UNIVERSITI PUTRA MALAYSIA

ISOLATION AND IDENTIFICATION OF BACTERIA FROM ACID SOIL (BEKENU SERIES) IN UNIVERSITI PUTRA MALAYSIA BINTULU SARAWAK CAMPUS

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By

NEELAVENI BALA MOHAN

A Project Report Submitted in Partial Fulfillment of the Requirement for the Degree of Bachelor of Science Bioindustry in the Faculty of Agriculture and Food Sciences Universiti Putra Malaysia Bintulu Sarawak Campus

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I am with thee, and no man shall set on thee to hurt thee

Acts 18:10

This book is specially dedicated to my parents,
Mr.Bala Mohan and Mrs.Roselene Alfred William

My siblings,
Mohanawani, Jon William, Arwin Daniel and Eunice william

And for the one, who is closely drawn to my heart,
Mr.Robert Devid

With all the Love and Courage
that I had from you I stand still,
I love you all.

ABSTRACT

The objective of the present study was to isolate and identify bacteria from acid soil, Bekenu series using morphological, biochemical and molecular techniques. Soil samples were collected from regions of Universiti Putra Malaysia Bintulu Sarawak Campus (UPMKB) in a simple random technique. A total of 11 soil bacteria samples were isolated and 7 of them could be molecularly identified. The rest 4 couldn't be identified due to time constrain. Morphological shapes of colonies were initially used to separate the grown bacteria of the samples. These bacterial isolates were Gram negative coccoid rod, Gram negative rods and Gram positive rods. Twelve biochemical tests were performed to study their biochemical characteristic. The test conducted are skim milk agar test, cellulose test, sprit blue agar test, starch agar test, Eosin Methylene Blue (EMB) agar test, Violet red bile agar test, MacCONKEY agar test, gelatin liquefaction test, Klinger Iron agar test, Catalase test and Lactose peptone broth test. Molecular technique comprises of genomic DNA extraction, polymerase chain reaction (PCR) amplification of 16S rRNA, DNA purification, gel electrophoresis and DNA sequencing were performed. DNA sequencing results were analyzed using online database Basic Local Alignment Search Tool (BLAST). Based on the 16S rRNA gene sequencing bacteria identified belongs to two major groups, Bacilli and β-proteobacteria. They were identified as *Chromobacterium violaceum*, *Cupriavidus basilensis*, *Bacillus subtilis*, *Bacillus cereus*, *Bacillus pycnus* and *Bacillus thuringiensis*. These studies demonstrate the great diversity of bacteria in acid soil

ABSTRAK

Objektif kajian ini adalah untuk memencil dan mengenalpasti bacteria daripada tanah berasid, siri bekenu menggunakan teknik morfologi, biokimia dan molekul. Sampel tanah diambil daripada kawasan Universiti Putra Malaysia Bintulu Sarawak Kampus (UPMKB) secara rawak. Sebanyak 11 bakteria berjaya dipencilkan dan 7 bakteria berjaya dikenalpasti. Selebihnya tidak dapat dikenalpasti kerana faktor masa. Mulanya, morfologi dan bentuk koloni digunakan untuk proses pemencilan bacteria yang telah tumbuh. Bakteria tersebut adalah Gram negatif coccoid rod, Gram negatif rod dan Gram positif rod. Dua belas ujian biokimia dijalankan untuk memahami ciri-ciri biokimia bakteria tersebut. Ujian- ujian biokimia tersebut adalah ujian 'skim milk', cellulose, 'Spirit Blue agar', kanji, Eosin Methylene Biru (EMB) agar, Violet red bile agar, MacCONKEY, gelatin, 'Klinger iron agar', Catalase dan 'Lactose peptone broth'. Kaedah molekul merangkumi amplifikasi 16S rRNA menggunakan reaksi berantai polimerase (PCR), purifikasi DNA, gel elektroporesis, dan penyusunan DNA. Penyusunan DNA dianalisa mengunakan Basic Local Alignment Search Tool (BLAST). Berdasarkan penyusunan 16S rRNA gen, bakteria yang telah dikenalpasti tergolong dalam kumpulan Bacilli dan β-proteobakteria. Bakteria tersebut ialah as *Chromobacterium violaceum*, *Cupriavidus basilensis*, *Bacillus subtilis*, *Bacillus cereus*, *Bacillus pycnus* and *Bacillus thuringiensis.* Kajian ini mendemonstrasikan kepelbagaian diversiti bakteria dalam tanah berasid.

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I praise the Lord, for his mercy and his blessing that I completed the task assigned to me. I thank him for his wisdom and strength that he had provided during the time I really needed it.

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APPROVAL

I certify that this research project report entitled "Isolation and Identification of Bacteria from Acid Soil (Bekenu Series) in Universiti Putra Malaysia Bintulu Sarawak Campus" has been examined and approved as a partial fulfillment of the requirement for the degree of Bachelor of Science Bioindustry in the Faculty of Agriculture and Food Sciences, Universiti Putra Malaysia Bintulu Sarawak Campus.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

µL	: microliter
%	: percentage
bp	: base pair
BLAST	: Basic Local Alignment Search Tool
DNA	: Deoxyribonucleic acid
g	: gravity
h	: hour
min	: minute
mL	: milliliter
mM	: mili molar
NA	: nutrient agar
NB	: nutrient broth
PCR	: polymerase chain reaction
RNA	: ribonucleic acid
rRNA	: ribosomal RNA
rpm	: Round per minute
V	: Volt

CHAPTER 1

INTRODUCTION

Soil is the naturally occurring, unconsolidated mineral and organic matter at the earth's surface that provides an environment for living organism (Voroney, 2007). Soil represents one of the most diverse and heterogeneous habitats for bacteria and archaea on the planet. Soil thus harbors a prokaryotic community with a much higher diversity than that present in the majority of the natural habitats (Gans *et al.*, 2005). Under favorable conditions of moisture, temperature, and nutrients, bacterial numbers range from 10^8 to 10^{10} per gram of soil (Paul and Clark, 1996).

Soils contain a great diversity of bacteria, with many of the organisms belonging to groups for which no cultivated representatives are known (Janssen *et al.*, 2002). Soil microbial diversity has developed in recent years. The morphological, physiological and ecological characteristics of microorganism are controlled by the genetic constitution of the organisms interacting with the environment they are part of (Torsvik and Ovreas, 2007).

In this present study, sample is taken from soil of the Bekenu series. Bekenu series is a member of Bekenu family which is fine loamy, siliceous, isohypthermic, red yellow to yellow Tipik Tualemkuts. It is characterized by being deep, well drained profile with brownish yellow to yellow subsoil color (Paramanathan, 2000). To date, soil of the Bekenu series have only been mapped in Sarawak.

Phenotypic characteristics of bacteria such as cell morphology, movement, flagellation, gram staining, cell wall composition and sporulation, are so few and indiscriminative. The use of physiological and biochemical characteristics to create phylogenetic classification is problematic, due to the speculation that any of these characteristics are more conversed than others. Recent advance in molecular biology provides clear foundation in comparing prokaryotic organism to each other on the basis of highly conserved genes and proteins (Torsvik and Ovreas, 2007).

This primary study of microbes in soil is very important as it is essential to understand agricultural and environmental science. The number, diversity and distribution of microorganism reflect overall soil productivity (Coyne, 1999). This project is significant as without a continuous research on microorganism in soil there will be no way to understand the richness of life that has evolved. It is hoped that this studies will be a stepping stone for future research

1.1 Objectives

The objectives of this research are listed as below:

1. To isolate soil bacteria
2. To study the morphological and biochemical properties of soil bacteria
3. To identify the soil inhabiting microbes using molecular method

CHAPTER 2

LITERATURE REVIEW

2.1 Bacterial Population in Soil

Soil provide a tremendous range of habitats, they support an enormous biomass, with an estimated 2.6 x 10^{29} prokaryotic cells alone, and harbor much of the earth's genetic diversity. A single gram of soil contains more than 10^{9} bacterial cells, organisms belonging to tens of thousands of different species (Voroney, 2007).

Thousands of different bacterial species can coexist within $1m^{3}$ of soil. Bacteria cultured from soils are frequently aerobic heterotrophs belonging to two major groups, Gram positive bacteria and Proteobacteria (Lal, 2006). *Anthrobacter*, *Bacillus*, and *Pseudomonas* are three of the most commonly isolated soil bacteria. *Anthrobacter* is classified among the actinomycetes. It is pleomorphic, slow growing, slightly motile, and grow on diverse substrates, which is one reason why it is frequently isolated from soil. *Bacillus* survives a pH range of 2 to 8 and a temperature range from -5°C to 75°C. *Pseudomonas* species represent 3% to 15% of soil isolates (Coyne, 1999).

2.2 Bekenu Series

The presence and abundance of different bacterial groups and their level of activity depends on soil properties, land use history and environmental condition such as temperature, moisture and nutrients (Lal, 2006).

Bekenu series is the common types of soil found in local Bintulu, Sarawak (Kevin *et al.*, 2007). It typifies the family and is developed over mixed sedimentary rocks. They have weak medium to coarse subangular blocky and consistence is friable. Texture in this soil is uniformly fine sandy clay loam. Typically they have pH-H_20 4.6 and pH-KCl 3.8. Organic carbon percentage in the soil is 2.51% and C: N ratio of 15 (Paramanathan, 2000).

2.3 Isolation Technique of Soil Bacteria

In nature most microbes are not found growing in isolation but in an environment that contains many different organisms. A pure culture that contains a single kind of microbes is required for studies. Microbes are too small to separate directly without sophisticated micromanipulation equipment; indirect method of separation must be used (Kirk *et al.*, 2004).

Serial dilutions are a series of dilutions. A series is used because it allows analyzing samples at different concentration. The use of serial dilution liquid culture techniques has proved to be effective for isolating numerically significant members of the community in soil. Another techniques used to isolate the bacteria is spread plate technique where previously diluted mixture of microorganism be used. During inoculation, the bacterial cells are spread over the surface of a solid agar medium with a sterile L-shaped bent rod (Cappuccino and Sherman, 1998).

Mechanical dilution which is streak plate method is another technique of isolation, the purpose of streaking a culture on a plate is to dilute the culture enough to get isolated colonies. These isolated colonies are needed to define different colonial morphologies and detect different biochemical characteristics.

In addition, streak plating is performed to purify bacterial culture before further analysis. This is due to, bacteria often exist as clumps or chains, stringent colony purification involves streaking isolated colonies at least twice first to isolate a colony then a second time to assure that the clump or chain that started the initial colony is homogenous (McLandsborough, 2005).

Advantage of the serial dilution agar plate techniques is, it allows only viable cells to be counted and it allows isolation of discrete colonies that can be subcultured into pure cultures which may then be easily studied and identified (Cappuccino and Sherman, 2001)

2.4 Identification of Soil Bacteria

2.4.1 Conventional Method

Phenotypic characterization has long been used for identification purposes these tests emphasize on growth habit and characteristic such as cell shape, cell size, and colony morphology (Klaenhammer and Kullen, 1999).

However, phenotypic tests are characterized by potential inherit problem such as not all strains within a given species may exhibit a common characteristic, the same strain may give different results upon repeated testing, the corresponding database does not enclose newly or not yet described species and finally the test result relies on individual interpretation and expertise (Bosshard *et al.*, 2004).

Moreover, small alterations in the execution of an assay may give false test results. Consequently, identification based on phenotypic tests does not always allow an unequivocal identification (Poyart *et al.*, 1998). When observed under the microscope, most soil bacteria appear round or rod-shaped, but physical similarity masks the vast diversity in their genetic composition and biochemical capabilities (Lal, 2006). Phenotypic properties can be unstable at times and expression can be dependent upon changes in environmental conditions such as growth substrate, temperature, and pH levels (Janda and Abbott, 2002)

Gram staining is a differential double-staining method which forms the basis of most examination and the preliminary identification of bacteria (Harrigan, 1998). Gram staining divides bacterial cells into two major groups, Gram positive (violet stained) and Gram negative (red stained). These Gram stain reaction is based on the difference in the chemical composition of bacterial cell walls (Cappuccino and Sherman, 2001). Gram positive bacteria will, due to as a result of their thicker peptidoglycan cell wall and absence of an outer cell membrane remain crystal violet and therefore not be counterstained with red safranin-O dye (Elsas *et al.*, 2007).

But, Gram staining is not always demonstrative of the true Gram nature of certain bacteria which can lead to misidentification (Bahrani-Mougeot *et al.*, 2008). This distinction is not perfect and can lead to misidentification because some bacteria such as the *Cyanobacteria* with a typical Gram-negative cell wall may stain Gram positive. While others, such as Gram positive *Arthrobacter* types are gram variable, staining red during growth and violet during stationery phase. In addition, some bacteria have no cell wall and in contrast to bacteria archea lack of peptidoglycan and their cell wall composition varies depending on the cell type (Elsas *et al.*, 2007).

All microorganisms have their own identifying biochemical characteristics. Their biochemical properties are controlled by cell's enzymatic activity, biochemical transformation that occur both outside and inside the cell are governed by biological catalysts called enzymes (Cappuccino and Sherman, 2001). Microorganism can be classified and distinguished by the ability to grow in different substrate and/ or production of different end products, produce different enzymes, use oxygen, or be motile (Harrigan, 1998).

2.4.2 Molecular Method

Molecular techniques offer new opportunities for determining and analyzing the structure and species composition of microbiological communities (Garbers *et al.*, 2004). In recent years, the availability of molecular tools to properly identify strains has drastically reduced confusion over strain identity. The genotypic identification procedures have increasingly received attention as an alternative or complement to conventional phenotypic methods (Bosshard *et al.*, 2003).

Ribosomal RNA (rRNA) is a good marker for phylogenetic studies involving microorganism. Recent advances in rRNA-based molecular techniques make it possible to identify different bacterial populations in soil (Selim, 2006). This is because rRNA which is responsible for protein synthesis is found in all organisms on Earth, microorganism as well as plants and animal.

The ribosome is also highly conserved because large changes in sequence effect the normal functioning of the critical and complex process of protein synthesis. By far the more common targets for characterizing microbial community are the rRNA genes because of their importance in establishing phylogenetic and taxanomic relationship (Woese *et al.*, 1990). Besides the 16S rRNA are sufficiently long that they contain a large amount of evolutionary information (Staley, 2002)

Small-subunit 16S rRNA gene sequencing is a widely accepted tool for used extensively for bacterial phylogeny for the identification of uncultivated bacterial pathogens and for the identification of cultural isolates (Bosshard *et al.*, 2003). The introduction of marker genes 16S rRNA with an easy detectable phenotype is very useful for it allows detection of specific bacteria strains in complex environments (Drancourt *et al.*, 2000).

At present, 52 phyla in the domain Bacteria are recognized, on the basis of their 16S rRNA gene sequence similarity (Elsas *et al.*, 2007). The 52 phyla are based on data obtained with isolates and on those obtained with direct molecular evidence. There are tens of thousands of 16S rRNA sequences derived from environmental samples, and new sequences are added to the database every day (Torsvik and Ovreas, 2007).

The molecular test is hoped to be more reliable in this project because small sub-unit 16S rRNA have the broadest specificity ranging from universal to species specificity. Regions of 16S rRNA show highly variable sequences that are specific for a species or genus (Bosshard, 2004). 16S rDNA sequencing has been Sequence determination and comparative database searches allow the unknown isolate to be assigned to a group of bacteria (Kolbert and Persing, 1999).

Polymerase Chain Reaction (PCR) is the widely used DNA-based method that is used to amplify 16S rRNA (Garbers *et al.,* 2004). The amplifying process occurs by using a pair of specific primers with about 20 base pair long. PCR steps include denaturation of double stranded DNA, primer anneling, elongation of strands with heat-stable *Taq* polymerase and amplication. After amplication for 30-35 cycles, a single gene can be amplified to millions of copies which are then separated in agarose gel for visual identification (Powell *et al.*, 2007).

CHAPTER 3

MATERIALS AND METHODS

3.1 Sampling

The experiment was conducted at the Universiti Putra Malaysia Bintulu Sarawak Campus. The type of soil used in these studies is Bekenu series. Sampling was done by using simple random sampling strategies. Soil sample were taken at the depth of 0-15 cm using soil auger.

3.2 Isolation of Soil Bacteria

Soil dispersion begins with the mixing of soil samples with distilled water. Suspension was made by adding 5 g of soil to 50 mL sterile distilled water (Kamil *et al.*, 2007). Then the solution were shaken at room temperature in a shaker for half and hour to disperse the bacteria from soil.

Suspension was filtered to separate the suspension from soil debris. The clean suspension then used in serial dilution, to obtain concentration of viable microbes from soil. Ten-fold serial dilution from 10^{-1} to 10^{-8} were done, diluted suspension placed on nutrient agar and gently rotated in a circular motion to achieve uniform distribution of microorganism. Finally, the plates were incubated at room temperature and allow bacteria to grow (Cappuccino and Sherman, 2001).

To get an isolated single colony, culture were streaked on a nutrient agar plate and incubated at room temperature for 24 h. Those single colony formed were sub-cultured into a slant agar for storage.

3.3 Isolate Identification

3.3.1 Morphology Test

Gram staining tests was performed on each purified isolates to compare the colony characteristic and those colonies showing different cell morphology or characteristic were identified (Witthuhn *et al.*, 2005).

The Gram staining procedure is as follows, a thin film was made using single fresh colony (within 24 h) obtained from the streak plate. Few drops of sterile distill water was used to smear the single colony on the slide. Slide was passed through flame several times to fix the cell.

Slide were covered with crystal violet solution and left for 1 min, rinsed with running tap water. Then covered with iodine solution and left on for 1 min. The iodine solution were drained and rinsed with tap water.

Next, 95% alcohol added drop by drops for one to five seconds to decolorize, immediately film were rinsed with water and finally counterstained with Safranin O for 45 seconds. Slide was rinsed with tap water and air dried with paper towel. Stained film was observed under oil immersion with 1000X magnification power and cellular morphology recorded to enable identification (McLandsborough, 2005).

3.3.2 Biochemical Tests

Biochemical tests were performed through several extra cellular enzyme tests, such as lactose, proteases, lipase and cellulose. Reaction and changes on medium determines the enzyme production of bacteria (Cappuccino and Sherman, 2005). Many microbes are being distinguished on the basis of differences in their ability to utilize or produce certain chemicals (Bauman, 2007).

3.3.2.1 Gelatin Liquefaction Test

Nutrient gelatin was prepared 200 mL to minimize wastage and contamination. To make 200 mL, 24 g of gelatin powder and 8 g of nutrient broth were suspended into 200 mL of sterilized distilled water in 400 mL Schott Duran bottle.

The mixture then warmed to 50 °C to completely dissolve the powder. Mixture was agitated frequently as gelatin hard to get dissolved completely. The mixtures were dispensed into tubes with each screw tubes were filled with approximately 7 mL mixture and sterilized at 121 °C for 15 min.

Inoculates with 18 to 24 h of growth were picked with a sterilized inoculating loop and were stabbed at about half an inch from the bottom of the screw tubes into nutrient gelatin. The tubes were incubated at room temperature.

At various intervals during the tubes were transferred to refrigerator for 30 min to examine liquefaction. Uninoculated nutrient gelatin tubes were used as control for comparison. The tubes must not be shaken during the transfer from room to refrigerator. The chilled tubes were inverted when reading the result to test for solidification or liquefaction. Observation was made up to 14 days.

3.3.2.2 Casein Hydrolyze Test

Four g of nutrient agar were weighed and suspended with 200 mL of sterilized distilled water in a 400 mL Schott Duran. In another separate 50 mL Schott Duran bottle, 40 mL of distilled water were prepared. Both bottles were sterilized at 121°C for 15 min.

Autoclaved nutrient agar was quickly transferred into oven at 40 °C to avoid solidification. Five g of skim milk powder were added into the autoclaved distilled water and heated up for a short boil for about 2 min in microwave and mixture was shaken to homogenate. Skim milk were cooled to 50 °C and added to the nutrient agar, approximately 15 mL of the mixture was poured into each petri dish to produce skim milk agar.

Fresh single colony with growth within 24 h was picked with sterilized inoculating loops. The bacteria was streaked with straight single line once onto the agar surface and incubated at room temperature for 24 h. Casein hydrolysis can be observed by placing the petri dish against a dark background. Clear zone around the grown bacteria indicates that the bacteria have hydrolyzed the proteins in skim milk.

3.3.2.3 Starch Hydrolyze Test

Soluble starch powder was weighed to 10 g and 20 g of nutrient agar suspended into transferred into 400 mL of distill water in a Schott Duran bottle. Distilled water and starch agar powder were mixed thoroughly. With frequent agitation, the solution were heated and boiled for 1 min to completely dissolve the powder.

The solution was autoclaved at 121 °C for 15 min. Approximately 15 mL of medium were poured into each petri dish to make starch agar plate. Single and fresh colony with the growth within 24 h was picked with sterilized inoculating loop. Bacteria were inoculated by streaking straight single line once on the media surface. Plates were incubated at room temperature for optimum growth for 24 h.

Plates with grown colonies then were flooded with 5 to 10 mL iodine and observed for the colourless zone around the colonies. Starch hydrolysis positive is indicated by a colourless zone surrounding colonies. A blue or purple zone indicates that starch has not been hydrolyzed.

3.3.2.4 Simmon's Citrate Agar Test

In 400 mL of distilled water, 9.36 g of Simmon's Citrate agar were suspended and mixed well. With frequent agitation the solution were heated in the microwave and mixed well until it completely dissolved.

The solution was autoclaved at 121 °C for 15 min. Approximately 15 mL of the autoclaved medium poured into each petri dish to make Simmon Citrate agar plate. Single colony with the growth within 24 h were picked using sterilized inoculating loop and inoculated into the plate by streaking one straight single line on media surface.

Plates are incubated at room temperature in the incubator and observed for the changes in colour around the colonies. Organism that utilizes citrate as a source of carbon only can grow on the media, citrate utilization indicated by colour changes from green to blue.

3.3.2.5 Kligler's Iron Agar test

Klinger Iron's agar powder were weighed for 20.8 g and suspended in 400 mL of distilled water. They were mixed well in a 500 mL Schott Duran bottle, and with frequent agitation the solution was heated to completely dissolve the powder.

During the heating process the cap of the Schott Duran bottle were loosen slightly to avoid increasing pressure inside the bottle. Medium were transferred into screw tubes with approximately 7 mL of solution each tubes. Tubes were autoclaved at 121 °C for 15 min.

The screw tubes were then cooled in a slanted position. Single and fresh colony with the growth of 24 h was picked with sterilized inoculating loop. Inoculates were stabbed into the butt and streaked once on the surface. Tubes were incubated with loosened caps for 24 h at room temperature.

Lactose fermenters produce yellow slants and butts while the lactose non-fermenters yield a red slant. Formation of Hydrogen sulfide is evidenced by a black color either throughout the butt, or a ring formation near the top of the butt. Gas production is detected as individual bubbles or by splitting or displacement of the agar.

3.3.2.6 Violet Red Bile Agar Test

In 400 mL of distilled water, 16.6 g of Violet Red Bile agar was suspended in a 500 mL Schott Duran bottle and the solution were mixed thoroughly. The solution were heated with frequent agitation and short boiled in the microwave oven to completely dissolve the powder.

Medium were then cooled to 50 °C and used immediately. Medium were dispensed into petri dish plate with approximately 15 mL each plate. Single and fresh colony with the growth within 24 h were picked using sterilized inoculating loops and were inoculated with streaking straight single line once on the media's surface. The plates were incubated for 24 h at room temperature.

Lactose fermenter is indicated by purple-red colonies, with or without a zone of precipitate around the colonies. Non-Lactose fermenters are colorless to transparent colonies.

3.3.2.7 Lactose Peptone Broth Test

In 500 mL Schott Duran bottle, 14 g of Lactose Peptone broth and 400 mL of distilled water were mixed thoroughly. The solution was warmed slightly in order to completely dissolve the powder. Approximately 7 mL of the solution were transferred into each screw tubes containing a Durham tube. The tubes then were autoclaved at 121 °C for 15 min.

Single and fresh colony with growth within 24 h was picked using sterilized inoculating loop. The colony was inoculated into the broth and incubated for 24 h at room temperature. Acid formation is demonstrated by a change in the color of the medium from reddish-purple to yellow. Gas production is demonstrated by the displacement of the medium from the Durham tubes.

3.3.2.8 Eosin Methylene Blue (EMB) Agar Test

Eosin Methylene Blue (EMB) agar powder was weighed to 15 g and mixed with 400 mL of distilled water in a 500 mL Schott Duran bottle. The solution was short boiled in microwave oven for 1 min to completely dissolve the EMB powder.

Medium was autoclaved at 121 °C for 15 min. Then, approximately 15 ml of the autoclaved EMB were poured into each petri dish plate and left to solidify. Single and fresh colony with growth within 24 h was picked using sterilized inoculating loop. Bacteria were inoculated with streaking straight single line once on the media's surface. Plates were incubated for 24 h at room temperature. Non-lactose fermenting organism produces colorless colonies while lactose-positive colonies are dark red in color.

3.3.2.9 MacCONKEY Agar Test

Twenty grams of MacCONKEY agar was weighed and suspended into 400 mL of distilled water in a 500 mL Schott Duran bottle. Solution was heated and mixed well with frequent agitation to completely dissolve the powder. Medium was autoclaved at 121 °C for 15 min.

After cooled down, the medium were poured into petri dish plate with approximately 15 mL per plate. Single and fresh colony with the growth within 24 h was picked using sterilized inoculating loop. Samples were inoculated into the agar with streaking straight single line once on the media's surface.

The plates were then incubated at room temperature for 24 h and observed for changes. Gram-positive bacteria will not grow on this medium. Lactose-negative colonies are colorless while lactose-positive are red in color (Fluornoy *et al.*, 1990).

3.3.2.10 Spirit Blue Agar Test

To prepare 400 mL of agar solution, 14 g of Spirit Blue agar was weighed and suspended into 400 mL of distilled water in a 500 mL Schott Duran bottle. The solution was mixed well to completely dissolve by heating and frequent agitation.

Thirty ml of lipoidial emulsion were prepared, with mixing 0.06 mL of Tween-80 and 6 mL of oil into warm distilled water. Solution was shaken until homogenous. Both solutions were autoclaved at 121 °C for 15 min.

At 50 °C in a sterile condition the lipoidal emulsion were mixed together with spirit blue agar medium, the medium were carefully shaken in a circular motion to homogenize without producing bubbles. Media prepared was distributed into petri dish with approximately 15 mL per plate.

Single and fresh colony with growth within 24 h was picked using sterilized inoculating loop. Samples were inoculated with streaking straight single line once on media's surface. Finally the plates were incubated in room temperature for 24 h and observed for the changes in the medium colour and growth of bacteria. Lipase production indicated with blue medium turns into whitish grey.

3.3.2.11 Catalase Test

A few drop of 3% hydrogen peroxide (H_2O_2) were placed on a petri dish. Using a sterilized inoculating loop, a single and fresh colony that grows within 24 h was picked and placed onto the 3% hydrogen peroxide. Immediate evolutions of gas or vigorous bubbles observed are positive results for this test.

3.3.2.12 Cellulose Test

Preparation of the medium was done by mixing cellulose powder, Congo red powder and nutrient agar. To prepare 400 mL of this medium, 8 g of nutrient agar, 0.752 g of cellulose powder and 0.08 g of Congo red powder were suspended into 400 mL of distilled water.

Mixtures were homogenized with frequent heating and agitation. Prepared solution was autoclaved at 121 °C for 15 min. After being cooled down, the medium were poured into petri dish with approximately 15 mL per plate.

Single colony with growth within 24 h was picked using sterilized inoculating loop. Bacteria were inoculated with streaking straight single line once on the surface of the agar. Finally the plates were incubated in room temperature for 24 h and observed for clearance zone. Ability of the bacteria to hydrolyze cellulose evidenced with clearing zone.

3.3.3 Molecular Test

3.3.3.1 DNA Extraction

Tween-20 solution was prepared by adding 50 mL of TE buffer (10 mM Tris, 1 mM, pH 8.0) and 1 mL of original Tween-20. The mixture was mixed with mild shaking at circular motion. Solution stored in a clean bottle.

Fresh colony with the growth within 24 h was picked and inoculated into 1.5 mL of sterilized nutrient broth. The nutrient broth was shaken in water bath shaker overnight at room temperature. An uninoculated nutrient broth was prepared as negative control. Any changes in the negative control show contamination.

The overnight grown culture of bacteria was transferred into a 1.5 mL eppendorf tubes and spin at 12,000 *g* for 10 min at 4 °C. Supernatant were removed and pellet were suspended with 200 µL of Tween-20 solution which were prepared earlier. Solution was vortexed thoroughly until the pellet and Tween-20 solution homogenized.

Solution was incubated at 95 °C for 30 min in heating block with agitation. Tubes were then placed in ice for 5 min and spun at 12,000 *g* for 10 min at 4 °C. Supernatant were transferred in a go into a new tube and stored in -20 °C prior for use.

3.3.3.2 Amplification using Polymerase Chain Reaction (PCR)

The total polymerase chain reaction (PCR) reaction volume was 20 µL and consist of 2 µL of bacterial pure DNA, 12.5 µL of distilled water, 2 µL of 10X *Taq* buffer with ammonium sulphate [$(NH_4)_2SO_4$)], 2 µL of 25mM magnesium chloride (MgCl), 1 µL of 0.5 µM primers (both forward and reverse), 0.4 µL of 10 mM deoxyribonucleotide triphosphate (dNTP) and 0.1 µL of 5 U/µL *Taq* polymerase mixed in 0.2 mL tube.

The PCR was performed in a thermal cycler with program included are an initial denaturation at 95 °C for 3 min, followed by 30 cycles of denaturation at 94 °C for 30 sec, annealing at 50 °C for 30 sec and extension at 72 °C for 1 min. The last cycle was followed by an extra extension step at 72 °C for 1 min (Ahmed *et al.*, 2007). After the run has finished the PCR product were stored at -20 °C.

3.3.3.3 Gel Electrophoresis

The PCR products were analyzed by 1% agarose gel electrophoresis at low voltage 90 V. To prepare 1% agarose gel, 0.25 g of agarose powder and 25 mL 0.5x Tris-acetate-EDTA (TAE) buffer were mixed together and short boiled in microwave with frequent agitation until the agarose dissolved completely. Fourty µL nile blue (2.5x) was added to the liquid gel mixture after the gel cooled to 60 °C.

Prepared agar was poured into the molding tray with the comb placed at appropriate depth and allowed to solidify for 30 min. The comb was removed after solidification. Solidified gel together with the gel mold was placed in electrophoresis tank. TAE buffer (0.5x) containing 300 µL of Nile blue was poured into the electrophoresis tank until it covers the surface of the gel.

PCR products (10 µL) were mixed with 5 µL of loading dye (bromophenol blue) and loaded into the well. The loading should be done fast to avoid the DNA to diffuse from the gel. Electrophoresis times were approximately 1 h at 100 V.

The negatively charged nucleic acid runs to the positive pole in an electric field. A standard molecular weight marker was typically run along with samples to enable the size of PCR product to be assigned at 1.5 kb during gel electrophoresis. Finally, gel electrophoresis images were captured with a gel imaging system.

3.3.3.4 Direct Precipitation

Once a band was obtained from 20 µL of PCR product, the volume was scaled up to 50 µL and amplified using PCR. 10 µL out of 50 µL were used to determine the existence of desired band at 1.5 kb.

The rest 40 µl were transferred into a 1.5 mL eppendorf tubes. Briefly, 40 µL of each samples were added with 4 µL of sodium acetate and 100 µl of ethanol and mixed well with inverting the tubes for few times.

Followed by, the tubes were kept in ice for 30 min and spin at 13,000 *g* for 15 min at 4 °C. The supernatant were discarded again and pellet was resuspended with 200 µL of 75% alcohol and vortexed for 1 min. Again, spin at 13,000 *g* for 5 min at 4 °C and supernatant were discarded.

Remaining ethanol was eliminated by air drying. The pellet was air-dried for at least 30 min in a half-open tube, resuspended in 20 mL of distilled water, and stored at -20 °C prior to be used. Five µL of the DNA was mixed with 2 µL 6x loading dye for gel electrophoresis.

3.3.3.5 DNA Sequencing

Band obtained from electrophoresis of DNA purification product were used to calculate the concentration of DNA. Calculation was made by using FluorChem 5500 software. Purified DNA product and DNA concentration was sent to First Base Laboratory Sdn. Bhd. for sequencing to determine the 16S rDNA nucleotide sequences.

Gene sequences once obtained are analyzed and compared with using online database Basic Local Alignment Search Tool (BLAST) accessed at http://bioinfo.unice.fr/blast/ (Altschul *et al.*, 1990).

CHAPTER 4

RESULTS

4.1 Isolation of Bacteria from Soil

Twenty isolates were made, some identical isolates were eliminated. Individual colonies were then picked based on their differences in morphology and cultured in a separated nutrient agar plates.

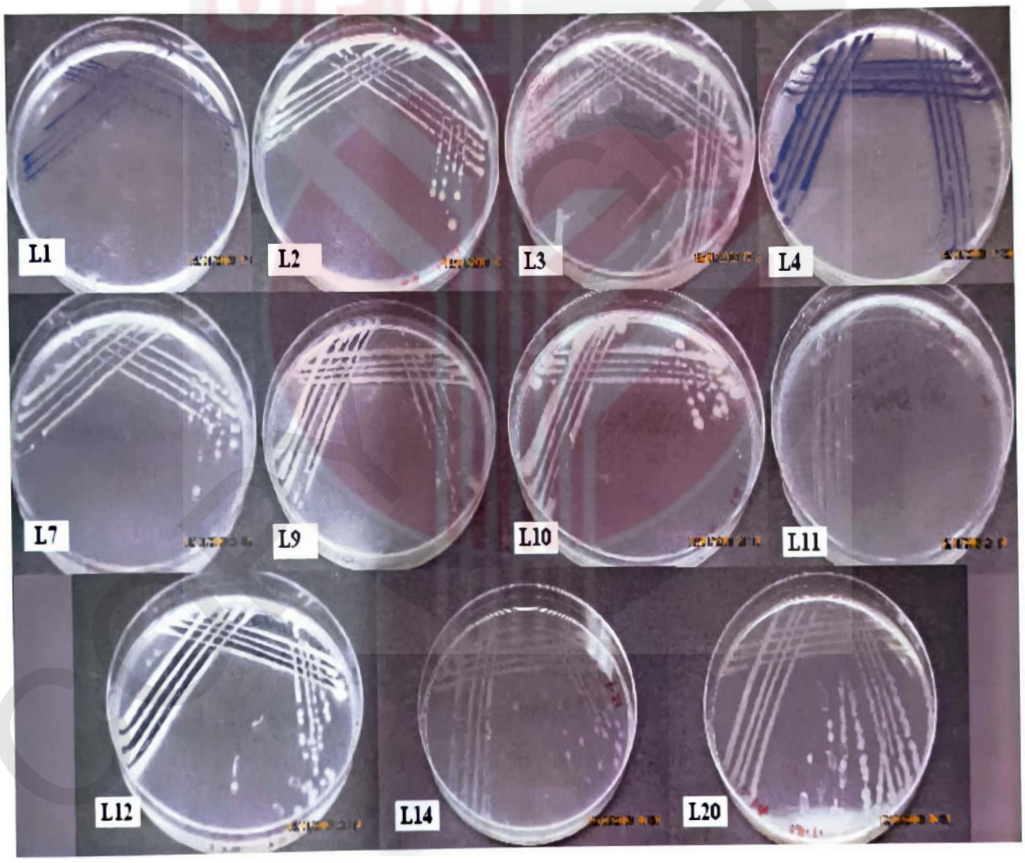

Figure 4.1: Isolated Individual Colonies

4.2 Identification of Bacterial Isolates

4.2.1 Colony and Cell Morphology

The isolated colonies of bacteria were identified on the basis of the morphology. Bacterial colonies were identified based on their distinct characteristics including colour, size, shape, elevation, texture and appearance of the colony's margin (Table 4.2).

4.2.2 Gram Staining

The results of Gram staining of bacterial isolates from soil are shown in Table 4.1.

4.2.3 Biochemical Tests

4.2.3.1 Gelatin Liquefaction Test

According to the method, gelatin inoculated with microbes that produces gelatinases, liquefies (Figure 4.2). The liquefaction test results are summarized in Table 4.3.

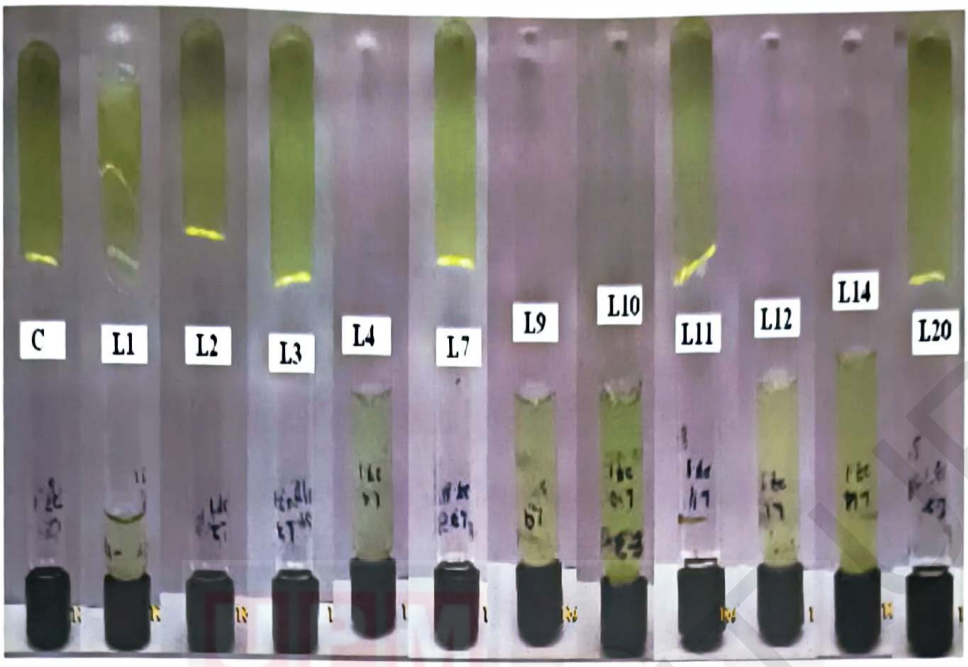

Figure 4.2: Gelatin Liquefaction Test

Notes:
C-control
Results obtained after 12 days incubation with interval of freezing at 4 °C.

Table 4.1: Results of Gram staining test on bacterial isolates from soil

Isolates	Gram stain	Colour	Shapes	Arrangements
L1	negative	Pink	Bacillus	Single bacillus
L2	positive	Purple	Bacillus	Streptobacilli
L3	positive	Purple	Bacillus	Single bacillus
L4	negative	Pink	Bacillus	Single bacillus
L7	positive	Purple	Bacillus	Filamentous
L9	positive	Purple	Bacillus	Single bacillus
L10	negative	Pink	Bacillus	Streptobacilli
L11	positive	Purple	Bacillus	Pleomorphic
L12	positive	Purple	Bacillus	Single bacillus
L14	negative	Pink	Bacillus	Single bacillus
L20	negative	Pink	Bacillus	Single bacillus

Table 4.2: Morphological characteristic of bacterial isolates

Isolates	Shape	Margin	Elevation	Size	Texture	Appearance	Pigmentation	Optical property
L1	Spindle	Entire	Convex	Small	Smooth	Rough	Purple	Opaque
L2	Circular	Entire	Convex	Moderate	Smooth	Dull	Cream	Opaque
L3	Rhizoid	Curled	Flat	Moderate	Rough	Dull	Cream	Translucent
L4	Circular	Entire	Pulvinate	Large	Smooth	Shiny and wet	Purple	Opaque
L7	Circular	Entire	Convex	Small	Rough	Dull	White	Opaque
L9	Circular	Entire	Pulvinate	Moderate	Rough	Dull	Cream	Opaque
L10	Circular	Undulate	Flat	Large	Rough	Dull	White	Translucent
L11	Irregular	Lobate	Flat	Large	Smooth	Shiny	Cream	Transparent
L12	Circular	Entire	Flat	Moderate	Rough	Dull	White	Opaque
L14	Spindle	Entire	Flat	Moderate	Smooth	Dull	White	Transparent
L20	Circular	Entire	Raised	Moderate	Smooth	Shiny	White	Opaque

4.2.3.2 Skim Milk Agar Test

Skim milk agar test is used to identify bacteria that hydrolyze casein. The ability to produce casein observed with presence of clearance zone (Figure 4.3) after incubation at room temperature. The skim milk agar test results with average clearance zone value are listed in Table 4.3.

L1, L2, L9, L12 and L20 shows clearance zone, L3, L4, L7, L10, L11 and L14 shows over grown cell that cause difficulty to observe the clearance zone.

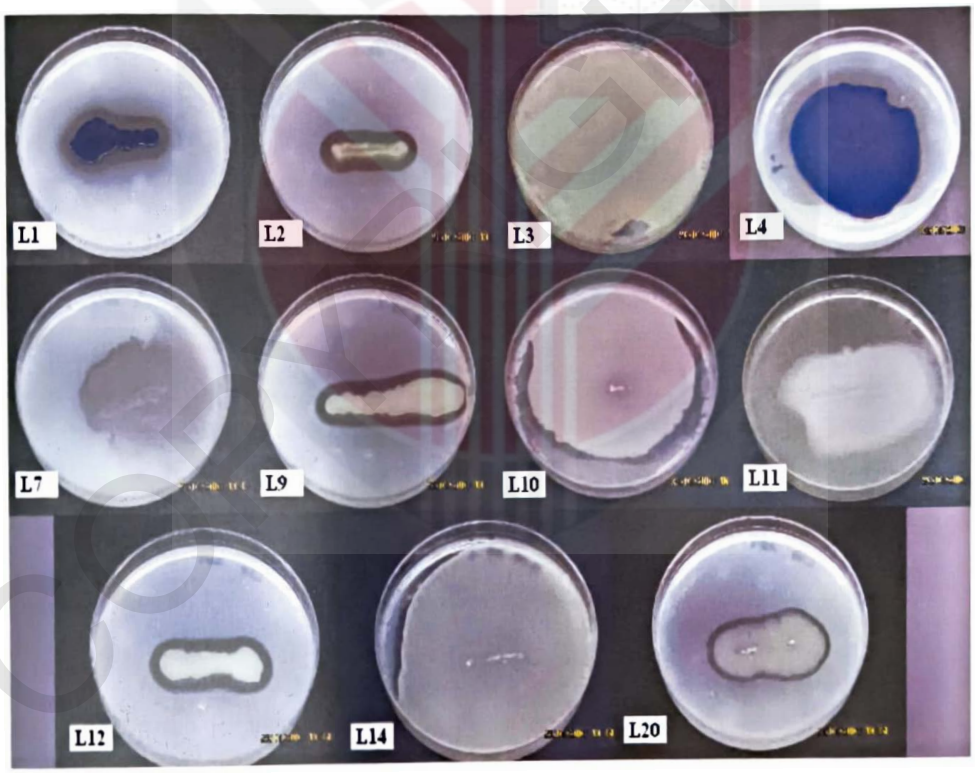

Figure 4.3: Skim Milk Agar Test

4.2.3.3 Starch Agar Test

Starch agar is used in differentiating microorganism based on starch hydrolysis. According to the method, when tested with iodine the plate shows clearance zone indicating the ability of the bacteria to hydrolyze starch. Inoculated plates were incubated for 24 h before testing with iodine.

Observation made immediately after flooding iodine on the surface (Figure 4.4). Only L9 and L12 shows clearance zone.The starch agar test results are summarized in Table 4.3.

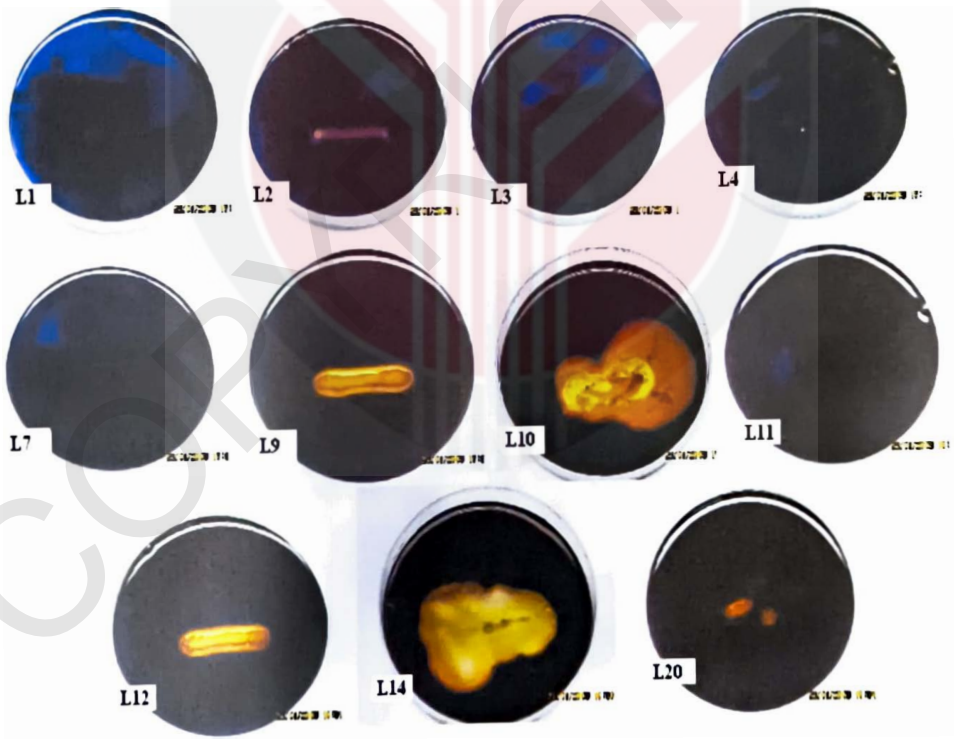

Figure 4.4: Starch Agar Test

4.2.3.3 Simmon's Citrate Agar Test

Simmon's citrate agar used to differentiate gram negative enteric bacilli on the basis of sodium citrate utilization. It is a selective agar where it allows only gram negative bacteria to grow and sodium citrate utilization is indicated by the color change of the agar from green to bluish.

Observation made after 24 h of incubation at room temperature (Figure 4.5). Only L1 and L3 show growth and color change. The starch agar test results summarized in Table 4.3.

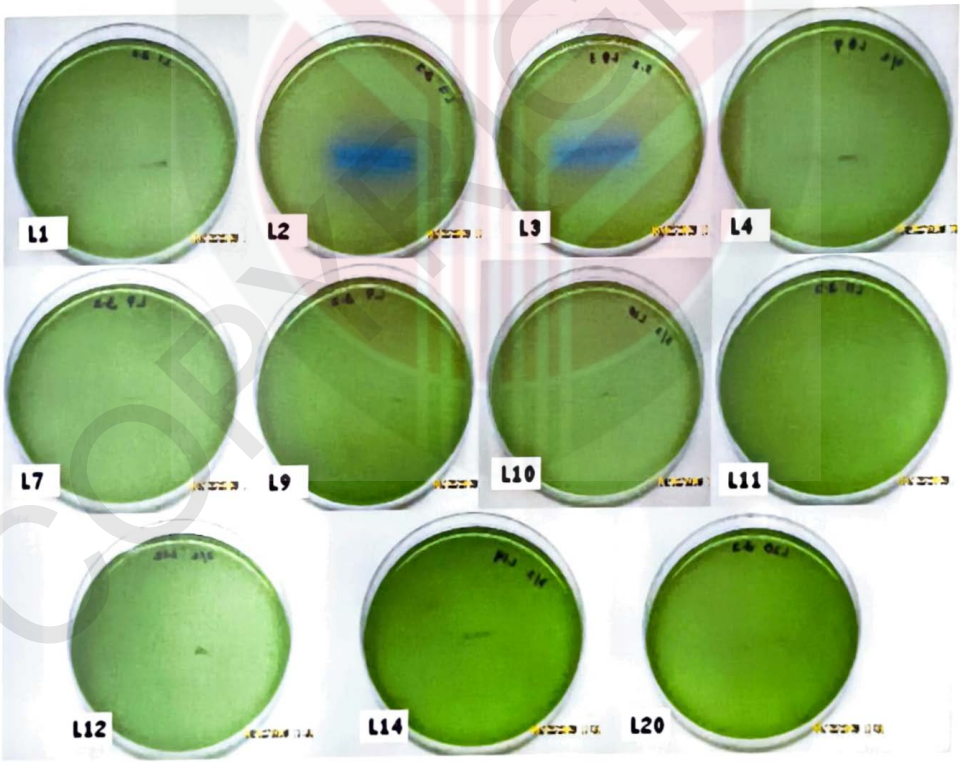

Figure 4.5: Simmon's Citrate Agar Test

4.2.3.4 Kligler's Iron Agar Test

Kligler Iron agar is used to differentiate bacteria that ferment dextrose and lactose and liberation of sulfide gas. Hydrogen sulfide production is evidenced by a black color through the butt in ring formation near the top of the butt. L11 shows hydrogen sulphide liberation.

Observation made through colour change (Figure 4.6). Lactose nonfermenters produce yellow slant (acid) which reverts back to red (alkaline) when supply of dextrose is exhausted and yellow butt whereas, lactose fermenters produce yellow slants and butts. L1, L2, L3, L4, L9, L10, L12, L14 and L20 are lactose non-fermenters. Both L7 and L11 are lactose fermenters.

A microbe that incapable of fermenting both lactose and dextrose produces red slants and butts. The surface of the L1 and L4 was purple due to the colony colour itself. This caused some difficulties in interpreting the observation.The Klinger Iron agar test results listed in Table 4.3

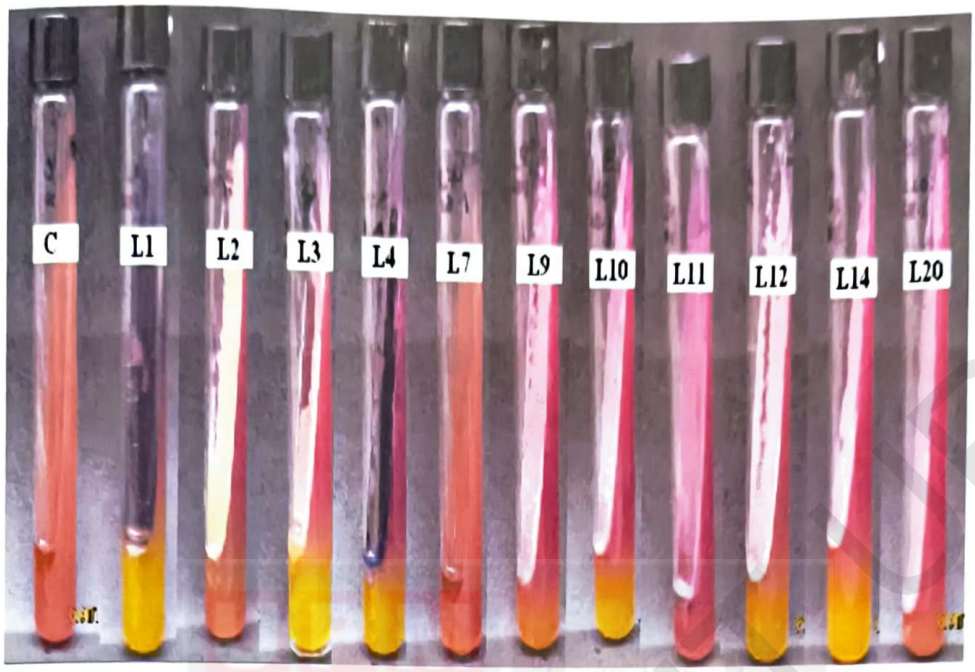

Figure 4.6: Klinger Iron Agar Test

Notes:
C-Control
Observation made after 24 h of incubation at room temperature

4.2.3.5 Violet Red Bile Agar test

This test is to identify gram positive bacteria and their ability to ferment lactose. The ability of the gram positive bacteria to ferment lactose is identified based on the colour of the colonies grown on the agar.

Lactose fermenters detected with their purple colonies and lactose non-fermenters are colorless to transparent colonies. The agar were inoculated and incubated for 24 h in room temperature before observation made (Figure 4.7). L1 and L4 couldn't be justified because they have purple pigment. L3 were identified as lactose non-fermenter and the rest of the colonies didn't grow. The Violet Red Bile agar test results summarized in Table 4.3.

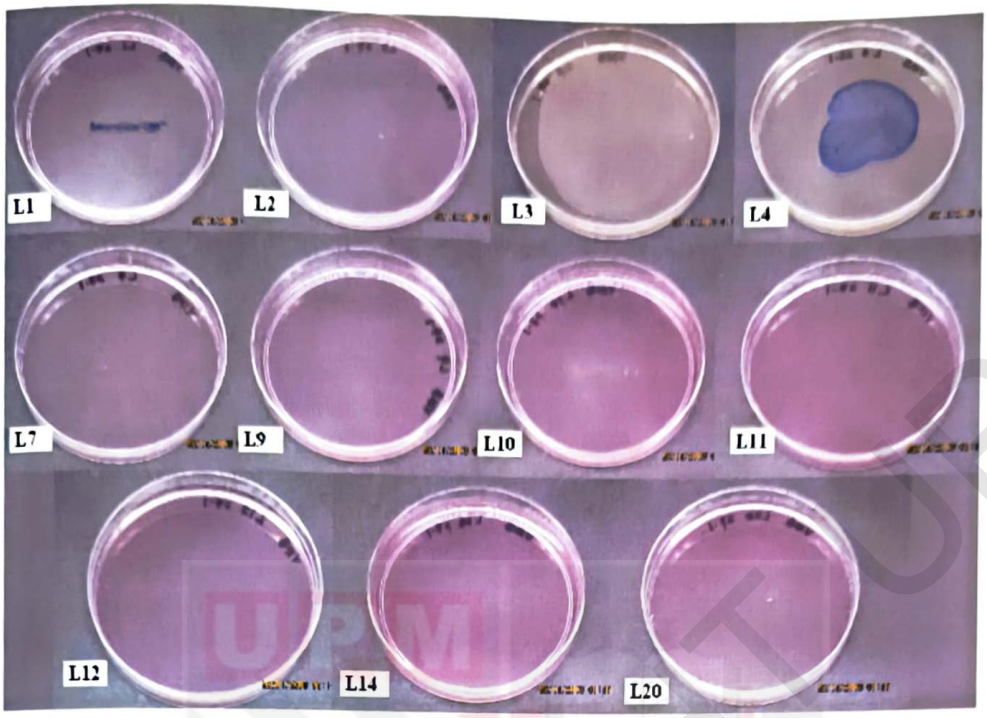

Figure 4.7: Violet Red Bile Agar Test

4.2.3.6 Lactose Peptone Broth Test

Lactose peptone broth test is used to identify coliform organisms. Presence of coliform organism was identified with the production of both acid and gas.

Acid formation is demonstrated by a change in color of the medium from reddish-purple to yellow. Gas production is demonstrated by the displacement of the medium from Durham tube. L2, L3 and L7 forms acid and releases gas. Observation made after 24 h of incubation at room temperature (Figure 4.8). The Lactose peptone broth test results listed in Table 4.3.

Figure 4.8: Lactose Peptone Broth Test

4.2.3.7 Eosin Methylene Blue (EMB) Agar Test

Eosin Methylene Blue (EMB) agar is used to identify coliform bacteria from water. An inoculated bacterium appears in different color. Observation made after incubation for 24 h at room temperature (Figure 4.9).

L2 and L3 colonies are pink with dark blue centre. L4 cannot be classified because the colonies have purple pigments. L9 and L14 colonies turned to pink with no dark center. The others plates has no growth. Observation made after incubation for 24 h at room temperature (Figure 4.9). The EMB test results listed in Table 4.3.

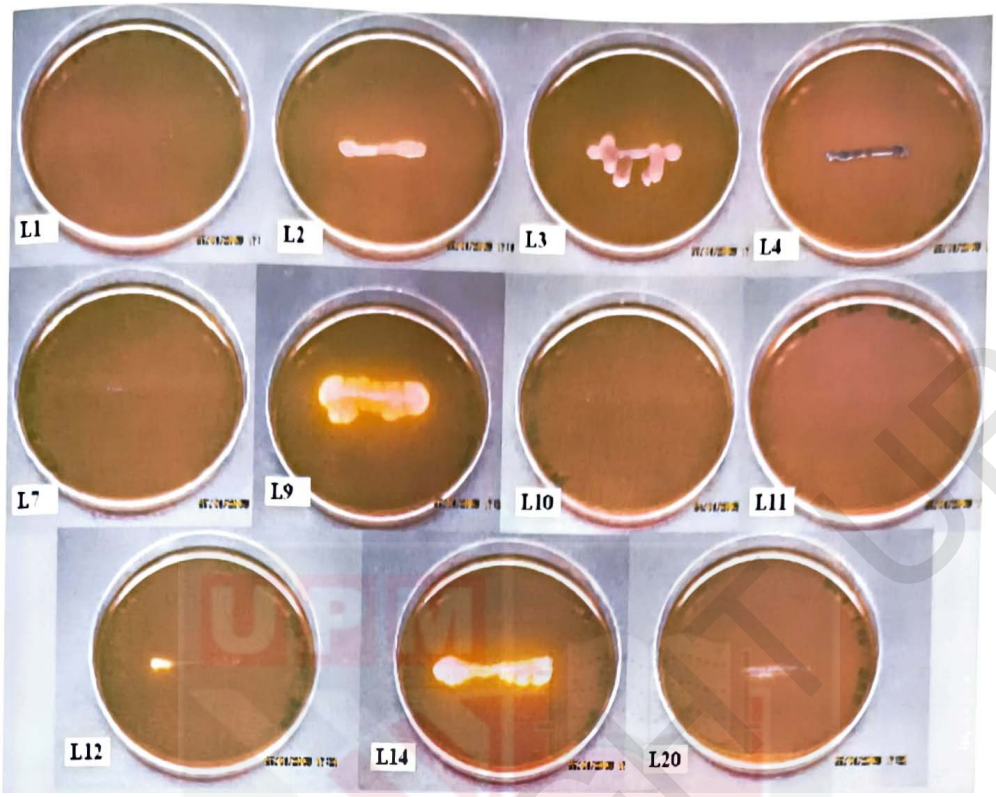

Figure 4.9: Eosin Methylene Blue (EMB) Agar Test

4.2.3.9 MacCONKEY Agar Test

MacCONKEY agar inhibits the growth of Gram-positive microbes and detects lactose degradation. The lactose degradation observed throughout color changes of the agar from red to orange. Observation made after 24 h of incubation at room temperature (Figure 4.10).

Inoculated bacteria L1, L2, L3 and L4 grown and changes of agar color are evidenced. The rest of the plates have shows no growth. The MacCONKEY test results listed in Table 4.3.

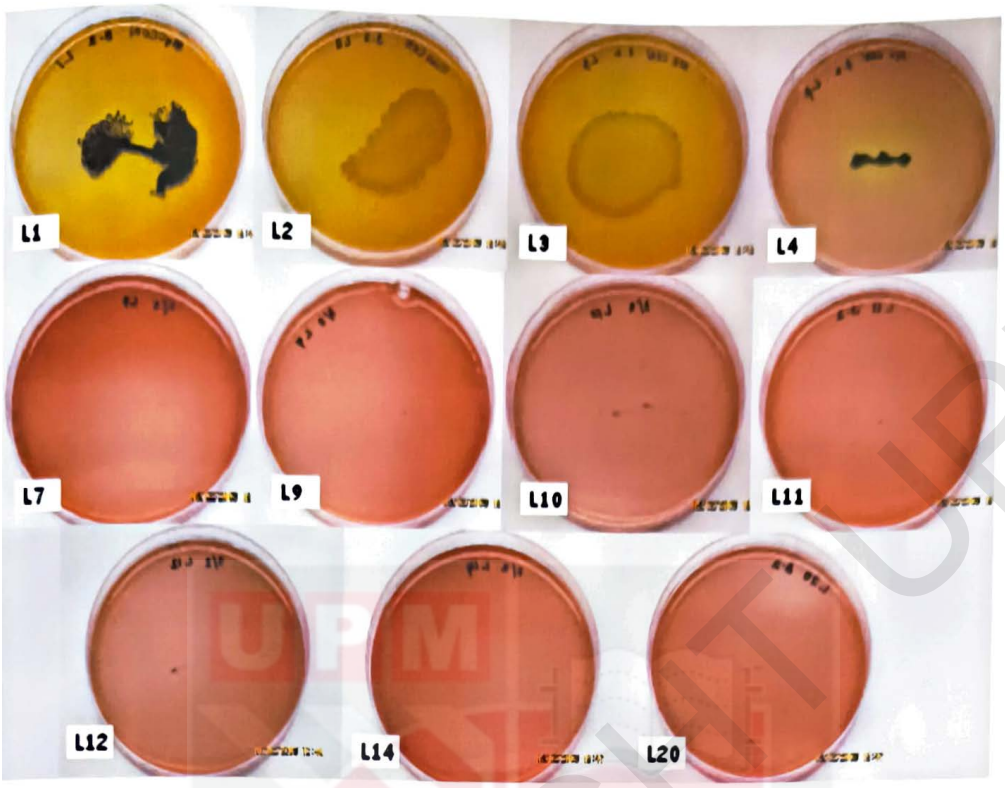

Figure 4.10: MacCONKEY Agar Test

4.2.3.10 Cellulose Agar Test

This test used to identify the microbes that utilize cellulose. Cellulose-utilizing bacteria were distinguishable on cellulose-agar by definite zones of clearing around the colonies.

Clearance zone evidenced in plates inoculated with L1, L4, L9, L12, L14 and L20. The other isolates don't show any changes. Observation made after 24 h of incubation at room temperature (Figure 4.11). The Cellulose agar test results with average clearance zone values are listed in Table 4.3.

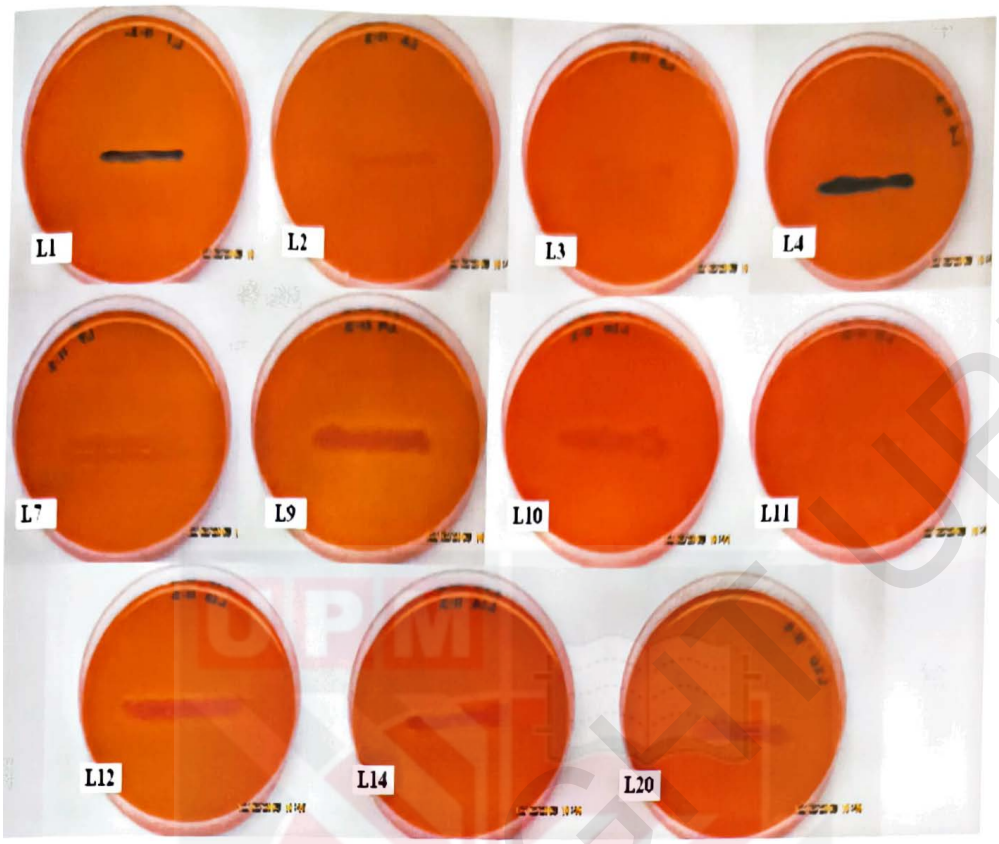

Figure 4.11: Cellulose Agar Test

4.2.3.11 Spirit Blue Agar Test

Spirit blue agar with lipase reagent is used for detecting lypolytic microbes. Inoculated bacteria metabolise the lipid in the medium and form colonies. This activity changes the agar from blue to whitish grey.

Observation was made after incubation for 24 h at room temperature (Figure 4.12). All the isolates have the ability to metabolise lipid, isolates from L4, L11, L12, L14 and L20 are over grown and shows clearance of the whole plate. The Spirit blue agar test results listed in Table 4.3.

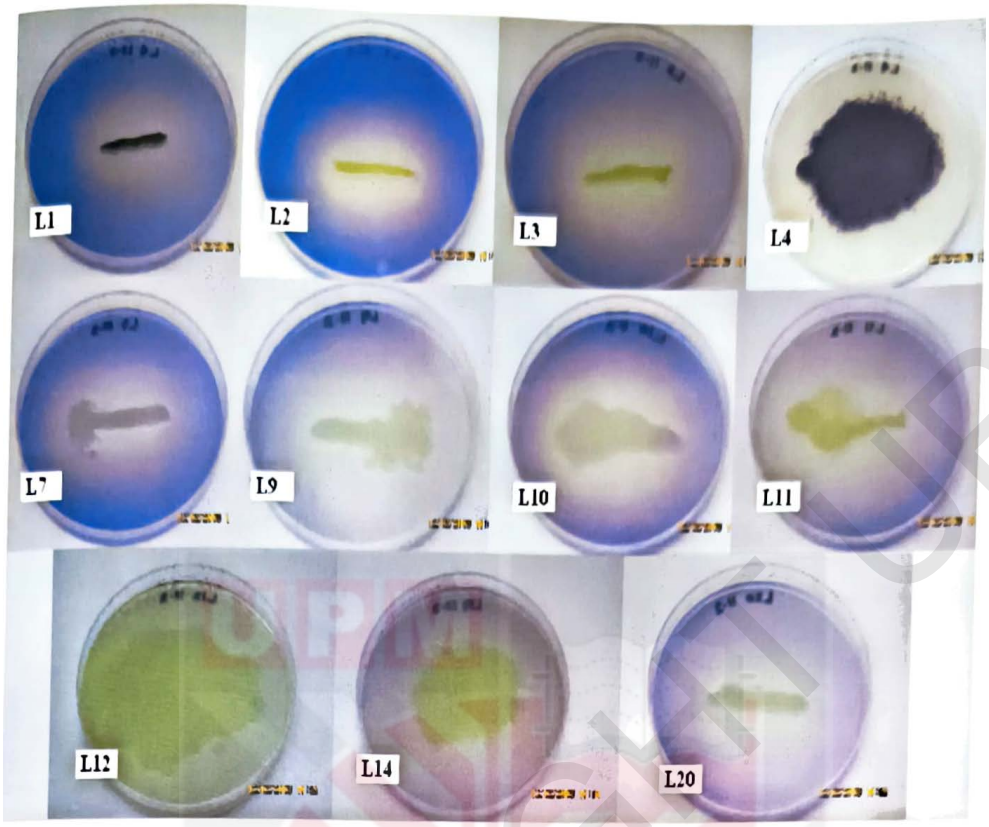

Figure 4.12: Spirit Blue Agar Test

4.2.3.12 Catalase Test

Catalase test was performed to detect the presence of catalase in the colonies. Gas bubbles appear on the 24 h old bacterial colonies once few drops of hydrogen peroxide added. This indicates the presence of catalase in the colonies.

Observation for the gas bubbles made immediately after gas bubbles appear (Figure 4.13). All the isolates produce catalase except L14, L1 and L11 shows gas bubbles but not vigorously but the rest of the isolates bubble vigorously. The results of catalase test listed in Table 4.3.

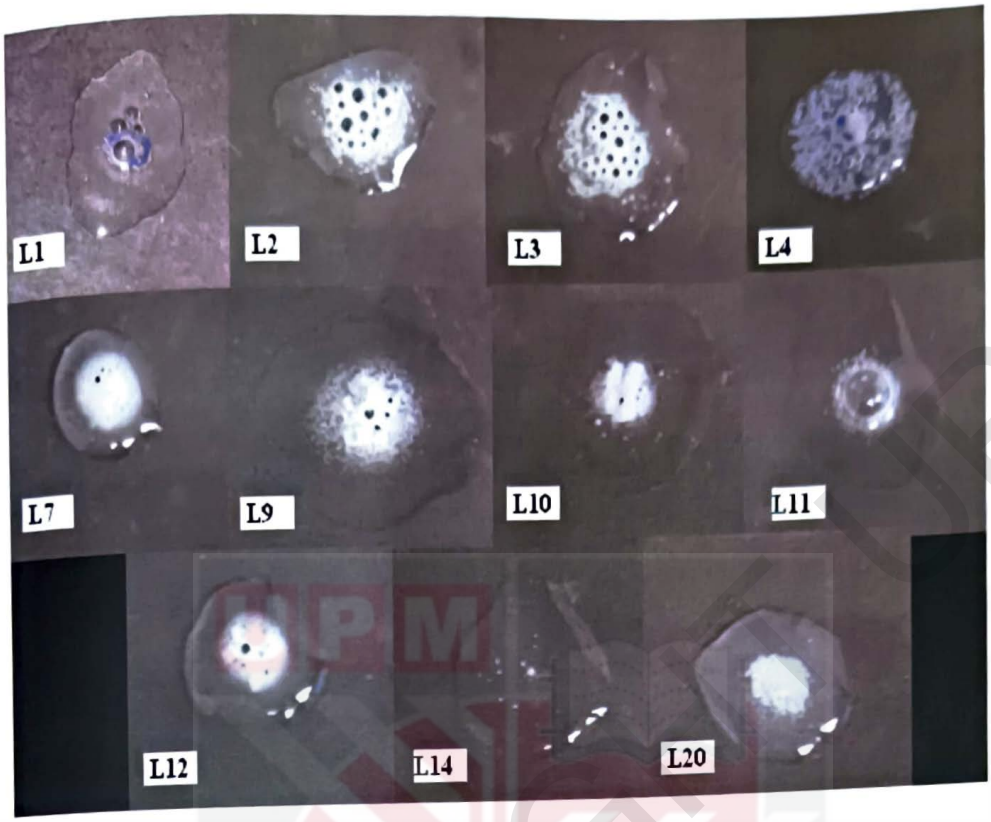

Figure 4.13: Catalase Test

Table 4.3: Biochemical characteristics of bacterial isolates from soil

Isolates	Clearance zone (cm)			Starch hydrolysis	Growth in				Gel .test	KIA test			Cat. test	LPB test	
	s.milk	Cell.	SBA		SCA	EMB	VRB	M.CON		Slant	butt	H_2S		Acid	Gas
L1	0.33	0.11	0.63	−	−	−	+	+	+	Red	Yellow	−	+	−	−
L2	0.48	−	0.75	−	+	+	−	+	−	Red	Yellow	−	++	+	+
L3	Crowded	−	0.78	−	+	+	+	+	−	Red	Yellow	−	++	+	+
L4	Crowded	0.17	Clear	−	−	+	+	+	+	Red	Yellow	−	++	−	−
L7	0.45	−	0.75	−	−	−	−	−	−	Red	Red	−	++	+	+
L9	0.38	−	Clear	+	−	+	−	−	+	Red	Yellow	−	++	−	−
L10	Crowded	−	1.0	−	−	−	−	−	+	Red	Yellow	−	++	−	−
L11	Crowded	−	Clear	−	−	−	−	−	−	Red	Red	+	+	−	−
L12	0.45	0.23	Clear	+	−	−	−	−	+	Red	Yellow	−	++	−	−
L14	Crowded	0.15	Clear	−	−	+	−	−	+	Red	Yellow	−	++	−	−
L20	0.28	0.15	1.35	−	−	−	−	−	−	Red	Yellow	−	−	−	−

Notes:

s.milk: skim milk test
Cell: Cellulose test
SBA: Spirit blue agar
SCA : Simmon's citrate agar
EMB: Eosin methylene blue
VRB: Violet red bile agar
M.CON: MacCONKEY agar
Gel: gelatin liquefaction test

KIA: Kligler Iron agar
KIA red: alkaline
KIA yellow: acid
H_2S: Hydrogen sulphide
Cat. test: Catalase test
Cat. test +: bubbles
Cat. test ++: bubbles vigorously
LPB test: Lactose peptone broth

LPB acid +: purple to yellow colour
LPB acid − : no change in colour
LPB gas −: gas deliberation
LPB gas +: no gas deliberation
+ : positive
−: negative

4.3 PCR Amplification of 16S rRNA gene

PCR results to identify the bacteria with 16S rRNA gene analyzed on agarose gel. Figure 4.14 shows gel electrophoresis results of the amplified 16S rRNA. Colonies with 16S rRNA gene is identified with the target gene marker. The obtained or desired PCR product size is 1500bp.

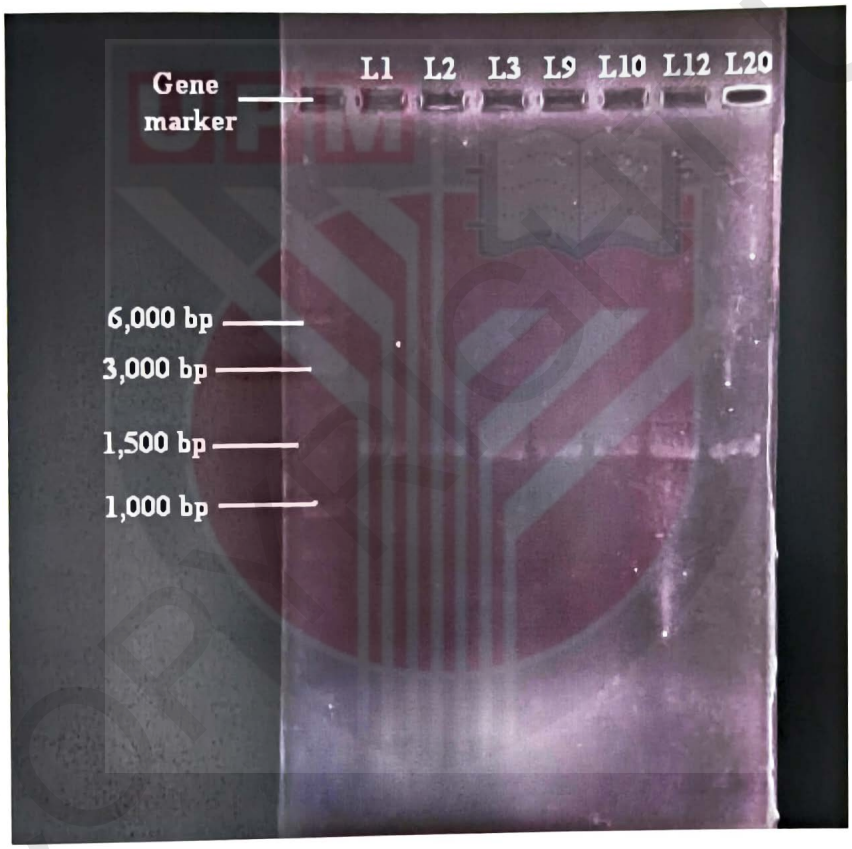

Figure 4.14: Amplified PCR Product of 16S rRNA gene from Soil Bacteria

4.4 DNA Sequencing Results

Table 4.4 summarizes the results of the DNA sequencing analysis for these bacterial isolates. The gene sequences were used against BLAST queries with the results indicating that the bacteria isolated from soil (Bekenu series) belong to two major groups.

Isolates L2, L3, L9 and L12 were grouped as Bacilli and isolates L1, L10 and L20 were grouped as β-proteobacteria.

Table 4.4: BLAST results for 16S rRNA gene sequence from soil

Isolate	Identification	BLAST Match Accession No	% similarity	Score (bits)	Identities
L1	*Chromobacterium violaceum*	AY117554	99%	2014	1052/1060
L2	*Bacillus subtilis*	EU257453	99%	2115	1102/1111
L3	*Bacillus pycnus*	AB271739	97%	1768	988/1016
L9	*Bacillus thuringiensis*	EF988334	98%	1800	968/984
L10	*Cupriavidus basilensis*	AY860224	97%	1461	802/821
L12	*Bacillus cereus*	DQ289989	99%	2052	1085/1095
L20	*Chromobacterium Violaceum*	AY117554	98%	1992	1048/1060

CHAPTER 5

DISCUSSION

The acid soil inhabiting bacteria were successfully isolated and characterized. In this study, 11 bacteria were isolated from acid soil (Bekenu series) based on their different and prominent morphology characteristics.

According to Prakash *et al.* (2007), identification of an unknown isolates need to be based on polyphasic approach. In this studies, based on polyphasic approach of deploying both conventional and molecular technique 63% (7 out of 11) bacteria isolates are successfully identified up to species level.

Based on PCR amplification of 16S rRNA and DNA sequencing analysis using online database, the bacteria isolates are able to be grouped in class Firmicutes and β-proteobacteria. L2 (*Bacillus subtilis*), L3 (*Bacillus pycnus*), L9 (*Bacillus thuringiensis*) and L12 (*Bacillus cereus*) belongs to the class Firmicutes. L1 (*Chromobacterium violaceum*), L10 (*Cupriavidus basilensis*) and L20 (*Chromobacterium violaceum*) belongs to the class β-proteobacteria. All the isolates have more than 97% of nucleotides similarity as this fact is also supported by Drancourt *et al.* (2000). His studies evidenced 97% similarity for the bacteria species identification using 16S rRNA sequence.

These results obtained from this primary studies is similar with previous findings by Elsas *et al.* (2007), whom also studied on soil bacteria by molecular methods using 16S rRNA emphasize that most bacteria isolated and identified from soil fall into 8 major phylogenetic group such as α, β, γ, δ and ε *Proteobacteria*, *Firmicutes*, *Actinobacteria* and *Bacteroidetes*.

Four out of seven isolates are identified to be bacilli. It is reported by US Environmental Protection Agency, 1997 (Lenzen *et al.*, 2003) and previous studies done by Aslim *et al.* (2002) supports the finding bacilli dominates soil with populations of 10^6 to 10^7 per gram of soils. This is also supported by Coyne (1999), stating that Bacillus species represent 7% to 67% of the soil isolates.

The molecular results obtained by online database are being confirmed and supported by biochemical characteristic exhibited by the isolates. Some discordant results are being discussed as following.

5.1 Gram Reaction and Morphology Characteristic

Based on the result that was obtained from DNA sequencing discordant results from gram reaction and morphology characteristic are discussed as follows.

Cultural and biochemical characteristics of the entire isolates and Gram reaction are variable. The phenotypic test results shows that the bacterial isolates were Gram negative coccoid rod (L10), Gram negative rods (L1) and Gram positive rods (L2, L3, L9,L12 and L20). The entire isolates Gram staining results are concordant with BLAST results exception for isolate L20.

Isolate L20 projects 98% of nucleotide homology of *Chromobacterium violaceum* but discordant with Gram staining results. According to Bergey's Manual of Determinative Bacteriology (Holt *et al.*, 1994) *Chromobacterium violaceum* are straight rods with cell diameter of 0.6 to 0.9 μm, Gram negative which produces butyrous violet colonies on solid media. These phenotypic projections of *Choromobacterium violaceum* were also supported by the findings by Lee *et al.* (1999) and Ezera *et al.* (2007).

From the morphology observation made on L20, these isolate were white colonies which were slowly turning into violet colonies. The possibility of two different morphology characteristic projected by L20 isolates explained further. According to Faber *et al.* (2001), phenotypic identification method are not universally applicable because they are subjected to variation in expression within the same isolates due to environmental pressures and acquisition of extra chromosomal DNA carrying genes that may alter phenotypic traits.

Gram reaction of L20 isolate was discordant with *Chromobacterium violaceum* and further explained by Elsas *et al.* (2007). According to his previous studies, distinction between Gram negative and Gram positive is due to differences in the nature of their cell walls and this distinction is not perfect as some bacteria may stain gram positive and vice versa.

Some bacteria stain red during growth and violet during stationery phase. In addition, some bacteria have no cell wall and their cell wall composition varies depending on the cell type. In addition, Gram staining reaction is related with some factors such as pH, bacterial culture time and stain time (Xu *et al.,* 2005). If the stain conditions are incorrect, it will give an incorrect result.

Ferguson *et al.* (2007) evidence that identification using phenotypic characterization alone is not sufficient and it should be combined with morphological, biochemical characteristics and 16S rRNA gene for an efficient and accurate identification system. Also supported by Paul and Clark (1989), stating that the morphological and staining properties of bacterial cells are useful taxonomically but unfortunately by themselves they often fail to differentiate species that are profoundly different physiologically.

5.2 Biochemical Characteristics of Bacterial Isolates

Genus Chromobacterium which are the soil organism (Holt *et al.*, 1994; Lee *et al.*, 1999; Shenoy *et al.*, 2002) are chemoorganotrophs having mainly a fermentative metabolism (Holt *et al.*, 1994) where acid, but no gas are produced from fermented

glucose, catalase positive and liquefies gelatin. L1 which was identified as *Chromobacterium violaceum* has the entire characteristic stated above but not L20. This discordant result may be caused by overgrowth by other bacteria or the presence of mutations in L20 (Xu *et al.*, 2005).

Biochemical test is a cultivation-dependent technique, used for identification of bacteria. As reported by previous studies the soil bacteria are extremely diverse (Paul and Clark, 1996; Janssen *et al.*, 2002; Gans *et al.*, 2005; Voroney, 2007) making it impossible to sustain growth of all the bacteria because syntropic relationship and other cell to cell interaction may be disrupted when cells are diluted and incubated on plates or in shaken liquid cultures.

Biochemical test using Simmon's Citrate agar requires growth to facilitate identification of *Chromobacterium violaceum* but the result obtained was discordant. This could be explained by referring to laboratory growth medium which does not accurately reflect the true condition of complex environment such as soil therefore vast majority microbial species cannot currently be grown in the laboratory (Cullimore, 2002). It has been estimated that less than 1% of all bacterial cells in soil can be cultured in different types of media (Amann *et al.*, 1995).

According to Bergey's Manual of Determinative Bacteriology (Holt *et al.*, 1994), cells of genus Bacillus is rod-shaped and straight. Cells stain gram positive. They are chemoorganotrophs with a respiratory metabolism. Following biochemical identification tests of utilization of citrate, gelatine liquefaction and starch hydrolysis, production of acid and gas from glucose (Klinger iron agar), production

of catalase, were determined to show that L2, L3, L9 and L12 belong the genus Bacillus (Holt *et al.*, 1994).

Biochemical test results of L3 (*Bacillus pycnus*) are agreeable with molecular results. But other isolates L2 (*Bacillus subtilis*), L9 (*Bacillus thuringiensis*) and L12 (*Bacillus cereus*) have some inconclusive results. This may be due to the competition and chemical welfare between bacteria where some bacteria not to grow especially on crowded agar plates (Fredricks *et al.*, 2005). Previous studies by Rabilloud *et al.* (2005) evidenced that biochemical results may have inconclusive results due to inefficient and very low expression levels of enzymes because of stressed bacteria.

L10 isolate was identified as *Cupriavidus basilensis* and extensive biochemical test of catalase, starch hydrolysis, lactose peptone broth test and no gas produced from glucose metabolism correlates with the genus cupriavidus (Holt *et al.*, 1994). Genus cupriavidus stains gram negative with coccoid rods shape (Barrett and Parker, 2006). Some limitation exhibit by biochemical test is that they are culture-dependent where mutation and contamination rates are very high. The interpretations of the results are dependent on individual expertise and skills (Bosshard *et al.*, 2003).

Most of the biochemical test relies on the changes of media colour. In some cases, like isolate L1 (*Chromobacterium violaceum*) which have violet pigmentation makes the results interpretation less efficient or hard. Conventional selection using routine biochemical tests generally take several weeks. Time required for confirmed result with biochemical test makes it impractical for routine monitoring of environmental

samples (Reynolds, 2004). An overgrown culture makes it hard to exhibit expected results.

5.3 Molecular Method

Some isolates were not able to be identified using molecular method because due to time constrain. The recent advances in molecular biology have enable identification of prokaryotic organisms on the basis of highly conserved genes and proteins 16S rRNA (Torsvik and Ovreas, 2007).

A number of reports have demonstrated that 16S rRNA gene sequence analysis improves the identification of bacteria compared to conventional method of identification (Li *et al.*, 2005). According to Fredricks *et al.* (2005), bacteria in complex microbial communities like soil can be efficiently identified by characterizing their ribosomal RNA (rRNA).

5.4 Significant of Locally Isolated Soil Bacteria

From these studies, it is learned that soil contains great diversity of bacteria. *Chromobacterium violaceum* commonly found in soil and water in tropical and subtropical climates is a rare cause of severe, often fatal human disease (Siqueira *et al.*, 2005). First human case was described in Malaysia in 1927 and less than 150 human cases have been reported worldwide, mainly in Asia, the United States, Australia and Africa. It can rarely cause human infections like septicemia, liver

abscess, skin lesions, dental infections, urinary tract infection and diarrhea (Shenoy *et al.*, 2002).

Curpriavidus basilensis is a versatile carbon metabolism and their property of resistance to heavy metal makes it as a good heavy metal pollutant degrader (Schoonbeek *et al.*, 2007).

Member of genus bacilli are known for their production of secondary metabolite. *Bacillus* species produce many kinds of antibiotics which share a full range of antimicrobial activity such as acitracin, pumulin and gramicidin (Al-Janabi, 2006). In addition, due to the fact that *Bacillus* species have produced antibiotics in the soluble protein structure and that these antibiotics have been found to be cheaper and more effective in studies conducted to date, these microorganisms are preferable for commercial production (Aslim *et al.*, 2002).

CHAPTER 6

CONCLUSION

Seven species which are *Chromobacterium violaceum*, *Cupriavidus basilensis*, *Bacillus subtilis*, *Bacillus cereus*, *Bacillus pycnus* and *Bacillus thuringiensis* successfully isolated and identified using 16S rRNA sequencing. Diverse morphological, biochemical and differentiating properties have been observed in the range of bacteria that have been successfully isolated from soil. Isolated strains must were classified on the basis of the 'polyphasic' approach where this pragmatic approach of deploying both conventional method and molecular tool was used to identify an isolate from soil and place them in an accurate position in the microbial world. It is clear that molecular biology tools are essential for better understanding of the composition and function of soil inhabiting bacteria. This tool helps to identify bacteria in a short period of time without much limitation compare to conventional time which are tedious and time consuming. The successful application of molecular technique based on novel 16S rRNA target gene would be a powerful tool to study the distribution and abundance of soil inhabiting bacteria in complex environment. Discovery of novel 16S rRNA gene sequence from environmental samples provides a window into a world of microbial diversity that is astonishing in its magnitude. Molecular identification potentially more precise than those conventional methods, and this method is also rapid but it is important to combine both methods for efficient and precise identification

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APPENDICES

APPENDIX 1

SAMPLING SITE

Sampling at the depth of 0-15 cm

APPENDIX 2

DNA sequencing

Isolate: L1
BLAST result: *Chromobacterium violaceum*

GATCCCACCGTGGTAGCGGCCTCCTTGCGGTTAGCCTACCCACTTCTGGT
GAAACTCACTCCCATGGTGTGACGGGCGGTGTGTACAAGACCCGGGAAC
GTATTCACCGCAGCATGCTGATCTGCGATTACTAGCGATTCCGACTTCAC
GCACTCGAGTTGCAGAGTGCGATCCGGACTACGATCGGTTTTCTGAGATT
AGCTCCACCTCGCGGCTTGGCAACCCTCTGTACCGACCATTGTATGACGT
GTGAAGCCCTGCTCATAAGGGCCATGAGGACTTGACGTCATCCCCACCTT
CCTCCGGTTTGTCACCGGCAGTCTCATTAGAGTGCCCAACTTAATGATGG
CAACTAATGACAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCA
CGACACGAGCTGACGACAGCCATGCAGCACCTGTGTTACGGCTCCCTTTC
GGGCACCAAGCCATCTCTGGCAAGTTCCGTACATGTCAAGAGCAGGTAA
GGTTTTTCGCGTTGCATCGAATTAATCCACATCATCCACCGCTTGTGCGG
GTCCCCGTCAATTCCTTTGAGTTTTAACCTTGCGGCCGTACTCCCCAGGCG
GTCAACTTCACGCGTTAGCTACGCTACCAAGGATTCAAACCCCCAACAGC
TAGTTGACATCGTTTAGGGCGTGGACTACCAGGGTATCTAATCCTGTTTG
CTCCCCACGCTTTCGTGCATGAGCGTCAGTGTCATCCCAGGGGGCTGCCT
TCGCCATCGGTATTCCTCCACATCTCTACGCATTTCACTGCTACACGTGGA
ATTCTACCCCCCTCTGACGCACTCTAGCTGTGCAGTCTCCAATGCCGTTCC
CAGGTTAAGCCCGGGGCTTTCACATCAGACTTGCACAACCGCCTGCGCAC
GCTTTACGCCCAGTAATTCCGAATAACGCTCGCACCCTACGTATTACCGC
GGCTGCTGGCACGTAGTTAGCCGGTGCTTATTCTTCAGGTACTGTCATCC
CCGCCGGGTATTAAACAAACGGGATTTCCTCCCCTGACAAAAATCCTTTA
CAACCCGAAAGGCTTTTTAAAAACCCCGGAAATGACTGGGAACAAGGGT
TGCCCCATTGACAA

Isolate: L2
BLAST result: *Bacillus subtilis*

GTCACCTTAGGCGGCTAGCTCCTTACGGTTACTCCACCGACTTCGGGTGT
TACAAACTCTCGTGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGT
ATTCACCGCGGCATGCTGATCCGCGATTACTAGCGATTCCAGCTTCATGT
AGGCGAGTTGCAGCCTACAATCCGAACTGAGAATGGTTTTATGGGATTGG
CTTGACCTCGCGGTCTTGCAGCCCTTTGTACCATCCATTGTAGCACGTGTG
TAGCCCAGGTCATAAGGGGCATGATGATTTGACGTCATCCCCACCTTCCT
CCGGTTTGTCACCGGCAGTCACCTTAGAGTGCCCAACTTAATGCTGGCAA
CTAAGATCAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGA
CACGAGCTGACGACAACCATGCACCACCTGTCACTCTGTCCCCCGAAGGG
GAACGCTCTATCTCTAGAGTTGTCAGAGGATGTCAAGACCTGGTAAGGTT
CTTCGCGTTGCTTCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCC
CCGTCAATTCCTTTGAGTTTCAGTCTTGCGACCGTACTCCCCAGGCGGAG
TGCTTAATGCGTTAGCTGCAGCACTAAAGGGCGGAAACCCTCTAACACTT
AGCACTCATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGC
TCCCCACGCTTTCGCGCCTCAGCGTCAGTTACAGACCAAAAAGCCGCCTT
CGCCACTGGTGTTCCTCCACATCTCTACGCATTTCACCGCTACACGTGGA
ATTCCGCTTTTCTCTTCTGCACTCAAGTTCCCCAGTTTCCAATGACCCTCC
ACGGTTGAGCCGTGGGCTTTCACATCAGACTTAAAAAAACCGCCTGCGCG
CGCTTTACGCCCAATAATTCCGGATAACGCTTGCCACCTACGTATTACCG
CGGCTGCTGGCACGTAGTTAGCCGTGGCTTTTTGGTTAGGTACCGTCAAG
GTACGAGCAGTTACTTTCGTACTTGTTCTTCCCTAACAACAGAGTTTTACG
ACCCGAAAGCCTTCATCACCCACGCGGGGTTGCTCCCGTCAAACTTTCGT
CCAATGCGGAAAGATTCTCTAAATGCCTGGCCTCCCGTAAG

Isolate: L3
BLAST result: *Bacillus pycnus*

TGTATCCCACCTTCGGCGGCTGGCTCCAAAAGGTTACCTCACCGACTTCG
GGTGTTACAAACTCTCGTGGTGTGACGGGCGGTGTGTACAAGGCCCGGG
AACGTATTCACCGCGGCATGCTGATCCGCGATTACTAGCGATTCCGGCTT
CATGTAGGCGAGTTGCAGCCTACAATCCGAACTGAGAACGGTTTTATGAG
ATTAGCGTGCTCTCGCGAGTTAGCGACTCTTTGTACCGTCCATTGTAGCA
CGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGACGTCATCCCCAC
CTTCCTCCGGTTTGTCACCGGCAGTCACCTTAGAGTGCCCAACTAAATGA
TGGCAACTAAGATTAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATC
TCACGACACGAGCTGACGACAACCATGCACCACCTGTCACCAATGTCCCC
GAAGGGAAAACTCTATCTCTAGAGCGGTCACTGGGATGTCAAGACCTGG
TAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATGCTCCACCGCTTGTGC
GGGCCCCCGTCAATTCCTTTGAGTTTCAATCTTGCGATCGTACTCCCCAGG
CGGAGTGCTTAATGCGTTAGCTGCAGCACTAAGGGGCGGAAACCCCCTA
ACACTTAGCACTCATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCT
GTTTGCTCCCCACGCTTTCGCGCCTCAGTGTCAGTTACAGACCAGACAGT
CGCCTTCGCCACTGGTGTTCCTCCAAATCTCTACGCATTTCACCGCTACAC
TTGGAATTCCACTATCCTCTTCTGCACTCAAGTTCCCCAGTTTCCAATGAC
CCTCCACGGTTGAGCCGTGGGCTTTCACATCAAACTTAAAAAACCACCTG
CGCGCGCTTTACCCCCAATAAATCCGGACAACGTTTGCCACCTACGTATT
ACCGCGGCTGTGGACCGACTTAACCTGGGCTTTCTAATAAGGTACCGTGG
AGGTACTTCATTTCTAACAACTTATCTTCCCTAAAAAAAAAATTTTACATC
CCAAAACTTTTTTTTACTTCAA

Isolate: L9
BLAST result: *Bacillus thuringiensis*

GTCACTTAGGCGGCTGGCTCCAAAAAGGTTA CCCAC GA TTCGGGTGT
TACAAACTCTCGTGGTGTGACGGGCGGTGTGTACAAGGC CGGGAA GT
ATTCACCGCGGCATGCTGATCCGCGATTACTAGCGATTCCAGCTT ATGT
AGGCGAGTTGCAGCCTACAATCCGAACTGAGAACGGTTTTATGAGATTA
GCTCCACCTCGCGGTCTTGCAGCTCTTTGTACCGTCCATTGTAGCACGTGT
GTAGCCCAGGTCATAAGGGGCATGATGATTTGACGTCATCCCCACCTTCC
TCCGGTTTGTCACCGGCAGTCACCTTAGAGTGCCCAACTTAATGATGGCA
ACTAAGATCAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACG
ACACGAGCTGACGACAACCATGCACCACCTGTCACTCTGCTCCCGAAGG
AGAAGCCCTATCTCTAGGGTTTTCAGAGGATGTCAAGACCTGGTAAGGTT
CTTCGCGTTGCTTCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCC
CCGTCAATTCCTTTGAGTTTCAGCCTTGCGGCCGTACTCCCCAGGCGGAG
TGCTTAATGCGTTAACTTCAGCACTAAAGGGCGGAAACCCTCTAACACTT
AGCACTCATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGC
TCCCCACGCTTTCGCGCCTCAGTGTCAGTTACAGACCAGAAAGTCGCCTT
CGCCACTGGTGTTCCTCCATATCTCTACGCATTTCACCGCTAAAATGGAA
TTCCACTTTCCTCTTCTGCACTCAAGTCTCCCAGTTTCCAATGACCCTCCA
CGGTTGAGCCGTGGGCTTTCACATCAAACTTAAAAAAACCACCTGCGCGC
GCTTTACCCCCAATAATTCCGGATAACGTTTGCACCTACGTATTACCCCGT
TTGCTGGCACGGAATTAACCGCGGTTTTCTGGTGAGTACCCGCAAGGTGC
GACTTATTCAATAAACTTTTTCTTCCTAAAAACAAAAATTTTACACCTAA
ACCCTTTCTTCACTTCAC

Isolate: L10
BLAST result: *Cupriavidus basilensis*

ACCTCCCGTGGTAATCGCCCTCCTTGCGGTTAGGCTAACTACTTCTGG A
AAACCCACTCCCATGGTGTGACGGGCGGTGTGTACAAGA CCGGGAACG
TATTCACCGCGGCATGCTGATCCGCGATTACTAGCGATTCCAGCTTCACG
TAGTCGAGTTGCAGACTACGATCCGGACTACGATGCATTTTCTGGGATTA
GCTCCCCCTCGCGGGTTGGCAACCCTCTGTATGCACCATTGTATGACGTG
TGAAGCCCTACCCATAAGGGCCATGAGGACTTGACGTCATCCCCACCTTC
CTCCGGTTTGTCACCGGCAGTCTCTCTAGAGTGCTCTTGCGTAGCAACTA
AAGACAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACA
CGAGCTGACGACAGCCATGCAGCACCTGTGTCCACTTTCCCTTTCGGGCA
CCTGATGCATCTCTGCTTCGTTAGTGGCATGTCAAGGGTAGGTAAGGTTT
TTCGCGTTGCATCGAATTAATCCACATCATCCACCGCTTGTGCGGGTCCC
CGTCAATTCCTTTGAGTTTTAATCTTGCGACCGTACTCCCCAGGCGGTCAA
CTTCACGCGTTAGCTACGTTACTGAAAAAATGAATCCCCAACAACTATTT
GACATCGTTTAGGGCGTGGACTACCAAGATATCTAATCCTGTTTGCTCCC
CACCCTTTCTTGCATGAACGTCAGTGACTTCCCACGAGGCTGCCTTCTCCA
TCATATTCCTCCACATCTCTACTATTTCACTGCTACACGTGGAATTCTACC
CCCCTCTTACATACTCTACCATTTCATCACAAACACAATTCCCATATAATC
TGCGGGGATTTCACTCTCTTCTTATATAAAACCACCTGCCCCACCTTTTAT
TCCCAAATTTCTATAAACATCCTACCCTACCTATTA

Isolate: L12
BLAST result: *Bacillus cereus*

GTCACCTTAGGCGGCTGGCTCCAAAAAGGTTACCCCACCGACTTCGGGTG
TTACAAACTCTCGTGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACG
TATTCACCGCGGCATGCTGATCCGCGATTACTAGCGATTCCAGCTTCATG
TAGGCGAGTTGCAGCCTACAATCCGAACTGAGAACGGTTTTATGAGATTA
GCTCCACCTCGCGGTCTTGCAGCTCTTTGTACCGTCCATTGTAGCACGTGT
GTAGCCCAGGTCATAAGGGGCATGATGATTTGACGTCATCCCCACCTTCC
TCCGGTTTGTCACCGGCAGTCACCTTAGAGTGCCCAACTTAATGATGGCA
ACTAAGATCAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACG
ACACGAGCTGACGACAACCATGCACCACCTGTCACTCTGCTCCCGAAGG
AGAAGCCCTATCTCTAGGGTTTTCAGAGGATGTCAAGACCTGGTAAGGTT
CTTCGCGTTGCTTCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCC
CCGTCAATTCCTTTGAGTTTCAGCCTTGCGGCCGTACTCCCCAGGCGGAG
TGCTTAATGCGTTAACTTCAGCACTAAAGGGCGGAAACCCTCTAACACTT
AGCACTCATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGC
TCCCCACGCTTTCGCGCCTCAGTGTCAGTTACAGACCAGAAAGTCGCCTT
CGCCACTGGTGTTCCTCCATATCTCTACGCATTTCACCGCTACACATGGA
ATTCCACTTTCCTCTTCTGCACTCAAGTCTCCCAGTTTCCAATGACCCTCC
ACGGTTGAGCCGTGGGCTTTCACATCAGACTTAAGAAACCACCTGCGCGC
GCTTTACGCCCAATAATTCCGGATAACGCTTGCCACCTACGTATTACCGC
GGCTGCTGGCACGTAGTTAAGCCGTGCTTTCTGGTTAGGTACCGTCAAGG
TGCCAGCTAATTCAACTAAAACTTGTTCTTCCCTAACAACAGAGTTTTAC
GACCCGAAAAGCCTTCTATCCCTCACCGCGGCGTTTCTCCGTCAAAATTT
CTTTCCATTTGGGGAAAATTTCCCTCTACGCCTGCCTCCCCGAAAAGAAT
CTGG

Isolate: L20
BLAST result: *Chromobacterium violaceum*

ATCCACCGTGGTAGCGGCCTCCTTACGGTTAGCCTACCCACTTCTGGTGA
AACTCACTCCCATGGTGTGACGGGCGGTGTGTACAAGACCCGGGAACGT
ATTCACCGCAGCATGCTGATCTGCGATTACTAGCGATTCCGACTTCACGC
ACTCGAGTTGCAGAGTGCGATCCGGACTACGATCGGTTTTCTGAGATTAG
CTCCACCTCGCGGCTTGGCAACCCTCTGTACCGACCATTGTATGACGTGT
GAAGCCCTGCTCATAAGGGCCATGAGGACTTGACGTCATCCCCACCTTCC
TCCGGTTTGTCACCGGCAGTCTCATTAGAGTGCCCAACTTAATGATGGCA
ACTAATGACAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACG
ACACGAGCTGACGACAGCCATGCAGCACCTGTGTTACGGCTCCCTTTCGG
GCACCAAGCCATCTCTGGCAAGTTCCGTACATGTCAAGAGCAGGTAAGG
TTTTTCGCGTTGCATCGAATTAATCCACATCATCCACCGCTTGTGCGGGTC
CCCGTCAATTCCTTTGAGTTTTAACCTTGCGGCCGTACTCCCCAGGCGGTC
AACTTCACGCGTTAGCTACGCTACCAAGGATTCAAACCCCCAACAGCTAG
TTGACATCGTTTAGGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTC
CCCACGCTTTCGTGCATGAGCGTCAGTGTCATCCCAGGGGGCTGCCTTCG
CCATCGGTATTCCTCCACATCTCTACGCATTTCACTGCTACACGTGGAATT
CTACCCCCCTCTGACGCACTCTAGCTGTGCAGTCTCCAATGCCGTTCCCA
GGTTAAGCCCGGGGCTTTCACATCAGACTTGCACAACCGCCTGCGCACGC
TTTACGCCCAGTAATTCCGATTAACGCTCGCACCCTACGTATTACCGCGG
CTGCTGGCACGTAATTAGCCGGGGCTTATTCTTCAAGTACTGTCATCCCC
GCCGGGTATTAACCAACGGGATTTTCTCCCTGACAAAAGTACATTTTACA
CCCCGAAGGCTTCCTTTCAAAACCCGCGGGAATGGCTTGAATCAGGGATG
GTCACCCATTTGTCAAAAA

APPENDIX 3

PCR product

Provided by manufacter	working concentration	Per sample	Master mix
10 x PCR buffer	1x	2 µL	10 µL
dNTP's	0.2	0.4 µL	2 µL
Primers – forward	0.5 (optimised)	1	5 µL
– reverse			5 µL
$MgCl_2$	2.5	2 µL	10 µL
Taq-polymerase	0.025	0.1 µL	0.5 µL
Distilled water (dH_20)	Top up	13.5 µL	67.5 µL

APPENDIX 4

Materials for gel electrophoresis

1) Working solution of the nile blue dye

Stock solution of nile blue dye = 1.6 mg/mL

= 0.16g/100mL

To prepare 40 ml of nile blue dye = $\frac{0.16 \times 40}{100}$

= 0.064 g of stock solution nile blue

2) Prepare 200ml of buffer solution with concentration of TAE is 0.5%

10 x TAE = (200 mL) (0.5)

=10 mL (hence topup 190mL to make 200 mL buffer solution)

3) Agarose gel

i. calculate the volume of the tray

= height x length x width

= 0.5 x 9 x 5.5

= 24.75~ 25 mL

To make 1% agarose gel, 0.25g of agarose boiled in 25mL of buffer solution

Calculate amount of dye inside agarose gel:

(1600µ/mL) (V1) = (1µg/mL) (25mL)

$(V1) = \frac{1 \times 25}{1600}$

= 0.0156 mL x 1000 µL

= 15.6 µL ~ 16 µL

4) Amount of dye inside 175ml of buffer solution

$$(1600\mu g/mL)\,(V1) = (1\mu g/mL)\,(175mL)$$

$$V1 = \frac{175}{1600}$$

$$= 0.1093 \times 1000\ \mu L$$

$$= 109\ \mu L$$

PUBLICATION OF PROJECT UNDERTAKING

This is to certify that I have no objection to publish the project entitled **"Isolation and Identification of Bacteria from Acid Soil (Bekenu Series) in Universiti Putra Malaysia Bintulu Sarawak Campus"** by the supervisor in a joint authorship. However, it has to be evaluated by the Faculty of Agriculture and Food Sciences, Universiti Putra Malaysia Bintulu Sarawak Campus and published in the form approved by the Faculty.

NEELAVENI BALA MOHAN

Date 5th May 2009.