ISOLATION OF ANTIBIOTIC PRODUCING BACTERIA FROM SOIL AND IDENTIFICATION BY 16S rRNA GENE SEQUENCING

By
Sehnin Fatima
Student ID: 15126022

A thesis submitted to the Department of Mathematics and Natural Sciences in partial fulfillment of the requirements for the degree of
Bachelor of Science in Microbiology

Mathematics and Natural Sciences

Brac University
September 2019

Declaration

It is hereby declared that

1. The thesis submitted is my/our own original work while completing degree at Brac University.
2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.
3. The thesis does not contain material which has been accepted, or submitted, for any other degree or diploma at a university or other institution.
4. I/We have acknowledged all main sources of help.

Student's Full Name & Signature:

Sehnin Fatima
ID: 15126022

Approval

The thesis/project titled “Isolation of Antibiotic Producing Bacteria from Soil and Identification by 16S rRNA Gene Sequencing “submitted by Sehnin Fatima (Student ID: 15126022), of Summer, 2019 has been accepted as satisfactory in partial fulfillment of the requirement for the degree of “Bachelor of Science in Microbiology” on 05-09-2019.

Examining Committee:

Supervisor:

_____

Fahim Kabir Monjurul Haque
Assistant Professor, Mathematics and Natural Sciences Department
Brac University

Program Coordinator:

_____

M Mahboob Hossain
Professor, Mathematics and Natural Sciences Department
Brac University

Departmental Head:

_____

A F M Yusuf Haider
Professor, Mathematics and Natural Sciences Department
Brac University

Abstract

Antibiotic resistance has become a challenging issue in medical treatment process. A large portion the novel antibiotics are derived from soil microbes as soil being a primary source. Hence, to isolate novel antibiotic agent, soil sample was screened in several steps and one isolate showed antimicrobial activity against the growth of two out of ten selected the test organisms (*Bacillus licheniformis* and *Bacillus subtilis).* The isolated bacteria was later identified as *Serratia sp.* Genotypic characterization was later carried out by amplifying the 16S rRNA gene of the isolate using universal primers in PCR mehtod. The amplified 16S rRNA gene of the isolate was sequenced using Sanger sequencing method and compared to the NCBI nucleotide database. The isolate had highest similarities with *Serratia marcescens* subsp. *sakuensis*. The isolate showed good antimicrobial activity but requires further characterization to determine its potentiality to be used as a new antibiotic for commercial production.

Keywords: Antibiotics; *Serratia marcescens*; Prodigiosin; Antibacterial activity; Microbial pigments; Secondary metabolites

Dedicated to

My Beloved Parents

Acknowledgement

Acclaim is to Allah, the most gracious, the most merciful for endowing me with the opportunity of this research course and supporting me throughout this journey to fulfill it successfully.

I truly thank **A F M Yusuf Haider,** the Chairperson of the MNS Department, Professor and **M. Mahboob Hossain,** Professor for allowing and encouraging me to complete my undergraduate thesis. I likewise thank the experts of BRAC University and Mathematics and Natural Sciences Department for organizing this magnificent learning open door for the entry level research.

My profound gratitude goes to my supervisor **Fahim Kabir Monjurul Haque**, Assistant Professor, MNS Department of BRAC University, for his constant supervision, constructive criticism, expert guidance, enthusiastic encouragement to pursue new ideas and never-ending inspiration throughout the entire period of my research work. I would like to thank and express my deepest gratitude for guiding me in my report writing and providing time to time suggestions regarding setting of experimental designs, interpretation of results and subsequent directions for the whole work without being a bit of impatient. It would have been impossible to complete this study without his cordial help.

I would like to extend my appreciation to **Akash Ahmed**, Lecturer, MNS Department of BRAC University for his suggestions and moral support during my work

I am also grateful to the respective Lab officers **Shamim Akhter Chowdhury**, **Asma Binte Afzal** and **Md. Nazrul Islam** for their great support in times of lab inquiries. I am extremely thankful and indebted to them for sharing expertise, and sincere and valuable guidance and encouragement extended to me.

My further gratitude goes to my friends and my thesis partners **Noshin Sayiara Shuchi** and **Arifa Taseen** for actively supporting me and for being with me throughout the study.

Sehnin Fatima

Table of Contents

List of Tables

List of Figures

Chapter 1

Introduction and Literature Review

1. Introduction and Literature Review

1.1 Background Information

Antibiotics can be expressed as any organic, chemical matter that can be harmful for growth and metabolism of other microbes even if present at a very low amount (Brun and Skimkets, 2000). Bacteria, fungi, actinomycetes have been a primary source of natural antibiotics since the start of antibiotic revolution. Soil is a homogenous source of different microbes depending on nutrient, moisture, temperature. Isolating & identifying such an organism which shows antibacterial and insecticidal activity may pave a new way towards modern medicine and agriculture. From ancient times, it is well understood that, natural products have a key role in the discovery and development of many antibiotics & insecticides. While many antibiotics are known to exist, efforts to discover new antibiotics still continue and so many species of *Streptomyces*, *Bacillus* and *Penicillium* have been studied continuously for their ability to yield antibiotics. *Bacillus* species are cheaper to find and produce, synthesized in soluble form so these are more convenient for commercial production. In this research, soil sample from a particular area will be processed & examined for the isolation, identification & effect check of antimicrobial activity. Genus along with the species of the bacteria can also be identified, also the genetic properties (plasmid or DNA characterization) which allow the organism to show such characteristic can be observed. In some microorganisms, other than antibiotic itself some other secondary metabolites such as terpenoids and steroids, fatty acid derived substances and polyketides, alkaloids, non-ribosomal poplypeptides and enzyme cofactors can also possess natural antimicrobial, antifungal, antitumor activity (Thirumurugan *et al*., 2018).These metabolites can act as competitive weapons used against other bacteria, fungi, amoebae, plants, insects, and large animals.

1.2 About Antibiotic; Discovery and Development

The discovery of antibiotic brought about a huge revolution in medicine history. In early 1900s, death of people due to infectious disease such as pneumonia, gonorrhea or rheumatic fever was greatly increasing. In such situation, discovery of ‘Antibiotic” was a necessity of time. A German physician and scientist named Paul Ehrlich discovered the very first antibiotics in 1909 which was synthetic arsenic-based drug (Salvarsan). It was initially used for treatment of syphilis. (Mohr, 2016)

Later, in 1928, Scottish Professor Alexander Fleming discovered antibiotic while working at St. Mary's Hospital, London. While sorting through petri dishes he observed on one dish a mold colony had contaminated it but around the blob of mold colony there was no bacterial growth present (Fleming, 1929). The zone immediately around the mold was later identified as a rare strain of *Penicillium notatum*—was clear, as if the mold had secreted something that inhibited bacterial growth. The substance that was overpowering the bacterial growth was named *Penicillin* and about ten years later its full potential as a drug had been disclosed. In the following years, further development of *Penicillin* did aid in the treatment for so many fatal diseases that were caused by bacteria. The success of *Penicillin* urged a widespread exploration among other microorganisms started for correspondingly useful compounds. During the following 20 years the most significant groups of antibiotics were discovered and these includes e.g. macrolides and tetracycline (Bérdy, 2005).

More than 75% of all antibiotics discovered during this time were originated from actinomycetes and mostly from various Streptomyces species. Actinomycetes possess a large quantity of genes in which different enzymes are encoded that are capable of producing secondary metabolites. These gene plays role in discovery for many novel antibiotics (Tiwari & Gupta, 2013). Other than that, scientists have done extensive study on numerous environmental samples throughout the last 50 years which lead to the re-discovery of many previously known substances. Many pharmaceutical companies started to look for cell wall inhibitors through rational screens (Silver, 2012).Considerable research has been carried out in order to find new antimicrobial producing bacteria isolated from environmental elements such as soil, water, sediments, wastes etc.

1.3 Action Mechanism of Antibiotics

The action mechanism is the biochemical way for testing pharmacological effectiveness of a drug. It may be associated with a specific target where the drug binds like an enzyme or a similar mechanism of a receptor. Mechanism of action refers to the biochemical process which takes place inside cell body at a molecular level.

The basic antibacterial activity includes protein synthesis, the inhibition or regulation of enzymes of cell wall synthesis or nucleic acid metabolism and repair. The fourth mechanism is the mechanism of cell membrane structure disruption. These cellular action mechanisms of antibiotics are most

effective in duplicating cells. In rare cases, there might be an overlap in these four functions between prokaryotic bacterial cells and eukaryotic mammalian cells. (Moore, 2015)

The major mechanisms of action of antibiotics are:

1. Inhibition of Cell Wall Synthesis
2. Inhibition of Protein Synthesis (Translation)
3. Alteration of Cell Membranes
4. Inhibition of Nucleic Acid Synthesis
5. Antimetabolite Activity

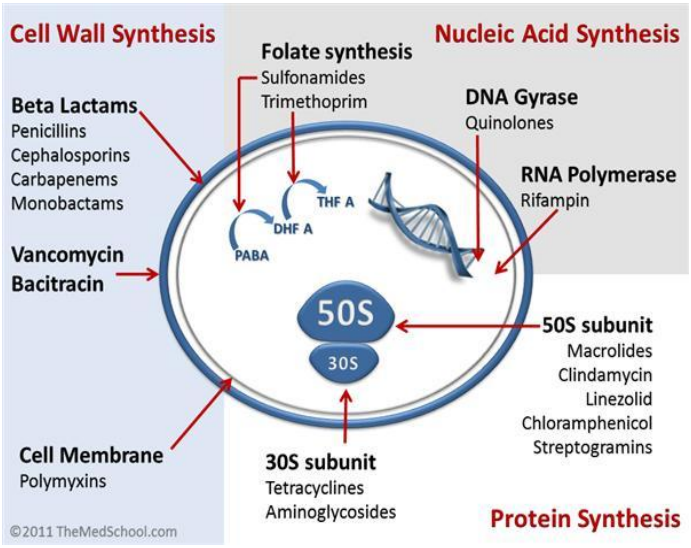


Figure 1.3.1: Action mechanism of antibiotics

1.4 Necessity of New Antibiotics

In recent times, the unnecessary usage of antibiotics is one major reason for the outburst of antibiotic-resistant bacteria and also multi-drug resistant bacterial generation. The infectious bacteria are no longer susceptible to even the strongest antibiotic and thus medication is becoming invalid. To overcome this devastating situation, discovering more efficient, novel antibiotics can be a possible way, by taking latest approaches in isolation of new, interesting bacteria, fungi. Another interesting technique can be positively using the resistance and enhance the self-protection mechanism antibiotic producers need to have, in order to avoid suicide. Due to repeated use of the same old antibiotics, the bacterial genes that are frequently getting exposed to those antibiotics have gained immortality in a sense and thus have no affect at all. The antibiotic resistant pathogens are

eventually merging with the antibiotics resistance genes. If this continues, there will be no effective antibiotics left for adequate treatment for deadly diseases. (Falagas *et al*., 2008)

1.5 Microbial Diversity

Microbial diversity represents a massive yet underexplored genetic and biological pool, which can be studied for novel genes, their products and metabolic pathways. Not all of the microbial consortium of certain environments can be cultured by standard laboratory techniques. There is a requirement for culture-independent methods to understand the genetic diversity, population structure and ecological roles of this microbiota (Krishnaveni, 2018). DNA is the most basic level of biodiversity which determinations the process of speciation and differentiates among all the other levels of biodiversity, including functional traits, species and ecosystems. Every different study needs to be designed according to the traits, environmental requirements needed for the microorganism that has to be studied on.

1.6 Strategic Sampling Plan

The basic problem in working with a sample is they represent only a very small fraction of the populations, not the overall representation of entire microbial pool of the resting environment. Hence, the initial steps of designing studies of microbial diversity could require considerable reflection and preliminary investigations. A second obvious challenge that one could expect for microbial biodiversity studies resides in multiple, independent tests of hypotheses. This is a challenge because the complexity of identifying microorganisms and bacteria in particular, can lead to considerable investment in time and labor. As a consequence, the number of strains or samples that can be analyzed is sometimes below the minimal number needed for a single unambiguous test of the hypothesis, and additional tests may be prohibitive.

1.7 Soil Microorganisms as Antibiotic Source

Soils are reservoir of a massive and diverse population of microorganisms due to its heterogeneous nature. Soil has capability to hold variation in biotic and abiotic conditions and so the microbial population in soil has capacity to adapt and develop strategies for survival and successful

reproduction against all odds (Chandra *et al*., 2017). Soil microbes (bacteria, fungi, Actinomycetes) are capable of producing a wide range of antibiotics natural defense system against other microorganisms living in their vicinity. These antibiotics are being developed in laboratories and used against life-threatening infections and diseases in humans, animals, and agriculture. For self-defense and nutritional antagonism bacteria can secrete substances which can kill or inhibit other bacteria, fungi. So to say, production of antimicrobials is one of the most potent strategies for this adaptation. Soil microorganism had always been the prime source for production of antibiotics and still continues to maintain its significance (Lihan *et al*., 2014). Unnecessary and uncontrolled use of antibiotics and disinfectants in medicine, agriculture, and fish culture and their release in environment comes with another alarming issue of multidrug-resistant pathogenic microbes and therefore some new, effective metabolites are needed so that it can be used as antibiotics which can show effective antibacterial activity against these resistant strains.

1.8 Antibiotics as Microbial Secondary Metabolites

One specific microbial strain is able to produce many metabolites. For instance, *Streptomyces griseus* and *Bacillus subtilis* can produce more than 50 types of bioactive secondary microbial metabolites that may include antibiotics, ergot alkaloids, naphthalenes, nucleosides, peptides, phenazines, quinolones, terpenoids, and others. *Serratia sp.* can produce two different group of secondary metabolites; prodiginines and serratamolides. Prodiginines are tripyrrole red pigments with different alkyl substituents with prodigiosin which has been examined to retain anticancer, antifungal and antibacterial activity (Williamson, 2006). Moreover, serratamolides are cyclodepsipeptides and biosurfactants with hemolytic, antibiotic and anticancer activities with serrawettin W1 as the most investigated metabolite.

Antibiotics are produced by microbial fermentation on microbial genera inhabiting soil that is undergoing morphological differentiation (Vining, 1990). It has been observed in an illustration, penicillin is produced only after the logarithmic growth phase of the cell (trophophase) is completed. Some bacteria can produce pigments, surfactants as secondary metabolites which possesses immunosuppressive, antifungal, antibacterial, anticancer, antiprotozoal, biosurfactant activity in a wide range (Fürstner, 2003). Microbial products (e.g. pigments, alkaloids, toxins, antibiotics, gibberellins, carotenoids, etc.) have minimal significance in the life of the microorganisms that produce them. The production of secondary metabolites is not common among microbes. Production

of secondary metabolite is generally inhibited during logarithmic growth and the suboptimal or stationary growth phases. Secondary metabolites synthesis is controlled by regulatory mechanisms which are being studied in present time (Malik, 1980).

1.9 Screening for Soil Organisms for Antibiotic Susceptibility Test

Soil microorganisms that produce antibiotics or other secondary metabolites which can perform as antibiotics can be isolated in different methods. Crowded plate technique can be one simple method to isolate any bacteria of that possess antimicrobial activity. This method aims at finding only those microorganisms that produces an antibiotic irrespective of its action against any specific organism. Hence, the sample is diluted only to such an extent that agar plates prepared from these dilutions will be crowded with individual colonies on agar surface, i.e. 300 to 400 colonies or more. The bacterial colonies that produce antimicrobial activity are indicated by clear zone of growth inhibition surrounding the colony. Such colony is later on sub cultured, purified, and afterwards microbial inhibition spectrum is tested against selective microorganisms (Okudoh, 2001).

Another technique mainly used is Wilkins's method. A Wilking medium containing a pH indicating dyes i.e. Bromo-thymol Blue which is green color at neutral pH but colorless at acidic pH. This method can separate antibiotic producer from the acid producer. Those colonies that produce antibiotics give zone of inhibition against sensitive organisms without changing color surrounding it. On the other hand, in case of acid producing colony, zone of inhibition might be observed due to fall in pH along with colorless area due high acid production which results in lower pH. In that case the color of dye is ultimately changed from green to colorless. This method also allows some advantages that the organism producing antibiotic against the microorganisms of choice can be secondary screened. This screening process uses dilutions of sample are applied to agar surface so as to get well isolated colonies (Okudoh, 2001). After the colonies have reached up to few millimeter in size the suspension of test organisms is spread over the surface of agar and incubated. Thus, zone of inhibition around the antibiotic producing colony indicates the antibiotic activity of potential isolates.

Chapter 2

Materials and Method

2. Materials and Methods

2.1 Study Place

This study was carried out in the BRAC University. The laboratory work needed for the completion of the research was done in the Microbiology and Biotechnology Laboratory of the Department of Mathematics and Natural Sciences of BRAC University.

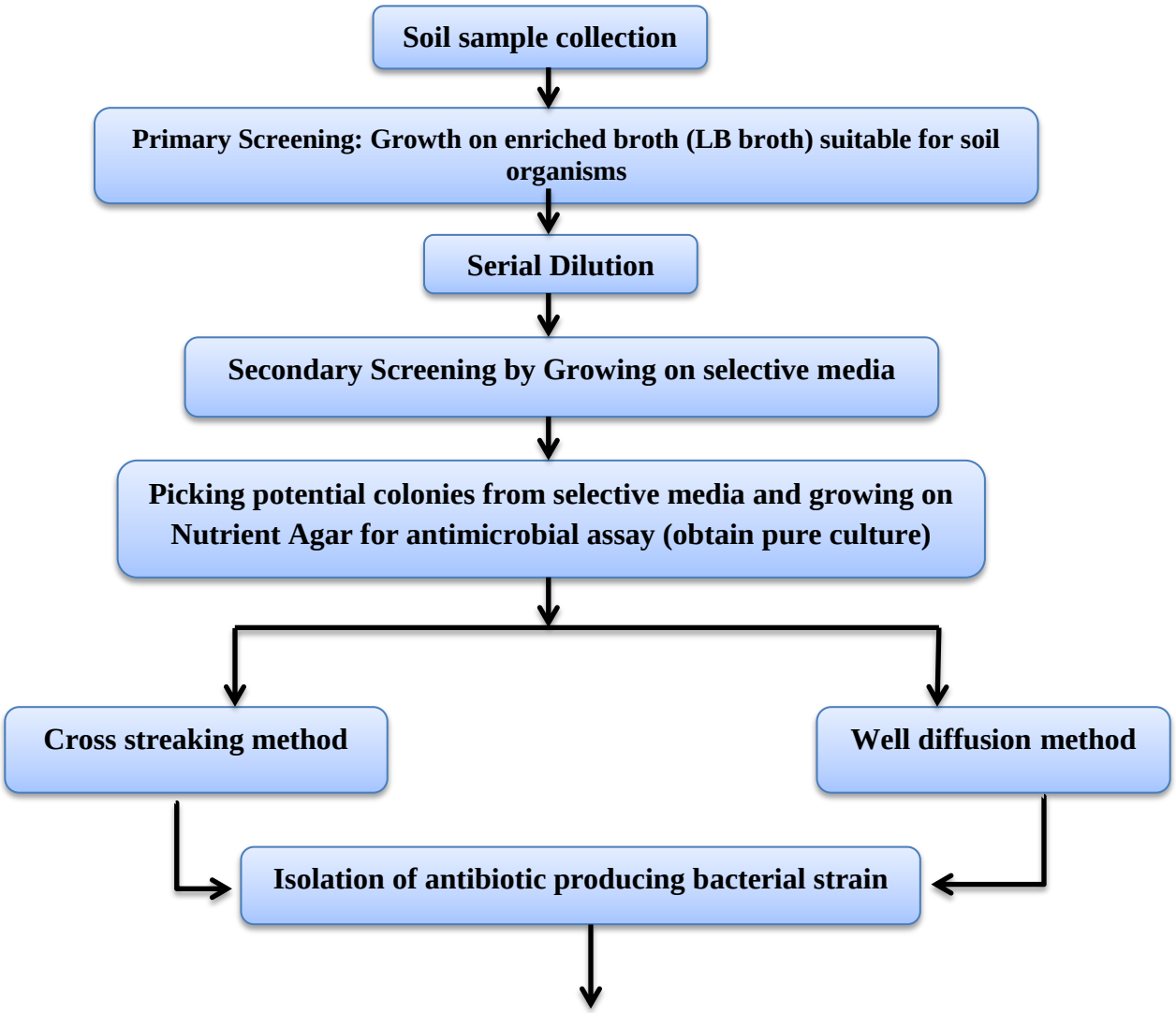
2.2 Study Duration

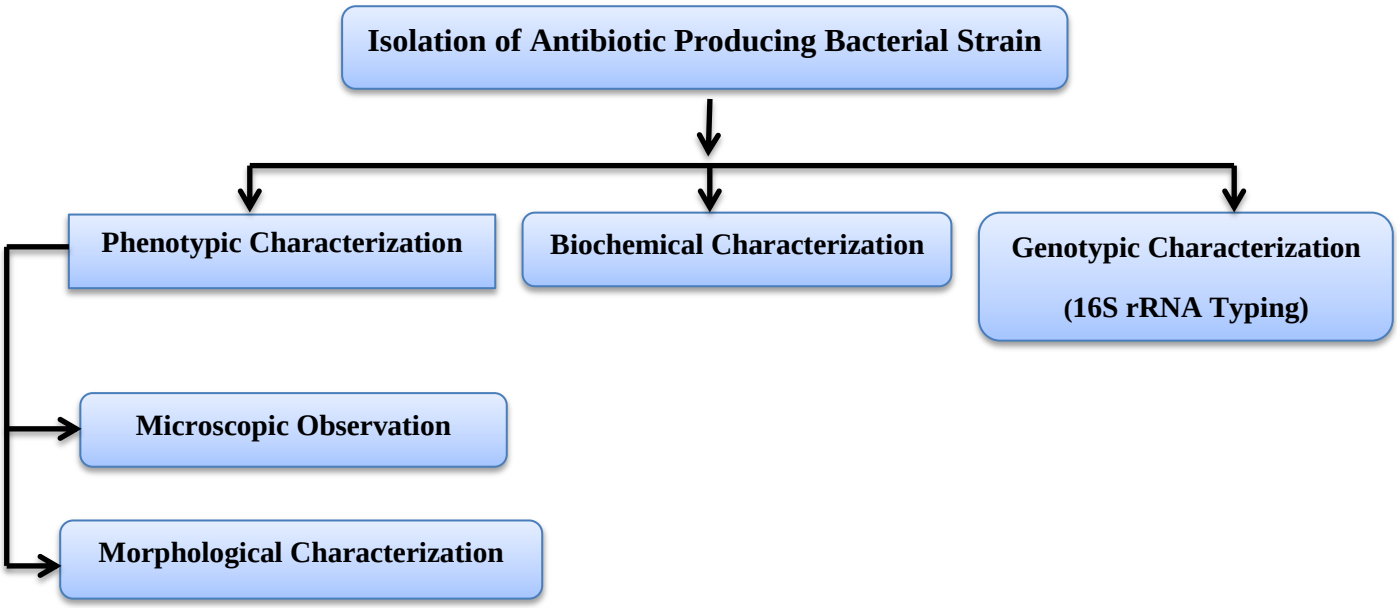
The duration of the study was from March 2018 to August 2018.

2.3 Sample Collection

The soil sample that was studied was collected from Shaheed Suhrawardy Medical College & Hospital. The samples were collected in sterile container and stored at 4◦C until use.

2.4 Flow Diagram of the Study Design





2.5 Handling of laboratory apparatus and glassware

All glassware was washed with mild detergent followed by rinsing with distilled water and then allowed to air dry. Petri dishes were heat sterilized at 180◦ in "hot air oven" for an hour in the sterilizer before use (Oven, Model: MH6548SR, LG, China). Micropipette tips, glass pipettes and microcentrifuge tubes were sterilized by autoclaving at 121◦C for 15 min at 15 PSI (SAARC).

2.6 Apparatus

The list of apparatus used is given below:

1. Incubator (Model: 0SI-500D, Digi System Laboratory Instruments Inc. Taiwan)
2. Laminar Airflow Cabinet (Model: SLF-V, Vertical, SAARC group, Bangladesh)
3. Autoclave Machine (Model: WIS 20R Daihan Scientific Co. Ltd, Korea)
4. Centrifuge Machine
5. Electric balance
6. Glassware
7. Laboratory Distillation apparatus- fractional distillatory set up
8. Microscope
9. Petri-dish,

10. Test-tubes
11. Micro-pipette
12. Bunsen burner

2.7 Serial dilution

Test tubes containing 9 ml of normal saline water were autoclaved before use. Ten folds serial dilutions of the soil sample were prepared in autoclaved saline water. Initially, 1 gram of soil was mixed with 9ml of normal saline water in a test tube. This makes a dilution of 10^{-1}which is further diluted in subsequent steps to obtain a final dilution of 10^{-10} .

2.8 Maintenance of pure culture

The colonies isolated from the selective media were streaked on to the Nutrient Agar medium, incubated at 37◦C for 24 hours and then stored at 4◦C until use (Immanuel *et al.,* 2006). For further stock of pure cultures, single colonies were stabbed onto vial containing T1N1 agar and then stored at room temperature in absence of light (30-37°C, upto 3-6 months).

2.9 Bacterial Culture Media

2.9.1 Nutrient agar

Nutrient Agar is a general purpose, basal nutrient medium used for the cultivation of microbes supporting growth of a wide range of non-fastidious organisms. It consists of peptone, beef extract and agar. To prepare it, 28 grams of nutrient agar powder was suspended in 1 liter of distilled water in a conical flask. It was mixed well and boiled for proper dissolving. The flask was then covered with aluminum foil and sealed with masking tape and sterilized in autoclave at 121°C for 15 minutes. Once autoclaved and lukewarm (cool but not solidified) it was poured onto petri dishes. The lid of each Petri dish was replaced and stored the refrigerator ($4°C$) with proper labeling of date and initials.

2.9.2 Mueller-Hinton Agar

Mueller-Hinton agar (MHA) is a medium to use for routine susceptibility testing using Kirby-Bauer disc diffusion method for non-fastidious bacteria (both aerobe and facultative anaerobe). It is a non-selective, non-differential medium. This means that almost all organisms plated on here will grow.

To prepare it, 38 grams of MHA agar powder was suspended in 1 liter of distilled water in a conical flask. After stirring on heat it was boiled. The flask was then covered with aluminum foil and sealed with masking tape and kept in autoclave at 121°C for 15 minutes for sterilization. Once autoclaved and lukewarm (cool but not solidified) it was poured onto petri dishes. The lid of each Petri dish was replaced and stored the refrigerator ($4°C$).

2.9.3 LB medium

LB Broth, also known as, LB medium, Lysogeny broth, Luria broth, or Luria-Bertani medium, is a commonly used nutritionally rich medium for culturing bacteria. To prepare this broth 25 grams of the medium was suspended in one liter of distilled water in a conical flask. It was then mixed well and dissolved by heating with frequent agitation. Boil for one minute until complete dissolution. Then it was sealed with aluminum foil and labeled properly and sterilized in autoclave at 121°C for 15 minutes. After sterilization it was stored at 4°C. The color of the prepared medium is clear amber, slightly opalescent.

2.9.4 Mannitol Salt agar

Mannitol Salt Agar is used as a selective media for the isolation of pathogenic Staphylococci from clinical and non-clinical samples. Many other bacteria except Staphylococci are inhibited by 7.5% sodium chloride. Mannitol is the fermentable carbohydrate source. For the preparation of MS agar, 111 grams of the agar was suspended in 1 liter of distilled water and then brought to the boil to dissolve completely. It was then sterilized by autoclaving at 121°C for 15 minutes. Once autoclaved and lukewarm (cool but not solidified) it was poured onto petri dishes. The lid of each Petri dish was replaced and stored the refrigerator ($4°C$). The color of the media is clear red prior to culture.

2.9.5 Cetrimide agar

Cetrimide Agar is a selective and differential medium used for the isolation and identification of *Pseudomonas aeruginosa* from clinical and non-clinical specimens. To prepare this media, 45.3 g of the agar was suspended and 10 ml of glycerol added in one liter of distilled water in a conical flask. It was then heated with frequent agitation and boiled for completely dissolve of the medium. The media was autoclaved at 121°C for 15 minutes for sterilization. Once autoclaved and lukewarm (cool but not solidified) it was poured onto petri dishes. The lid of each Petri dish was replaced and

stored the refrigerator ($4°C$). The color of the media is clear red prior to culture. The color of the media is clear straw color.

2.9.6 MacConkey agar

MacConkey agar a selective and differential culture medium for bacteria which selectively isolates *E. coli* and other Gram-negative and enteric bacilli and differentiate them based on lactose fermentation. For media preparation, 49.53 grams of the agar was suspended in 1 liter distilled water in a conical flask. It was then boiled till dissolved completely. After that, the media was sterilized by autoclaving at 121°C for 15 minutes. After it had cooled down, the media was poured onto petri dishes. The lid of each Petri dish was replaced and stored the refrigerator ($4°C$). The color of the media is clear, pale red prior to culture.

2.9.7 Bacillus cereus agar

Bacillus Cereus Agar Base with added supplements is used as a selective medium for the isolation and enumeration of *Bacillus cereus* from food samples. To prepare this media, 20.5 grams of agar is suspended in 475 ml distilled water. It was heated to boiling to dissolve the medium completely. Th The media was then sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes and cooled to 45-50°C and aseptically added rehydrated contents of 1 vial of Polymyxin B Selective Supplement [;'[[(FD003) and 25 ml of sterile Egg Yolk Emulsion (FD045). All the additives were mixed well and poured into sterile Petri plates.

2.10 Identification of the bacteria

2.10.1 Morphological characterization of the bacteria

To obtain isolated discrete colonies NA plate was streaked following aseptic techniques. The plates were then incubated at 37◦C for 24 hours. After incubation, the bacterial colonies were taken under observation for size, pigmentation, form, margin, elevation and texture (Cappuccino & Sherman, 2005).

2.10.2 Microscopic observation of the bacteria

The potential bacteria were observed under microscope in order to study their properties.

2.10.2.1 Gram stain

At first, the slides taken were properly labeled. Then using sterile technique, a drop of saline was placed on the slide and a small amount of the bacteria were then transferred to the drop of saline with a sterile cooled inoculating loop. A smear was then prepared by mixing and spreading the bacteria by means of a circular motion with the loop. The smear was then allowed to air dry followed by heat fixation. The Gram staining process was then carried out before the smear over-dried. The smear was flooded with Crystal Violet and let stand for 1 minute. Then, the smear was gently washed with tap water. It was then flooded again with the Gram's Iodine mordant and let stand for 1 minute followed by gentle wash with tap water. After that, the smear was decolorized with 95% Ethyl Alcohol and gently washed with tap water. Finally, it was counterstained with Safranin for 45 seconds and gently washed with tap water. The slide was then blot dried with bibulous paper and covered with cover slip and then examined under oil immersion 100X Achromat objective lens (Cappuccino & Sherman, 2005).

2.10.3 Biochemical characterization of the bacteria

Several biochemical tests were carried out in order to have a presumptive identification of the potential bacteria chosen before. Most of the methods were done according to the microbiology laboratory manual (Cappuccino & Sherman, 2005). The biochemical tests performed were;

Carbohydrate fermentation (Sucrose, fructose, glycerol, maltose and D-xylose), Triple sugar iron agar test, IMViC test (Indole production test, Methyl red test, Voges- Proskauer test, Citrate utilization test), Urease test, Nitrate reduction test, Catalase test, Oxidase test, Casein hydrolysis test, Motility test, Gelatin hydrolysis test, Mannitol Salt Agar, Starch hydrolysis, Blood agar.

2.10.3.1 Carbohydrate Utilization test

Phenol red sucrose, fructose, glucose, maltose and mannitol broths were prepared by autoclaving at 15 psi 121◦C for 15 minutes (Autoclave, SAARC) in separate test tubes. Using sterile technique, small amount of the experimental bacteria from 24 hour pure culture was inoculated into the broths by means of loop inoculation. All the tubes were incubated for 24 hours at 37◦C (Cappuccino & Sherman, 2005).

2.10.3.2 Triple Sugar Iron Agar test

Triple sugar iron slants were prepared in the test tubes and autoclaved at 15 psi 121◦C. Using sterile technique, small amount of the experimental bacteria from 24 hour pure culture was inoculated into the tubes by means of a stab and streak inoculation method. The tubes were incubated for 24 hours at 37◦C (Cappuccino & Sherman, 2005).

2.10.3.3 Indole Production test

Tryptophan broth of 5 ml in each test tube was prepared by autoclaving at 15 psi, 121◦C. Using sterile technique, small amount of the experimental bacteria from 24 hour pure culture was inoculated into the tubes and the tubes were incubated for 48 hours at 37◦C. In order to test for indole production, 5 drops of kovac's reagent was added directly into the tubes (MacWilliams, 2009).

2.10.3.4 Methyl red test

MR-VP broth of 6 ml in each test tubes were prepared by autoclaving at 15 psi 121◦C. Using sterile technique, small amount of the experimental bacteria from 24 hour pure culture was inoculated into the tubes and the tubes were incubated for 24 hours at 37◦C. After 24 hours 3.5 ml from the culture tubes were transferred to clean test tubes for Voges- Proskauer test and the remaining broth were re-incubated for additional 24 hour. After 48 hour incubation 5 drops of methyl red indicator was added directly into the remaining aliquot of the culture tubes to observe the immediate development of a red color (Cappuccino & Sherman, 2005).

2.10.3.5 Voges Proskauer test

To the aliquot of MR-VP broth after 24 hour incubation, 0.6 ml (12 drops) of 5% alpha naphthol (Barrit's reagent A) was added followed by 0.2 ml (4 drops) of 40% KOH (Barrit's reagent B).
The tube was gently shaken to expose the medium to atmospheric oxygen (30seconds to 1 minute) and the medium was allowed to remain undisturbed for 10-15 minutes. The test was read, but not beyond, one hour following the addition of the reagents (McDevitt, 2009).

2.10.3.6 Citrate utilization test

Simmons citrate agar slants of 2 ml in each vial were prepared by autoclaving at 15 psi, 121◦C. Using sterile technique, small amount of the experimental bacteria from 24 hour pure culture was inoculated into the vials by means of a streak inoculation method with an inoculating needle and the vials were incubated for 48 hours at 37◦C (Cappuccino & Sherman, 2005).

2.10.3.7 MIU (Motility- Indole- Urease) test

MIU media was prepared by autoclaving at 15 psi, 121◦C. the media was cooled to about 50-55◦C and 100ml of urease reagent was added aseptically to 900 ml base medium. After that, 6ml solution was transferred to each sterile test tube and allowed to form a semi solid medium. Using sterile technique, small amount of the experimental bacteria from 24 hour pure culture was inoculated into the tubes by means of a stab inoculation method with an inoculating needle and the tubes were then incubated for 24 hours at 37◦C (Acharya, 2015).

2.10.3.8 Catalase test

A microscopic slide was placed inside a petri dish. Using a sterile inoculating loop, a small amount of bacteria from 24 hour pure culture was placed onto the microscopic slide. 1 drop of 3% H2O2 was placed onto the organism on the microscopic slide using a dropper and observed for immediate bubble formation (Reiner, 2010).

2.10.3.9 Oxidase test

A small piece of filter paper was soaked in Gaby and Hadley oxidase test reagent and let dry. Using an inoculating loop, a well isolated colony from pure 24 hour culture was picked and rubbed onto filter paper and observed for color change (Shields & Cathcart, 2010).

2.10.3.10 Gelatin hydrolysis test

All the ingredients of the nutrient gelatin medium were mixed and gently heated to dissolve. 6 ml from the media was dispensed in test tubes. The test tubes with the medium were then autoclaved at 121◦C, 15 psi. The tubed medium was allowed to cool in an upright position before use. Using sterile technique, a heavy inoculum of 24 hour old culture bacteria was stab inoculated into the tubes

with an inoculating needle. The tubes were then incubated at 37◦C and observed up to 1 week (Cruz & Torres, 2012).

2.10.3.11 Mannitol Salt Agar test

Using sterile technique, a plate of MSA agar was streaked by picking a loopful colony of 24 hour old pure culture to obtain isolated colonies. The plates were then incubated at 37◦C for 24 hours (Shields & Tsang, 2013).

2.10.3.12 Starch hydrolysis test

Using sterile technique, a starch agar plate was streaked by picking a loopful colony of 24 hour old pure culture with an inoculating loop. The plates were then incubated at 37◦C for 48 hours and the hydrolysis was observed using gram's iodine (Cappuccino & Sherman, 2005).

2.10.3.13 Casein hydrolysis test

Distilled water and agar solution was taken in separate conical flasks and both were autoclaved at 121◦C, 15 psi. Skim milk powder (28 g/L) was then added to the autoclaved distilled water aseptically and boiled for 1 minute to dissolve completely. After that, the milk solution was mixed with agar solution. The media was dispensed into sterile plates while liquid and left for a while to solidify. Using sterile technique, a milk agar plate was inoculated by 24 hour old culture by means of streak plate method. The plates were then incubated at 37◦C for 24 hours (Sturm,
2013).

2.10.3.14 Blood agar test

Blood agar base was prepared in a conical flask and autoclaved at 121◦C, 15 psi. The agar base was allowed to cool at 45-50◦C and 5% (vol/vol) sterile de-fibrinated sheep blood that had been warmed to room temperature was added and gently mixed avoiding air bubbles. The media was then dispensed into sterile plates while liquid and left for a while to solidify. Using sterile technique, a blood agar plate was streaked by picking a loopful colony of 24 hour old pure culture with an inoculating loop. The plates were then incubated at 37◦C for 24 hours. After incubation, the plates were observed for gamma, beta and alpha hemolysis (Aryal, 2015).

2.10.4 Genotypic characterization

In order to achieve the complete identification of the bacteria via molecular analysis the following procedures were carried out:

2.10.4.1 Total DNA preparation

To prepare total DNA of the sample microorganism, one loopful of bacteria (freshly grown culture, 24 hours) was aseptically transferred to a 1.5 ml micro-centrifuge tube containing 1ml of nuclease-free water. Then the tube was heated in a water bath at 95°C for 5 minutes. By cooling down the micro-centrifuge tube for a while, it was then centrifuged (Centrifuge; Eppendorf, Germany) at 5,000 RPM for 2 minute in order to separate the cells. 5µl of the supernatant was used for PCR. The total DNA was stored at 4°C until further use.

2.10.4.2 Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) technique is a process to amplify genes (Kary Mullis, 1990). PCR involves a process of heating and cooling called thermal cycling which is carried out by machine. The new fragments of DNA that are made during PCR also serve as templates to which the DNA polymerase enzyme can attach and start making DNA. The result is a huge number of copies of the specific DNA segment produced in a relatively short period of time.

There are three main stages:

1. **Denaturing:** In this stage the double-stranded template DNA is heated to separate it into two single strands.
2. **Annealing:** The temperature is lowered to enable the DNA primers to attach to the template DNA.
3. **Extending:** The last stage takes place when the template is raised and the new strand of DNA is made by Taq polymerase enzyme.

These three stages are repeated 20-40 time, doubling the number of DNA copies each time. A complete PCR reaction can be performed in a few hours, or even less than an hour with certain high-speed machines. After completion, the tubes were kept at -20°C until further use.

A reaction mixture was prepared containing 10µl of 5X reaction buffer that contains 25mM MgCl2, 1µl of 10mM dNTPs and 33µl of nuclease free water. Then each of the DNA primers was added in

an amount so that the final concentration of DNA primers become 0.2mM in the final reaction mixture. Afterwards, 2µl of DNA template was added along with 1µl of Taq polymerase. The final reaction volume should be 50 µl. All the steps were performed on ice bath.

Table 1: Forward and reverse primers for 16S rRNA gene for Isolate 5

Primer		**5´ - 3´**
27F	Forward primer	AGAGTTTGATCCTGGCTCAG
1492R	Reverse primer	GGTTACCTTGTTACGACTT

Table 2: PCR reaction mixture for 16S rRNA gene for 50 µl set up

Ingredients	**Volume added**
5X Buffer	10 µl
MgCl2	2 µl
DNTPs (10mM)	1 µl
Forward primer	0.5 µl
Reverse primer	0.5 µl
Taq Polymerase	1 µl
Nuclease-free water	33 µl
Template DNA	2 µl
Total	50 µl

Table 3: Cycling parameters for PCR for 16S rRNA gene

PCR step	**Temperature**	**Time**
Initial denaturation	95°C	5 minutes
Denaturation*	95°C	30 seconds
Annealing*	51°C	30 seconds
Extension*	72°C	2 minutes
Final extension	72°C	10 minutes

**** 35 cycles**

2.10.4.3. Detection of amplification by electrophoresis analysis

The DNA samples which were amplified by the PCR process were checked by horizontal electrophoresis in 1.0% agarose slab gel in Tris - Acetate EDTA (TAE) buffer. Agarose was dissolved in 1x Tris-acetate EDTA (TAE) buffer to give a final concentration of 1.0% agarose and was heated to dissolve in a microwave oven for about 30 seconds and then allowed to cool down to about 50◦C. EtBr (staining solution) was added into the agarose gel before pouring int tray (10µl EtBr in 150ml TAE). Then it was poured on the tray previously set with the comb and allowed to solidify. 6µl aliquot of the PCR product was mixed 2µl of loading dye and was loaded into the individual well of the gel. 1 Kb plus DNA marker (Invitrogen, USA) was used to identify the amplification size. The EtBr stained DNA bands were observed on a UV transilluminator at 365 nm (UV Transilluminator, Wealtec).

2.10.4.4 Purification of PCR products

Amplified PCR products were purified by using the purification kit Wizard SV Gel and PCR Clean-Up System.

2.10.4.5 Sequence analysis of 16s rRNA gene

These purified PCR products were sequenced by Sanger sequencing method using ABI Genetic Analyzer (Model: 3700) and was performed by 1st BASE Laboratories, Malaysia.

2.10.4.6 Sequence trimming and submission

The forward and reverse sequences of the DNA were screened for removal of non-essential vector sequences using the program BLASTn by the software Seqman (Lasergene). The corrected partial sequence was then submitted to NCBI, Nucleotide databank for alignment with the closest species (Singh *et al.,* 2015).

2.10.4.7 Downloading homologous sequences

The corrected partial sequence in FASTA format was subjected to BLASTn to retrieve homologous sequences belonging to divergent species available at the NCBI database based on higher percentage

identity and E value (<10-5). A total of 10 divergent species and their respective sequences were downloaded and saved in FASTA format (Singh *et al.,* 2015).

2.10.4.8 Multiple sequence alignment

The sequences of 10 divergent species along with the query sequence in FASTA format were subjected to multiple sequence alignment using MEGA6 software (Tamura et al., 2013) to identify the evolutionary conserved regions of 16S ribosomal RNA gene among species (Singh *et al.,* 2015).

2.10.4.9 Phylogenetic inference

The MEGA6 software (Tamura *et al.,* 2013) was used for phylogenetic tree construction for determining the closest species. The phylogenetic tree of the sequences was inferred using maximum likelihood ratio (with 500 bootstrap replication) using the nucleotide substitution type and Tamura-Nei model, with uniform rates for rates among sites and with complete deletion of the gaps/ missing data (Singh *et al.,* 2015).

Chapter 3

Results

3. Results

3.1 Primary screening on enriched media

LB medium was used as an enriched medium for cultivating the potential soil microorganisms. Conical flasks containing LB medium after incubation of 24-48 hours in shaker incubator at 120 rpm were observed to be opaque. The significant change in the color and opacity of the broth indicated microbial growth.

3.2 Secondary screening on selective media

Cetrimide agar, Bacillus cereus (BC) agar, MacConkey agar, Mannitol salt agars were used for secondary screening of the soil sample from the LB medium. 20-30µl of the LB broth containing bacterial culture was transferred to each plate and cultured through spread plate technique. There was significant growth observed on each media plates.

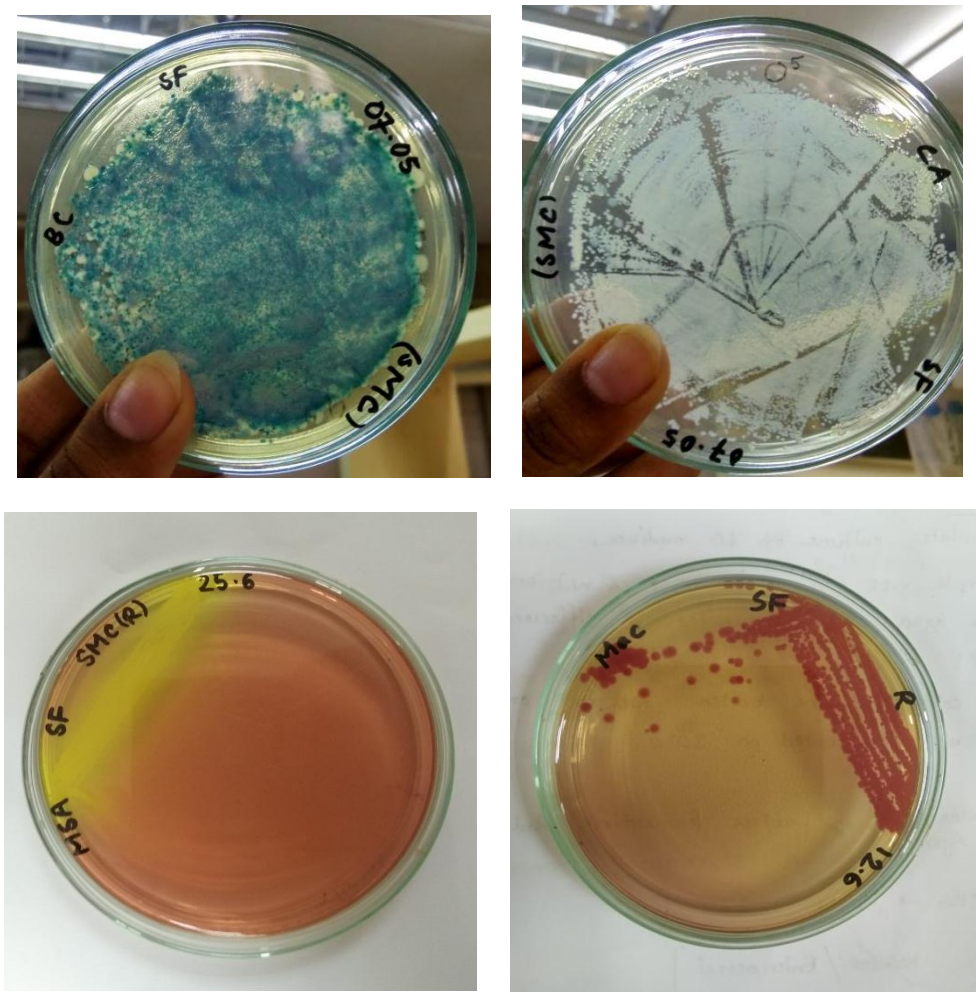


Figure 3.2.1: Screening on selective media

3.3 Selection of potential colonies and pure culture obtained on Nutrient agar plates

Observing the colony morphology, form, color, size; distinct isolated colonies were selected from Cetrimide agar, BC agar, MacConkey agar and Mannitol salt agar. The selected colonies were then cultured on nutrient agar plates to obtain pure culture for further isolation of antimicrobial activity producing bacterial strain.

3.4 Antibiotic production test

3.4.1 Cross streak on Mueller-Hinton Agar plate (1st Stage)

Mueller-Hinton agar was used for cross streaking of the potential bacterial colonies isolated from prior screening against total 10 test organism strains. Among all, against 2 of the test strains were observed to be sensitive against one of the organism that was isolated from the soil sample.

Table 3.4.1.1: Record of cross streaking pattern for antibiotic production test

SL of potential colonies from soil sample / **Test Organism**	1	2	3	4	5	6	7	8	9	10
Eshcherichia coli	R	R	R	R	R	R	R	R	R	R
Proteus vulgaris	R	R	R	R	R	R	R	R	R	R
Klebsiella varicola	R	R	R	R	R	R	R	R	R	R
Salmonella typhi	R	R	R	R	R	R	R	R	R	R
Bacillus subtilis	R	R	R	R	**S**	R	R	R	R	R
Bacillus cereus	R	R	R	R	R	R	R	R	R	R
Bacillus licheniformis	R	R	R	R	**S**	R	R	R	**S**	R
Salmonella paratyphi	R	R	R	R	R	R	R	R	R	R
Staphylococcus aureus	R	R	R	R	R	R	R	R	R	R
Pseudomonas aeruginosa	R	R	R	R	R	R	R	R	R	R

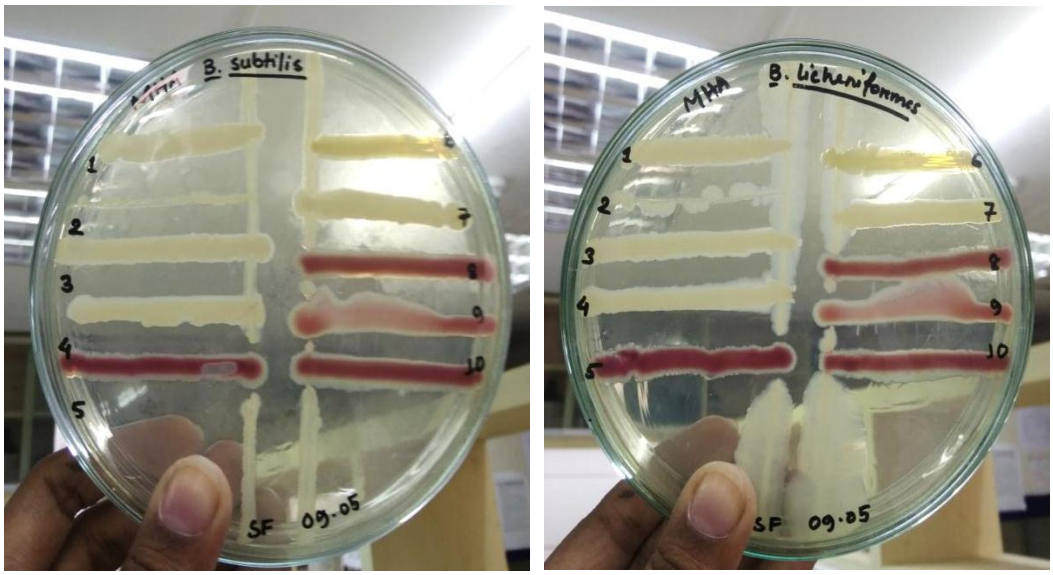


Figure 3.4.1.1: Cross streak method for antibiotic production test

3.4.2 Agar well diffusion method on Mueller-Hinton agar (2nd Stage):

Mueller-Hinton agar was used for agar well diffusion technique (Kriby-Bauer method). The sample organisms which showed sensitivity again test organisms where taken for further confirmation of antibiotic productivity test. On the MHA plates, lawns of test organisms were done by standardizing with 0.5 McFarland standard solutions (0.5 CFU/ml) (Bauer, 1966). Several suspension of potential bacterial isolate was prepared in different formulation to observe better results. The results were observed after 24 hours of incubation at 30° C. The diameters of the zone of inhibition were measured and recorded in a table.

Table 3.4.2.1: Measure of zone of inhibition in agar well diffusion method

Test Organism	**Zone of inhibition (Diameter in mm)**				
	Sample R (Isolate 5) (grown on nutrient agar) in normal saline solution	Sample R (Isolate 5) (grown on Cetrimide agar) in normal saline	Raw soil sample inoculated on LB broth	Sample R (Isolate 5) Inoculated on LB broth	Supernatant collected from LB broth inoculation of Sample R (Isolate 5)
B. licheniformis	13	13	11	8	10
B. subtilis	17	18	-	13	11

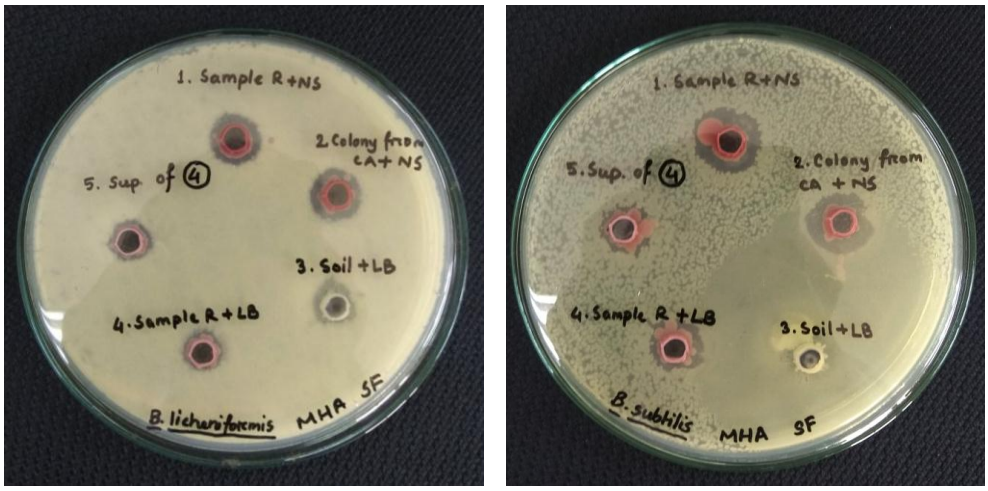


Figure 3.4.2.1: Agar well diffusion method for antibiotic production test

3.5 Phenotypic Characterization

3.5.1 Morphological Characterization

The potential antimicrobial activity producing microorganism was grown in nutrient agar plate and well isolated colony was evaluated for cultural characterization. The plates were observed in following manner:

Table 3.5.1.1: Colony Characteristics of potential bacteria

Parameters	Result
Form	Circular
Margin	Entire
Elevation	Convex
Pigmentation	Reddish orange
Texture	Smooth, mucoid

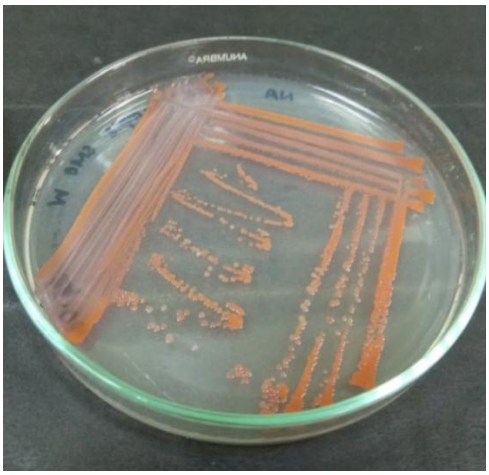


Figure 3.5.1.1: Overnight culture of sample R (Isolate 5) in nutrient agar plate

Another significant observation was the microorganism showed slow pigment production at 37°C as compared to the plate that was kept in 30°C, although both of the plates showed similar colony morphology and significant growth. This indicated that the microorganism had temperature influence on pigment production.

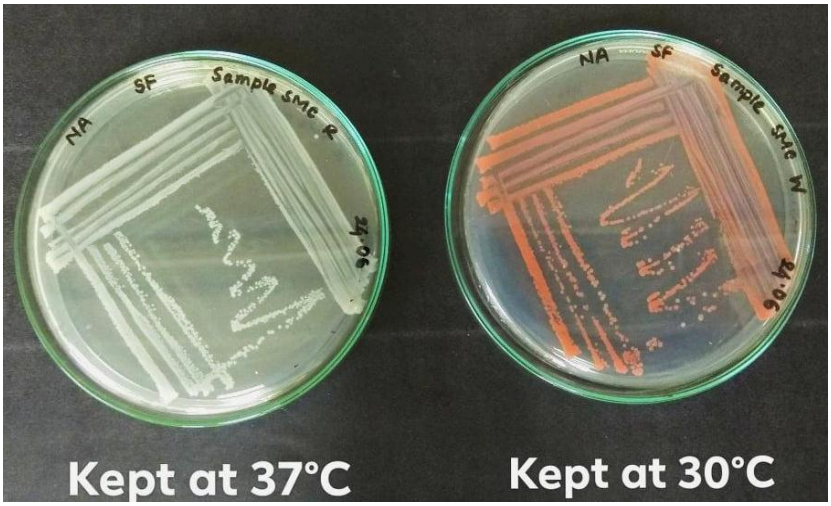


Figure 3.51.2: Temperature influence on pigment production in overnight culture of isolate 5

3.5.2 Microscopic Identification

3.5.2.1 Gram staining

After proper gram staining the slides were observed under light microscope with oil immersion objective lens. The bacterial cells were observed to be Gram-negative rod shaped.

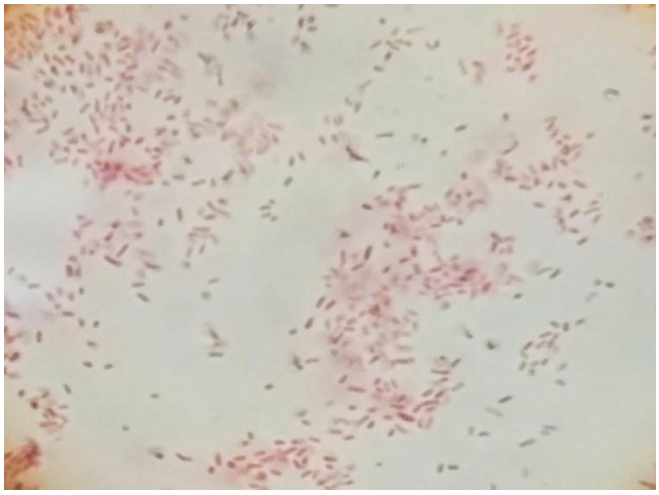


Figure 3.5.2.1.1: Gram staining of isolate 5

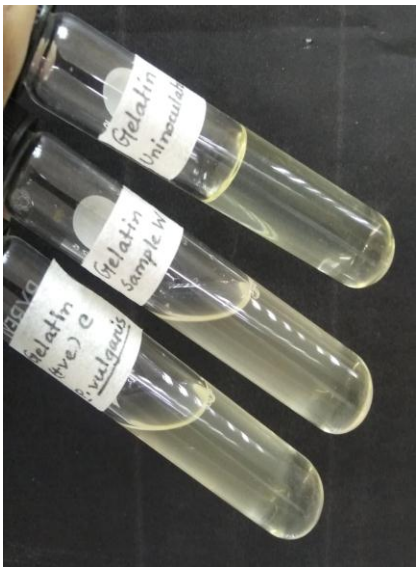
3.6 Biochemical Identification

Several biochemical tests were carried out in order to have a presumptive identification of the potential bacteria chosen before. Most of the methods were done according to the microbiology laboratory manual (Cappuccino and Sherman, 2005). The biochemical tests that were performed, among those glucose utilization, sucrose utilization, maniitol utilization, citrate utilization, catalase test, gelatin hydrolysis showed positive results whereas lactose utilization, H2S production, indole test, methyl red test, motility test, urease test, oxidase test, starch hydrolysis, 6.5% NaCl tolerance test showed negative results. All the biochemical tests were done in triplicate to avoid any false positive or false negative results. The results of the tests are given in the following table 3.6.1:

Table 3.6.1: Biochemical tests

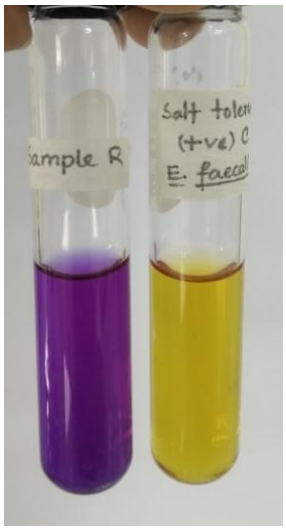
SL	Biochemical Test	Results/Remarks
1	Glucose Utilization	+
2	Sucrose Utilization	+
3	Lactose Utilization	-
4	Mannitol Utilization	+
5	Triple Sugar Iron (TSI)	Alkaline slant (red) and acid butt (yellow) without gas and Hydrogen sulfide production
6	H2S production	-
7	Indole	-
8	Citrate	+
9	Methyl Red	-
10	Voges-Proskauer	-
11	MIU (Motility-Indole-Urease)	(-) , (-) , (-)
12	Nitrate Reduction	+ (to nitrite)
13	Catalase	+
14	Oxidase	-
15	Starch hydrolysis	-
16	Gelatin hydrolysis	+
17	Casein hydrolysis	+
18	Lipid hydrolysis	+
19	Blood agar	hemolytic
20	Salt tolerance (6.5%)	-

Indole test



Gelatin hydrolysis test

Methyl red test

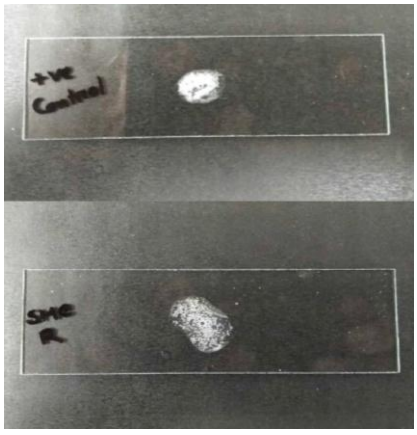


Salt tolerance test

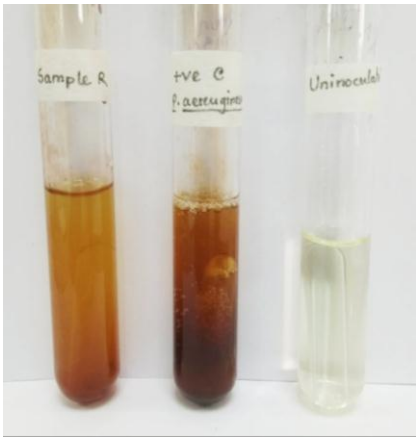
Casein hydrolysis test

Glucose fermentation test

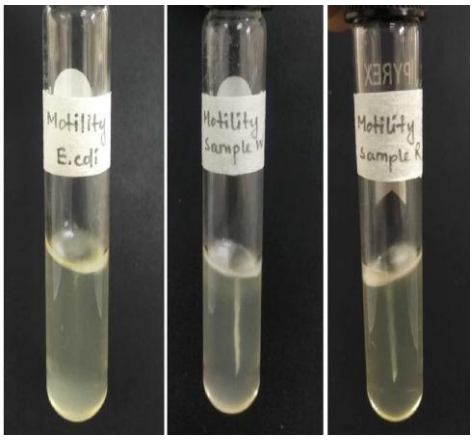
Citrate test



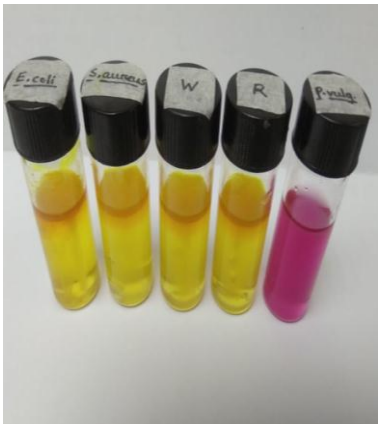
Catalase test



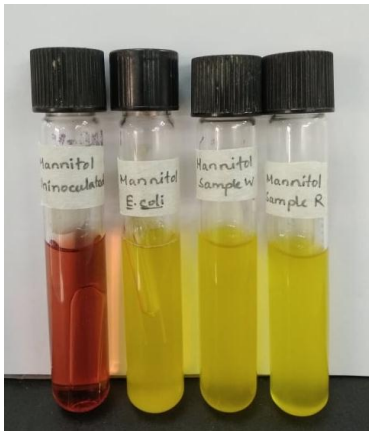
Nitrate reduction test



Motility test



Urease test



Mannitol fermentation test

Figure 3.6.1: Biochemical tests

3.7 Genotypic Characterization

3.7.1 Estimation of DNA band size

Amplification of 16S rRNA gene was carried out with two universal primers: 27F - AGAGTTTGATCCTGGCTCAG and 1492R- GGTTACCTTGTTACGACTT and then the amplified genes were run in PCR. For confirmation of successful PCR, agarose gel electrophoresis was done to have an estimation of PCR product band size. It was observed that the size of the band was ~1200 bp.

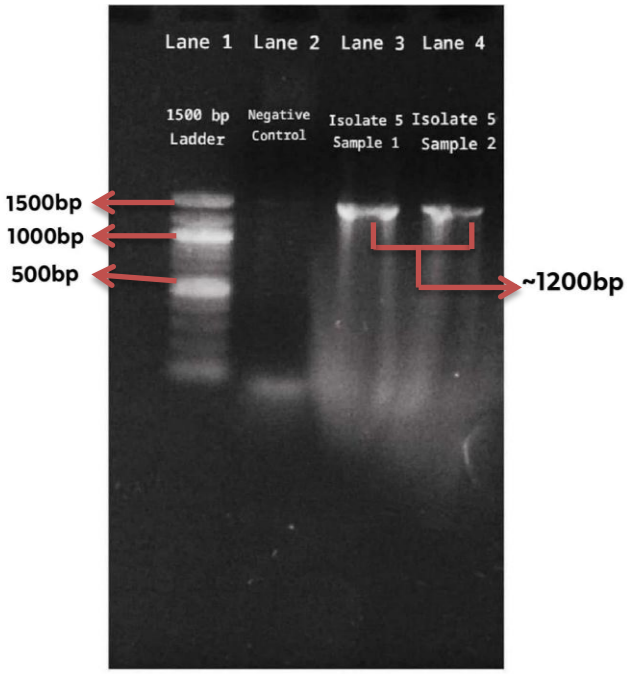


Figure 3.7.1.1: Agarose gel electrophoresis result of the amplification of 16S rRNA gene of the isolate 5. The DNA band size was estimated to be around 1200 bp according to the 1500bp ladder.

3.7.2 Sequence analysis of 16S rRNA gene

The method Sanger sequencing was followed using ABI Genetic analyzer by 1st BASE Laboratories in Malaysia. The trimmed and cleaned query sequence is given in table 3.8.2.1:

Table 3.7.2.1: Trimmed and corrected DNA sequence of 16S rRNA gene of isolate 5 (Sample R) (FASTA format)

Query sequence (1059 bp)	TGGTAAGCGCCCTCCCGAAGGTTAAGCTACCTACTTCTTTTGCAACCCACTCCCATGGTGTGACGGGCGGTG TGTACAAGGCCCGGGAACGTATTCACCGTAGCATTCTGATCTACGATTACTAGCGATTCCGACTTCATGGAG TCGAGTTGCAGACTCCAATCCGGACTACGACGTACTTTATGAGGTCCGCTTGCTCTCGCGAGGTCGCTTCTCT TTGTATACGCCATTGTAGCACGTGTGTAGCCCTACTCGTAAGGGCCATGATGACTTGACGTCATCCCCACCTT CCTCCAGTTTATCACTGGCAGTCTCCTTTGAGTTCCCGGCCGAACCGCTGGCAACAAAGGATAAGGGTTGCG CTCGTTGCGGGACTTAACCCAACATTTCACAACACGAGCTGACGACAGCCATGCAGCACCTGTCTCAGAGTT CCCGAAGGCACCAATCCATCTCTGGAAAGTTCTCTGGATGTCAAGAGTAGGTAAGGTTCTTCGCGTTGCATC GAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCATTTGAGTTTTAACCTTGCGGCCGTAC TCCCCAGGCGGTCGATTTAACGCGTTAGCTCCGGAAGCCACGCCTCAAGGGCACAACCTCCAAATCGACATC GTTTACAGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCACCTGAGCGTCAGTCTTC GTCCAGGGGGCCGCCTTCGCCACCGGTATTCCTCCAGATCTCTACGCATTTCACCGCTACACCTGGAATTCTA CCCCCCTCTACGAGACTCTAGCTTGCCAGTTTCAAATGCAGTTCCCAGGTTGAGCCCGGGGATTTCACATCTG ACTTAACAAACCGCCTGCGTGCGCTTTACGCCCAGTAATTCCGATTAACGCTTGCACCCTCCGTATTACCGCG GCTGCTGGCACGGAGTTAGCCGGTGCTTCTTCTGCGAGTAACGTCAATTGATGAACGTATTAAGTTCACCAC CTTCCTCCTCGCTGAAAGTGCTTTACAACCCGAAGGCCTTCTTC

The query sequence was then submitted to NCBI, nucleotide databank and subjected to BLASTn. The top 20 homologous sequences are given below depending on their higher percentage identity and E value (<10-5) from the NCBI database.

Table 3.7.2.2: Top 20 sequences of NCBI list with higher percentage of identity (99%) and 0.0 E value.

Description	Max Score	Total Score	Query Cover	E Value	Percent Identity	Accession
Serratia marcescens subsp. sakuensis strain KRED 16S ribosomal RNA, partial sequence	1949	1949	99%	0.0	99.91%	NR_036886.1
Serratia nematodiphila DZ0503SBS1 16S ribosomal RNA, partial sequence	1943	1943	99%	0.0	99.81%	NR_044385.1
Serratia marcescens strain NBRC 102204 16S ribosomal RNA, partial sequence	1938	1938	99%	0.0	99.72%	NR_114043.1
Serratia marcescens subsp. marcescens ATCC 13880 strain JCM 1239 16S ribosomal RNA, partial sequence	1936	1936	99%	0.0	99.62%	NR_113236.1
Serratia marcescens subsp. marcescens ATCC 13880 strain DSM 30121 16S ribosomal RNA, partial sequence	1927	19327	99%	0.0	99.53%	NR_041980.1
Serratia rubidaea strain DSM 4480 16S ribosomal RNA, partial sequence	1912	1912	99%	0.0	99.24%	NR_114716.1
Serratia rubidaea strain JCM1240 16S ribosomal RNA, partial sequence	1905	1905	99%	0.0	99.15%	NR_024644.1
Serratia rubidaea strain NBRC 103169 16S ribosomal RNA, partial sequence	1890	1890	99%	0.0	98.68%	NR_114232.1
Serratia ficaria strain DSM 4569 16S ribosomal RNA, partial sequence	1860	1860	99%	0.0	98.40%	NR_041979.1
Serratia ficaria strain NBRC 102596 16S ribosomal RNA, partial sequence	1857	1857	99%	0.0	98.30%	NR_114155.1

Description	Max Score	Total Score	Query Cover	E Value	Percent Identity	Accession
Serratia odorifera strain PADG 1073 16S ribosomal RNA, partial sequence	1855	1855	99%	0.0	98.30%	NR_037110.1
Serratia entomophila strain DSM 12358 16S ribosomal RNA, partial sequence	1855	1855	99%	0.0	98.30%	NR_025338.1
Serratia ficaria strain JCM1241 16S ribosomal RNA, partial sequence	1855	1855	99%	0.0	98.30%	NR_112005.1
Serratia odorifera strain NBRC 102598 16S ribosomal RNA, partial sequence	1851	1851	99%	0.0	98.20%	NR_114157.1
Serratia ureilytica strain NiVa 51 16S ribosomal RNA, partial sequence	1844	1844	99%	0.0	98.11%	NR_042356.1
Enterobacter tabaci strain YIM Hb-3 16S ribosomal RNA, partial sequence	1832	1832	99%	0.0	97.92%	NR_146667.2
Cedecea lapagei strain DSM 4587 16S ribosomal RNA, partial sequence	1832	1832	99%	0.0	97.92%	NR_126319.1
Serratia odorifera DSM 4582 16S ribosomal RNA, partial sequence	1832	1832	99%	0.0	97.20%	NR_114578.1
Gibbsiella dentisursi strain LEN33 16S ribosomal RNA, partial sequence	1831	1831	99%	0.0	97.92%	NR_118387.1
Cedecea davisae DSM 4568 16S ribosomal RNA, partial sequence	1829	1829	99%	0.0	97.83%	NR_025243.1
Cedecea lapagei strain DSM 4587 16S ribosomal RNA, partial sequence	1821	1821	99%	0.0	97.27%	NR_126317.1

All the 20 subject sequences were downloaded in FASTA format and then the top 8 sequences of different species of *Serratia* along with two other sequences of different genus same were aligned with the query sequence to construct the phylogenetic tree with ClustalW using the maximum likelihood method with 500 bootstrap replication (MEGA 6).

3.7.3 Phylogenetic inference

Phylogenetic tree was constructed subjecting 10 nucleotide sequences (one of which was the query sequence) to maximum likelihood method based on the Tamura-Nei model (Tamura & Nei, 1993) with 500 bootstrap replicates (Felsenstein, 1985) using MEGA 6 (Tamura *et al.*,2013).The evolutionary tree demonstrated that all the sequences belonging to the same family or order formed cluster. Therefore, the sequences were merged and represented by the same leaf (terminal OTU) for better resolution of the phylogeny. All the *Serratia* sequences formed one clad (bootstrap value 100). Higher bootstrap value of the query sequence with the sequence of *Serratia marcescens* subsp. *sakuensis* strain indicated higher consistency of the given data for taxonomical bipartioning. While the bootstrap values did not indicate how accurate the tree is but it indicated the stability of the branching pattern. The higher bootstrap value of the braches of *Serratia marcescens* subsp. *sakuensis* with the query sequence clearly signified the stability of the branching pattern. Thus, it was identified to be the *Serratia marcescens*. Also, the distant branches of the two sequences belonging to the two different genuses clearly signify having a distant relationship with the query sequence (Singh *et al*., 2015).

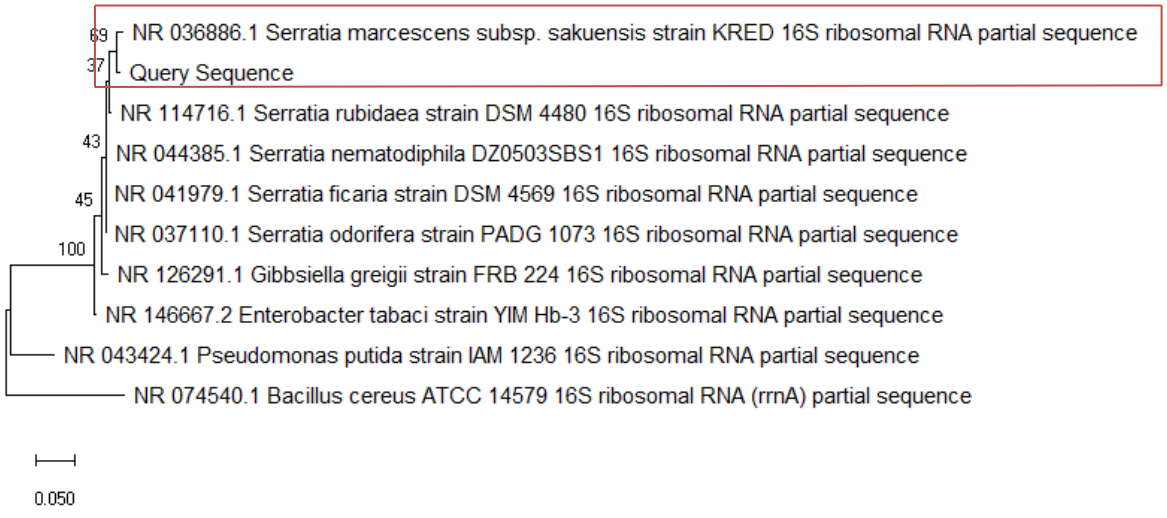


Figure 3.7.3.1: Phylogenetic analysis by Maximum Likelihood method illustrating the query sequence to be identified *S. marcescens* as with a bootstrap value of 69.

Chapter 4

Discussion

4.Discussion

The purpose of the study was aimed at isolation and identification of such a novel bacteria that possesses antimicrobial activity which can be developed and used further, to overcome the recent emergence of antibiotic resistant bacterial strains.

To begin with the study, through primary and secondary screening 10 different colonies was isolated for potential antimicrobial production test. In the primary screening, 1 gram of raw soil sample was diluted in 9 ml normal saline solution to get one fold of dilution. From the solution, 100µl was then inoculated into LB broth medium. LB broth medium is an enriched medium for cultivation of a wide variety of soil organisms. It supports growth of all the soil organisms including fastidious ones and so the chances of getting potent bacteria get higher. It was then incubated for 24-48 hours at 37°C and 120 rpm (in a shaker incubator). After incubation, the LB broth was serially diluted in normal saline and spread plated onto different selective media plates. In this secondary screening process, the selective media that were used were; *Bacillus cereus* agar (selective for *B. cereus* and other *Bacillus sp*.), Cetrimide agar (selective for *Pseudomonas* sp.), MacConkey agar (differential for Lactose-fermenting bacteria) and Mannitol Salt agar. The individual colonies taken from each selective media were then picked and grown on nutrient agar plate for pure culture obtaining.

After that, antibiotic susceptibility test was done for the picked colony isolates against selected test organisms. In the first stage, cross streak method was done where the test organism was streaked vertically and the isolate organisms were streaked horizontally. The streaks were done in such a way so that the test organism line would touch each of the isolate line and thus if there was any growth inhibition it would be clear to observe. The test was done in Mueller-Hinton agar plate as it is established and widely used for routine susceptibility test. In each plate, 1 test organism was streaked against 10 isolates. After incubation for 48 hours at 30°C, the plates were observed. In observation, it was seen that the isolate 5 & 9 (two of the isolates were later identified to be the same organism and so isolate 9 was neglected) was able to give a clear zone around the test organism streak line. Isolate 5 was observed for growth inhibition against *B. licheniformis* and *B. subtilis*. It was also observed that the isolate 5 would appear dark red color on MHA plates. Very few bacteria would give red pigment on MHA plate and so it was recorded for further characterization.

In the second stage of antibiotic susceptibility test, agar well diffusion method (Kriby-Bauer method) was done for further confirmation and to have idea about measurements of the zone of inhibition. On the MHA plates, lawns of test organisms were done by standardizing with 0.5 McFarland standard solutions (0.5 CFU/ml). Several suspensions of potential bacterial isolate 5 were prepared in different formulation to observe better results. The different prepared suspensions were: isolate 5 (grown on nutrient agar) in normal saline solution, isolate 5 (grown on Cetrimide agar) in normal saline, raw soil sample inoculated on LB broth, isolate 5 inoculated on LB broth, supernatant collected from LB broth inoculation of Isolate 5. The results were observed after 24 hours of incubation at 30°C. For the above mentioned order of suspension samples, the diameter zone of inhibition for *B. licheniformis* was 13 mm, 13 mm, 11 mm, 8 mm and 10 mm respectively. For *B. subtilis* the diameter of zone of inhibition was 17 mm, 18mm, 0, 13mm and 11mm respectively. It was observed that the isolate on normal saline suspensions would give better zone of inhibition. In this way antibiotic production of the sample isolate was confirmed.

After confirmation of antibiotic production, morphological and microscopic observation was carried out. A freshly grown culture plate was taken for morphological characterization and slide preparation. The isolated colonies were examined carefully and it was recorded that the colonies were in circular form, entire margin, convex elevation and smooth, mucoid texture. The colonies were pigmented as reddish orange. It was also examined that the pigment production in the plates that were kept on relatively low temperature, 30°C were more intensified than the plates kept at 37°C. The antibiotic production was also associated with the pigment production as the antibiotic susceptibility test gave better results in the plates kept at 30°C. It was assumed that the isolate 5 had an optimal temperature of 28-30°C.

For smear preparation, one drop of normal saline was taken onto a clean glass slide. Then taking a small amount of organism from a fresh culture plate with an inoculating loop, a thin layer on smear was prepared by mixing the organism with the saline in circular motion. After the smear was done, it was then left to dry a bit and then heat fixed to avoid the smear getting washed out while gram-staining. Then, to perform gram-staining, few drops of Crystal Violet were taken onto the smear and let it stand for 1 minute. Then, the smear was gently washed with tap water and then flooded again with the Gram's Iodine mordant and let stand for 1 minute followed by gentle wash with tap water. After that, the smear was decolorized with 95% Ethyl Alcohol and gently washed with tap water. Finally, it was counterstained with Safranin for 45 seconds and gently washed with tap water. The slide was then blot dried with bibulous paper and covered with cover slip. The slide was

then observed in oil immersion lens and it was examined that the bacteria was gram-negative, rod shaped.

After the microscopic observation was done, a series of biochemical tests were designed to identify the potential gram-negative bacteria. The biochemical tests that were performed, among those glucose utilization, sucrose utilization, mannitol utilization, nitrate reduction, citrate utilization, catalase test, gelatin hydrolysis showed positive results whereas lactose utilization, H2S production, indole test, methyl red test, motility test, urease test, oxidase test, starch hydrolysis, 6.5% NaCl tolerance test showed negative results. All the biochemical tests were triplicated to avoid any false positive or false negative results. Since, it was hard to fully determine the species only on the basis of performances of biochemical tests given by isolate 5 as it is also considered as presumptive identification, the bacterial genus were tentatively identified to be *Serratia.*

Having an initial idea about the genus, it was essential to identify the species of the isolated potential bacteria. So, a molecular analysis had to be done for further confirmation. To start with the genotypic characterization, a polymerase chain reaction (PCR) was done to amplify the 16S rRNA gene with the help of universal primer pair 27F and 1492R. The reason for using the 16S rRNA sequencing method here was because it is usually present in almost all bacteria, the function of the 16S rRNA gene over time has not changed, suggesting that random sequence changes are a more accurate measure of time (evolution) and the 16S rRNA gene (1,500 bp) is large enough for informatics purposes for species identification. After the PCR reaction was done, a gel electrophoresis was run to confirm the PCR reaction by examining the visible band on the gel. It was observed that the band size was ~1200bp. The PCR product was then purified using kit Wizard PCR Clean-Up System and preserved at -20°C prior sending for sequencing.

Afterwards, the PCR product of the isolate 5 was sent to 1st BASE Laboratories in Malaysia for sequencing. The Sanger sequencing method was followed using ABI Genetic analyzer. A continuous stretch of 1059 nucleotide long sequence of 16S rRNA gene was used to search for similar sequences using online search tool called BLAST (http://www.ncbi.nlm.nih.gov/blast/). The isolate number 5 had closest sequence identity mostly with *Serratia marcescens* subsp. *sakuensis* having 99.91 % identity along with few other species belonging from the same genus in the top 20 list given by NCBI database.

The sample was collected from a hospital area where a huge variety of opportunistic pathogens are common to be found. These opportunistic bacteria are responsible for Hospital-Acquired Infection (HAIs) or nosocomial infection in immune-compromised patients. *Serratia marcescens* is also an opportunistic bacterium which can be found naturally in soil and water and produces a red pigment at room temperature. It is associated with urinary and respiratory infections, endocarditis, osteomyelitis, septicemia, wound infections, eye infections, and meningitis. Transmission is by direct contact. Droplets of *S. marcescens* have been found growing on catheters, and in supposedly sterile solutions (Buckle, 2015).

Serratia marcescens is a member of the genus *Serratia*, which is a part of the family *Enterobacteriaceae*. As members of the *Enterobacteriaceae* family, *Serratia* spp are motile, non-endospore forming Gram-negative rods (Ciragil *et al.,* 2006). In the laboratory *Serratia* are routinely isolated from bloodstream and wound sites using blood agar culture or from respiratory and urinary sites using selective culture methods. Common selective agar cultures include MacConkey agar which categorizes *Serratia* isolates with the other non-lactose fermenting *Enterobacteriaceae* or chromogenic agars, which classifies them into a broad *Klebsiella*, *Enterobacter*, *Serratia* and *Citrobacter* (KESC) grouping (Carricajo *et al.*, 1999).

Serratia marcescens has been reported to produce antibiotic activity against several pathogenic bacteria. It was researched that "Prodigiosin" – the pigment that S. marcescens produce is an antibacterial agent. Prodigiosin is reported to be an intracellular compound which exhibited a broad inhibitory spectrum activity against 13 out of 18 test bacteria (Ibrahim et al., 2014). In another study, it had been experimented that prodigiosin could be a potential antibacterial agent against bacteria such as *Staphylococcus* and may exhibit blocking of DNA gyrase pockets. Pharmcophore modelling and docking analysis of Prodigiosin from Serratia marcescens have illustrated the potential of the molecule to inhibit various pathogens like HBV, HIV, Plasmodium vivax and H1N1 through protein-ligand binding (Suba et al., 2013). *Serratia sp.* can produce two different group of secondary metabolites; prodiginines and serratamolides. Prodiginines are tripyrrole red pigments with different alkyl substituents with prodigiosin which has been examined to retain anticancer, antifungal and antibacterial activity (Williamson, 2006). Moreover, serratamolides are

cyclodepsipeptides and biosurfactants with hemolytic, antibiotic and anticancer activities with serrawettin W1 as the most investigated metabolite.

In this study, it was found that *S. marcescens* showed antimicrobial activity against *B. licheniformis* and *B. subtilis*. These bacteria are mainly responsible for food spoilage, intoxication, food poisonsing, nausea, vomiting, and abdominal cramps. As *S. marcescens* showed bactericidal and bacteriostatic effect, it can be used in food industry, agriculture and in veterinary if promising level of antimicrobial activity can be achieved. Further study and development of the *S. marcescens and "Prodigiosin"* is necessary as well as further characterization to determine its potentiality to be used as a new antibiotic for commercial production.

Chapter 5

References

References

1. Aryal, S. (2015). Blood agar composition-preparation-uses and pictures. Retrieved from Microbiology Info: http://www.microbiologyinfo.com/blood-agar-compositionpreparation-uses-and- pictures/

2. Atlas, R. M and Bartha, R. (1998). Microbial Ecology- Fundamentals and Applications. 4th edition. Benjamin/Cummings Science Publishing company.

3. Brun, Y.V., Skimkets, L.J. (2000). Prokaryotic development. ASM Press, Pp. 11-31.

4. Bérdy, J. (2005). Bioactive Microbial Metabolites. Journal of Antibiotics, 58(1), 1–26.

5. Bauer, A. W., Kirby, M. M., Sherris, J.C. and Truck M. (1966). Antibiotic susceptibility testing by a standardized single disk method. Am J Clin Pathol., 45: 493-6.

6. Carricajo, A., Boiste S., Thore, J., Aubert, G., Gille. Y., Freydière, AM. (1990) Comparative evaluation of five chromogenic media for detection, enumeration and identification of urinary tract pathogens. Eur J Clin Microbiol Infect Dis 1999; 18: 796-803.

7. Ciragil, P., Gul, M., Aral, M., Ekerbicer, H. (2006). Evaluation of a new chromogenic medium for isolation and identification of common urinary tract pathogens. Eur J Clin Microbiol Infect Dis 2006; 25: 108-111.

8. Chandra, N., Kumar, S. (2017). Antibiotics Producing Soil Microorganisms. In: Hashmi M., Strezov V., Varma A. (eds) Antibiotics and Antibiotics Resistance Genes in Soils. Soil Biology, vol 51. Springer, Cham.

9. Cruz, E. D., & Torres, J. M. (2012). Gelatin hydrolysis test. Retrieved from Microbe Library: http://www.microbelibrary.org/library/laboratory-test/3690-gelatin-hydrolysistest.

10. Cappuccino, J. G. and Sherman, N (2005). Microbiology A Laboratory Manual seventh edition.

11. Ding, M., Williams, R. P. (1983). Biosynthesis of prodigiosin by white strains of *Serratia marcescens* isolated from patients. *J Clin Microbiol* **17**, 476–480 (1983).

12. Eckelmann, D., Spiteller, M., Kusari, S. (2018). Spatial-temporal profiling of prodiginines and serratamolides produced by endophytic *Serratia marcescens* harbored in *Maytenus serrate*. *Scientific Reports*volume 8, Article number: 5283 (2018).

13. Fleming, A. (1929). On the antibacterial action of cultures of a *Penicillium* with special reference to their use in the isolation of *B. influenzae*. British Journal of Experimental Pathology, 10, 226–236.

14. Fürstner, A. (2003). Chemistry and biology of roseophilin and the prodigiosin alkaloids: a survey of the last 2500 years. Angew Chem Int Ed 42, 3582–3603.

15. Falagas, M. E., Grammatikos, A. P., Michalopoulos, A. (2008). Potential of old-generation antibiotics to address current need for new antibiotics, Expert Review of Anti-infective Therapy, 6:5, 593-600, DOI: 10.1586/14787210.6.5.593.

16. Ibrahim, D., Nazari, T.F., Kassim, J., Lim, S. H. (2014). Prodigiosin-an antibacterial red pigment produced by Serratia marcescens IBRL USM 84 associated with a marine sponge Xestospongia testudinaria. J App Pharm Sci, 2014; 4 (10): 001- 006.

17. Kumar, S., Stecher, G., Li, M., Knyaz, C., and Tamura, K. (2018). MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. *Molecular Biology and Evolution* 35:1547-1549.

18. Lihan, S., Lin, C. S., Ahmad, I., Sinang, F. M., Hua, N. K., Sallehin, A. A. (2014) Antimicrobial producing microbes isolated from soil samples collected from Nanga Merit Forest in Sarawak, Malaysian Borneo. Euro. J. Exp. Bio., 2014, 4(1):494-501.

19. Mohr, Kl. (2016) History of Antibiotics Research. Curr Top Microbiol Immunol; 398:237-272 doi: 10.1007/82_2016_499.

20. Moore, D. (2015). Antibiotic Classification and Mechanism. http://www.orthobullets.com/basic-science/9059/antibioticclassification-and-mechanism.

21. MacWilliams, M. P. (2009). Indole Test Protocol. Retrieved from Microbe Library: 10. http://www.microbelibrary.org/component/resource/laboratory-test/3202-indole-testprotocol.

22. McDevitt, S. (2009). Methyl red and Voges Proskauer test Protocol. Retrieved from Microbe Library: http://www.microbelibrary.org/component/resource/laboratorytest/3204-methyl-red-and-voges-proskauer-test-protocols.

23. Nahler, G. (2009). Polymerase chain reaction (PCR). Dictionary of Pharmaceutical Medicine, 141-142. doi: 10.1007/978-3-211-89836-9_1075.

24. Reiner, K. (2010). Catalase test protocol. Retrieved from Microbe library: 10http://www.microbelibrary.org/library/laboratory+test/3226-catalase-test-protocol.

25. Sandhya, M. V. S. (2014). ISOLATION OF ANTIBIOTIC PRODUCING BACTERIA FROM SOIL Page No 46 to 51, Vol-6, Issue-1, Jan-Mar-2015, India. International Journal of Applied Biology and Pharmaceutical Technology. 6. 46 to 51.

26. Sethi, S., Kumar, R. and Gupta, S. (2013). Antibiotic production by Microbes isolated from Soil. *Int J Pharm Sci Res* 2013: 4(8); 2967-2973. doi: 10.13040/IJPSR. 0975-8232.4(8).2967-73.

27. Singh, A. P. (2012). Studies on Isolation and Characterization of Antibiotic Producing Microoragnisms from Industrial Waste Soil Sample. The Open Nutraceutucals Journal, 5(1), 169-173. Doi: 10.2174/1876396001205010169.

28. Silver, L.L. (2012). Rational Approaches to Antibacterial Discovery: Pre-Genomic Directed and Phenotypic Screening. In: Dougherty T., Pucci M. (eds) Antibiotic Discovery and Development. Springer, Boston, MA. Available from: doi.org/10.1007/978-1-4614-1400-1_2.

29. Sauera, P., Gallob, J., Kesselováa, M., Koláře, M., & Koukalováa, D. (2005). UNIVERSAL PRIMERS FOR DETECTION OF COMMON BACTERIAL PATHOGENS CAUSING PROSTHETIC JOINT INFECTION. Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub., 149 (2), 285–8.

30. Suba, K., Stalin, A., Girija, A., Raguraman, R. (2013). Homology modeling and docking analysis of Prodigiosin from Serratia marcescens. Biotechnology, 2013; 55:12897–12902.

31. Tiwari, K. & Gupta, R. K. (2013). Diversity and isolation of rare actinomycetes: an overview. Critical Reviews in Microbiology, 39(3), 256–294.

32. Thirumurugan, D., Cholarjan, A., Raja, S. S. S., Vijaykumar, R. (2018). An Introductory Chapter: Secondary Metabolites. Reviewed: June 26th 2018. Doi: 10.5772/intechopen.79766.

33. Tsang, A. Y., & Shields, P. (2013). Mannitol Salt Agar Plate protocol. Retrieved from Microbe Library: http://www.microbelibrary.org/component/resource/laboratorytest 3034-mannitol-salt-agar-plates-protocols.

34. Tamura, K. and Nei, M. (1993). Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Molecular Biology and Evolution* 10:512-526.

35. Vining, L.C. 1990. Functions of Secondary Metabolites. Annual Review of Microbiology. 44: 395–427.

36. Williamson, N. R., Fineran, P. C., Leeper, F. J. & Salmond, G. P (2006). The biosynthesis and regulation of bacterial prodiginines. Nat Rev Microbiol 12, 887–899.

Appendix

List of Abbreviations

LB	Luria Bertani
NA	Nutrient Agar
MHA	Muller-Hinton Agra
MSA	Mannitol Salt Agar
TSI	Triple Sugar Iron
MIU	Motility Indole Urease
NS	Normal Saline
rRNA	Ribosomal RNA
DNTP	Deoxyribonucleotide Triphosphate
PCR	Polymerase Chain Reaction
EDTA	Ethylene diamine tetraacetic acid
TAE	Tris-acetate-EDTA
CFU	Colony Forming Unit
HAI	Hospital Acquired Infection
rpm	Revolutions per minute
NCBI	National Center for Biotechnology Information
BLAST	Basic Local Alignment Searching Tool
MEGA	Molecular Evolutionary Genetics Analysis
bp	Base pair
kb	Kilo base pair