1999). World Health Organization (WHO) (2000) has reported that over 20% of new tuberculosis cases are now multi-drug resistant. Thus, there is a need to procure new antibiotics with different mechanisms of action to augment the currently used ineffective antibiotic.

Antibiotic-producing microorganisms can be collected from various sources including soil and marine environment. Three quarter of the earth surface are enclosed by marine environment, and this forms rich source of diversity for natural products (Abdel-Lateff, 2004). In fact, it has been claimed that marine environment is unique in terms of its specific composition in both organic and inorganic substances, as well as temperature ranges, and pressure conditions (Abdel-Lateff, 2004). Therefore, it is essential to explore the diversity of marine environment to discover new novel compounds that can help to revive antibiotics capability for treatment of diseases.

Penicillium sp., a fungus that produces the first discovered antibiotic, has been found to have potential of producing antibiotics other than penicillin. Several secondary metabolites that showed antibiotic activity have been successfully isolated from *Penicillium*. These include vermiculinol and vermiculidiol from *Penicillium vermiculatum* (Massias *et al.*, 1988) and (+)-aristolochene from *Penicillium roqueforti* (Demyttenaere *et al.*, 2001). The rich sources of secondary metabolites from *Penicillium* sp. will be advantagous to fight the ongoing emergence of antibiotic resistant microorganisms. For this purpose, more intensive study on *Penicillium* sp. has to be done to isolate antibiotic compounds with different mechanism to kill pathogenic microorganisms.

Objectives:

1. To characterize the fungal isolates
2. To screen for antimicrobials activities from *Penicillium* sp.
3. To extracts non-penicillin antibiotics from *Penicillium* sp.
4. To determine the antibacterial activities of extracted antibiotics

2.0 LITERATURE REVIEW

2.1 Antibiotic-resistance microorganism

The occurrence of disease-causing microorganisms that acquire the resistance to antibiotic has become worldwide concerns, giving new challenges to medicinal world. Overuses of antibiotics are believed to be the cause for the emergence of these antibiotic-resistance microorganisms. Antibiotics are used for widespread purposes, even for the treatment of a non-life threatening ailment such as colds and sore throat (Vora, 2002). In addition, farmers also used antibiotics for their livestock, but not for the treatment of sick animals. The antibiotics are applied for the purpose to promote the growth of their farm animals (Hughes & Heritage, n.d). This phenomenon has added antibiotic pool into the environment and causes more exposure for the microorganisms to the antibiotics. As the results, the emergences of antibiotic-resistant microorganisms develop faster.

The emergences of antibiotic-resistant bacteria are occurring rapidly. The World Health Organization (2000) has reported that over 20% of new tuberculosis cases are now multi-drug resistant. Commonly, the isolated bacteria from antibiotic-contaminated environments such as hospital, sewage, effluents and wastewater show highest level of resistance (Fontaine &Hoadley, 1976; Toranzo *et. al*, 1984; McPherson & Gealt, 1986; Chandrasekaran *et. al*, 1998).

Bacteria posses the capacity to adapt to its new environmental condition by resistance mechanism (Scientific Committee on Emerging and Newly Identified Health Risks, 2009). This feature helps bacteria to survive in the presence of antibiotics. Numerous studies have been done to identify the mechanism of resistance (Roberts, 1996). Furthermore, the resistance gene can be transferred to other bacteria as well (Chandrasekaran *et al.*, 1998). Spratt (1994) stated that the transfer of resistance genes among the bacteria occurs through

horizontal transfer of plasmid-encoded gene. This feature helps bacteria to survive in harsh condition where their lives are threatened by the presence of lethal substances which is antibiotic.

2.2 Antibiotic compounds

Antibiotics are the substances that can kill and inhibit growth of microorganisms (Walsh, 2003). Antibiotic which inhibit bacteria growing are term as bacteriostatic, whereas the type that kill and lowering bacterial amount term as bacteriocidal (Walsh, 2003). The discovery of antibiotics by Alexander Fleming in 1928 was happen by chance, where he accidentally found a ring around the mould growing on his plate (Guy, 2005). The mould, which was later identified as *Penicillium* are believed to contain a substance that can inhibit bacterial growth. The application of antibiotics in healthcare has saved billions of lives from bacterial infection (Zhang *et al.*, 2009).

Antibiotics are either natural products or made synthetically. Fungi or bacteria produce antibiotic to defense themselves from live-threatening condition and also for life-sustaining (Walsh, 2003). In a condition where the microorganisms have to compete for their food, antibiotic compounds are synthesized and excreted out to eliminate their competitor. This phenomenon was observed in the production of pestalone, an antimicrobial compounds produce by marine fungus *Pestalotia* sp. only when they were co-cultured with the marine bacterium (Cueto *et al.*, 2001).

2.3 Marine fungi

Ocean covers about two third of the earth surfaces. The wide area of marine environment offers a plenty of resources to be explored for their natural products. Most of the natural products isolated from marine environments are believed to have a huge potential as pharmaceutical, cosmetics, enzymes and nutritional supplements (Kansoh *et al.*, 2010). Many have successfully isolates novel compounds from marine environment. For example, fungus found in marine environment, *Aigialus parvus* has been shown to produce number of bioactive compounds, an antibiotic, which was found to suppress the sporulation of *L. laevis* (Jones, 2000). Other study by *Heo et al.* (2009) shows that phlorotannins from *Ecklonia cava* have photoprotective effect against the photo-oxidative stress induced by UV-B irradiation. Therefore, marine environment deserved the attention from researchers for their diversity.

With the growing concern on the emergence of antibiotic-resistant pathogen, intensive efforts have been undertaken to screen for new antibiotics. Two groups have been identified as the major-antibiotic producing organisms which are bacteria and fungi (Neumann, 2008). However, the antibiotic-producing organisms are still dominated by fungus group (Ho *et al.*, 2003). Fungi are one of the most diversified groups of organism (Ho *et al.*, 2003) with estimated number reaching 1.5 million, but 95% have yet to be discovered (Hawksworth, 1991, 2000; Ho *et al.*, 2003). To date, lots of new antibiotic have been isolated from diverse species of marine fungi. Cueto *et al.* (2001) have found a new antibiotic, pestalone that was produce by *Pestalotia* sp. which was isolated from the surface of the brown algae. Thus, the screenings of new antibiotic from marine fungi are absolutely needed to cope with the rapid emergence of antibiotic-resistance microorganisms.

2.4 Thin Layer Chromatography

Thin layer chromatography (TLC) is a technique used to identify sample's components by separating the components (Clark, 2007). TLC comprises of two phases, liquid mobile phase and the solid stationary phase. Alumina or silica is commonly used for stationary phase that is highly polar. The mobile phase is a solvent, called the eluent, will move up through capillary action. The movement of the eluent along the plate is quantified by R_f value.

The basic principle of TLC is the same to other chromatographic method which is based on the principle of separation (HubPages Inc., n.d). The mobile phase which is the solvent will dissolved the compound and carried them together as the solvent continues to move upwards. There are two factors that control how fast the compounds to get carried up the plate; the solubility of the compound in the solvent and the affinity of the compound to the stationary phase (Clark, 2007). The compounds will be separated during the movement as the high affinity compounds travel slowly than the other compounds.

This technique is the most common technique used for separation of components because it offers several advantages over other chromatography methods (HubPages Inc., n.d). TLC offers a simple process with short development time and helps in visualization of separated compound spots easily. Besides that, this method helps to identify the individual compounds and can be used to isolate most of the compounds. In addition, the purity standards of the given sample can be assessed easily. Most importantly, this method is a cheap chromatography technique.

3.0 MATERIALS AND METHODS

3.1 Preparation of Culture Media

All media in this study were supplemented with 10% of seawater. Potato Dextrose agar (PDA), Malt Extract Agar (MEA) and Czapek Dox agar (CDA) were prepared by adding known volume of sterilized seawater into Schott bottle containing agar media. The media was boiled with stirring on a magnetic stirrer hotplate and then autoclaved at 121°C, 15 psi for 20 minutes. Next, the media were cooled in oven at 60°C and then poured onto the Petri dishes in the laminar hood to prevent contamination. After the media have solidified, the plates were stored at 4°C in the cold room.

3.2 Sample Processing

Three mangrove seedlings and three tubes with the seawater from sampling site were collected from Bako National Park. Mangrove seedlings and tubes were washed using autoclaved seawater. The samples were collected by swabbing the surface of mangrove seedlings and tubes, and inoculated onto agar plates. The mangrove seedlings were cut into small pieces and put into small universal bottle containing autoclaved seawater. The universal bottle was then vortex and 100 µl of the solution was pipetted onto the agar plates. Aliquots of 100 µl of the seawater taken from sampling site were also pipette onto the agar plates and spread over the surface.

3.3 Identification and Characterization of Fungi

Selected fungal isolate, which showed the most potential of producing antibiotics in the antibacterial screening were selected for identification and characterization.

3.3.1 Macroscopic Examination

The growth characteristics of selected fungal isolates were determined by macroscopic observation after the fungal colony has grown to full plate. Macroscopic observation were made on the colour of the mycelia mat, reverse colour, margin of the colony, mycelia mat characteristics, and the colour change of culture media (Maza *et al.*, 1997).

3.3.2 Microscopic Examination

The identification of fungi was carried out through microscopic examination using slide-culture method (Maza *et al.*, 1997). A small piece of agar with fungal mycelium was cut out from a fungal culture, and was aseptically transfer onto a sterilized microscope slide. The sample was then covered with a cover slip, which was supported by plasticins. The culture slides were placed in Petri dish sealed with parafilm and then incubate at room temperature for 3 to 5 days to allow sporulation. The slides were then examined under the microscope (Olympus BX51) for the presence of spores, spore structure and the hyphae structure. The identification of fungi was based on The Saccardo System of Classification with the aid of descriptions found in Illustrated Genera of Imperfect Fungi (Barnett & Hunter, 1972).

3.3.3 Molecular Identification of Fungal Isolates

3.3.3.1 DNA Extraction of Fungal Isolates

Isolation of fungal DNA were done following CTAB method as was described by Cubero *et al.* (1999). About 3-100 mg of samples were dispensed into 1.5 ml tubes and placed in a

container with liquid nitrogen for 5-10 minutes. The tubes were then removed from the container and clean liquid nitrogen was added to the tube and a sterile pre-cooled sharp glass bar was used to grind the material.

Extraction buffer (1% w/v CTAB, 1M NaCl, 100 mM Tris, 20 mM EDTA, 1% w/v polyvinyl polypyrolidone, PVPP) of about 0.5 ml were added to the pounded material. The buffer was pre-warmed before addition to avoid CTAB precipitation. PVPP was added to the buffer immediately prior to use. The tubes were mixed and then heated in a waterbath for 30 minutes at 70°C before adding one volume of chloroform: isoamyl alcohol (24:1 v/v) followed by centrifuge at 10000 rpm for 5 minutes at room temperature. The upper aqueous phase was collected in a new tube. Two volumes of precipitation buffer (1% w/v CTAB, 50 mM Tris-HCl, 10 mM EDTA, 40 mM NaCl) were added to the supernatant and mixed well by inversion for 2 minutes. The mixture was then centrifuged at 13000 rpm for 15 minutes at room temperature and the pellet were collected.

The pellet was resuspended in 350 µl of 1.2 M NaCl, to which one volume of chloroform: isoamyl alcohol (24:1) was added. This was mixed vigorously and centrifuged at 10000 rpm for 5 minutes at room temperature. The aqueous phase was transferred to a new tube and 0.6 volume of isopropanol was added. The mixture was inverted several times and the tube was placed at -20°C for 15 minutes. The final pellet was collected by centrifugation for 20 minutes at 13000 rpm at 4°C. The final pellet were washed with 1ml of 70% ethanol and recollected by centrifugation at 13000 rpm for 3 minutes at 4°C. The pellet was drained and dried at 50°C and then suspended in 25 µl TE buffer (10 mM Tris pH 7.4, 1 mM EDTA). Five micro liter of extracted DNA concentration is determined using 1% agarose gel analysis.

3.3.3.2 Polymerase Chain Reaction (PCR)

The extracted DNA from fungal isolates were subjected to PCR amplification using specific primers ITS1F (Gardes &Bruns, 1993; Cubero *et al.*, 1999) and ITS4 (White *et al.*, 1990; Cubero *et al.*, 1999). The PCR amplification was programmed to 40 cycles of a denaturation at 94°C for 1 minute, annealing at 53°C for 45 seconds and elongation at 72°C for 1 minute.

Table 1: Thermal cycling profile

Parameter	Temperature (°C)	Time	No. of cycles
Denaturation	94	1 minute	
Annealing	53	45 second	40
Extension	72	1 minute	

The condition for DNA amplification was 2.5 mM $MgCl_2$, 0.4 mM each dNTP, 0.2 µM each primer, 0.2 U Tag and 1-5 ng DNA. The final reaction volume will be 25 µl. Amplified PCR products were determined through 1% agarose gel in Tris-acetate-EDTA buffer (Sambrook *et al.*, 1989) and stained with ethidium bromide.

3.4 Antibacterial Screening of Fungal Cultures

The fungal isolates were sub-cultured from slant agar onto PDA. After 5 days incubation at room temperature, the fungal isolates were sub-cultured to new plates with maximum of four isolates per plate. Three-day cultures of the fungi were used for screening against the test bacteria using agar overlay technique. Selected fungal isolates were screened for the presence of inhibition zone against the test bacteria.

Antibacterial screening was performed against four bacteria species: one Gram-positive bacteria, *S. aureus* and three Gram-negative bacteria, consisting of *E. coli*, *S. typhi* and *E. aerogenes*. The test bacteria were prepared on NA and incubated at 37°C for overnight. Single colonies were then inoculated into nutrient broth (NB) and incubated at the same condition. The final concentration of the test bacterial suspension was adjusted (Ahmed *et al.*, 2008) to optical density (OD) of 0.168 at 550 nm. Test bacterial suspension was then added to the soft agar. The soft agar media will be overlaid onto agar plates that are seeded with the fungal isolates and incubate at room temperature for 24 hours. After incubation, the plates are check for the presence of inhibition zone of growth inhibition around the bacterial spots as a result of antibacterial activities.

3.5 Antibiotics Extraction

The fungal isolates that show antibacterial activity against the test bacteria were selected for antibiotic extraction. A total of eight plates were prepared three colonies per plate. These colonies were grown on solid agar until almost full plate and then let to dry at room temperature to remove most of the water that present in the agar. Dried agar were then peels off from Petri dish and cut into small pieces.

Subsequently, agar pieces were submerged in two different organic solvents, which were hexane and dichloromethane (DCM) of 100 ml using two different conical flasks to dissolve different antimicrobial substances from marine fungi isolates. Each 250 ml conical flask was specific for one type of organic solvent. Each extract was then filtered and poured into 15 ml universal bottles. Initially, the universal bottles containing extracts were left to dry at room temperature for one month. In addition, organic solvents were left to dry using water

bath and incubator. The various extracts of solvents were tested for the presence of antimicrobial activity.

3.6 Antibiotic Assay of Crude Extracts

3.6.1 Test Bacteria Extraction

The antibacterial activities of extracted antibiotics were assessed against four bacteria species: Gram-positive bacteria, *S. aureus* and Gram-negative bacteria, consisting of *E. coli*, *S. typhi*, *E.aerogenes*. The test bacteria were prepared on Mueller-Hinton agar (MHA) and incubate at 37°C for overnight as described by Valgas *et al.* (2007). Single colonies were then inoculated into Mueller-Hinton broth (MHB) and incubated at similar condition. The final concentration of the test bacterial suspension was adjusted (Ahmed *et al.*, 2008) to OD = 0.168 at 550 nm.

3.6.2 Antibacterial Screening of Extracted Antibiotics: Disk Diffusion Assay

This step was performed according to method described by Choudhury *et al.* (2005). Hundred microliters of the standardized test bacteria was swab onto MHA. When dried, seven antibiotic free filter discs of 6 mm diameter were arranged on the Petri dish containing agar. The extract was weight and reconstituted in 100 µl of 5% methanol and 900 µl of sterile distilled water, and then diluted to final concentration of 0.5 mg/ml, 0.25 mg/ml, 0.125 mg/ml, 0.0625 mg/ml and 10 µl of solutions were transferred into each filter disc. Ten microliter of sterile distilled water was used as negative control while 10 µl of 2.5x dilution of penicillin-streptomycin used as positive control. The Petri dish were incubated at 37°C for 18-24 hours. Formation of inhibition zone were observed and measured. The antibacterial

activity was expressed as the mean of inhibition diameters (nm) produced as described by Choudhury *et al.* (2005).

3.7 Thin Layer Chromatography

Thin layer chromatography (TLC) was carried out as described by Volland (2005). Initially, fractionation of extract using TLC was conducted in glass container. The stationary phase were chromatography plate covered with silica gel while the mobile phase were based on which solvents extracts that shows the most antibacterial activities.

Initially, 6 µl of extract (1mg/ml) was drop at 0.5 centimeter (cm) from the chromatography plate base. The plate was then allowed to air-dry before inserted into glass container containing filter paper. Each run was stop when the solvent front reach 0.5 cm from the chromatography plate end and the solvent end-point was marked with straight line using pencil. Then, the developed TLC plates were air-dried.

The presence of spots and bands were visualized under UV irradiation at 254 nm and marked with red colour pencil. After that, the chromatograms were dip into 10% of H_2SO_4 in vanillin solution follow with heating at 110°C using hair-dryer until no bands appear. The bands were marked with green colour pencil. The R_f value for each spot on the chromatogram were calculated (Harbone, 1973) using the formula:

R_f = (distance of sample travelled) / (distance of solvent travelled)

3.8 Bioautography Assay of Crude Extracts

Bioautography assay was performed against four bacteria species: one Gram-positive bacteria, *S. aureus* and three Gram-negative bacteria, consisting of *E. coli*, *S. typhi* and *E. aerogenes*. The test bacteria were prepared on NA and incubated at 37°C for overnight. Single colonies were then inoculated into nutrient broth (NB) and incubated at the same condition. The final concentration of the test bacterial suspension was adjusted (Ahmed *et al.*, 2008) to optical density (OD) of 0.168 at 550 nm. Test bacterial suspension was then added to the soft agar.

The soft agar were overlaid onto agar plate with the developed TLC plate on the agar and incubated at 37°C for 24 hours. After incubation, the agar was sprayed with MTT to detect the presence of inhibition zone. The plate were left for 4 hours before cutting out TLC plate with agar on the plate and transferred into new Petri dish.