Drug Discovery in Soil Bacteria from Unique Ecosystems of India and Indonesia

Victor Tran

A THESIS

Submitted to

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In partial fulfillment of

The requirements for the

Degree of

Honors Bachelors of Science in General Science

With an Option in Pre-Pharmacy

and

Minor in Chemistry

and

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Abstract

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Abstract approved: _____
Dr. Taifo Mahmud

Natural products isolated from bacteria can be developed into new therapeutic agents. The soil bacteria actinomycetes, specifically the *Streptomyces* species, are prolific producers of antibiotics. This thesis is composed of two projects which focus on novel drug discovery from soil bacteria collected from the Indonesian Black Water Ecosystem (the ICBB strains) and soil bacteria from the abandoned Hundung Cement Factory in India (the MBRL 201 and 251 strains). The ICBB strains were cultured in Yeast Peptone Glucose Malt Extract and Modified Bennet media and the MBRL strains were cultured in Yeast Malt Extract Glucose medium. Following a week of cultivation, the culture broths were centrifuged. The supernatant was extracted sequentially with EtOAc and BuOH. The remaining aqueous solution after the extraction process was lyophilized and prepared for biological activity testing. The mycelia of the MBRL strains were also subjected to further extraction using a mixture of MeOH and acetone. Biological assays were carried out against several microbial and fungal organisms. Active extracts from the ICBB strains were noted, but due to time constraints no follow-up experiments were carried out. On the other hand, the active extracts from the MBRL strains were extensively investigated using bioassay-guided isolation and purification.

On the basis of the sequence of their 16S rRNA genes, MBRL 201 and 251 strains were identified to be *Streptomyces* sp. and *Pseudomonas* sp., respectively. Active extracts from these strains were subjected to bioassay-guided fractionations. The MBRL 251 BuOH extract and the MBRL 201 EtOAc extract were investigated most extensively because the TLC and bioassay results indicated the presence of bioactive natural products in these extracts. The MBRL 251 BuOH extract was fractionated and purified with a series of open column chromatography. The active compound was analyzed using mass spectrometry, ^{1}H and ^{13}C Nuclear Magnetic Resonance (NMR) spectroscopy. In addition, ANTIBASE®, a searchable database that contains descriptive, physico-chemical and spectroscopic data, was used to quickly identify known compounds. The results revealed that the bioactive compound in MBRL 251 is phenazine-1-carboxylic acid. The MBRL 201 extract was fractionated with preparative thin layer chromatography and purified with size exclusion chromatography. However, at this time, further experiments are necessary to determine the chemical structure of the active MBRL 201 compound.

Keywords: drug discovery, natural products, phenazine-1-carboxylic acid

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Honors Baccalaureate of Science in General Science Project of Victor Tran

presented on May 31st, 2013

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I understand that my project will become part of the permanent collection of Oregon State University, University Honors College. My signature below authorizes release of my project to any reader upon request.

_____

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CHAPTER 1

INTRODUCTION

1.1. The History of Natural Product Discovery

Humans have constantly sought ways to survive against the onslaught of diseases. While there have been many scientific and therapeutic advancements in medicine since the Paleolithic era, the discovery of antibiotics did not happen until the 1930s.[i] One of the most defining events that revolutionized modern medicine was the accidental discovery of penicillin.[ii] In 1928, Alexander Fleming, a microbiologist who studied bacteria that he isolated and grew on petri dishes, noticed that one of his dishes was contaminated with a mold that inhibited the growth of bacteria. Fleming managed to isolate the product, called penicillin, and found that it was active against other bacteria. His discovery of penicillin was significant because it introduced antibiotics into modern medicine and encouraged other scientists to venture into the unknown and work with other bacteria and fungi to discover potentially new therapeutic agents that could cure a myriad of infectious diseases.

Fleming's discovery of penicillin led to a "golden epoch" of antimicrobial discovery in the 1940s and 1950s where many significant antibiotics, such as aminoglycosides, cephalosporins, macrolides, and tetracyclines were isolated[iii]. Approximately 70% of the compounds isolated during this time were from soil bacteria. Soil bacteria (actinomycetes) have been known as a prolific source of the antimicrobial and anticancer compounds used in the clinics. Of all the actinomycetes, the genus *Streptomyces* has produced the greatest number and structural diversity of these drug leads. As of 2011, out of the approximately 11,000 known natural product compounds derived from microbes; the *Streptomyces* species make up 74% of them, whereas the rare strains of actinomycetes represent 26% of the compounds.[iii] Despite their tremendous potential, it has become

increasingly difficult to isolate novel strains of these bacteria and subsequently discover new classes of bioactive natural products. The difficulty lies in the fact that readily cultured species, mostly *Streptomyces* strains, are repeatedly isolated, resulting in the re-isolation of known natural products more often that the discovery of new ones. Nevertheless, there remains enormous potential to discover more drug leads from this prolific resource. It has been suggested that less than one part in 10^{12} of the earth's soils have been screened for actinomycetes, and only a small fraction (1-3%) of the total number of natural products produced by *Streptomyces* species have been characterized.[iv]

The importance of discovering new novel natural products is based on the rising concerns of the growing presence of multi drug-resistant infectious strains of bacteria and fungi that cause disease. In developed countries, the luxury of antibiotics has led to a misuse of antibiotics leading to the emergence of multi-drug resistant strains of bacteria and fungi. This evolution of antibiotic resistance is due to a variety of reasons. Foremost, among non-hospital patient cases, approximately 50% of the 130 million antibiotics prescribed annually by physicians are considered to be unnecessary interventions[v]. In addition, industrial farms have increased their antibiotic use on livestock from 22 million in 2001 to 29.9 million in 2011, which further encourages drug resistance in infectious agents.[vi] The diseases caused by these infectious agents cannot be cured with the current antibiotics that the medical institutions have at their disposal, so it is essential to find new lead compounds that can be potentially be used as future therapeutic agents.

Besides human diseases, plant pathogens are also an enormous problem in developing countries that heavily rely on agriculture for subsistence. World hunger affects over 900 million people every day, with India holding more than 24%[vii] of those whom are malnourished.[viii] While the media has concentrated the public's attention on what climate change can do to growing crops, it should also be noted that some bacteria and fungi can also affect the crop yield, and thus reducing

the overall year's worth supply of food. Rice fungi, such as *Rhizoctonia solani*, found in India plague a farmer's crop yield exacerbating the hunger experienced by millions of rural families every day.

As part of an effort to discover new bioactive natural products from soil bacteria, our investigation focuses on a number of *Streptomyces* and non-*Streptomyces* soil bacteria that were isolated from unique ecosystems in India and Indonesia. This work is part of a collaborative project between Dr. Mahmud's laboratory and scientists in those countries.

1.2. Natural Production Isolation and Structure Elucidation Techniques

1.2.1. Liquid-liquid Partitioning

One of the common natural product isolation techniques firstly used is liquid-liquid partitioning. The liquid media containing the bacterial secondary metabolites are poured into the appropriate separatory funnel. The broth is then extracted with a series of solvents that vary in polarity to yield different types of compounds. The major rule that applies in the liquid-liquid partitioning is "like dissolves like". The use of non-polar solvents, such as hexane or ethyl acetate (EtOAc), will yield non-polar extracts and the use of more polar solvents, such as butanol (BuOH), will extract more polar compounds.[ix] For liquid-liquid partitioning and the remaining following techniques, experimental procedures will be documented further below during the investigation explanation.

1.2.2. Thin-Layer Chromatography (TLC)

Thin-layer chromatography is usually the first lab technique used for small scale observations to determine the best solvent systems to isolate the compound(s) of interest. Its concepts then can be applied to larger scale chromatographic studies, such as open column or preparative chromatography. TLC and all other chromatographic techniques have two phases: a stationary phase and a mobile phase.[x] The stationary phase supports the substance by binding it to its own surface[xi]. In this case, the stationary phase is a thin layer of silica gel that covers a sheet of aluminum metal or glass. The mobile phase is always the solvent system that carries the compounds through the stationary phase. Based on the solvent system, components of the substance will move at different speeds due to polarity, thus separating crude extracts into multiple compounds. The TLC plate provides information about the components of the crude extracts and its affiliated best solvent system used to isolate each fraction.

1.2.3. Open Column Chromatography (OCC)

The concepts behind TLC can be applied in a larger scale through this type of lab technique. This technique is named OCC because the vertical glass column used is open to the environment. Silica gel is used to pack the column and the solvent system determined by initial TLC screenings can be used in a larger scale for this fractionation process. The more crude extract that one wants to separate, the more silica gel and a longer vertical glass column are required.

1.2.4. Preparative Thin Layer Chromatography (PTLC)

Like open column chromatography, PTLC uses the concepts behind thin-layer chromatography in order to separate the crude cocktail into multiple fractions. A PTLC plate is essentially a thicker version of a TLC plate, but the silica gel is manufactured in a way that makes it easy to scratch off with a sharp tool. Compounds move based on the solvent system determined earlier by a TLC screening used to separate the crude extract. In this technique, one scratches off certain areas of the PTLC plate depending on what compounds one wants to isolate. The compound of interest is then extracted using specific solvents. Due to the limited thickness and length of a PTLC plate, one cannot fractionate as much crude as one can with an open column.

1.2.5. High Performance Liquid Chromatography

This is another lab technique that employs the concepts behind polarity to separate the crude into multiple fractions.[xii] HPLC is a finer-tuned version of open column chromatography that uses high pressure to push the solvent out. Samples are injected into the HPLC system where they then run through a stationary phase column and then a sensitive detection machine for identification. The machine detects how much ultra-violet light is absorbed by the organic compounds and then displays a graph on the computer screen. This recording graphs out retention time of the organic compound mixture on the x-axis and the UV light wavelength absorption on the y-axis. Thus, one can collect different fractions of their sample by looking at the varying spikes on the graph. There are also a few limitations with this experiment. Foremost, not all compounds can be detected at the same wavelength. HPLC also can't detect the difference between two compounds that come out at the same retention time.

1.2.6. Size Exclusion Chromatography

TLC, OCC, PTLC, and HPLC all use polarity in order to fractionate crude extracts. Size exclusion chromatography uses the size of molecules in order to isolate and purify the compound of interest. Inert porous dextran beads or other materials are packed into a vertical glass column, making up the stationary phase. When the extract is placed in the column, the large molecules bounce around the beads, but won't go into them, so they come out first. Smaller molecules get trapped inside the dextran beads and have to navigate through some tunnels before coming out of the column, so they are the last to come out[xiii].

1.2.7. Electrospray Ionization (ESI) Mass Spectrometry

Mass spectrometry measures the molecular mass of a sample subjected to the analysis of a mass spectrometer.[xiv] For ESI, a sample is injected into a chamber where it is subjected to high atmospheric pressure, which removes their electrons, creating molecular cations. These molecules eventually break into smaller particles and are accelerated to have the same kinetic energy. Afterwards, they go through the magnetic field of the analyzer tube, in which they are deflected. The larger and more charged the ion is, the more it got deflected. The analyzer then detects the abundance and the mass–to-charge ratio of the molecules. Mass spectrometry has two types of ion modes: positive and negative. Both mass spectra help identify the mass of known and unknown compounds.

1.2.8. Nuclear Magnetic Resonance (NMR)

Nuclear magnetic resonance is another technique used to elucidate the chemical structure of compounds.[xiv] NMR helps determine the carbon-hydrogen layout of an organic structure. Analytes that are run through NMR are immersed in magnetic fields, in which their atomic nuclei spin in certain directions. The NMR spectroscopy detects the energy difference between these low and high energy spins and plots them on a spectra (signal frequency vs. intensity). Neighboring atoms that are in different "environments" (electron withdrawing groups, bond differences, etc.) show up distinctly on the spectra and can help determine various parts of an organic structure.

CHAPTER 2

PRELIMINARY BIOACTIVITY SCREENING OF EXTRACTS FROM INDONESIAN SOIL BACTERIA

2.1. INTRODUCTION

The black water rivers of the Black Water Ecosystem (BWE) are acidic, nutrient deficient, and contain high levels of tannins, which have very different characteristics in comparison to freshwater environments[xv]. Tannins are plant polyphenols that bind and precipitate proteins.[xvi] They are known to be astringent, toxic when consumed in high concentrations, and bitter tasting. Microorganisms living in such unique conditions may produce novel natural products.

In collaboration with the Indonesian scientists at the Indonesian Center for Biotechnology and Biodiversity (ICBB), we set out a research program to discover novel bioactive natural products from soil bacteria of the Black Water Ecosystem in Indonesia. As part of that effort, I cultured a number of non-*Streptomyces* strains isolated from Indonesia, produced their extracts, and tested their biological activities against a number of bacterial and fungal organisms.

2.2. EXPERIMENTAL MATERIALS AND METHODS

Organism Collection:

Non-*Streptomyces* strains were collected from the Black Water Ecosystem in Kalimantan, Indonesia[xvii]. Their deposits were stored as 1 mL 20% glycerol stocks in a -80 ºC freezer. The

following strains (with their affiliated ICBB codes) were screened in preliminary bioassay examinations to look for potential bioactive natural products.

Table 2.1: Non-*Streptomyces* actinomycetes strains identified from BWE

Isolate	Taxonomy	Ident. %	Accession No.
8177	*Micrococcus* sp.	98	AM990780
8311	*Amycolaptopsis albidoflaxus*	97	AB327251
8313	*Nocardia paucivorans*	99	AJ437309
8316	*Amycolaptopsis albidoflaxus*	96	AB327251
8336	*Amycolaptopsis halotolerans*	97	DQ000199
8351	*Amycolaptopsis* sp.	97	AY129762
8355	*Kribbella swartbergensis*	97	AY995147

Solid Culture

The ICBB strains were streaked in a crisscross pattern (shown in Fig. 2.1) with sterile round toothpicks on YMGA plates (Yeast extract (4 g/L), malt extract (10 g/L), glucose (4 g/L), agar (15 g/L), pH adjusted to 7.3 with 1M NaOH). Plates were then incubated for 3-5 days at 30 °C. The plates were then moved to a 4 °C refrigerator for later use.

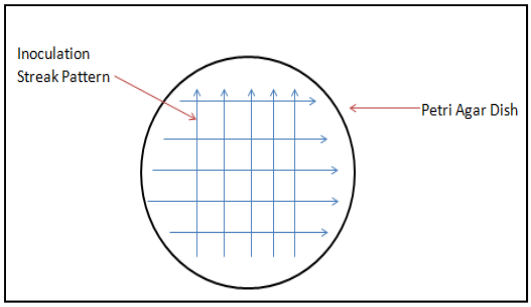


Figure 2.1. Inoculation Streak Pattern

Liquid Culture

Each strain was inoculated in a 125 mL Erlenmeyer flask containing YPGM (50 mL) liquid medium [Yeast extract (3 g/L), malt extract (3 g/L), peptone (5 g/L), and glucose (10 g/L) and a 125 mL flask containing Modified Bennet medium (50 mL) [Glucose (10 g/L), yeast extract (1 g/L), beef extract (1 g/L), soytone (2 g/L), metal ion solution (1 mL)]. They were cultivated on a rotary shaker at 200 rpm and 30 °C for 7 days.

Compound Extraction

The liquid cultures of the ICBB strains were centrifuged at 5000 rpm for 20 minutes in 50 mL conical vials. The supernatant for each culture was extracted sequentially three times with ethyl acetate (EtOAc) and twice with butanol (BuOH). The mycelia were discarded in the biohazard waste and autoclaved. This was because the resources and energy required to investigate the metabolites in each of the strains' bacterial cells would most likely not payoff. 10 mL aqueous broth remaining from the liquid extraction was frozen at -20 °C and was lyophilized. The EtOAc and BuOH extracts were transferred to 20 mL weighed vials and dried separately with a rotary evaporator. Vials containing the extracts were weighed again to determine the mass of each sample. For each strain, a total of six extracts were obtained (three from each media): two EtOAc, two BuOH, and two aqueous extracts.

Bioassay Screening

The six extracts were subjected to agar diffusion assays against bacteria, *Bacillus subtilis* (BS), *Escherichia coli* (EC), *Mycobacterium smegmatis* (MS), *Staphylococcus aureus* (SA), and *Pseudomonas aeruginosa* (PA), and the fungus *Rhizoctonia solani* (RS). The organic layer extracts, EtOAc, and BuOH, were diluted with methanol (MeOH) to a concentration of 10 mg/mL and the aqueous extracts were diluted with sterile de-ionized water to a concentration of 10 mg/mL. Antibiotics apramycin sulfate (for MS and SA) and ampicillin (for BS, EC, and PA) diluted with sterile de-ionized water to a concentration of 1 mg/mL were used as positive controls. Diffusion discs were impregnated with 20 µL of respective extract or antibiotics. Bioassay plates were then left in the hood for 20 minutes to let the solvents evaporate. Subsequently, they were incubated for

24 hours at 30 °C and then stained for better examination with (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) 98% (1 mg/mL, de-ionized water).

2.3. RESULTS AND DISCUSSION

The EtOAc, the BuOH and the aqueous extracts produced from the YPGM and Modified Bennet's media small scale cultivation (50 mL) were tested for biological activity against bacterial pathogens *Bacillus subtilis* (BS), *Escherichia coli* (EC), *Mycobacterium smegmatis* (MS), *Staphylococcus aureus* (SA), and *Pseudomonas aeruginosa* (PA), and the fungus *Rhizoctonia solani* (RS). The following legend is used to read Table 2.2, which summarizes the bioassay results.

Legend to read following table results:
EtOAc: Ethyl Acetate Extract
BuOH: Butanol Extract
Aq: Aqueous Extract
Y: YPGM Media
B: Modified Bennet Media
BS: *Bacillus subtilis*
EC: *Escherichia coli*
MS: *Mycobacterium smegatis*
PA: *Pseudomonas aeruginosa*
RS: *Rhizoctonia solani*
SA: *Staphylococcus aureus*
+: Active against
-: Not active against

Table 2.2: Identification Data Table of Metabolite Codes and Details on Assays

Sample Name	Crude Extract Yield (mg)	Bioactive Comments
8311 EtOAc Y	3.2	-: BS, EC, MS, PA, PS, SA (all results)
8311 EtOAc B	5.0	
8311 BuOH Y	200	
8311 BuOH B	117	
8311 Aq. Y	79.4	
8311 Aq. B	90.0	

8313 EtOAc Y 8313 EtOAc B 8313 BuOH Y 8313 BuOH B 8313 Aq. Y 8313 Aq. B	56.9 12.9 57.2 45.4 372.5 64.4	**8313 BuOH B +: SA and PS** -: all other results
8316 EtOAc Y 8316 EtOAc B 8316 BuOH Y 8316 BuOH B 8316 Aq. Y 8316 Aq. B	4.4 6.1 9.0 92.9 102.8 40.1	**8316 BuOH Y+: SA** -: all other results
8336 EtOAc Y 8336 EtOAc B 8336 BuOH Y 8336 BuOH B 8336 Aq. Y 8336 Aq. B	5.6 7.3 28.8 8.4 71.6 48.4	**8336 BuOH Y +: SA** -: all other results
8351 EtOAc Y 8351 EtOAc B 8351 BuOH Y 8351 BuOH B 8351 Aq. Y 8351 Aq. B	3.3 1.4 30.2 6.1 203 41.5	**8351 BuOH Y +: SA** **-:** all other results
8355 EtOAc Y 8355 EtOAc B 8355 BuOH Y 8355 BuOH B 8355 Aq. Y 8355 Aq. B	2.9 36.5 17.4 14.4 68.1 54.7	8355 EtOAc Y +: PS -: all other results

These strains were inoculated in 50 mL of media (small scale production) as a preliminary screening to find out which of them have bioactive metabolites. ICBB strains 8313, 8316, 8336, 8351 all each had a BuOH extract active against *S. aureus*. ICBB 8336 and 8351 BuOH extracts had the largest zone of inhibition against this particular pathogen, which suggests a high possibility for a promising natural product isolation in a large scale production. It should also be noted that some of these results are not 100% definitive for determining that certain ICBB strains do not produce bioactive natural products because the cultivation scale was relatively small. There may have been bioactivity, but there wasn't enough that could be observed by the method used.

CHAPTER 3

NATURAL PRODUCT DISCOVERY FROM INDIAN SOIL BACTERIA

3.1. INTRODUCTION

A preliminary study carried out by our collaborator, Dr. Debananda Ningthoujam, in the Department of Biochemistry, Manipur University, India, revealed that a number of bacterial strains isolated from the state of Manipur, India, produce excellent antifungal agents that inhibit the growth of several rice fungi, *e.g.*, *Bipolaris oryzae, Fusarium oxysporum, and Curvularia oryzae.* Among them are one *Streptomyces* strain and one non-actinomycetes strain, identified as MBRL 201 and MBRL 251, which were isolated from soil samples of the abandoned Hundung Cement Factory in Manipur (Figures 3.1 and 3.2).

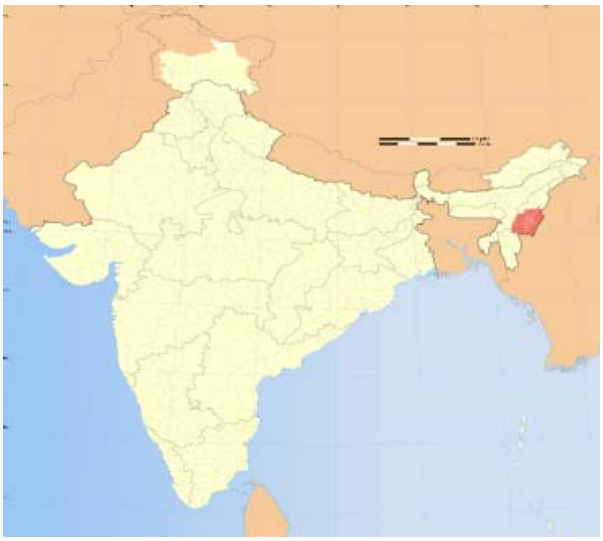


Figure 3.1. Map of India. The red color spot indicates the state of Manipur

Figure 3.2. Abandoned Hundung Cement Factory

In order to identify the active compounds produced by these bacteria, we carried out chemical investigations using bioassay-guided fractionation and isolation.

3.2. EXPERIMENTAL MATERIALS AND METHODS

3.2.1. Classification of Bacterial Strains

To classify the MBRL 201 and MBRL 251 strains, amplification and sequencing of the 16S RNA genes in these strains were carried out.

Genomic DNA Extraction: MBRL 201 and MBRL 251 were each individually inoculated in YMG medium (3 mL) in a falcon tube (15 mL) for 3 days in a rotary shaker at 30 °C. Afterwards, the culture broth was centrifuged at 5000 rpm for 15 minutes to separate the supernatant from the cells. The supernatant was discarded and the cells were kept for DNA extraction. Sodium-Tris-EDTA (STE) buffer (500 μL) was added to the cells. The STE buffer was used to wash out any remaining

medium residue entrapped in the bacterial cell pellets. The cells with the STE buffer were then transferred to an Eppendorf tube (2 mL) and re-centrifuged at 7000 rpm for 10 minutes. The supernatant was discarded and additional STE buffer (200 μL) was added to the cells. In this case, the STE buffer acted as a pH buffer to prevent any future pH fluctuations that might disrupt the DNA extraction process. Lysozyme (50 mg/mL, 10 μL) was added to the cells to break down the cell walls. The mixture was incubated at 37 ºC for 1 hour. After the incubation process, 2.5% sodium dodecyl sulfate (200 μL) and proteinase K (20 mg/mL, 8 μL) were added to dissolve the lipids and proteins that form the cell membranes. This helped break down the cells and expose the DNA chromosomes of the bacteria. This mixture was then placed in a hot plate at 57 ºC overnight.

After the mixture of cells and solvents were removed from the hot bath, phenol-chloroform (500 μL) was added to the Eppendorf tube. The non-polar chloroform extracted all the hydrophobic lipids and proteins, keeping only the DNA in the aqueous solution of STE buffer. The Eppendorf tube was then shaken vigorously for 30-45 seconds. When a white emulsion formed, the tube was centrifuged at 9000 rpm for 10 minutes. The supernatant, separated from the white band of proteins, was then transferred to another Eppendorf tube and mixed with 3M sodium acetate (40 μL). An equal amount of chilled 100% ethanol was then added to precipitate the DNA. The Eppendorf tubes were then placed in a -20 ºC freezer for 10 minutes, followed by centrifugation at 14,000 rpm for 10 minutes. Finally, the supernatant was removed and 70% EtOH (1 mL) was added to wash the DNA and take away any remaining chemical residues.

Polymerase Chain Reaction (PCR): PCR was performed using the isolated DNA as template using the forward primers 27F (5'AGAGTTTGATCMTGGCTCAG3') and the reverse primers 1492R

(5'GGTTACCTTGTTACGACTT3')[xviii]. *Taq.* DNA Polymerase (Invitrogen™) was used for the PCR experiments.[xix]

Sequencing and Identification: After the DNA was purified using the MicroElute Cycle-Pure – spin protocol and purification kit provided by Micron Omega Ltd., it was sent to the Center for Genome Research and Biocomputing for sequencing. The nucleotide sequence received was then introduced into the online National Center of Biotechnology Information DNA identification database.

3.2.2. Solid Culture

The MBRL strains were streaked on solid agar the same way as the ICBB strains.

3.2.3. Preliminary Antifungal Assay

An antifungal assay was tested against the *R. solani* to reconfirm the bioactivity of MBRL 201 and MBRL 251. A colony of *R. solani* was placed in the center of a YMGA plate and a 1 cm^2 area was streaked with MBRL 201 on one side and MBRL 251 on the other side with sterile toothpicks.

3.2.4. Small Scale/Large Scale Liquid Culture

Each strain was inoculated individually in two 250 mL Erlenmeyer flasks filled with YMG liquid media (125 mL) (YMGA recipe without the agar, pH adjusted to 7.28 - 7.32 with 1N NaOH). This was a small-scale cultivation of 250 mL. They were cultured on a rotary shaker at 200 rpm and 30 ^oC for 7 days. For large-scale culture, which was done following the biological activity screenings

of small scale extracts, each strain was inoculated individually in 10 Erlenmeyer flask (500 mL) filled with of YMG liquid media (100 mL). The culture protocol was the same as the small scale one.

3.2.5. Preparation of Extracts

The orange-brown liquid culture of MBRL 201 and the dark green-yellow liquid culture of MBRL 251 were centrifuged at 5000 rpm for 20 minutes in centrifuge bottles (1L). The supernatant for each culture was then extracted three times with EtOAc and two times with BuOH. The cell cake was transferred to another flask (500 mL) and extracted with Acetone-MeOH (1:1, 250 mL) by leaving it in static conditions for 24 hours before using vacuum filtration to extract the cake metabolites. Aqueous solution remaining from the liquid extraction (100 mL) was frozen at -20 °C and lyophilized. EtOAc and BuOH extracts were transferred to previously weighed vials (20 mL) and dried separately with a rotary evaporator. Vials containing the extracts were weighed again to determine the mass of each sample. The extraction process produced four extracts for each strain.: 1 EtOAc, 1 BuOH, 1 cake, and 1 aqueous extract. The following extraction scheme summarizes the liquid-liquid fractionation procedure for large scale.

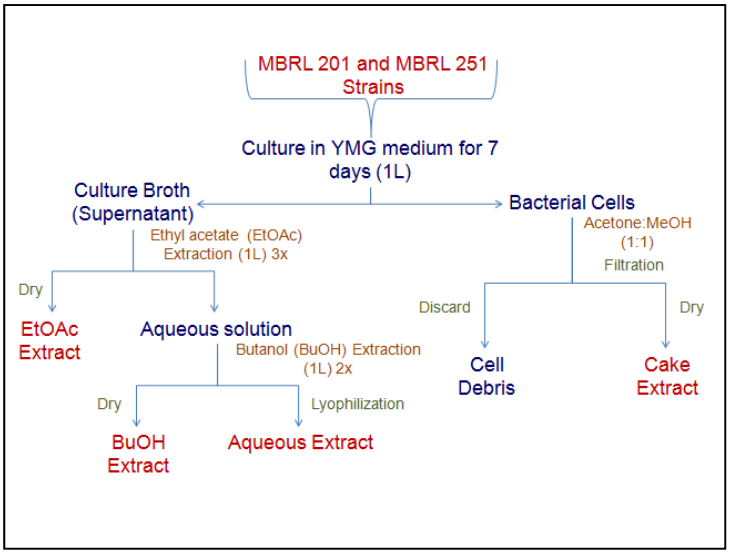


Figure 3.3. Large Scale Extraction Scheme for MBRL 201 and MBRL 251

3.2.6. Biological Activity Tests

Agar diffusion assays against bacteria, *B. subtilis, S. aureus*, and *P. aeruginosa*, and the fungus *R. solani* were used to observe the bioactivity of the 4 extracts of each strain. Like the ICBB strain protocol, the organic layer and cake extracts were diluted with MeOH to a concentration of 10 mg/mL and the aqueous extracts were diluted with sterile de-ionized water to a concentration of 10 mg/mL. Positive controls used were antibiotics apramycin sulfate and ampicillin diluted with sterile de-ionized water in a 1 mg/mL concentration. Diffusion discs were then impregnated with the respective antibiotics or extracts (20 µL). Bioassay plates were then left in the hood for 20 minutes to let the solvents evaporate completely before they were incubated for 24 hours at 30 ° C.

Bioassay plates were stained for better examination with MTT diluted in 1 mg/mL concentration with de-ionized water.

3.2.7. Natural Product Isolation from MBRL 251

Bioactive extracts (EtOAc, BuOH, and cake) were subjected to thin-layer chromatography to determine the best solvent system to use for large scale compound fractionation. Extracts were dissolved with MeOH to a 10 mg/mL concentration and spotted 3 times with a capillary tube (3 mm). TLC plates were run through two solvent systems: chloroform ($CHCl_3$)-MeOH (10:1) and $CHCl_3$-MeOH (20:1). TLC plates were observed under 254 nm of ultraviolet (UV) light and then stained with cerium sulfate/ammonium molybdate solution (CAM) to observe for compounds that cannot be seen under UV light.

TLC bioassays in $CHCl_3$-MeOH (10:1) were carried out against SA and BS to determine where the bioactive fractions were. The BuOH and cake extracts were the ones tested first because they looked like the easiest to fractionate. A open column chromatography [$CHCl_3$-MeOH (15:1 with increasing polarity)] was run for 171.9 mg of the BuOH extract. The column gave four fractions and one MeOH wash fraction. Another open column chromatography was run using $CHCl_3$-MeOH-5% NH_4OH (3:6:1) to better separate the MeOH wash fraction into pure components.

Fractions collected from the open column runs were tested against SA. Bioactive fractions were further purified with additional open column chromatographies in the same solvent system with increasing polarity. The pure compound was analyzed with mass spectrometry in positive and negative ion mode to determine its molecular weight. ^{1}H and ^{13}C NMR were also run. The data acquired from the mass spectrometry and NMR were introduced into the ANTIBASE natural product database to help elucidate the compound's chemical structure. A final TLC and bioassay

against SA and RS (because the highest bioactivity was seen with these test organisms) were done with the isolated compound to reconfirm its purity

3.3. RESULTS AND DISCUSSION

3.3.1. Identification of MBRL 201 and MBRL 251

MBRL 201 and MBRL 251 are soil bacteria that we received from our collaborator at Manipur University, India. Preliminary studies showed that these bacteria produced secondary metabolites that have potent antifungal activity against a number of fungal pathogens in rice plants. However, there is no information about the classification of these bacteria. Upon receiving these bacteria, we cultured them on solid agar and liquid media and isolated their chromosomal DNA for identification. On the basis of the sequence of their 16S rRNA genes, MBRL 201 strain was identified as *Streptomyces* sp. and MBRL 251 strain was identified as *Pseudomonas* sp.

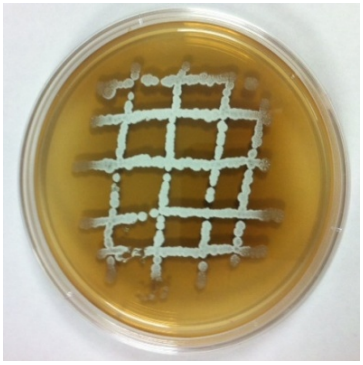


Figure 3.4. Agar plate of MBRL strain 201

Figure 3.5. Agar plate of MBRL strain 251

3.3.2. Biological Activities of MBRL 201 and MBRL 251

To reconfirm the bioactivity of the MBRL strains, an antifungal assay was tested against the plant pathogen *Rhizoctonia solani*. Figure 3.6 shows that while both bacterial strains have bioactivity, MBRL 251 secreted a yellow compound, presumably the compound of interest that killed the fungus. Interestingly, upon contact to *R. solani* this secondary metabolite induces the fungus to secrete a red pigment.

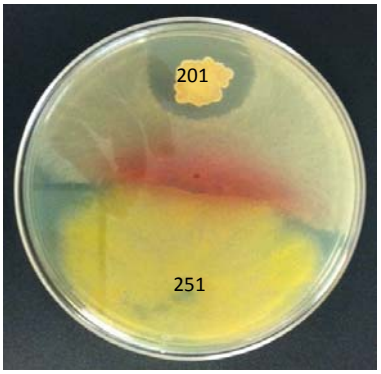


Figure 3.6. Antifungal assay against *Rhizoctonia solani* , MBRL 201 small zone of inhibition and MBRL 251 large zone of inhibition with yellow compound

3.3.3. Investigation of Bioactive Natural Products in *Pseudomonas* sp. MBRL 251

Based on this interesting result, we decided to first focus on MBRL 251 for further studies. In addition to its antifungal activity, we also explored its ability to produce antibacterial compounds. Therefore, extracts of MBRL 251 were tested against *B. subtilis*, *S. aureus*, and *P. aeruginosa.* The results showed that MBRL 251 EtOAc extract was active against *S. aureus* and *E.coli.* On the other hand, the MBRL 251 BuOH extract was active against *B. subtilis*, *S. aureus,* and *P. aeruginosa*. The cake extract was active against *S. aureus.*

Since there was not enough extract obtained from the small scale culture for chromatographic experiments, the liquid culture was scaled up to 1 L. The strain was re-cultured in YMG medium (1L) and extracted following the fractionation scheme mentioned on page 18. The extracts were then subjected to agar diffusion assays to screen for their biological activity. The following table summarizes these results:

Table 3.1: Extract Agar Diffusion Bioassays against RS, SA, PA, and BS

Extract Name	Extract Yield	Bioactivity Comments	Bioassay Codes
MBRL 251 EtOAc	189.5 mg	High + RS, SA - PA, BS	1
MBRL 251 BuOH	171.8 mg	High +SA Weak + BS - RS, PA,	2
MBRL 251 MeOH/Acetone Cake	56.0 mg	+SA	3
MBRL 251 Aqueous	10.6 mg	- All strains	4

Notes: Bioassay codes correlate to what is on this table throughout the results and discussion until noted otherwise

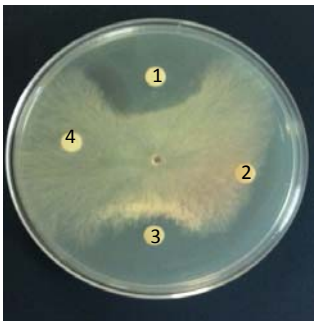


Figure 3.7.
R. solani

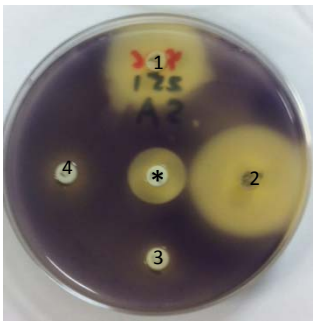


Figure 3.8.
S. aureus

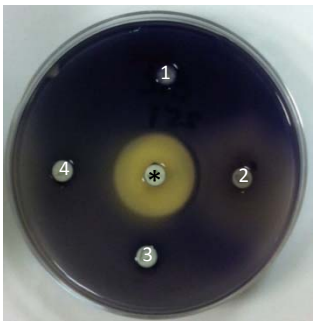


Figure 3.9.
P. aeruginosa

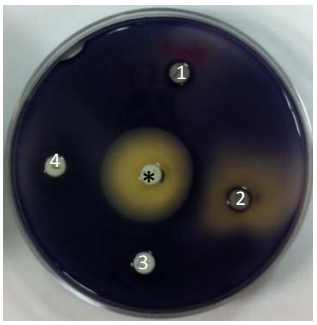


Figure 3.10.
B. subtilis

The most notable results were the organic layer extracts EtOAc and BuOH. Both had excellent bioactivity against SA. On the other hand, the aqueous extract was not active against any strain. The BuOH extract also no longer had bioactivity against *B. subtilis* (Figures 3.7 – 3.10).

Biologically active extracts were then subjected to thin layer chromatography. Two solvent systems, $CHCl_3$-MeOH (10:1) and $CHCl_3$-MeOH (20:1), were used in this experiment and the spots were detected by 254 nm UV light (Figures 3.11 and 3.12). The TLCs were then stained with CAM solution and heated, but there were no significant changes other than the most nonpolar and polar (top and bottom) spots turned blue.

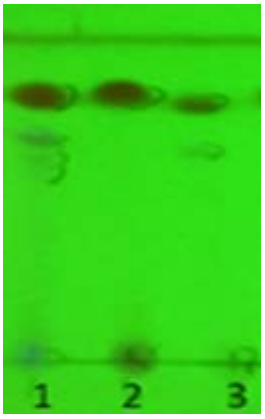


Figure 3.11. TLC Analysis of MBRL 251 extracts [$CHCl_3$-MeOH (10:1)]. 1, EtOAc extract; 2, BuOH extract; and 3, cell-cake extract.

Figure 3.12. TLC Analysis of MBRL 251 extracts [$CHCl_3$-MeOH (20:1)]. 1, EtOAc extract; 2, BuOH extract; and 3, cell-cake extract.

The most UV active compounds were marked with a pencil to estimate when they might come out during a large scale chromatographic study. The EtOAc extract has seven UV active spots, butanol has four (hard to see in picture) and the cake extract has four. The butanol extract looked like the easiest to separate into multiple fractions and it shared two compounds with the EtOAc extract, so it was focused on for this investigation.

The MBRL 251 BuOH extract was loaded into an open silica gel column [$CHCl_3$-MeOH (15:1 with increasing polarity)] and a total of 36 initial fractions were collected. After comparing the TLCs under UV light and CAM stain, similar fractions were combined and the total number of fractions was reduced to five. Although fraction 1 through fraction 5 were observed to have the same pure compound, except fraction 4, they were kept separate because of some had impurities. The MeOH wash fraction was run using $CHCl_3$-MeOH-5% NH_4OH (3:6:1). A total of 33 fractions were collected and after comparing the TLCs, the total number of fractions reduced to four. Two

fractions, 28M and 29M from the MeOH wash were UV and CAM active. BuOH fractions 1, 2, 28M, and 29M (1 mg/mL concentration) were tested against *S. aureus*. Fractions 1 and 2 (containing the top spot observed on TLC) showed bioactivity, but 28M and 29M (containing the bottom spot observed on TLC) did not. Figure 3.13 shows the results for the biological activity test for the fractions.

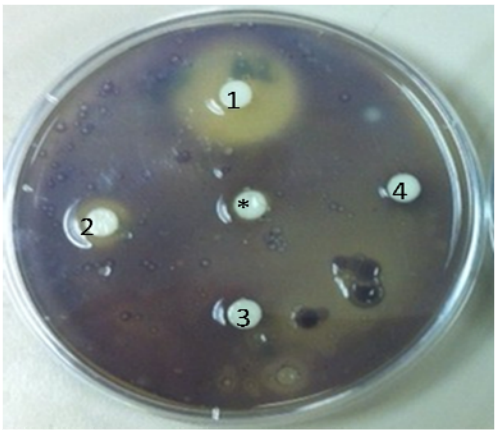


Figure 3.13. Fraction Biological Activity Test

Fraction 1 had high bioactivity, but was also impure, so it was run through another open column chromatography in a $CHCl_3$-MeOH (15:1) solvent system, which yielded two different fractions (1*2F-1*4F (pure) and 1*5F-6F (a mixture). The following fractionation scheme summarizes the purification process of the active compound:

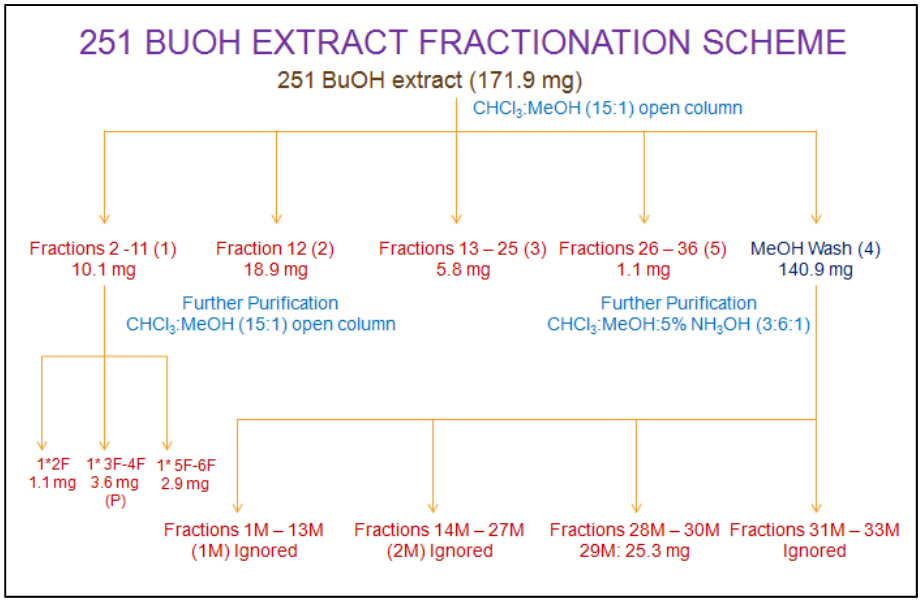


Figure 3.14. MBRL 251 BuOH extract fractionation scheme

The purity of the compound was confirmed by TLC (Figure 3.15), MS, and NMR (see below).

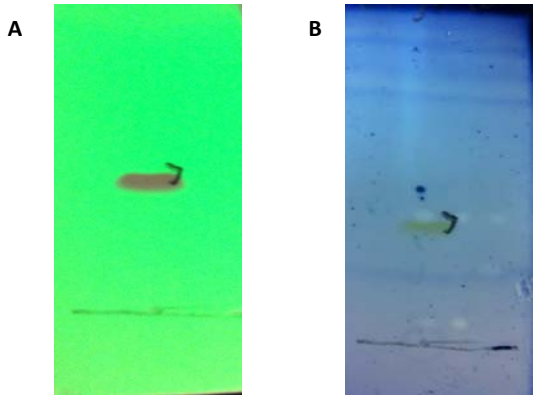


Figure 3.15. TLC analysis of MBRL 251 pure compound detected by UV light (A) and cerium sulfate/ammonium molybdate (CAM) (B). Solvent system: $CHCl_3$-MeOH (30:1).

To determine the molecular weight of the pure compound 1* 3F-4F, the compound was analyzed by ESI mass spectrometry in positive- and negative-ion modes. The MS spectra showed m/z 225.07 $[M + H]^+$ and 470.80 $[2M + Na]^+$ in the positive ion mode and m/z 222.8 $[M - H]^-$ and

468.80 $[2M - 2H+Na]^-$ in the negative ion mode, suggesting the molecular weight of the active compound to be 224 (Figures 3.16 and 3.17).

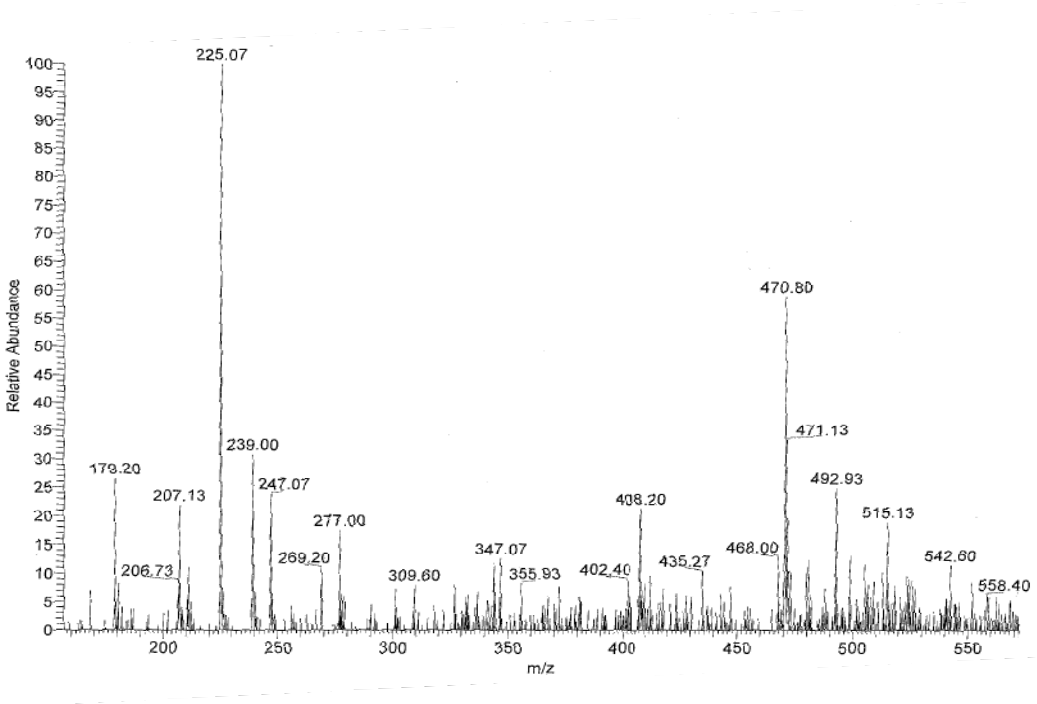


Figure 3.16. Positive Ion Mode ESI Mass Spectrum of MBRL 251 Compound

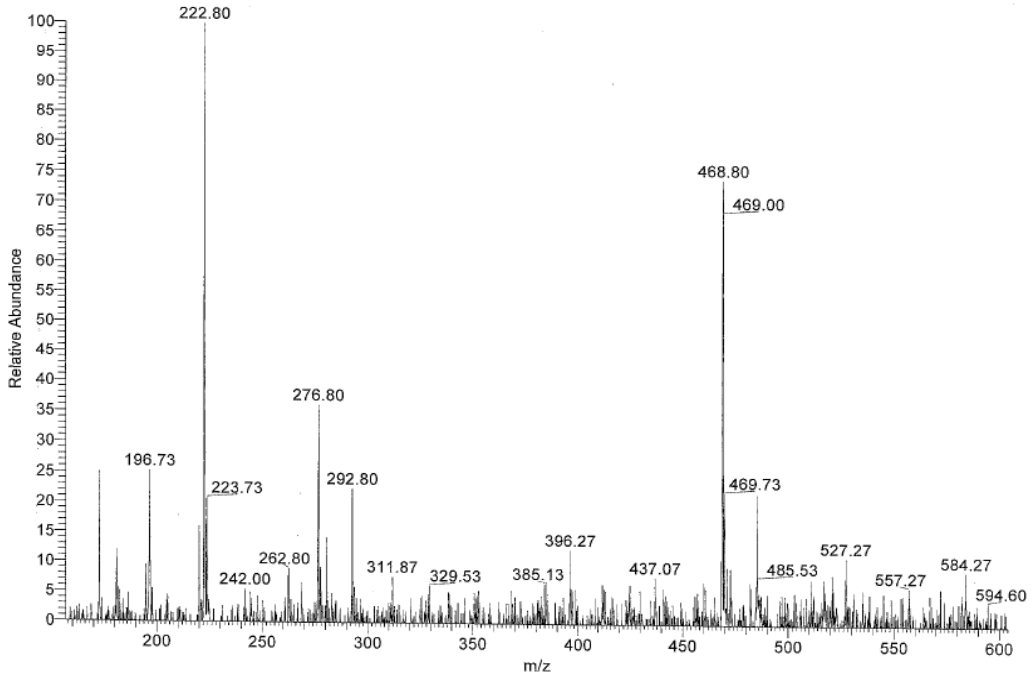


Figure 3.17. Negative Ion Mode ESI Mass Spectrum of MBRL 251 Compound

To establish how many carbons and hydrogens the pure compound has, ^{1}H and ^{13}C NMR experiments were carried out. The spectra are provided below (Figures 3.18 and 3.19).

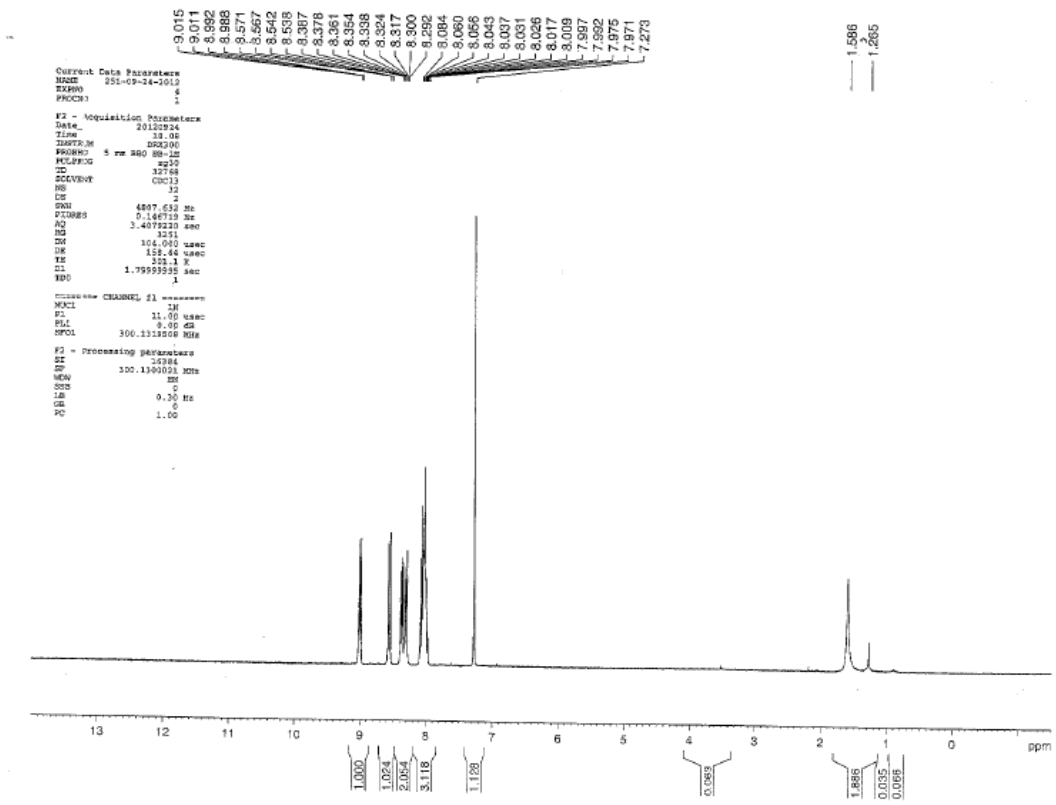


Figure 3.18. ^{1}H NMR Spectrum of MBRL 251-Butanol-Pure Compound in $CDCl_3$

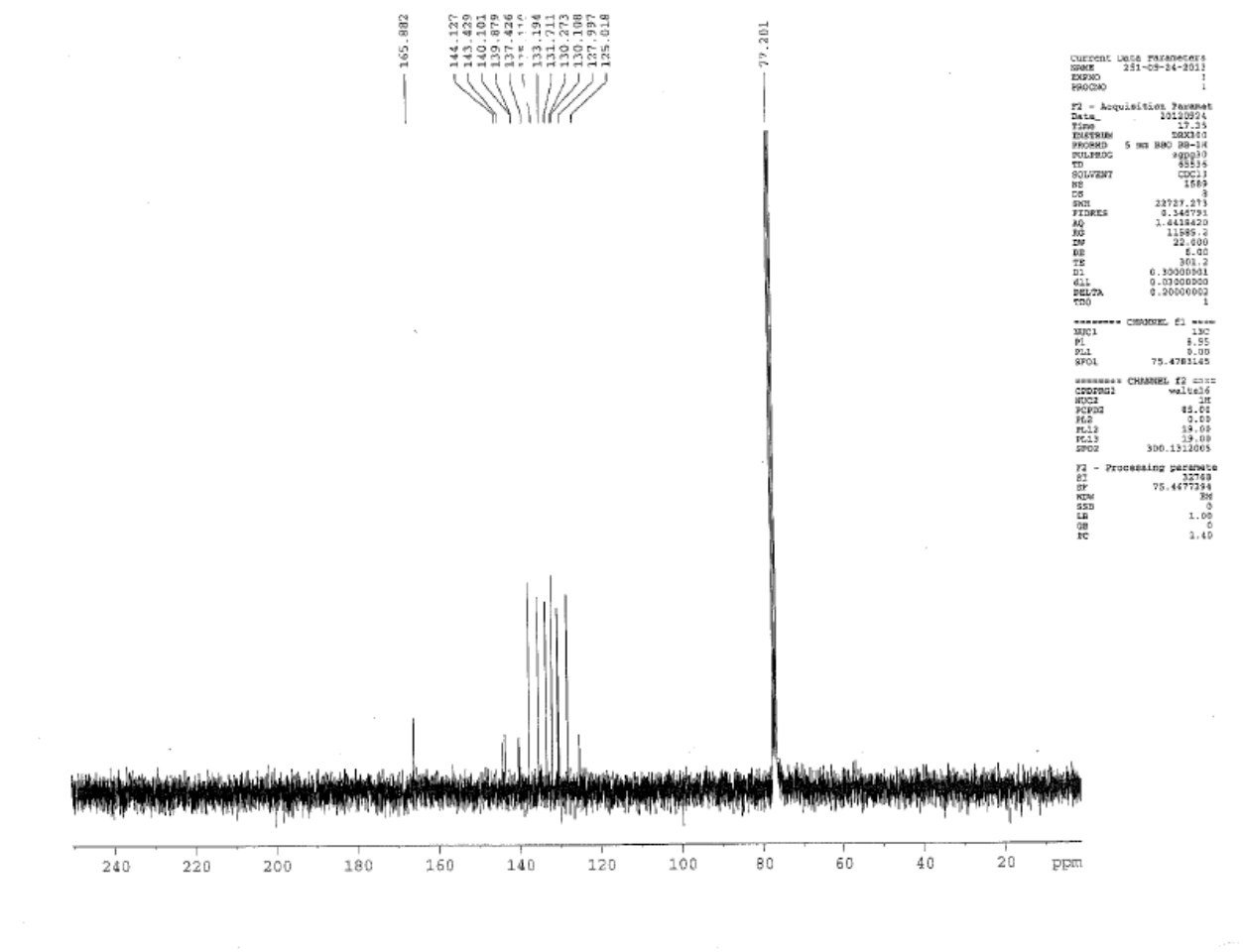


Figure 3.19. ^{13}C NMR Spectrum of MBRL 251 Compound in $CDCl_3$

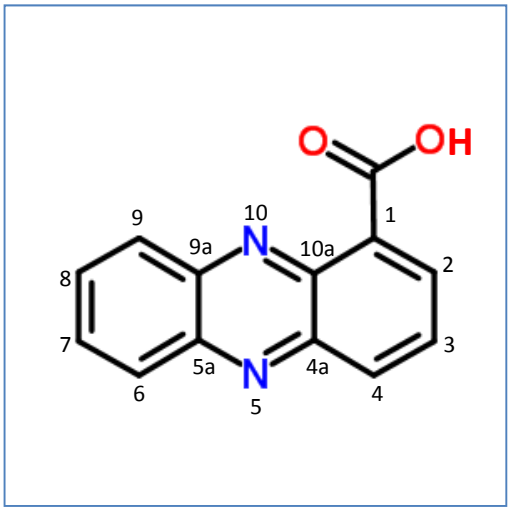
Table 3.2. ^{1}H and ^{13}C NMR data for MBRL 251 compound dissolved in $CDCl_3$

Carbon number	^{13}C signal	^{1}H signal (J in Hz)
1	125.0	-
2	128.0	8.58 (dd, J = 8.7, 1.4, 1H)
3	130.1	7.98 – 8.12 (m, 1H)
4	130.2	9.02 (dd, J = 7.1, 1.4, 1H)

4a	131.7	-
5a	133.2	-
6	135.1	8.39 (dd, *J* = 7.1, 2.2, 1H)
7	137.4	7.98 – 8.12 (m, 1H)
8	139.9	7.98 – 8.12 (m, 1H)
9	140.1	8.33 (*J* = 7.1, 2.1, 1H)
9a	143.4	-
10a	144.1	-
COOH	165.9	-

Notes: Format of table and carbon numbering were adopted from of Lee et al, dd = doublet of doublets, m = multiplet[xx]

On the basis of its ^{1}H and ^{13}C NMR data, it was predicted that the MBRL 251 compound contains at least two aromatic rings and a carboxylate group. The mass spectrometry and NMR data were then subjected to a chemical structure search using ANTIBASE®, a searchable database that contain descriptive, physico-chemical and spectroscopic data, including NMR data, for over 38,700 bacterial and fungal compounds. The chemical compound that matched the information provided was phenazine-1-carboxylic acid (Figure 3.20), which is a known natural product produced by some strains of *Pseudomonas*.



[xxi]

Figure 3.20 Chemical structure of natural product isolated from MBRL 251

Table 3.3. Phenazine-1-carboxylic acid natural product information[xx]

Synonyms	Molecular Formula	Molecular Weight	Solubility	Use	Appearance
1-carboxylic acid phenazine, phenazine-alpha-carboxylic acid, tubermycin B	$C_{13}H_8N_2O_2$	224.21	Chloroform	Pesticide M18, low toxicity	Bright yellowish brown crystals

To confirm if phenazine-1-carboxylic acid is indeed responsible for the antibacterial and antifungal activity of MBRL 251 extract, we tested the pure compound for their antibacterial and antifungal activities using agar diffusion assays. The antibacterial assay was carried out against *S. aureus* with apramycin sulfate as the positive control antibiotic (Figure 3.21). Both discs were injected with 20 µL of compound solution in 1 mg/mL concentration. The top disc impregnated with phenazine-1-carboxylic acid shows a large zone of inhibition, which indicates its strong potency against *S. aureus*.

Similarly, the antifungal assay was carried out using agar diffusion assay with *Rhizoctonia solani* as the test organism. Previously, we have shown that product(s) of MBRL 251 induced the fungus *R. solani* to produce a red pigment along the area of contact. Similar phenomenon was also observed when phenazine-1-carboxylic acid was used as the test compound (Figure 3.22), suggesting that phenazine-1-carboxylic acid is the compound responsible for inducing the red pigment and the antifungal activity of MBRL 251.

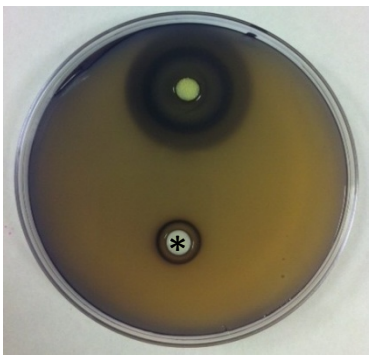


Figure 3.21. SA Pure Fraction Bioassay

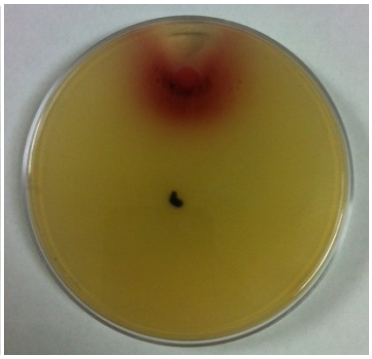


Figure 3.22. PS Pure Fraction Bioassay

3.3.4. Investigation of Bioactive Natural Products in MBRL 201

To investigate the bioactive compounds in MBRL 201, EtOAc, BuOH, and aqueous extracts from a small scale culture of MBRL 201 were tested against *E. coli*, *P. aeruginosa*, *S. aureus*, and the fungus *R. solani*. The results showed that EtOAc extract had minor bioactivity against *S. aureus* and *R. solani*. On the other hand, the BuOH extract showed bioactivity against all the bacteria. The cake extract showed only bioactivity against SA. The aqueous extracts were not active.

Like MBRL 251, bioactive extracts (EtOAc, BuOH, and cell cake) were analyzed on TLC plates to figure out the optimal solvent system to use for large-scale fractionation. Extracts were dissolved in MeOH to a 10 mg/mL concentration and spotted 3 times with a capillary tube. The TLC were developed using two solvent systems $CHCl_3$-MeOH (10:1) and Hexane-EtOAc-MeOH (3:5:1). Compounds of interest (spots) on the TLC plates were observed under UV light 254 nm. The plates were then stained with CAM and heated to observe compounds that cannot be seen under UV light.

There were many problems that occurred during the fractionation process of the MBRL 201 extracts. The extracts were only soluble in MeOH, but if they stayed in that solvent for more than several hours, the extracts would degrade and lose bioactivity. As a result, dried extracts were always stored at -80^{o}C to avoid compound degradation. Also, the extracts had a great tendency to stick with silica gel, so it was difficult to pull off all the compounds stuck to it during chromatographic experiments. A number of open column chromatography were attempted using $CHCl_3$-MeOH, (20:1) but none of the fractions showed activity in the bioassays. This result may be due to the active compound(s) is tightly bound to the column or the compound was decomposed by the time it reached the end of the open column.

Since chromatographic studies that worked for MBRL 251 extracts did not work for MBRL 201 extracts, other methods were explored. 40 mg of EtOAc extract was run through a preparative thin layer chromatography (PTLC silica gel 60 F_{254} 0.5 mm thickness) in Hexane-EtOAc-MeOH (3:5:1). The PTLC plate was observed under 254 nm UV light and compounds of interest were circled. Each fraction bound to the silica was scratched off, transferred to conical vials (15 mL) and extracted twice with 50% EtOAc-50% MeOH. The conical vials were then centrifuged at 5000 rpm for 7 minutes. The supernatant was taken and dried for each fraction separately. An agar diffusion assay of the fractions was then carried out against *P. aeruginosa*. We found one active fraction, which contain a major compound (with minor impurities) that gives m/z 281.13 $[M-H]^-$ and m/z 305.11 $[M+Na]^+$ in ESI-MS, indicating the molecular weight of compound to be 282 (Figure 3.23). However, there was not enough material for us to carry out NMR experiments, therefore, MBRL 201 was re-cultivated with 1L of medium and the extracts were prepared.

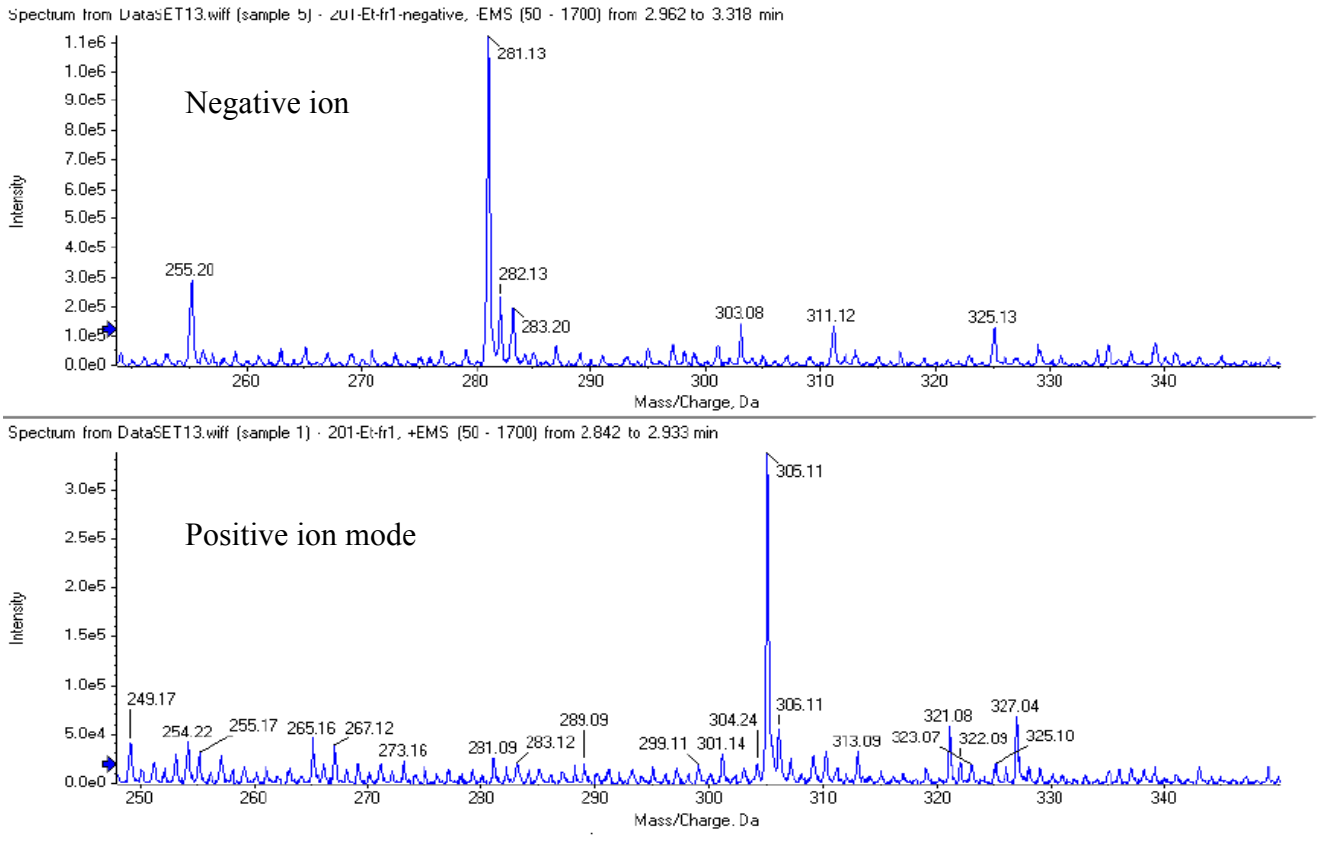


Figure 3.23. Negative and Positive Ion Mode ESI Mass Spectra Active MBRL 201 Compound

The crude extracts were tested again against *R. solani* to confirm the previous antifungal assay that showed a small zone of inhibition. They were also tested against *P. aeruginosa* and *S. aureus* because these pathogenic bacteria appeared to be highly sensitive to the compounds in the crude compounds of MBRL 201. Table 3.4 displays all the results observed from the diffusion disc bioassays.

Table 3.4: Crude Extract Diffusion Disc Bioassays (against RS, SA, PA)

Extract Name	Crude Extract Yield	Bioactivity Comments	Bioassay Codes
201 EtOAc	189.5 mg	High+ PA, SA Low + RS	1
201 BuOH	171.8 mg	High +SA Low+ PA - RS	2

201 MeOH/Acetone Cake	56.0 mg	Low + SA, PA	3

Notes: This is the yield from the first 1L large scale production. Due to many difficulties with the natural production isolation of this strain, multiple batches were grown. However, their yields will not be reported in this report because it is irrelevant to the main purpose of this table, which is show which extracts were bioactive. Bioassay codes used for TLCs as well.

Figures 3.24. – 3.26. accompany the results reported in Table 3.4. * is the positive control.

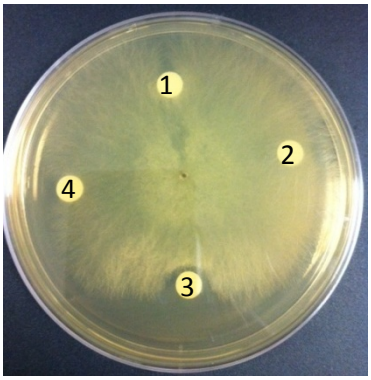


Figure 3.24.
RS Bioassay

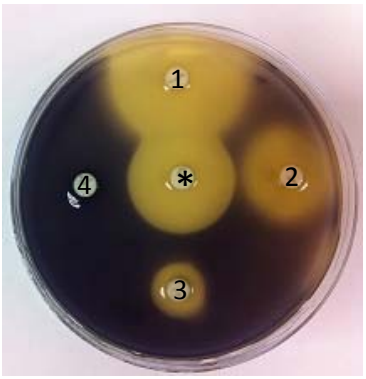


Figure 3.25.
PA Bioassay

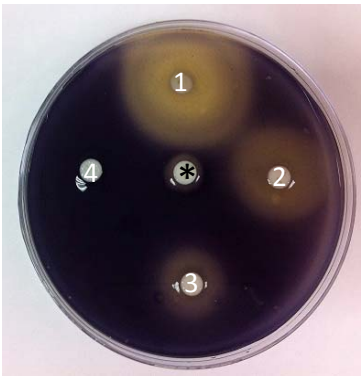


Figure 3.26.
SA Bioassay

TLCs were run for active compounds using $CHCl_3$-MeOH (10:1) and Hexane-EtOAc-MeOH (3:5:1). Figures 3.27. show how many spots are active under UV light and Figure 3.28. show which compounds are active in CAM stain. Figure 3.27. shows the compound that is present in both the EtOAc and BuOH extracts.

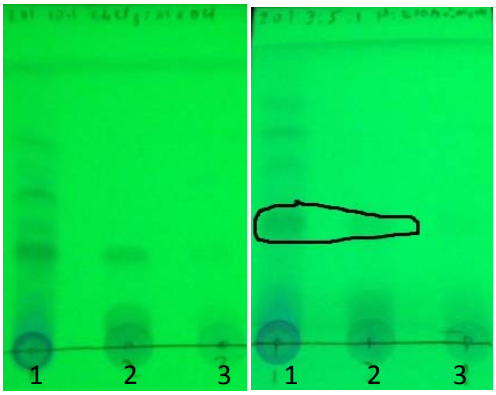


Figure 3.27. TLC Analysis of MBRL 201 extracts [$CHCl_3$-MeOH (10:1) and Hexane-EtOAc-MeOH (3:5:1)]. 1, EtOAc extract; 2, BuOH extract; and 3, Cell-cake extract detected by UV light

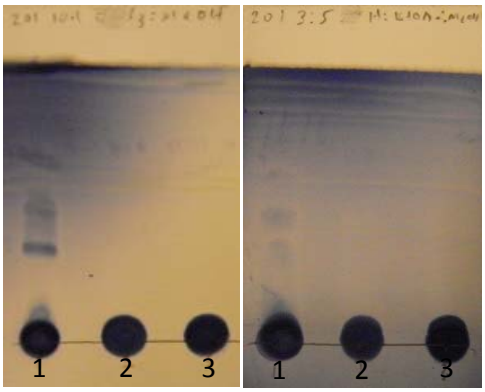


Figure 3.28. TLC Analysis of MBRL 201 extracts [$CHCl_3$-MeOH (10:1) and Hexane-EtOAc-MeOH (3:5:1)]. 1, EtOAc extract; 2, BuOH extract; and 3, Cell-cake extract detected by CAM

Since there were many spots in these extracts, a TLC bioassay was carried out against *P. aeruginosa* (due to its high sensitivity to the extracts) to get an idea where the bioactive compounds might be. Figure 32 shows that there are at least three bioactive compounds in the EtOAc extract and there are at least two bioactive compounds in the BuOH extract.

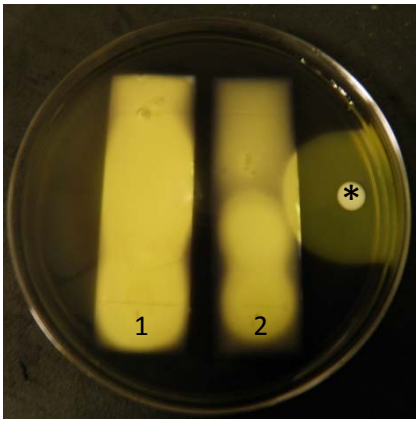


Figure 3.29. PA TLC Bioassay 1, EtOAc extract; 2, BuOH extract

EtOAc extract from the 1L liquid culture was subjected to PTLC for fractionation because it was the most promising extract (run twice through a preparative thin layer chromatography again with the same solvent system, Hexane:EtOAc:MeOH (3:5:1)). Eight fractions of interest were marked and scratched off. They were extracted twice with 100% MeOH to ensure all of the compounds could be extracted from the silica gel. Another agar diffusion assay was then carried out against *P. aeruginosa*, *S. aureus*, and *R. solani*.

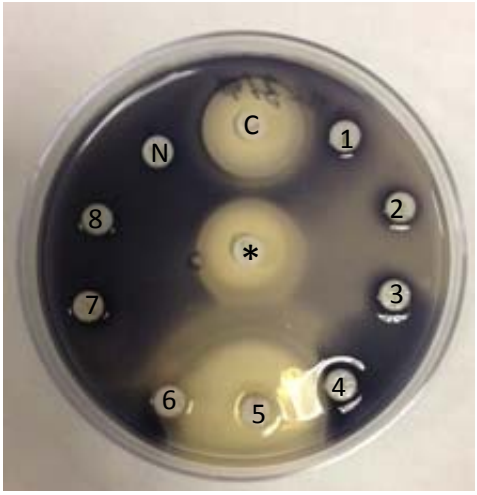


Figure 3.30. MBRL 201 fraction bioassay, C, crude standard; N, negative control, MeOH; *, Positive Control

The extract, which acted as a standard, showed high levels of bioactivity. Fraction 5 showed a big zone of inhibition in a 1 mg/mL concentration, which demonstrates its high potency against the bacteria (Figure 3.30.). Another significant observation of this bioassay is that the other two fractions (the more nonpolar spot, and the most polar spot at the bottom) that showed bioactivity during the TLC bioassay no longer exhibited any activity. This is most likely because this type of extraction process facilitates compound degradation or the active compound did not move up cleanly during the TLC analysis, which suggests other chromatographic experiments may be required to isolate those polar compounds.

The active fraction was then loaded to a size exclusion Sephadex LH-20 column. Collected fractions were observed under UV light and examined with mass spectrometry. Another biological assay against *P. aeruginosa* was also run to test for bioactivity of the fractions. The results from the mass spectrometry and bioassays were used to determine which fractions were to be combined.

^{1}H NMR experiments with several fractions were performed on an 500 MHz NMR instrument, but the data contained impurity peaks, so it was difficult to discern which hydrogens belonged to the active compound. At this stage, we have not been able to determine the chemical structure of the active compound. The main problem with this natural product isolation is it is very difficult to acquire enough active and pure compound for NMR analysis due to problems noted above. Further experiments are required to determine the chemical structure of this bioactive compound.

CHAPTER 4

CONCLUSION

This thesis focuses on novel drug discovery from soil bacteria collected from the Indonesian Black Water Ecosystem (the ICBB strains) and soil bacteria from the abandoned Hundung Cement Factory in India (the MBRL strains). The ICBB strains collected in Indonesia were cultured in a small scale for preliminary biological activity against various bacteria and fungi. On the other hand, strains MBRL 201 and MBRL 251 from India were heavily investigated for bioactive secondary metabolite production.

On the basis of its 16S rRNA gene sequence, MBRL 251 was identified as a Gram - bacterium from the genus of *Pseudomonas*. The BuOH extract of MBRL 251 was studied extensively because of its promising bioactivity against *Staphylococcus aureus* and *Rhizoctonia solani*. This specific extract was also easier to separate than the other bioactive extracts. Using bioassay-guided separation and purification involving various chromatographic techniques we were able to isolate the active compound and characterize its chemical structure. The active compound of MBRL 251 was determined to be phenazine-1-carboxylic acid.

MBRL 201 was identified as a bacterium from the genus of *Streptomyces*. The EtOAc extract of MBRL 201 was investigated because of its high potency against *P. aeruginosa*. However, due to the low production yield and compound stability issues, we have not been able to determine the chemical structure of the active compound. While the investigation managed to isolate one active compound from each strain, there remains enormous potential for other natural products to be found in these bacteria since changes to the growing environment may induce the bacteria to produce different compounds and those compounds can affect various pathogens in different ways.

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