BACTERIAL DIVERSITY OF ELEPHANT DUNG USING 16S rRNA GENE SEQUENCE ANALYSIS

by

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A report submitted in fulfillment of the requirements for the degree of Bachelor of Applied Science (Natural Resources Science) with Honours

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DECLARATION

I declare that this thesis entitled "Bacterial Diversity of Elephant Dung Using 16S rRNA Gene Sequence Analysis" is the result of my own research except as cited in the references. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.

Signature : _____

Name : Tan Shan Wen

Date : _____

APPROVAL

"I hereby declare that I have read this thesis and in our opinion this thesis is sufficient in terms of scope and quality for the award of the degree of Bachelor of Applied Science (Natural Resources Science) with Honors"

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Date	:	……..

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Bacterial Diversity of Elephant Dung using 16S rRNA Gene Sequence Analysis

ABSTRACT

The objective of this study was to identify the bacterial diversity of dung from Asian elephant (*Elephas maximus*). The important uses of elephant dung have been identified through various studies, but the knowledge for the bacterial diversity of elephant dung is very limited and insufficient. In this study, 16S rRNA gene sequence was used to identify the bacterial distribution and diversity of elephant dung. Thirteen pure bacterial isolates labelled as ED1, ED2, ED3, ED4, ED5, ED6, ED7, ED8, ED9, ED10, ED11, ED12, and ED13 were obtained and basic characterisation of bacteria by Gram staining was performed. Polymerase Chain Reaction (PCR) of the bacteria isolates was done by targeting 16S rRNA gene. Unpurified Polymerase Chain Reaction (PCR) products of ED2, ED5 and ED7 were sent for purification and sequencing. The obtained sequences were compared with the sequences available in GenBank database. BLAST was used to perform identification of bacteria based on the percentage of similarity. Phylogenetic analysis was done by constructing a phylogenetic tree by using Neighbor-Joining (NJ) method. The obtained sequences were BLAST and the results of their phylogenetic analysis showed that ED2 has 90% similarity with *Exiguobacterium profundum*, ED5 has 98% similarity with *Flavobacterium lutescens*, and ED7 has 99% similarity with *Micrococcus luteus*. *Micrococcus luteus* is cellulolytic bacteria which can produce cellulose enzyme to degrade the cellulose found in plant cell wall. The results obtained from this study provided a preliminary result and further studies of elephant dung is needed to be done for more understanding of microbial diversity of elephant dung. The findings of this study focus on determining the ecological significance of bacteria found in elephant dung.

Pengenalan Keanekaragaman Bakteria dari Najis Gajah dengan Menggunakan Analisis gen 16S rRNA

ABSTRAK

Matlamat kajian ini adalah untuk megenalpasti keanekaragaman bakteria dari najis gajah asian (*Elephas maximus*). Kegunaan utama najis gajah telah dikenal pasti melalui pelbagai kajian, tetapi pengetahuan untuk kepelbagaian bakteria najis gajah adalah sangat terbatas dan tidak mencukupi. Dalam kajian ini, gen 16S rRNA telah digunakan untuk mengenal pasti pembahagian bakteria dan kepelbagaian bakteria dari najis gajah. Sejumlah tiga belas isolat bakteria tulen yang bernama ED1, ED2, ED3, ED4, ED5, ED6, ED7, ED8, ED9, ED10, ED11, ED12, and ED13 telah diperoleh dan pencirian bakteria asas oleh pewarnaan Gram telah dilakukan. Reaksi Rantai Polimerase (PCR) dari isolat bakteria telah dilakukan dengan mensasarkan pada gen 16S rRNA. Produk Reaksi Rantai Polimerase (PCR) bernama ED2, ED5 and ED7 yang tidak belum dibersihkan telah dihantar untuk pembersihan dan penjujukan DNA. Urutan yang diperoleh dibandingkan dengan urutan yang tersedia dalam pangkalan data GenBank. BLAST telah digunakan untuk mengenal pasti bakteria berdasarkan peratusan persamaan. Analisis Phylogenetic dilakukan dengan membina pokok phylogenetic melalui kaedah Neighbor-Joining (NJ). Urutan yang diperoleh dari BLAST dan hasil analisis phylogenetic telah menunjukkan bahawa ED2 mempunyai kesamaan sebanyak 90% dengan *Exiguobacterium profundum*, ED5 mempunyai persamaan sebanyak 98% dengan *Flavobacterium lutescens*, dan ED7 mempunyai kesamaansebanyak 99% dengan *Micrococcus luteus*. *Micrococcus luteus* adalah bateria yang dapat menghasilkan enzim selulosa untuk merendahkan selulosa yang terdapat di dinding sel tumbuhan. Keputusan yang diperolehi dari kajian ini memberikan keputusan awal dan kajian lanjut mengenai najis gajah sangat diperlukan untuk lebih memahami keanekaragaman mikroba najis gajah. Penemuan kajian ini menumpukan pada kepentingan ekologi bakteria yang terdapat dalam najis gajah.

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LIST OF ABBREVIATIONS

16S	16 Svenberg unit(s)
AGE	Agarose gel Electrophoresis
BLAST	Basic Local Alignment Search Tool
CTAB	Cetyltrimethylaammonium bromide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
HCl	Hydrochloric acid
IUCN	International Union for Conservation of Nature
$MgCl_2$	Magnesium chloride
mM	Millimolar
n.d.	No date
NaCl	Sodium chloride
NCBI	National Center for Biotechnology Information
PCR	Polymerase Chain Reaction
pmol/ µL	Picomol per microliter
rRNA	Ribosomal ribonucleic acid
SDS	Sodium dodecyl sulfate
TAE	Tris-acetate-EDTA
TE	Tris-EDTA
Tris	tris(hydroxymenthyl)aminomethane
µL	Microliter(s)

LIST OF SYMBOLS

ºC	Degree(s) Celcius
%	Percent
≤	Less than or equal to
≥	Greater than or equal to

CHAPTER 1

INTRODUCTON

1.1 Background of Study

Asian elephant (*Elephas maximus*), is known as the largest terrestrial animal in the world is distributed in Southeast Asia. Asian elephant mainly feeds on vegetation such as grasses, bushes, fruits, tree bark and roots. Elephants are large herbivores which will consume about 200-250kg of food daily that allow them to produce about 50kg of dung. Elephant dung can be useful in many ways as it can be used to repel mosquitos, process into eco-friendly paper and use as a mild pain killer and remedy for a bleeding nose (Brough, 2015). The important uses of elephant dung have been identified through various studies, but the knowledge for the bacterial diversity of elephant dung is very limited and insufficient. Recent studies of elephant dung are mainly focused on the specific uses of bacteria such as cellulose degrading bacteria that can be found in the elephant dung via isolation (Saha, 2015) and study related to biohydrogen production by elephant dung (Fangkum & Reungsang, 2011). Based on these studies, bacteria that are commonly found are from Genus *Lactobacillus* and *Bifidobacterium,* and also *Escherichia coli* and *Salmonella*.

This study was carried out in order to gather more information about the bacterial distribution and diversity that can be found in elephant dung. Hereby, the

results of this study showed the diversity of bacteria found in Asian elephant (*Elephas maximus*) identified using 16S rDNA gene sequence analysis.

1.2 Problem Statement

There is limited study about the types of bacteria and no detailed analysis of elephant dung has been done yet. Therefore, lack of knowledge and data regarding to elephant dung has led to this study to be carried out.

1.3 Objectives

1. To isolate the bacteria found in the elephant dung.
2. To characterize the bacteria by using Gram staining method and microscopy technique.
3. To identify the bacterial diversity of elephant dung using 16S rDNA gene sequence analysis.

1.4 Scope of Study

The scope of study is about the study of bacterial diversity. This study is needed to carry out in order to gather more information about the bacterial abundance and diversity that can be found in elephant dung.

1.5 Significance of Study

The significant of study is about the gathering of more information about the diversity of bacteria that can be found in elephant dung. Besides, this study is also important in providing elephant dung bacteria database and identification of the role of beneficial bacteria in elephant dung which can help in further studies.

1.6 Study Area

The study area is located at Gunung Basor, Jeli, Kelantan. It is located at latitude 5° 35' 57.1" (5.5992°) north and Longitude: 101° 48' 31.3" (101.8087°) east. Figure 1.1 shows the map of Gunung Basor, Jeli, Kelantan.

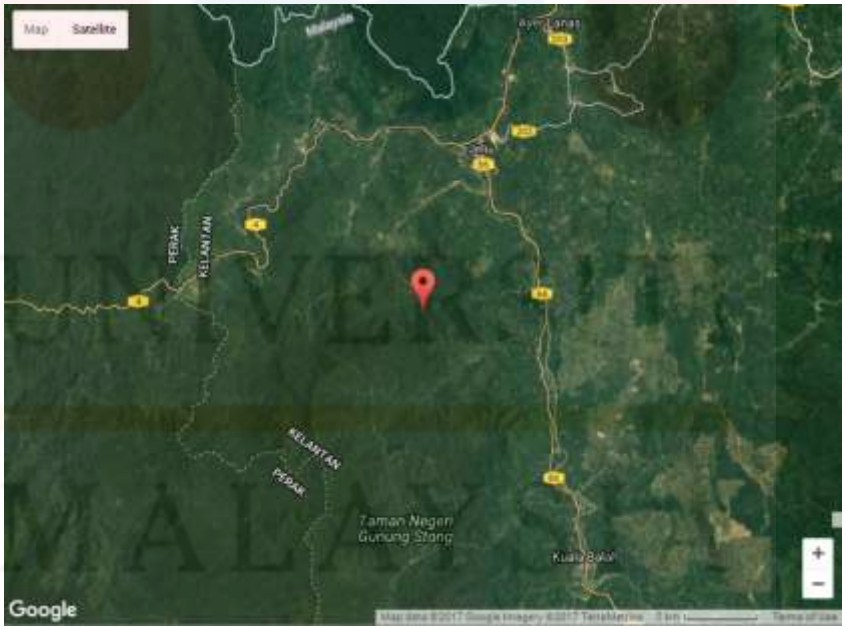

Figure 1.1: Map of Gunung Basor, Jeli, Kelantan

CHAPTER 2

LITERATURE REVIEW

2.1 Asian Elephant

Asian elephant (*Elephas maximus*) is known as the largest terrestrial mammal on the Earth. Asian elephants have ears which are smaller in size and more rounded on top and flat along the bottom compared with African elephant that have ears that shaped like African continent. The Asian elephant has pink patches on their forehead, ears and their skin colour is ranges from dark grey to brown. The habitat for Asian elephant is forests, which is tropical and subtropical with moist and dry broadleaf (WWF, 2017). The life span of Asian elephant in the wild is about 60 to 70 years. They usually have the height of about 6.6 to 9.8 feet, and weighted for range from 2.25 to 5.5 tons by depending on their body sizes (NATGEO, 2017).

However, due to anthropogenic and environmental issues such as habitat loss, human-wildlife conflict, poaching and capture, this has threatened the population of Asian elephant. They have been listed as Endangered by the IUCN as their population size is reducing for at least 50% over the last three generations (IUCN, 2008). Figure 2.1 shows the picture of Asian elephant.

Figure 2.1: The Asian elephant (*Elephas maximus*) (Source: Diane, 2010)

2.2 Diet and Eating Habits of Asian Elephant

Asian elephants are herbivores which consume grasses, roots, fruit and bark. They will spend for about 16 to 18 hours per day on feeding, which allow them to consume about 149 to 169 kg of vegetation daily. Asian elephants need to eat large amount of food daily as they can only digest and make use of 40% of what they have consumed (Meyer, 2015). This is mainly due to poor efficiency of elephant's digestive system at absorption of nutrients (EREC, 2013). For water, they need to consume at least 70 liters of water per day. Besides, in order to balance their diet, they will dig up the soil into pieces by using their tusks, and then consume it so they are able to obtain salt and minerals from it. Tree bark is the favourite food source for elephants. However, a recent study about the food habits of Asian elephants in a rainforest of Peninsular Malaysia showed that elephants prefer grass more, which

may cause changes in the bacterial diversity found in elephant dung (Yamamoto-Ebina *et al.*, 2016).

There are previous study shows that the host diet will influence bacterial diversity (Ley *et al.*, 2008). Asian elephants are herbivores which only consume on plants. Therefore, it is predicted that cellulose degrading bacteria can be found in elephant dung as the bacteria can produce cellulase to degrade the cellulose that contain in the plant cell wall (Saha, 2015).

2.3 Deoxyribonucleic acid (DNA) Extraction from Elephant Dung

In this study, elephant dung is used to study the bacterial diversity and also the phylogenetic relationship of the bacteria that found in the elephant dung. Deoxyribonucleic acid (DNA) is found inside the nucleus of every cell in an individual's body (Paul, 2011) and it is most abundant in tissue and fluids. It is difficult to obtain tissue and fluids or blood from elephant as it may harm and cause stress to the animal. Researchers find out a more suitable method to collect DNA samples of mammals which is called non-invasive genetic sampling (Schuttler, 2014). Non-invasive genetic sampling is a method that enables researchers to obtain DNA without having any contact with the animals. Sources such as dung, hair, urine, and saliva are collected to obtain the DNA (Waits & Paetkau, 2005). The dung should be freshly collected (within 24 hours) as the bacteria activity may degrade the DNA.

Deoxyribonucleic acid (DNA) extraction is the process that involved separation of membranes, proteins, and other cellular material that are found in the cell (Elkins, 2013). In this study, the DNA is extracted from the cells that contained in elephant dung. In order to conduct further analysis of DNA, it needs to be

extracted or isolated and purified to a certain extent. It is an essential technique in molecular biology as it can be applied in construction of genomic or sequencing library and PCR analysis (Surzycki, 2000).

The first and the well-known DNA extraction method was first described by Marmur in 1961 (Bazzicalupo & Fancelli, 1997). The methods of DNA extraction varies depending on the bacterial species from which the DNA must be extracted, sources for DNA extraction, sample size, and storage time of sample. Despite there are many different method in extracting DNA, there are similarities among them.

The basic steps in DNA extraction are (i) lysis or break open the cells, (ii) separation of DNA from the other cell components, (iii) precipitation of DNA with alcohol, (iv) purification of DNA and (v) analysis the integrity of DNA (Appalasamy, 2017). During the lysis step, the salt solution containing detergents and enzyme such as Proteinase K is used to dissolve cellular proteins and release DNA through breaking down the cell and nucleus. After lysis step, organic extraction such as phenol and chloroform is used to separation of DNA from the other cell components. During purification step, alcohol such as ethanol or isopropanol is used to rinse DNA in order to remove any remaining unwanted material and cellular debris. The isolated DNA precipitation is then purified with alkaline buffer, which aids in ensuring the optimum quality of DNA. The qualitative analysis of extracted DNA can be analyzed by Agarose Gel Electrophoresis (Shahriar *et al.*, 2011).

According to Elkins (2013), the DNA extraction method consists of four commonly used procedures which are organic extraction, inorganic extraction (Chelex or silica methods), solid phase extraction methods and differential extraction. In this study, organic extraction is used to lyse the cell by using enzymes or reagents

such as lysozyme, Proteinase K and Sodium Dodecyl Sulfate (SDS). In order to obtain an effective DNA extraction, the bacteria's cell wall composition is determined via Gram staining. This is to differentiate the gram type of bacteria into gram positive and gram negative, where methods used for DNA extraction for gram positive bacteria may not be suitable for gram negative bacteria. This is important as it affects the amount and the quality of the DNA extracted that is further used for molecular tests (De Oliveira *et al.*, 2014).

2.4 Polymerase Chain Reaction (PCR)

Polymerase Chain Reaction (PCR) is an automated technique that commonly used in molecular biology for the amplification of specific DNA sequences. This technique is originally developed in 1983 by Kary Mullis. This technique can amplify the quantity of the DNA by synthesizing the new strand of DNA which complementary to the template strand by using enzyme *Taq* DNA polymerase. *Taq* DNA polymerase has the ability of adding nucleotide base onto a preexisting 3'-OH group with the aids of primers.

During PCR reaction, the repetition of 20 to 40 cycles will be done by an automated thermal cycler, which enable to generate billions of copies of specifies DNA sequences only in few hours (Karya, 2015). The wide variety of applications of PCR is vital in genotyping, sequencing, pathogen detection and also paternity testing.

Typically, there are three main steps for PCR cycle, which are denaturation, annealing and extension. During PCR reaction, the automated thermal cycler will heat and cool the reaction mixture rapidly, which allows the three main steps to be

carried out. First is denaturation step, which involved the separation of DNA stands from each other, allowing to produces two single strands of DNA for further replication in the next step of cycle. During this step, the reaction mixture is heated to 94°C for 15 seconds to 2 minutes. Next is annealing step, which allows the primers to anneal to specific DNA sequences at the end of the target sequence. During this step, the temperature is reduced to between 52°C to 58°C for 30 seconds. Lastly the cycle is concludes with extension step. During extension step, the temperature is increased to the range of 70-74°C for 1-2 minutes, which DNA polymerase such as *Taq* polymerase is used to extent the primers by adding nucleotides to produces a new strand of DNA, hence the quantity of DNA in the reaction is doubled (Lorenz, 2012).

2.5 16S ribosomal RNA (rRNA) Gene Sequence

16S ribosomal RNA (rRNA) gene is the gene that can be found in all bacteria. It is the gene that encodes the smaller subunit of the bacterial ribosome (30S subunit) in the RNA component. The reasons that enables 16S rRNA gene sequences to be widely used in study of bacterial phylogeny and taxonomy includes (i) it can be found in all bacteria; (ii) it contains highly conserved region that used for species identification; and (iii) the large 16S rRNA gene sequence (1500 bp) for informatics purposes (Patel, 2001).

16S rRNA gene sequencing is a technique to distinguish and compare bacteria that present within a given sample. Despite all bacteria have highly conserved 16S rRNA genes, there are still variations appeared in the gene sequence, which those variations can be used in bacterial identification. However, not all

regions in 16S rRNA gene are highly conserved (Dale & Park, 2010). It is important to design an universal PCR primers which is target on the conserved regions of 16S rRNA gene as this enable to amplify the gene in a wide range of different bacteria from a single sample (LCSciences, 2017). PCR primers that recognize conserved sequences can be used to amplify the region, and the sequence of the conserved region in between is used for comparative taxonomy, by comparing the sequence obtained with sequence in GenBank (Clarridge, 2004). In this study, bacterial identification of elephant dung will be carried out by obtaining the pure PCR product with contains of 16S gene. Then, the gene were sequenced and aligned against the DNA data base of bacteria (Barghouthi, 2011).

Although 16S rRNA gene sequencing is useful in bacterial identification, there are still limitations such as difficulties in obtaining recognition of novel taxa, high similarity of species that shared identical 16S rRNA gene sequence and too few sequences stored in GenBank (Janda & Abbott, 2007).

2.6 Phylogenetic Analysis

Phylogenetic is the study of the evolutionary relationships of species. The phylogenetic can be analyzed based on the sequence of a common gene or protein. The evolutionary relationships can be represented as branching and treelike diagram which is known as phylogenetic tree. The phylogenetic tree consists of nodes that represent taxonomic units and branches that represent the time estimate of the evolutionary relationships among the taxonomic units (Choudhuri, 2014b). There are five main steps for phylogenetic tree construction which are: (i) Selection of suitable molecular marker, (ii) Multiple sequence alignment, (iii) Model of evolution

selection, (iv) Phylogenetic tree construction, and (v) Reliability of the topology of phylogenetic tree (Choudhuri, 2014a).

There are two major methods for construction of phylogenetic tree, which are distance-based that includes unweighted pair group method with arithmetic mean (UPGMA) and neighbor joining (NJ); and character-based that includes maximum parsimony (MP) and maximum likelihood (ML). Among these methods, neighbor joining (NJ) is the most commonly used method to construct the phylogenetic tree. The principle of this method is to find pairs of operational taxonomic units (OTUs) that minimize the total branch length at each stage of clustering of OTUs starting with a star like tree (Saitou & Nei, 1987a). Recent study shows that there is one time saving method called Bayesian inference method, as this method use user-friendly software to obtain full probability distribution for all parameter values (Douady *et al.*, 2003). However, the computational cost for Bayesian method is high due to its used of large number of parameters in model.

The reliability of the topology of phylogenetic tree is determined by the bootstrap method. Bootstrapping is a computationally performed statistical analysis which involves repeated resampling (with replacement) from the original sample to create many new subsets of pseudosamples that are subjected to the same analysis as the original sample to obtain many bootstrap trees. The topology of these bootstrap trees is compared with that of the original tree to statistically assess the reliability of the original phylogenetic tree. The bootstrap value (%) is then indicated on the braches. The topology of the branch is considered accurate if the bootstrap value is 95% or higher (Choudhuri, 2014b).

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Equipment

The equipment that were used in this study were light microscope, electronic balance, vortex, agarose gel electrophoresis tank, hot stir plate, PCR machine, autoclave machine, pH meter, incubator, laminar flow, fume hood, microwave oven, water bath, freezer, chiller, microcentrifuge, dry bath incubator, Nanodrop Spectrophotometer and UV transilluminator.

3.1.2 Apparatus

The apparatus that were used in this study were zipper bags, petri plates, surgical masks, gloves, inoculating loop, petri dish, Bunsen burner, capped test tubes, micropipette, hockey stick, pipette, slides, conical flasks, media bottles, bijou bottles, beakers, measuring cylinder, cover slides, spatula, micropipette tips (blue, yellow, white), 0.2ml PCR tubes, and 1.5ml microcentrifuge tubes.

3.1.3 Reagents and Chemicals

The reagents and chemicals that were used in this study were nutrient agar powder and nutrient broth powder. For gram staining, 70% ethanol (EtOH), Crystal Violet, Gram's Iodine, acetone and Safranin were used. For DNA extraction, Tris (hydroxymenthyl) aminomethane (Tris) base, ethylenediaminetetraacetic acid (EDTA), chloroform, isoamyl alcohol, sodium chloride (NaCl), sodium dodecyl sulfate (SDS) powder, Cetyltrimethylaammonium bromide (CTAB), lysozyme, Proteinase K, Sodium acetate, isopropanol and 70% ethanol were used. For AGE, TAE buffer, RedSafe™ Nucleic Acid Staining Solution (iNtRON, USA), agarose gel powder, 1kb DNA ladder and loading dye were used. For PCR, PCR reagents containing DNA template, forward and reverse primers, PCR buffer, $MgCl_2$, autoclaved ddH_2O, dNTPs, and *Taq* DNA polymerase were used.

3.2 Methods

3.2.1 Sampling

The sample of elephant dung was collected at Gunung Basor, which is located at Jeli, Kelantan, Malaysia. The elephant dung collected was then kept by putting the elephant dung into the sterile zipper bag and store at freezer at -20ºC.

3.2.2 Preparation of Nutrient Agar and Broth

The type of media that was used for bacteria culturing in this study were nutrient agar and nutrient broth. The nutrient agar medium and nutrient broth were

prepared as described by the manufacturer (Oxoid). First, 1000ml of nutrient agar medium was prepared by using 28g of nutrient agar powder. Then, the nutrient agar powder was transferred to a 2000ml beaker. Next, 1000ml of distilled water was measured and added to the beaker that contained nutrient agar powder. The solution was then boiled and stirred for 1 minute by using a hot plate. The solution was then poured into a 1000ml media bottle and autoclaved at 121ºC for 15 minutes (Oxoid, n.d.). The nutrient agar solution was allowed to cool down to 50 ºC-55 ºC before it was used. This is to reduce the amount of steam condensation on the Petri plate lids after the agar has been poured. The agar solution was poured to sterilized petri plates. The agar was allowed to cool and harden, the plates were then stored in an inverted position.

The preparation of nutrient broth was done by adding 13g of nutrient broth powder to 1000ml of distilled water. The solution was mixed well by stirring the solution using a glass rod. The solution was then transferred into 1000ml of media bottle and autoclaved at 121ºC for 15 minutes. The nutrient broth was cooled and kept in cabinet under room temperature.

3.2.3 Preparation of Elephant Dung Suspension

The serial dilution method was used to prepare elephant dung suspensions. The purpose of using serial dilution method is it can reduce dense culture of cells to a more usable concentration which can be further used to isolate into pure culture of bacteria (Ben-David & Davidson, 2014).

First, ten sterile capped test tubes were prepared and labelled as 10^{0}, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, and 10^{-9}. About 1 g of elephant dung was weighed and

added in sterile capped test tube containing 10 ml of sterilized distilled water (Farouq *et al.*, 2012). The sample was shaken to thoroughly mix with the dilution fluid. This is the 10^0 dilution. Each of the capped test tube contained 9 ml of sterile distilled water. Micropipette was used to transfer 1 ml of the 10^0 dilution to 10^{-1} test tube, and further 1 ml of sample from 10^{-1} dilution is transferred to 10^{-2} dilution blank, then from the 10^{-2} to the 10^{-3}, then from the 10^{-3} to the 10^{-4}. The procedure was repeated until the 10^{-9} dilution (Singh, 2014).

3.2.4 Spread Plate Method

Spread plate method is a technique to plate the liquid sample that containing bacteria, which enable to produce a plate with viable count of isolated bacterial colonies (Sanders, 2012). The sterilized hockey stick was used to spread the sample over the surface of the plate. The hockey stick was sterilized by dipping it into a beaker of 70% ethanol. The hockey stick was then placed in a flame of Bunsen burner for a few seconds to ignite and burn off the ethanol. First, 0.1 ml of 10^0 dilution was transferred to nutrient agar plate and spread by a sterilized hockey stick. Triplicate of spread plate were done for each of the dilution. The steps were repeated by replacing 10^0 dilution with 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8} and 10^{-9} dilution. The plates were inverted and incubated at 37ºC for 24 hours in the incubator.

3.2.5 Streaking Method

In this study, a four quadrant streak method was used. The inoculating loop was sterilized by using Bunsen burner. The sterilized inoculating loop was used to scoop up a single well-isolated colony. The plate was divided into four areas which

were area A, B, C and D. The plate was streak from area A to area B, from area B to area C, from area C to area D. The inoculum was smeared forwards and backwards (zig-zag pattern) over the edge of the nutrient agar plate (area A) which is shown in Figure 3.1. The loop was removed and the lid was replaced. The inoculating loop was sterilized using Bunsen burner and the plate was turned through 90º anticlockwise before the next streak on the plate. After the loop has cool down, the second streak was performed with sterile loop beginning at one end of the first streak pattern (area B). The plate was streaked from area A across the surface of the agar in 3-4 parallel lines to area B. The loop was sterilized and the step was repeated with third streak beginning in the second streak (area C). The loop was sterilized then a fourth streak was performed streak beginning in the third streak and extending into the middle of the plate (area D). The loop was sterilized and the plate was sealed and incubated in an inverted position (Amrita, 2011). Figure 3.1 shows the process of streaking method.

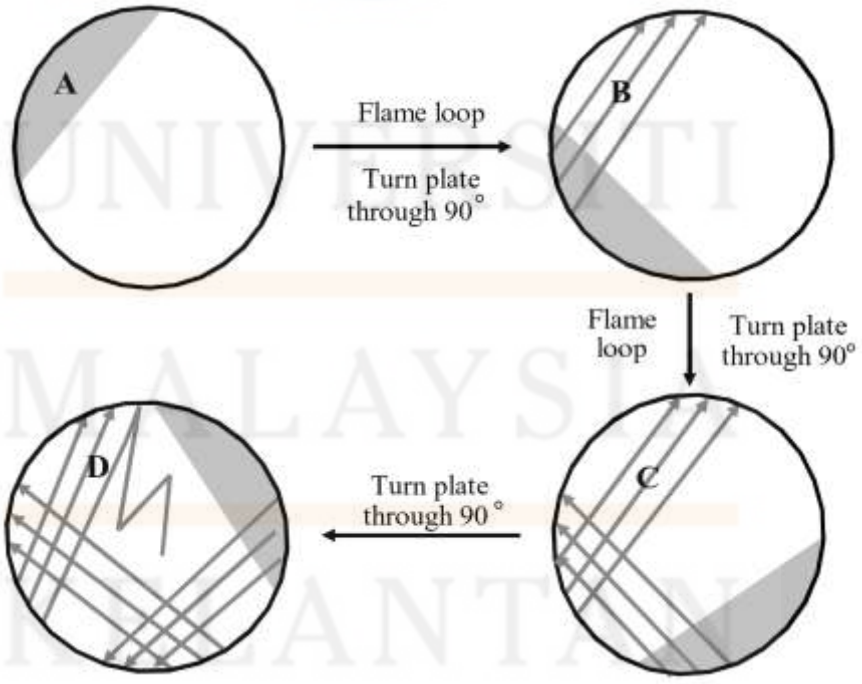

Figure 3.1: The four quadrant streak method (Source: UMK, n.d.)

3.2.6 Gram Staining

Gram staining is a technique that used to distinguishes the bacteria into Gram positive and Gram negative based on the cell wall structure of bacteria (Monica, n.d.). First, a drop of saline solution was placed on the center of the slide with a sterile inoculating loop. Then, the sterilized inoculating loop was used to transfer a minute amount of a bacteria colony from the plate and it was gently stirred into the drop of saline on the slide to create a smear. The smear was then undergoes heat fixing by passing the entire slide through the flame of Bunsen burner for few times with the smear-side up. The smear was then stained by covered with crystal violet stain and leaved for 1 minute. Then, the slide was rinsed carefully with distilled water. Next, the smear was stained with Gram's Iodine for 1 minute. The slide was gently rinsed with distilled water. The smear was rinsed carefully with acetone. Then, the slide was rinsed with distilled water. The smear was counter-stain with Safranin for at least 10 seconds. Lastly, the slide was rinsed with distilled water and air dried before putting it on the microscope (Ann & Marise, 2005). The smear was viewed using a light microscope under oil immersion.

3.2.7 Bacteria Identification by 16S rRNA Gene Sequence

i. DNA Extraction of Elephant Dung

The single colony from the pure bacteria culture was picked and growth in bijou bottles that contained 10ml of nutrient broth. The nutrient broth was incubated for 24 to 48 hours in incubator at 37ºC. 1ml of the overnight bacteria culture was transferred to 1.5ml micro centrifuge tube. The culture was centrifuged at 10000 rpm for 5 minutes. The supernatant was discarded and another 1ml of the overnight

bacteria culture was added into the same micro centrifuge tube. This step was repeated for 3 to 5 times in order to obtain pellet with higher concentration of bacteria cells.

Then, 500 µL of TE buffer was added to resuspend the pellet. 500 µL of cell suspension was transferred to a clean micro centrifuge tube. 20 µL of 50 mg/ml lysozyme was then added and it was incubated for 30 minutes at 37ºC. Next, 40 µL of 10% SDS and 4 µL of 20 mg/ml of Proteinase K were added and mixed well. The cell pellet was incubated for 1 hour at 37ºC. 100 µL of 5M NaCl and 100 µL of CTAB/NaCl solution were added to the mixture and the cell pellet was incubated at 65ºC in water bath for 10 minutes. Then, 500 µL of chloroform/isoamyl alcohol (24:1) was added into the tube and mixed well. The mixture was centrifuged at 13000 rpm for 15 minutes. The supernatant was transferred to a clean micro centrifuge tube. Next, 0.6 volume of ice-cold isopropanol and 0.1 volume of 3M Sodium acetate were added. The tube was inverted for a few times until a white DNA precipitate was formed. The tube was then stored in freezer at -20ºC for 30 minutes. After freezing, the tube was centrifuged at 14500 rpm for 20 minutes. The supernatant was removed and the pellet was washed by 1ml of ice-cold 70% ethanol, then it was centrifuged at 14500 rpm for 5 minutes. The supernatant was removed and the pellet was left for air dry through evaporation in laminar flow.

Lastly, the pellet was resuspended in 50 µL of TE buffer (Appalasamy, 2017). About 5 µL of extracted DNA was run in 1.0% agarose gel for 45 minutes at 80V. The result was visualized under UV illuminator. The remaining extracted DNA was kept in -20ºC freezer for PCR (William *et al.*, 2012).

ii. Agarose Gel Electrophoresis (AGE) of Extracted DNA of Elephant Dung

In order to prepare agarose gel, a 100ml stock of 50x TAE buffer was prepared. 24.2g of Tris-base, 5.71ml of glacial acetic acid and 10ml of 0.5M EDTA solution pH 8.0 were mixed to prepare the 50x TAE buffer. The volume was brought up to 100ml with distilled water. The solution was stored in 100ml media bottle and kept in room temperature.

The 1.0% agarose gel was prepared by using the 1x TAE buffer. 1x TAE buffer was prepared by diluting 20ml of 50x TAE buffer into 980ml of distilled water. To prepare 50ml of electrophoresis agarose gel, 0.50g of agarose powder was mixed with 50ml of 1x TAE buffer in conical flask and it was microwaved for 60 seconds until the agarose powder has completely dissolve in TAE buffer. 5µL of RedSafe™ Nucleic Acid Staining Solution was added to the gel and mixed gently. The gel was poured into a gel mold and an appropriate comb was placed into the gel mold to create the wells. The comb was removed after the gel was solidified and the gel was placed in an electrophoresis tank. Proper amount of 1x TAE buffer was added to cover the surface of the gel. Then, DNA sample was prepared by adding 1µL of 6x loading dye with 5µL of DNA sample. 1µL of 6x loading dye and 5µL of 1kb DNA Ladder was mixed and loaded into the well to serve as molecular weight marker. The DNA sample was run in 1.0% AGE for 45 minutes at 80V. The result of the electrophoresis was viewed under Ultraviolet (UV) illuminator.

iii. Polymerase Chain Reaction (PCR) of DNA for 16S rRNA Gene Amplification

A total volume of 50µL of PCR reagents (Table 3.2) containing DNA template, forward and reverse primers, 1x PCR buffer, $MgCl_2$, autoclaved ddH_2O, dNTPs, and *Taq* DNA polymerase (Promega, USA) were prepared and mixed in the 0.2ml PCR tube. The process of PCR was begin based on the optimized PCR condition which were: (i) Initial denaturation at 95ºC for 2 minutes, (ii) Further denaturation at 95ºC for 15 seconds, (iii) Annealing at 65.6ºC for 30 seconds, (iv) Elongation at 72ºC for 90 seconds and (v) Final elongation at 72ºC for 2 minutes. The optimization of PCR was done with annealing temperature with the range between 50ºC to 60ºC and 60ºC to 70ºC to obtain the optimum annealing temperature.

The PCR amplification of 16S rRNA gene sequence was conducted by using the set of 16S rRNA Primers shown in Table 3.1. Thirty rounds of PCR cycles was carried out by using the PCR thermal cycler. The products were analyzed by AGE and the results were observed under UV illuminator (Daniel *et al.*, 2013). Table 3.2 shows the composition of PCR reagents while Table 3.3 shows the optimized PCR conditions.

Table 3.1: The 16S Primers

Primer name and orientation	Sequence	Melting temperature, Tm (ºC)
16S Forward primer	5'-GAG TTT GAT CCT GGC TCA G-3'	52.9
16S Reverse primer	5'-AGA AAG GAG GTG ATC CAG CC-3'	56.7

Table 3.2: The Composition of PCR reagents

PCR reagents (Stock concentration)	Amount for single PCR reaction	
	Concentration	Volume in one PCR tube (µl)
Autoclaved ddH_2O	-	32.0
5x PCR buffer	1x	5.0
$MgCl_2$ (25mM)	2mM	4.0
dNTPs (10mM)	0.25mM	2.0
Forward primer	10 pmol/ µL	2.0
Reverse primer	10 pmol/ µL	2.0
Taq DNA polymerase (5U/µL)	1 U / 25µL	1.0
DNA template (0.1µg/µL)	<0.5µg/ 50µL	2.0
Total		50

Table 3.3: Optimized PCR conditions

Process	Temperature (°C)	Duration	No. of cycles
Initial Denaturation	95	2 minutes	1
Denaturation	95	15 seconds	30
Annealing	65.6 (Optimized)	30 seconds	30
Extension	72	90 seconds	30
Final Extension	72	2 minutes	1

iv. DNA Sequencing and Phylogenetic Tree Analysis

100µL of unpurified PCR products together with 100µL of forward and reverse primers were sent to Apical Scientific Sdn. Bhd. (Malaysia) for purification and sequencing by using the forward and reverse primers. The type of service chosen for DNA sequencing was DNA sequencing Service Plus. The reverse sequence had to be reverse complemented before combining both of the forward and reversed sequence. The FASTA format of the forward and reverse complement sequence of each sample was combined using online sequence merger (EMBOSS) at http://www.bioinformatics.nl/cgi-bin/emboss/merger. The combined sequences were then analyzed by using BLAST. The nucleotide sequences was uploaded and alignment was done by comparing them against the NCBI nucleotide database (Madden, 2013). Then, the phylogenetic analysis was done by constructing a phylogenetic tree by using MEGA7 by applying Neighbor-Joining (NJ) method with Kimura 2-parameter model with 10000 bootstraps value.

CHAPTER 4

RESULTS AND DISCUSSIONS

4.1 Bacterial Cultivation

In this study, nutrient agar and nutrient broth were used to culture the bacteria from dilution of 10^{0}, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8} and 10^{-9} dilution. A total of 13 bacterial colonies were isolated from the elephant dung. The bacteria isolates were labelled as ED1, ED2, ED3, ED4, ED5, ED6, ED7, ED8, ED9, ED10, ED11, ED12, and ED13. Thirteen bacteria isolates were obtained from spread plates that contained colonies which have different morphology such as shape, elevation, margin and color. The 13 pure bacterial colonies were obtained by performing dilution-streaked onto the fresh nutrient agar plates. There was one bacteria isolated from 10^{-1} dilution, one bacteria isolated from 10^{-2} dilution, three bacteria isolated from 10^{-3} dilution, one bacteria isolated from 10^{-4} dilution, two bacteria isolated from 10^{-5} dilution, two bacteria from 10^{-7} dilution, two bacteria from 10^{-8} dilution and one bacteria from 10^{-9} dilution (Table 4.1). The morphology of bacteria isolates on nutrient agar plates were recorded in Table 4.1.

Nutrient agar was chosen for bacterial cultivation as it can provide nutrients to support growth of variety of types of bacteria. In this study, the cultured bacteria were not yet identified at the beginning. Hence, nutrient agar is the most suitable culture medium for growing unidentified bacteria as it is frequently used for bacteria

isolation. The preliminary step in bacteria identification is through observation of bacteria colony morphology. Colony morphology such as shape, elevation, margin and color can be used to identify bacteria (Reynolds, 2018). Bacteria that have different appearance are usually different in species or genera.

Based on the result in Table 4.1, most of the bacterial colonies appear white or creamy yellow in color, and they were mostly circular in shape. Figure 4.1 shows the bacteria morphology of sample ED3, which has orange colored bacteria and circular in shape. However, there were limitations in identification of bacteria species through observing bacteria morphology under light microscope because bacteria may have similar colony morphology. Hence, further analysis or test such as Gram stain was performed for better result of bacterial identification.

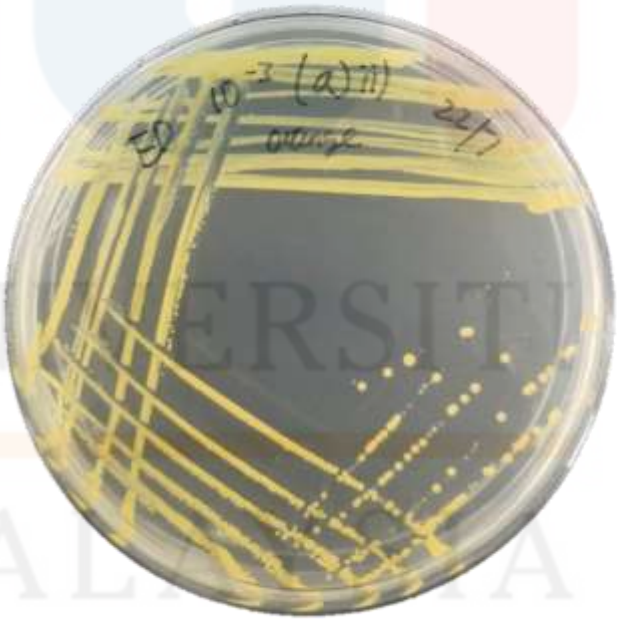

Figure 4.1: The morphology of bacteria colony from sample ED3 in yellow color

Table 4.1: The Morphology of Bacteria Colony.

Sample	Dilution factor	Shape	Elevation	Margin	Color
ED1	10^{-1}	Circular	Flat	Entire	Pale orange
ED2	10^{-2}	Circular	Raised	Entire	Milky white
ED3	10^{-3}	Circular	Raised	Entire	Orange
ED4	10^{-3}	Irregular	Convex	Undulate	Yellowish
ED5	10^{-3}	Circular	Convex	Entire	Light yellow
ED6	10^{-4}	Circular	Flat	Entire	Creamy yellow
ED7	10^{-5}	Circular	Raised	Entire	Creamy yellow
ED8	10^{-5}	Circular	Convex	Entire	Orange
ED9	10^{-7}	Circular	Flat	Entire	Milky white
ED10	10^{-7}	Circular	Raised	Entire	Light orange
ED11	10^{-8}	Irregular	Raised	Undulate	Milky white
ED12	10^{-8}	Circular	Convex	Entire	Creamy yellow
ED13	10^{-9}	Circular	Convex	Entire	Light yellow

4.2 Gram Staining

Gram staining is one of the basic methods used in bacteria identification. The 13 pure bacteria colonies were subjected to Gram staining in order to differentiate the bacteria into Gram positive and Gram negative. The thickness of peptidoglycan wall in bacteria is the key factor that determines the Gram type of the bacteria (Monica, n.d.). The Gram positive bacteria stain purple or violet while Gram negative bacteria stain pink. Gram positive bacteria is has thicker peptidoglycan layer which can retain crystal violet stain during decoloring process while Gram negative bacteria has thin peptidoglycan layer which make the bacteria unable to retain the crystal violet during decoloring process. However, Gram negative appear pink as it can retain the counter stain Safranin (Coico, 2005).

The aim of doing Gram staining in this study is to determine the method used in DNA extraction. The methods used for bacterial genomic DNA extraction for Gram negative bacteria may differ with Gram positive bacteria. The composition of Gram positive cell wall is composed of thick peptidoglycan, teichoic acid, polysaccharides and other protein, while Gram negative cell wall has a thinner and simpler peptidoglycan layer (Oliveira *et al.*, 2014). The successfulness of DNA extraction of bacteria may affected by the different composition found in the bacteria cell wall.

Gram staining can be used as a preliminary step in bacteria characterization and identification. However, it is possible that the Gram staining results do not match with the final identification of bacteria (Thairu *et al.*, 2014). Limitations such as misidentification of the bacteria's morphology, prolonged exposure to the decolorizing agent and appearance of Gram variable may reduce the accuracy in bacteria identification (Wang, 2018). Hence, specific tests which are bacterial DNA

extraction and Polymerase Chain Reaction (PCR) were done for further identification of bacteria.

Table 4.2 shows the results of Gram staining performed on the 13 bacteria isolates from the elephant dung. The results show that there are seven Gram positive isolates and six Gram negative isolates. The Gram positive appear purple while the Gram negative bacteria appear pink. Figure 4.2 shows the result of Gram stain of sample ED8 and ED11. Figure 4.2 shows that sample ED8 is Gram negative bacteria which stained pink while sample ED11 is Gram positive bacteria which stained purple.

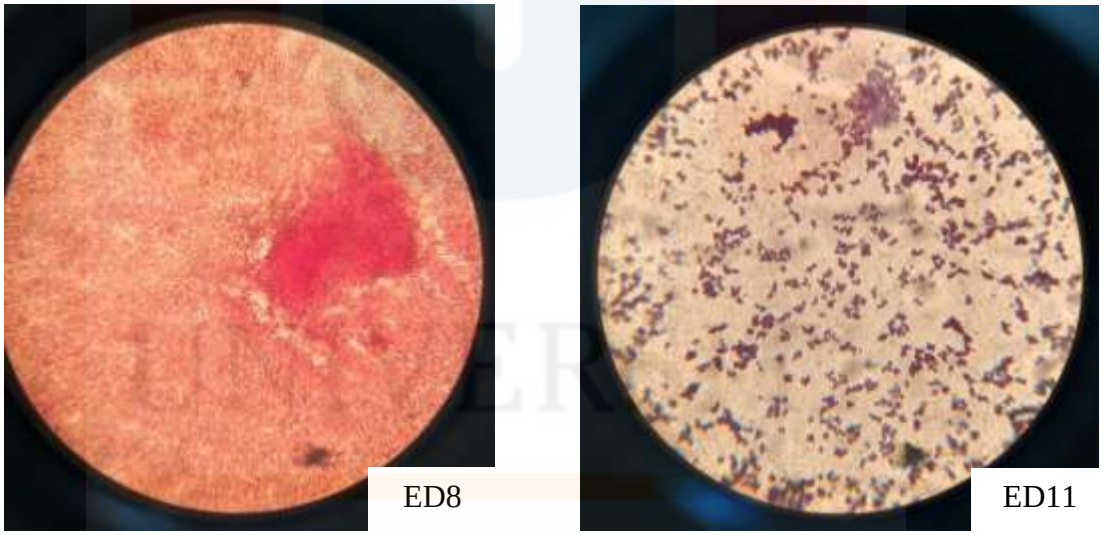

Figure 4.2: The result of Gram staining of sample ED8 and ED11.

Table 4.2: The results of Gram Staining (Monica, n.d.)

Sample	Shape	Color	Gram Stain
ED1	Bacilli	Pink	Gram negative
ED2	Cocci	Purple	Gram positive
ED3	Cocci	Purple	Gram positive
ED4	Cocci	Pink	Gram negative
ED5	Bacilli	Pink	Gram negative
ED6	Cocci	Purple	Gram positive
ED7	Bacilli	Pink	Gram negative
ED8	Bacilli	Pink	Gram negative
ED9	Cocci	Purple	Gram positive
ED10	Bacilli	Purple	Gram positive
ED11	Bacilli	Purple	Gram positive
ED12	Bacilli	Pink	Gram negative
ED13	Bacilli	Purple	Gram positive

4.3 Bacterial Genomic DNA Extraction

Extraction of genomic DNA from bacteria in this study was done by using the Cetyl Trimethylammonium Bromide (CTAB) method prepared by William in 2012. CTAB is a cationic detergent, which can complexes with polysaccharides and the residual protein. The usage of CTAB enables to remove polysaccharides and residual protein during emulsification and extraction. CTAB can forms insoluble complexes with nucleic acids, which can be used to precipitate them from solution (Jones, 1953).

Six out of thirteen bacteria isolates were chosen for DNA extraction. The selected samples of bacteria were ED2, ED5, ED6, ED7, ED9 and ED10. The bacteria cells were cultured in nutrient broth and were incubated in an incubator at 37ºC for 24 to 48 hours. The overnight cultured bacteria in broth were used as the source for extraction purpose as it contained higher density of bacteria cells. This enables to produce a higher yield in DNA extraction.

The extracted DNA from sample ED2, ED5, ED6, ED7, ED9 and ED10 were visualised on 1.0% TAE agarose gel electrophoresis pre-stained with RedSafe™ and observed under a UV illuminator. 1 kb DNA ladder was loaded into the gel as it act as a molecular weight size marker so that the approximate size of the molecule during electrophoresis can be identified. It is suitable for DNA which has the size from 250bp to 10000bp. Visualization of extracted DNA by agarose gel electrophoresis (AGE) is important as it enable to estimate the DNA concentration of the sample. Agarose gel electrophoresis can separate DNA fragments according to their sizes, where smaller negatively charged DNA fragments will migrate faster toward to the anode (Lee *et al.*, 2012). Genomic DNA usually have expected base pair at 23 kb, it is high molecular weight which will appeared as band at the top part

of the agarose gel. The larger the molecular weight, the slower the migration rate of DNA.

Figure 4.3 shows the results of agarose gel electrophoresis for 6 selected genomic DNA samples. Lane 1 to Lane 6 consisted of 1µL of 6x loading dye with 5µL of DNA sample and electrophoresed on 1.0% agarose gel at 80V for 45 minutes. The results in Figure 4.1 shows the extracted genomic DNA runs at a high molecular weight and bands were formed above 1kb ladder. This indicates that the DNA of the 6 selected samples was successfully extracted and can be used in PCR analysis. However, smearing of bands were found and it may indicates that the extracted DNA samples might have undergo degradation, contained high amount of salt-buffer and contaminated by protein (Abdel-Latif & Osman, 2017).

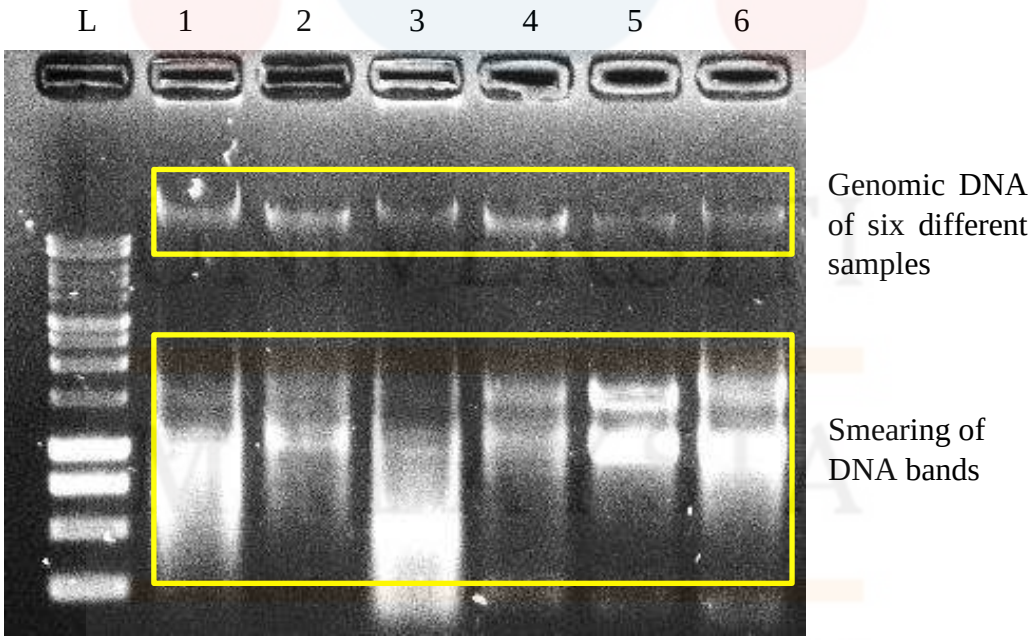

Figure 4.3: DNA Extraction by CTAB method. Numbering of lane is from left to right. Lane L, 1kb DNA ladder; Lane 1, ED2; Lane 2, ED5; Lane 3, ED6; Lane 4, ED7; Lane 5, ED9; Lane 6, ED10.

4.4 Quantification of Bacterial Genomic DNA

The amount and purity of extracted bacterial genomic DNA were quantified before performing PCR amplification. In this study, NanoDrop Spectrophotometer was used to determine the quality and purity of each extracted DNA sample. Quantification of DNA is important as can detect the presence of contaminants such as protein, RNA and salts which may interfere in PCR amplification.

The purity of the DNA samples can be determined based on the result of 260/280 nm ratio. Nucleic acids have maximum absorbance at 260 nm while contaminants such as protein, phenol and RNA have high absorbance at 280 nm. The purity of nucleic acid is higher when it yields 260/280 nm ratio of 1.8 to 2.0 (Abdel-Latif & Osman, 2017). Lower or higher value of the ratio might indicate the presence of contamination of protein and phenol (Desjardins & Conklin, 2010). Based on the results showed in Table 4.3, the 260/280 nm ratio of DNA samples were in the range of 1.99-2.25. The ratio which is higher than 2.0 indicates that the DNA sample may slightly contaminated by RNA. Some of the DNA samples have ratio more than 2.0, and it indicates that RNA contamination of DNA samples was detected as RNase was not used during DNA extraction. The smearing of bands for sample ED2, ED5, ED6, ED7, ED9 and ED10 were showed in Figure 4.3.

Table 4.3 shows the nucleic acid concentration for the 13 isolates obtained from the elephant dung. Normally the concentration of bacterial DNA for PCR is about 100-250 ng per 50µL PCR reaction. The DNA concentration for sample ED1, ED3, ED6 and ED12 is too high for PCR amplification, it may lead to non-specific amplification and formation of smear to occur. Hence, DNA samples with high nucleic acid concentration are needed to be diluted to the desired concentration. The nucleic acid concentration for sample ED11 and ED13 is too low, which shows that

the DNA of bacteria was not successfully extracted and it cannot be used for PCR amplification.

The results of DNA yield and purity range for all extracted DNA samples was showed in Table 4.3.

Table 4.3: The DNA yield and purity range for all extracted DNA samples

Sample Name	Nucleic acid Concentration (ng/ μL)	A260	260/280
ED1	1696.852	33.937	2.19
ED2	461.344	9.227	2.04
ED3	1902.852	38.057	2.25
ED4	402.862	14.057	2.02
ED5	320.935	6.419	2.13
ED6	886.122	17.722	2.08
ED7	132.739	2.755	1.99
ED8	465.881	9.138	2.05
ED9	460.104	9.202	2.15
ED10	534.910	10.698	2.11
ED11	11.455	0.229	1.99
ED12	760.719	15.214	2.22
ED13	15.279	0.306	2.01

4.5 Polymerase Chain Reaction (PCR)

4.5.1 Optimization of PCR

Optimization of PCR conditions was performed before the PCR amplification by using 16S rRNA gene. This step was performed to determine the optimum annealing temperature for the primers to work at the optimum level. Optimization was carried out by using genomic DNA extracted from sample ED2. The melting temperatures of forward and reverse primers based on the manufacturer were shown in Table 3.1. Generally, the annealing temperature of PCR amplification was 5ºC below the melting temperature of primers (Roux, 2009). Hence, gradient PCR was done to determine the optimum annealing temperature. The optimum annealing temperature is the temperature where the resulting bands are sharp and shows high intensity. Figure 4.4 and Figure 4.5 shows the results from agarose gel electrophoresis for PCR optimization.

The result of agarose gel using the set of temperature gradient (50ºC to 60ºC) was showed in Figure 4.4. According to Figure 4.4, the bands formed were placed at the expected size (1500 bp), however multiple bands and smears were observed and it was not good enough to be used as the optimum annealing temperature for the actual PCR amplification of 16S rRNA gene. Multiple bands indicate that nonspecific amplification has occurred and the annealing temperature might need to be increased. Hence, a higher set of temperature gradient (60ºC to 70ºC) was used for the following amplification and the result was shown in Figure 4.5. According to Figure 4.5, there was only single band formed at Lane 7 at 65.6ºC and no multiple bands were observed. Thus, 65.6ºC was the temperature that selected to perform actual PCR amplification of 16S rRNA gene.

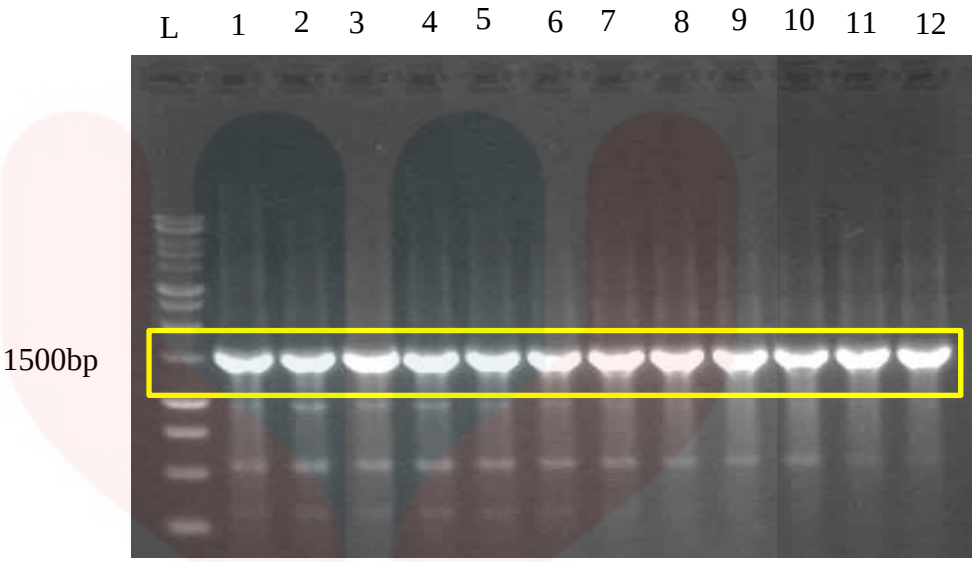

Figure 4.4: Optimization of PCR conditions with gradient temperature of 50ºC to 60ºC. Numbering of lane is from left to right. Lane L, 1kb DNA ladder. Lane 1: 50.0ºC; Lane 2: 50.3ºC; Lane 3: 50.9ºC; Lane 4: 51.8ºC; Lane 5: 53.1ºC; Lane 6: 54.4ºC; Lane 7: 55.6ºC; Lane 8: 56.9ºC; Lane 9: 58.2ºC; Lane 10: 59.1ºC; Lane 11: 59.7ºC; Lane 12: 60.0ºC.

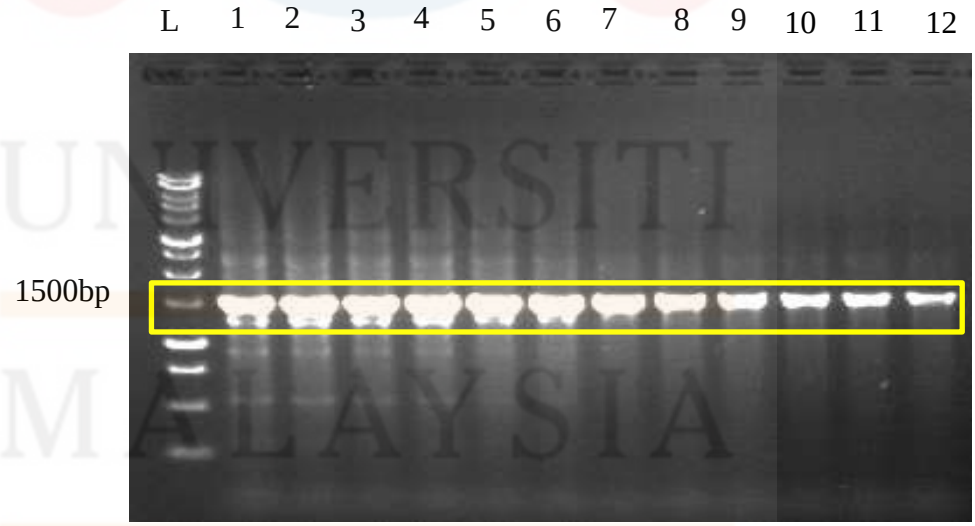

Figure 4.5: Optimization of PCR conditions with gradient temperature of 60ºC to 70ºC. Numbering of lane is from left to right. Lane L, 1kb DNA ladder. Lane 1: 60.0ºC; Lane 2: 60.3ºC; Lane 3: 60.9ºC; Lane 4: 61.8ºC; Lane 5: 63.1ºC; Lane 6: 64.4ºC; Lane 7: 65.6ºC; Lane 8: 66.9ºC; Lane 9: 68.2ºC; Lane 10: 69.1ºC; Lane 11: 69.7ºC; Lane 12: 70.0ºC.

4.5.2 Amplification of 16S rRNA gene

In this study, sample ED2, ED5 and ED7 were selected to perform PCR amplification of 16S rRNA gene with 16S forward primer and 16S reverse primer. The estimated expected size for PCR product from amplification of 16S rRNA gene was approximately 1500bp (Maciel *et al.*, 2009). The optimum annealing temperature at 65.6ºC was set to perform PCR amplification by using a PCR machine. Agarose gel electrophoresis was used to visualize the products from the amplification by running the products in 1.0% AGE for 45 minutes at 80V and 1 kb DNA ladder was used to determine the size of the products. The result of the electrophoresis was viewed under Ultraviolet (UV) illuminator.

The genomic DNA from sample ED2, ED5 and ED7 was amplified with PCR with the annealing temperature of 65.6ºC and the results of amplification were shown in Figure 4.6 and Figure 4.7. According to Figure 4.6 and Figure 4.7, it showed the product bands from sample ED2, ED5 and ED7. The product bands formed from both of the sample were bright and clear. The product bands from sample ED5 was the brightest among the three samples.

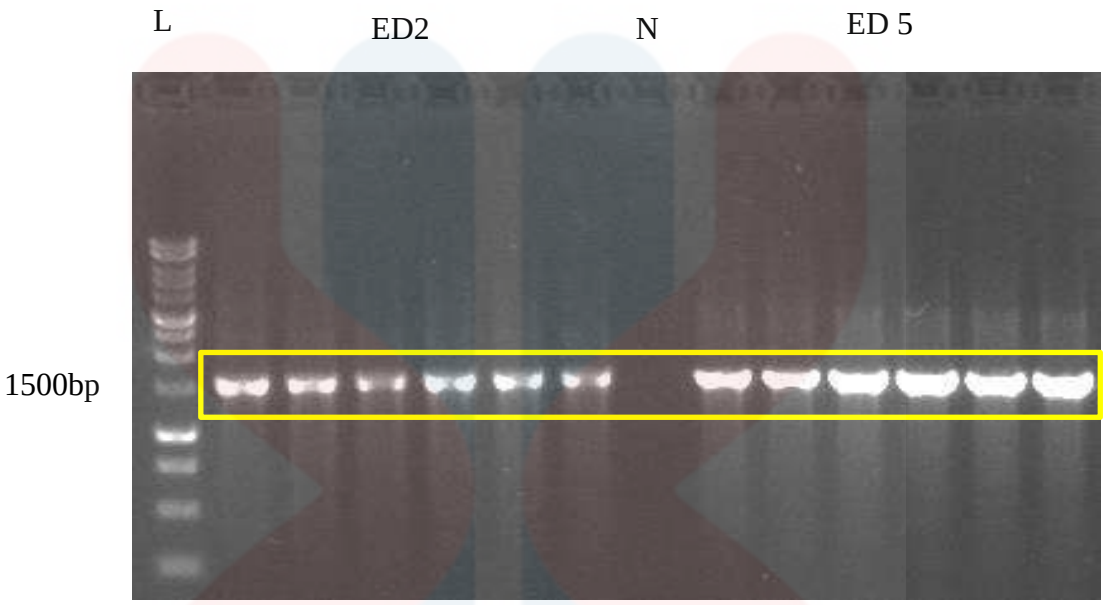

Figure 4.6: Amplification of 16S rRNA gene from sample ED2 and ED5. Lane L is 1kb DNA ladder. Lane N is negative control (ddH_2O).

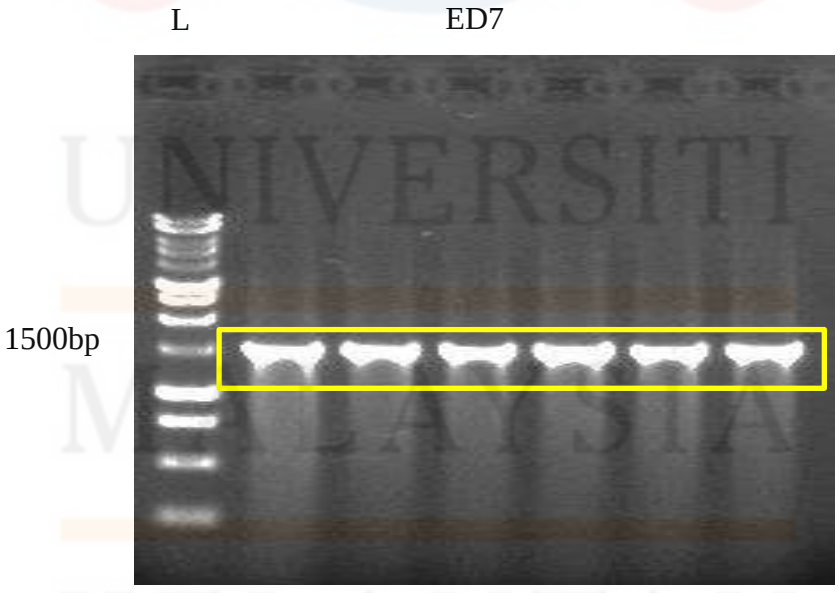

Figure 4.7: Amplification of 16S rRNA gene from sample ED7. Lane L is 1kb DNA ladder.

4.6 Phylogenetic Analysis

All three unpurified PCR samples which were ED2, ED5 and ED7 had sent for purification and sequencing. The FASTA format of forward and reverse sequences of each samples were obtained and both of the sequences were combined by using online sequence merger (EMBOSS).

The DNA sequencing quality was determined by visualizing the DNA sequence chromatogram. The beginning and the end of the sequence usually contained high level of background noise and unknown base (N sign). This may indicates that the samples might have contaminated template with ethanol residual during DNA purification. DNA sequence with poor result of chromatogram containing noisy background and unknown base may cause problems during BLAST. If the noises are too strong, the software has difficulty in resolving the base peaks from background noise. This results in poor sequence reliability and may reduce the accuracy of percentage of similarity in BLAST.

Based on the chromatogram showed in APPENDIX D, ED2 produced weak sequencing result present in high noise background while for the sequenced samples for ED5 and ED7 showed fairly good sequencing as evenly-spaced peaks with low noise background and less N sign. The alignment between samples sequences with sequences obtained from the GenBank database was performed by using Clustal W. The alignments were then used to construct phylogenetic tree with MEGA7.

Table 4.4 shows the result of identification of bacteria isolates from elephant dung. A total of 3 species of bacteria were identified from the elephant dung. The bacteria were identified based on the results that showed the highest percentage of similarity from the GenBank database and the result was showed in Table 4.4. The

criteria in identifying the bacteria species shows that the bacteria genus level can be identified if the similarity of rate is ≥ 97%, while the bacteria species level can be identified if the similarity of rate is ≥ 99% (Drancourt *et al.*, 2000). ED2 has matching identity of 90%, ED5 has matching identity of 98% and ED7 has the matching identity of 99%. Identification of ED7 was the only sample that can be done up to species level.

Table 4.4: Identification of bacteria isolates from elephant dung, by comparing the percentage of similarity with closest match from GenBank.

DNA Samples	Closest match from Nucleotide GenBank	Accession number	% Similarity
ED2	*Exiguobacterium profundum*	KU245731.1	90%
ED5	*Flavobacterium lutescens*	KX698103.1	98%
ED7	*Micrococcus luteus*	EU440972.1	99%

In this study, MEGA7 was used to construct the phylogenetic tree. There are four types of phylogenetic trees that can be constructed by using MEGA7, which are Maximum Parsimony (MP), Maximum Likelihood (ML), Unweighted Pair Group Method with Arithmetic Mean (UPGMA) and Neighbor Joining (NJ). Neighbor joining method was chosen for this study as it based on the principle of minimum evolution which study the distance between each pair of taxa (Saitou & Nei, 1987b). The evolutionary distance between sequences was studied by Neighbor Joining (NJ) with Kimura 2 parameter model. *Thermococcus alcaliphilus* was chosen as outgroup in constructing phylogenetic tree.

According to the result showed in Table 4.4, the sample ED2 had matching identities of 90%. Thus, identification of ED2 can only be done based on the result showed in BLAST, and the isolate was assumed as *Exiguobacterium profundum.* *Exiguobacterium profundum* is a Gram positive, facultatively anaerobic and moderately thermophilic bacteria which is classified under family Bacillaceae (Crapart *et al.*, 2007). However, further analysis which is phylogenetic analysis about this species was not been done due to the lack of accuracy of information. The phylogenetic tree for sample ED5 and ED7 were showed in Figure 4.8 and Figure 4.9 respectively.

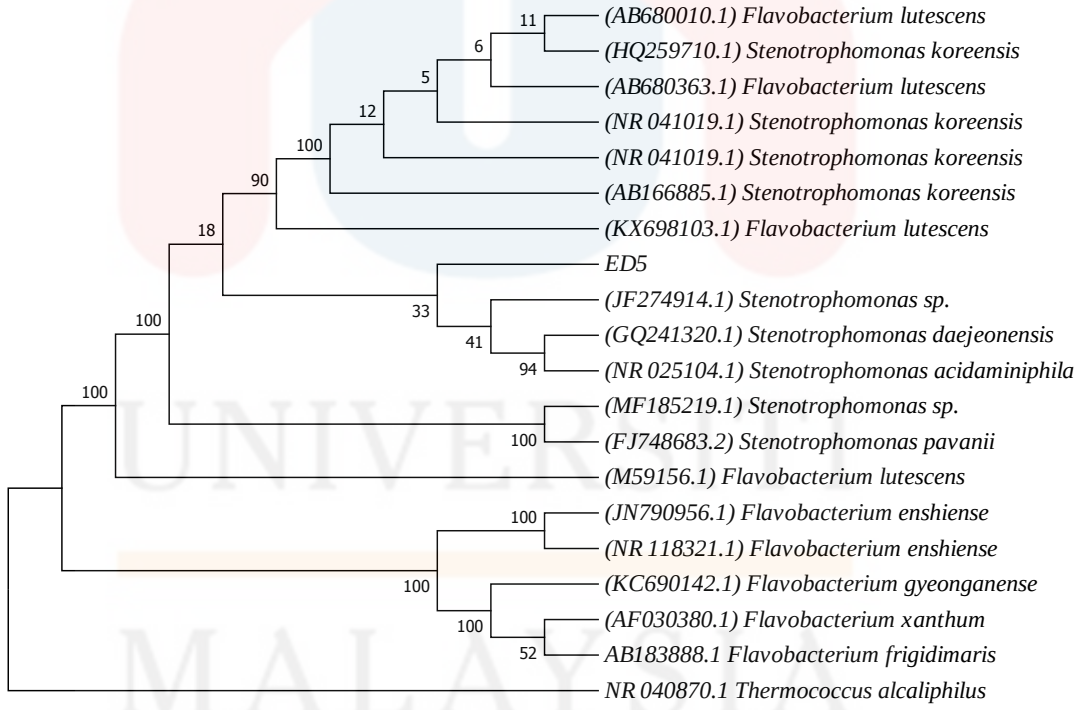

Figure 4.8: The phylogenetic relationship between ED5 with the closest sequences found in GenBank. The sequences in GenBank with highest similarity with ED5 showed by BLAST with the percentage of 98% and 96% were used to compare with ED5.

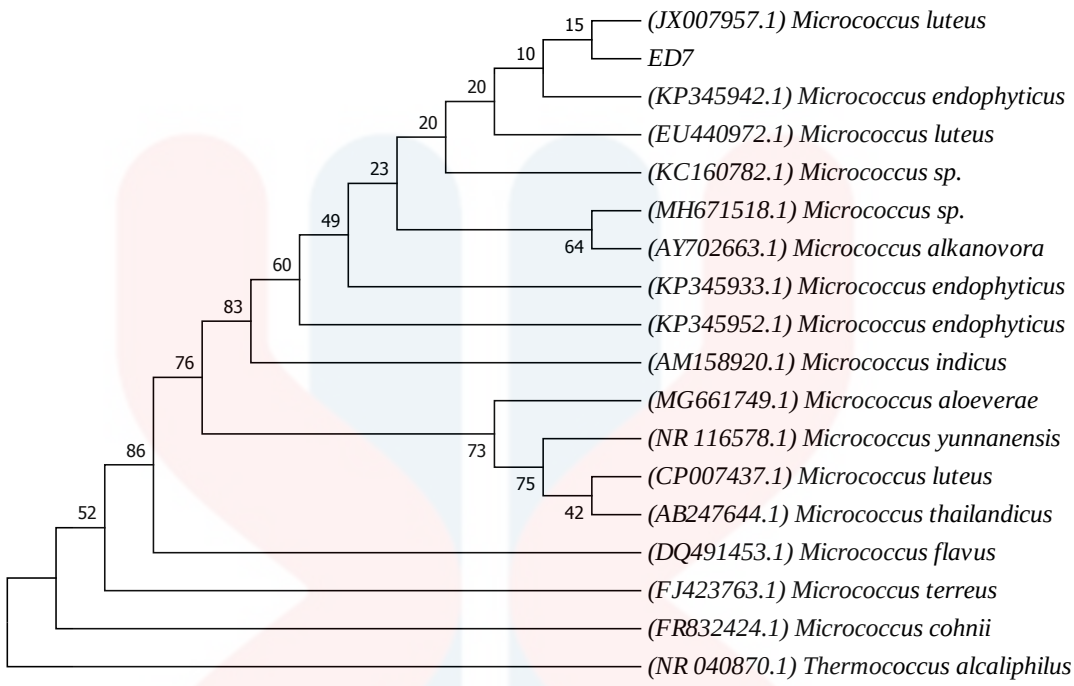

Figure 4.9: The phylogenetic relationship between ED7 with the closest sequences found in GenBank. The sequences in GenBank with highest similarity with ED7 showed by BLAST with the percentage of 99% were used to compare with ED7.

Flavobacterium spp. is the genus of Gram negative, aerobic, and rod-shaped bacteria (Bernardet, 1989) which is classified under the family Flavobacteriaceae. There are a few numbers of species of bacteria which is classified under this genus such as *F. endophyticum*, *F. aureus*, *F. ferrugineum* and *F. lutescens*. *Flavobacterium* spp. can be found in wide variety of environment including soils, fresh and marine aquatic environments, foods and beverages (Betts, 2006). Since the elephant dung had contacted with soils, this may indicates that the bacteria was transferred from the soils to elephant dung. The ecology of *Flavobacterium* sp. shows that some flavobacteria are able to grow at environment with the temperature of 37°C. Hence, *Flavobacterium* sp. can be found in diverse habitats including soils, and freshwater streams. Study by Loch and Faisal on 2015 showed that *Flavobacterium* sp. are pathogenic to living organisms including plants, reptiles and

mammals. It can cause flavobacterial diseases which caused the fish body to display dirty-white or yellowish areas (Loch & Faisal, 2015).

Flavobacterium sp. can be related to elephant as a recent study done by Vyas and Kumar showed that *Flavobacterium lutescens* is one of the cellulose producing bacterial that isolated from cattle dung (Vyas & Kumar, 2018). The study stated that *Flavobacterium lutescens* was one of the isolates that has the ability to produce cellulose enzyme to perform cellulose activity. Hence, *Flavobacterium lutescens* is known as cellulose producing bacteria. Besides, *Flavobacterium* sp. was found to be one of the hydrocarbon degrading bacteria isolated from cow dung and might have contributed in bioremediation of automobile workshop (Adams *et al.*, 2014). Thus, *Flavobacterium* sp. found in elephant dung might be able to be used in bioremediation of petroleum.

Micrococcus luteus is a Gram positive, aerobic, non-endospore forming and coccus shaped bacteria which is classified under family Micrococcaceae. *M. luteus* was first known as *Micrococcus lysodeikticus* and was discovered by Alexander Fleming in 1928 (Benecky *et al.*, 1993). *M. luteus* can be found in the environments which include soil, water, air and also on the skin of humans and animals (Betts, 2006). It was found to be one of the cellulolytic bacteria that was isolated from the gut of some termites and it might contributed in cellulose degradation within termites (Sexana *et al.*, 1993). Since Asian elephants are herbivores which only consume on plants, *M. luteus* found in elephant dung is to produce cellulose to degrade the cellulose that contain in the plant cell wall.

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

In conclusion, 13 pure bacterial colonies were successfully isolated by using spread plate and streaking method. The result of Gram staining of 13 bacteria isolates had also been identified. There were total seven Gram positive isolates and six Gram negative isolates. The Gram positive bacteria stained purple while the Gram negative bacteria stained pink. Three out of thirteen colonies of pure bacterial colonies were chosen to perform bacteria identification by using 16S rRNA gene sequence analysis. The results of comparisons of the sample sequences with the GenBank database showed that the three isolates ED2, ED5, and ED7 were closely related to *Exiguobacterium* sp., *Flavobacterium* sp. and *Micrococcus luteus* respectively. However, further research on these isolates still need to be done to further identify the species and the ecology of the bacteria. Based on previous studies, *Flavobacterium* sp. was found to be one of the hydrocarbon degrading bacteria while *Micrococcus luteus* was identified as cellulolytic bacteria.

5.2 Recommendations

In order to obtain higher accuracy of bacteria identification, additional test such as biochemical tests need to be carried out instead of just performing Gram staining of bacteria. Biochemical tests enable to provide more information about the characteristic of the bacteria isolates. Next, the pure isolated bacteria colonies can be preserved by using freeze drying method. The bacteria can be recovered from freeze drying and further studies can be conducted in order to obtain more detailed information about the bacterial diversity of elephant dung. Further studies on the bacteria isolated from elephant dung are needed as it enables to provide more understanding on the ecological significance of bacteria in elephant dung. The health status of the elephant and their living condition can be identified if the microbial diversity of the elephant dung is successfully determined.

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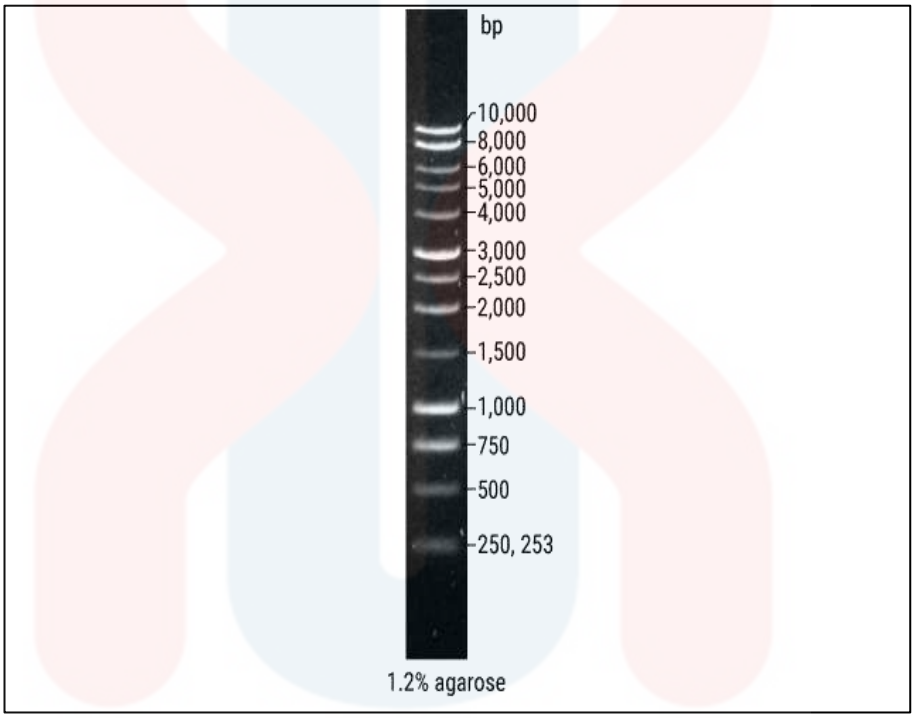
APPENDIX A

List of Chemical Used

No.	Chemical Used
1.	70% ethanol (EtOH)
2.	Crystal Violet
3.	Gram's Iodine
4.	Acetone
5.	Safranin
6.	Tris (hydroxymenthyl) aminomethane (Tris) base
7.	Ethylenediaminetetraacetic acid (EDTA)
8.	Chloroform
9.	Isoamyl alcohol
10.	Sodium chloride (NaCl)
11.	Sodium dodecyl sulfate (SDS) powder
12.	Cetyltrimethylaammonium bromide (CTAB)
13.	Proteinase K
14.	Sodium acetate
15.	Isopropanol
16.	TAE buffer
17.	RedSafe™ Nucleic Acid Staining Solution
18.	PCR buffer
19.	$MgCl_2$
20.	dNTPs
21.	*Taq* DNA polymerase (Promega, USA)

APPENDIX B

The 1 kb DNA Ladder manufactured by Promega, United States

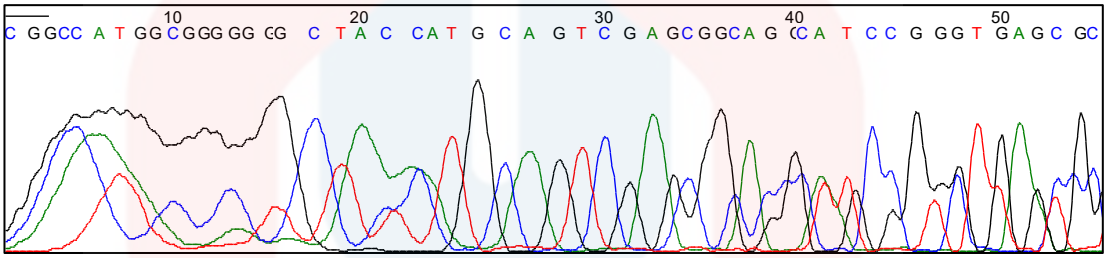


APPENDIX C

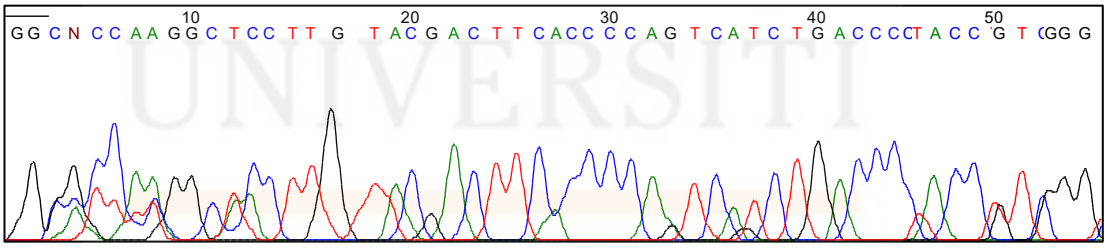
The chromatogram of forward and reverse 16S rRNA gene sequences of 3 isolated bacteria samples from elephant dung.

Note: APPENDIX C only shows a portion of chromatogram for each 16S forward and reverse sequences ranging from 1 to 55 nucleotides for 3 isolates.

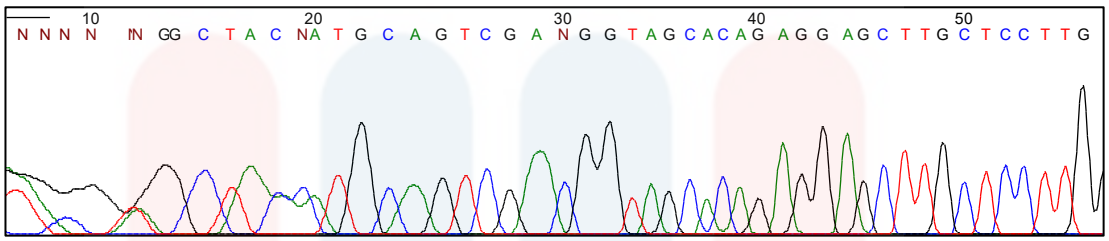
ED2: Forward



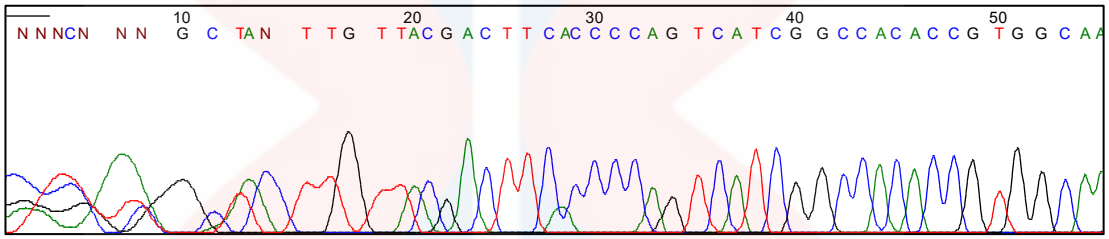
ED2: Reverse



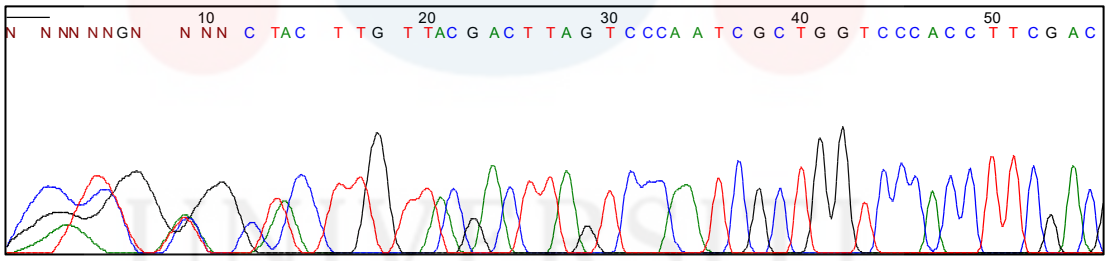
ED5: Forward



ED5: Reverse



ED7: Forward



ED7: Reverse