Isolation of *Methylobacterium* sp. (Pink Pigmented Facultative Methylotrophs) from Paddy (*Oryza sativa*) and Oil Palm (*Elaeis guineensis*) Leaves

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A thesis submitted in fulfilment of the requirements for the degree of Bachelor of Applied Science (Bio-industrial Technology) with Honours

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DECLARATION

I hereby declare that the work embodied in this report is the result of the original research and has not been submitted for a higher degree to any universities or institutions.

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I certify that the report of this final year project entitled "Isolation of *Methylobacterium* sp. (Pink Pigmented Facultative Methylotrophs) from Paddy (*Oryza sativa*) and Oil Palm (*Elaeis guineensis*)" by LEE JIA MEAN, matric number F15A0069 has been examined and all the corrections recommended by examiners have been done for the degree of Bachelor of Applied Science (Bio-industrial Technology) with Honours, Faculty Of Bioengineering And Technology, Universiti Malaysia Kelantan.

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LIST OF ABBREVIATIONS

PPFM	Pink Pigmented Facultative Methylotrophs
AMS	Ammonium mineral Salt
mxaF	Methanol dehydrogenase gene
MMOs	Methane monooxgenases
pMMO	Intra-cytoplasmic membranes
sMMO	Soluble methane monooxygenase
ISR	Induced Systemic Resistance
DCM	Dichloromethane
PCR	Polymerase Chian Reaction
IAA	Auxin
C1	Single Carbon
$NADH_2$	Reduced Nicotinamide Adenine Dinucleotide
PQQ	Pyrrolo-Quinoline Quinone
ATP	Adenosine Triphosphate
RuMP	Ribulose Monophosphate Pathway
AHLs	N-acyl-homoserine lactones
QS	Quorum Sensing
16S rRNA	16S ribosomal Ribonucleic acid
DNA	Deoxyribonucleic acid
GTE	Glucose-Tris-HCL EDTA
TE	Tris-HCL-EDTA
TAE	Tris-acetic acid-EDTA
BLAST	Basic Local Alignment Search Tool

LIST OF SYMBOLS

℃	Degree celcius
g	Gram
mol	Mol
ml	Milliliter
µl	Microliter
%	Percentage
rpm	Revolution per minute
cm	centimeter
k	Kilo
V	Volt
bp	Base pair

Isolation of *Methylobacterium* sp. (Pink Pigmented Facultative Methylotrophs) from Paddy (*Oryza sativa*) and Oil Palm (*Elaeis guineensis*)

ABSTRACT

Methylobacterium sp. commonly known as Pink Pigmented Facultative Methylotrops (PPFM) which able to utilize methanol and other C1 compounds as their sole carbon source. This genus of bacteria are ubiquitous in environments due to their physiology plasticity which enable them to colonize different environment. In this study, phyllosphere *Methylobacterium* sp. was isolated from two types of samples which are paddy leaves and oil palm leaves. Isolation was done on ammonium mineral salt supplemented with 0.5% methanol. 10 isolates labelled as EPPD1, EPPD2, EPPD3, ENPD1, ENPD2, ENPD3, EPPM1, ENPM1, ENPM2, and ENPM3 were selected for further identification and characterization based on their colony morphology. All of the isolates showed the same cellular morphology which are Gram negative and rod shape. The isolates were then undergone molecular identification by 16S rRNA sequence analysis. The genomic DNA of the isolates were extracted and proceed to polymerase chain reaction to amplify the DNA fragment by using primers 68F and 1392R. The band can be observed at range of 1000 bp to 1500 bp. Based on BLAST analysis, two *Methylobacterium* sp. were identified. The isolates EPPD1, EPPD4, ENPD1, ENPD3, ENPM1, ENPM2 and ENPM3 were identified as *Methylobacterium radiotolerans* while ENPM2 was identified as *Methylobacterium aerolatum*. The isolates EPPD3, EPPM1 and ENPD2 were failed to be identified due to the low quality and concentration of DNA samples.

Keywords: *Methylobacterium* sp., PPFM, isolation, phyllosphere, leaf surface

Pengasingan Methylotrophs Fakultatif Berpigmen Merah Jambu dari Daun Padi (*Oryza sativa*) dan Kelapa Sawit (*Elaeis guineensis*)

ABSTRACT

Methylobacterium sp. dikenali sebagai Methylotrops Fakultatif berwarna Merah Jambu (PPFM) boleh menggunakan methanol dan kompaun C1 lain sebagai sumber karbon tunggal. Genus bakteria ini boleh didapati dari mana-mana persekitaran disebabkan oleh fleksibiliti fisiologi mereka yang memudahkan mereka menumbuh di dalam persekitaran yang berbeza. *Methylobacterium* sp. di permukaan daun telah diasingkan dari dua jenis sampel iaitu daun padi dan daun kelapa sawit. Pengasingan dilakukan pada media amonium mineral garam ditambah dengan 0.5% metanol. 10 isolat dipilih untuk mengenal pasti dan menciri berdasarkan morfologi koloni mereka. Semua isolat menunjukkan morfologi sel yang sama iaitu Gram negatif dan bentuk rod. Oleh itu, isolat telah menjalani pengenalan molekul oleh analisis urutan 16S rRNA. DNA genomik isolate telah diekstrak dan meneruskan PCR untuk mengamplifikasikan genomik DNA dengan menggunakan primer 68F dan 1392R. Jalur ini boleh dilihat pada jarak 1000 bp hingga 1500 bp. Berdasarkan analisis BLAST, dua *Methylobacterium* sp. telah dikenalpasti. Isolat EPPD1, EPPD4, ENPD1, ENPD3, ENPM1, ENPM2 dan ENPM3 telah dikenalpasti sebagai *Methylobacterium radiotolerans* manakala ENPM2 dikenal pasti sebagai *Methylobacterium aerolatum*. Isolat EPPD3, EPPM1 dan ENPD2 gagal dikenalpasti disebab oleh kualiti dan kepekatan sampel DNA yang rendah.

Kata kunci: *Methylobacterium* sp., PPFM, pengasingan, permukaan daun

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Methylotrophs is a diverse group of methanotrophs. Methanotrophic bacteria were first isolated by Söhngen in 1906 and further studied and characterized by Whittenbury *et.al* in 1970. In 1976, Patt, Cole, & Hanson had discovered methylotrophic bacteria. Methylotrophs categorized as new genus due to their ability oxidize the other C1 compounds other than methane such as methanol, halomethane, sulphur containing methylated compounds or other carbon bonds lacking multi-carbon compounds (dimethyl ether) in their metabolic pathways (Chistoserdova *et al.*, 2003; Hanson & Hanson, 1996).

Methylobacterium sp is one of the well-known methylotrophs that had been studied lately. The bacteria of *Methylobacterium* genus are classified as the member of class α-proteobacteria, order *Rhizobiales* and family of *Methylobacteriaceae*. These genus of

bacteria consist of various pink pigmented facultatively methylotrophs (PPFMs) and non-pigmented facultatively methylotrophs (NPFM) which are not obligate to methanol and methane.

They are ubiquitous in the environments due to their physiology plasticity which enable them to colonize in different environment (Hanson & Hanson, 1996). *Methylobacterium* sp. can be found in variety of natural environment. They generally present in oxic environment, plant phyllosphere and rhizoshpere, in the soil, and lake. Methylothrophs are well known in inhabit in the plant phyllosphere and associated with mutualistic relationship with the plants they colonized and frequently reported on agronomic plant species such as maize, cotton and soybean (Madhaiyan, Poonguzhali, Senthilkumar, Lee, & Lee, 2011; Pattanashetti, 2012; Iguchi, Yurimoto, & Sakai, 2015; Minami *et al.*, 2016;). In this study, phyllosphere *Methylobacterium* sp. (PPFM) is isolated and identified from paddy (*Oryza sativa*) and oil palm leaves (*Elaeis guineensis*) since these two plants are the major crops in Malaysia.

Agricultural sector is an important sector in developing a country's economy (Azer *et al.*, 2016). Hence, crops yield will be the main factor in farming activities. In order to maintain crop yield, modern technique is applied such as application of chemical pesticide and fertilizer are widely used in farming as they are efficient in controlling pest and enhance crop yield. However, the increases of using chemical fertilizer and pesticide will lead to the increases of heavy metal level in soil (Arruda & Azevedo, 2009) and decreases soil avaibility for plant. Many researches had conducted to find out solution to overcome this issue (Watanabe, 2001; Rajendran, Muthukrishnan, & Gunasekaran, 2003; Pathak, Devi, Sarma, & Lal, 2014;). Therefore, *Methylobacterium* sp. was found that have the

ability that tolerance to heavy metal like nickel, zinc and cadmium (Idris *et al.*, 2006; Kist, 2012), and increase soil avaibility for plant by degrading toxic organic compounds (Dourado, Neves, Santos, & Araújo, 2015).

Besides, *Methylobacterium* sp. is also used to produce biofertilizer in order to replace the application of chemical fertilizer. Biofertilizer is a fertilizer that combines with targeted bacteria strain to produce environmental friendly fertilizers with various function (Amna, 2012). *Methylobacterium* sp. show significant result in improving plant growth which includes branching, rooting, seed germination and promoting leaves formation (Holland, 1997) by solubilizing inorganic compound and fixing atmospheric nitrogen which leads to increasing phytohormone level in plants and nutrient level (Kumar, Tomar, Lade, & Paul, 2016). Since most studies had highlighted the importance and uses of methylotrophs on the agronomic crops, isolation, screening, characterization and identification of *Methylobacterium* sp (PPFM) was done and is going to be studied as it has high potential in agronomic and biotechnological field.

In this experiment, *Methylobacterium* sp. from leaf surface of paddy leaves and oil palm leaves were isolated on ammonium mineral salt medium supplemented with 0.5% methanol. Molecular identification was done to identify the species of the bacterial isolates. There was total of 10 isolates successfully isolated. From molecular identification by 16S rRNA analysis, the isolates were identified as *Methylobacterium radiotolerence* and *Methylobacterium aerolatum*.

1.2 Objectives

i. To isolate endophytic and epiphytic *Methylobacterium* sp. from paddy and oil palm leaves.

ii. To identify the isolated bacteria by morphology and 16S rRNA analysis.

CHAPTER 2

LITERATURE REVIEW

2.1 Methylotrophs

Methylotrophs is a group of microorganism has the ability in carbon assimilation through RuBP and serine pathways. It can be bacteria, archaea, fungi or yeast. It comprises obligate and facultative aerobic eubacteria, which possess the ability to use C1 compounds as a source of carbon and energy (Mosin & Ignatov, 2014), while methanotrophs are methane-utilizing bacteria, which is a sub-group of methylobacterium. Both are able to utilize C1 compounds but almost all of the methanotrophs are obligate C1 utilizer. On the ability to utilize carbon, methylotrophs are divided into two sub-groups: obligate and facultative methylotrophs (Tzygankov, 1986). Obligate methylotrophs are able to grow only on methane and C1 compounds as substrates. Conversely, facultative methylotrophs are able to grow not only on methanol and methylamine, but also on methane and some simple multi-carbon compound such as ethanol and acetic acid (Mosin & Ignatov, 2014).

According to Mosin *et al.* (2014), *Methylobacterium sp.* is one of the genera under obligate methylotrophic bacteria which use methanol and methane as it substrate.

2.2 Metabolic Pathways in Methylotrophs

Methylotrophy, the ability to utilize reduced carbon substrates containing no carbon-carbon bonds (C1 substrates) as their sources of carbon and energy is an example of a specialized mode of microbial metabolism (Chistoserdova and Lidstrom 2013). Methylotrophs have this ability mainly due to the presence of enzyme methanol dehydrogenase gene (*mxaF*). The pathway of methanol assimilation in all methylotrophs is similar. If encounter with methane, it will first oxidized into methanol catalyzed by methane monooxgenases (MMOs), a $NADH_2$-dependent enzyme. There are two types of MMOs, intra-cytoplasmic membranes (pMMO) and soluble methane monooxygenase (sMMO) (Anthony, 1986). sMMO can gain electron from cytochrome or $NADH_2$ while pMMO gain electron only from $NADH_2$. These two enzymes incorporate with cytochrome c and $NADH_2$ to break the covalent bond of O_2 and reduced one oxygen atom to H_2O and another oxygen atom oxidized methane to form methanol. Methanol that synthesized through this process called endogenous methanol. Exogenous and endogenous methanol is then oxidized by periplasmic methanol dehydrogenase (MDH), which is a cytochrome c dependent enzyme to form formaldehyde (Anthony, 1986). The formaldehyde subsequently undergoes enzymatic oxidation by the relevant enzyme: formaldehyde dehydrogenase and formate dehydrogenase into formate and CO_2 as end product. The pathway is shown in Figure 2.1.

Most of the energy required for methane metabolism is produced through this pathway for methylotrophs. The energy efficiency of the oxidation of C1 compounds by relevant dehydrogenases is determined by the electron acceptor along the respiratory chain. In oxidative metabolism of C1 compounds are involved $NADH_2$, flavins, quinones, cytochromes a, b, c. Oxidation of methanol to formaldehyde, catalyzed by methanol dehydrogenase containing as prosthetic group the residue of coenzyme PQQ (pyrrolo-quinoline quinone), is accompanied by the transfer of electrons in the mitochondrial respiratory chain at the level of cytochrome c. This process leads to the synthesis of ATP molecule (Mosin & Ignatov, 2014).

Formaldehyde is the key intermediate metabolic product as they represent the branching point at which carbon is either oxidized to CO_2 or assimilated into cell carbon. There are 2 alternative pathways which assimilate formaldehyde into energy for cell activities or biomass either through ribulose monophosphate pathway, RuMP (Type I methanotrophs, γ- proteobacteria) or serine pathway (Type II methanotrophs, α-proteobacteria) (Hanson & Hanson, 1996). *Methylobacterium sp*. assimilate methanol via serine cycle (Šmejkalová, Erb, & Fuchs, 2010) as shown in Figure 2.1.

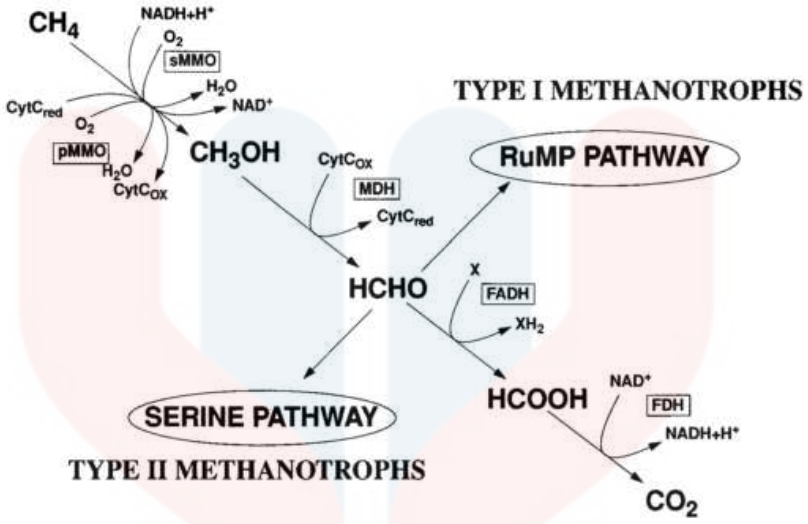


Figure 2.1: Methane oxidation pathway and formaldehyde assimilation pathway. CytC: cytochrome c; FADH: formaldehyde dehydrogenase; FDH: formate dehydrogenase. Adapted from (Hanson & Hanson, 1996)

2.3 Role of Methylotrophs in Carbon Cycle

Methane is an organic gas emits from agricultural activities, livestock farming and others human activities. The increases of atmospheric methane causes global warming as methane absorbs heat radiation more efficient than CO_2 even though the concentration of methane in atmosphere is lower than CO_2 (Lelieveld, Crutzen, & Brühl, 1993). Paddy filed and wetland contribute most emission of methane gas to atmosphere, the flooded condition creates anaerobic environment and favour the methanogens to generate methane in the soil. Thus methylotrophs play role in reducing the release of this organic gas into atmosphere by oxidizing it into CO_2 aerobically on the soil surface before methane emits to the atmosphere (Iguchi *et al.*, 2015). The metabolic pathway of methane to CO_2 is mention as above. The CO_2 is then enter the carbon cycle begin with photosynthetic organisms. Alternative

assimilation pathways of methane in methylotrophs can fully utilized by converting into energy (Kumar, Saxena, & Tomar, 2017).

2.4 Interaction between *Methylobacterium sp.* And Plant

2.4.1 Colonization on Plant Phyllosphere

Methylobacterium sp. always associate with plants, they actively colonize plant phyllosphere (leaf and stem part) and rhizosphere (root part). Bacteria recognize the plant they colonize by the exudates secreted from plants such as sugars, flavonoids, amino acid and organic acid. Different exudates attract different type of beneficial microorganisms to colonize. For epiphytic methylothrophs, they are major in colonizing the plant phyllosphere. Plant emits methanol into atmosphere due to the synthesis of cell wall. During plant growth, pectin is breakdown by penctinmethylesterase causing the formation of methanol and release out from stomata. Mehtylothrophs have a selective advantage on host colonization because methanol is not the only carbon source. Methylothrophs take this advantage by expressing gene related to methylotrophy, resulting more efficient in colonization than the other plant associated bacteria. Plant colonization of endophytic methylotrophs may be initiated quorum sense system by detecting signaling molecules such as N-acyl-homoserine lactones (AHLs). Once the AHLs concentration is high enough to initiate QS system, biofilm will be formed by the bacteria to colonize their habitat (Dourado *et al.*, 2015).

Plant phyllosphere is a harsh environment for the other microorganisms as it exposes high intensity of UV, fluctuation of temperature, low nutrient avaibility. The most abundant genera in the phyllosphere were found to belong to *Methylobacterium*, *Sphingomonas* and *Pseudomonas* (Delmotte *et al.*, 2009) while *Methylobacterium sp*. are

more interested for study because it play important role in the methanol cycle by utilizing methanol emmited by plants (Trotsenko, Ivanova, & Doronina, 2001). *Methylobacterium* sp. synthesis carotenoids gives a distinctive pink pigmentation to its morphology enables them to tolerate extreme light and high UV condition. This is one of the reasons they can colonize on plant phyllosphere (Kaparullina *et al.*, 2017).

2.4.2 Promotion of Plant Growth

One of the features of *methylobacterium sp*. is they promote plant growth. When these bacteria colonized a plant, utilization of plant exudates results in accumulation of secondary metabolites by bacteria. These metabolites can influence plant growth and devevlopment. Phytohormone is the major secondary metabolites that stimulate growth in all plants. Cytokinin and auxin are the essential growth hormone for plant. Cytokinin regulates cell division and elongation in shoots and roots, auxin regulates the development of plant roots. Ethylene is another hormone that regulates growth and development of plant roots. This hormone has antagonistic properties with auxin (IAA). High level of ethylene can lead to stress condition in plants, deleterious effect on plant growth, causes early anscission and senescence. IAA production by methtlotrophs will increase if the ethylene synthesis increases. Thus, methylotrophs are important in modulating plant growth and development by balancing the IAA and ethylene level (Castillo *et al.*, 2015).

2.4.3 Inhibition Plant Pathogen

The presence of endophytic microorganisms could protect plant host from pathogenic microorganisms. *Methylobacterium* sp. provide protection by synthesising antimicrobial component, competes nutrient with pathogens as *Methylobacterium* sp. can colonize strongly in phyllosphere or induced systemic resistance (ISR). ISR is an activated resistance process that activated by volatile organic compound released by bacteria, its action is not direct killing or inhibition of the pathogen, but through the induction of plant disease resistance. Effect on colonization of endophtic methylotrops can protect it host by controling the microbial community on plant (Rosenblueth & Martínez-Romero, 2006).

2.5 Application of *Methylobacterium* sp.

2.5.1 Bioremediation

Bioremediation is a process that used to treat contaminated media including water, soil, and sea water by using naturally occurring organisms to breakdown the harmful contamination substances into less toxic or non-toxic form.

Methylobacterium species are found able to degrade organic pollutants. Most common toxic pollutant are halogenated organic compounds (dichloromethane) contain in waste water from industry. Plant associated *Metylobacterium extorquents* DM4 able to degrade these volatile and toxic compounds by converting Dichloromethane (DCM) into formalaldehyde which is an intermediate product use in metabolic pathway (Ventorino *et al.,* 2014). Next, after explosion occurred, there will be residual of explosive component such as 2,4,6-trinitrotoluene (TNT), hexahydro-1,3,5-trinitro-1,3,5-triazene (RDX) and

octahy- dro-1,3,5,7-tetranitro-1,3,5-tetrazocine (HMX), *Methylobacterium* sp. also use to treat toxic explosive contaminated sites (Aken *et al.*, 2004). Furthermore, soil and groundwater contamination by pollutant MTBE (methyl-tert-butyl ether) also had been concerned. It is an oxygenating component added into vehicle fuel to enhance engine performance and to reduce emission of air pollutant component. Zhang *et al*., (2008) had reported that *Methylobacterium* sp. and other methylotrophs showed high efficiency in degrading MTBE. The capabilities of *Methylobacterium sp*. have high potential in bioremediation of contaminated environment (Hanson, Tsien, Tsuji, Brusseau, & Wackett, 1990).

2.5.2 Biofertilizer

Besides oxidize single carbon compound, *Methylobacterium* sp. that isolated from soil or mud efficient in fixation of atmospheric nitrogen due to the presence of nitrogenase. The application of chemical fertilizer in agricultural activities will increased the salinity of the soil; by inoculating nitrogen fixation bacteria into the soil could reduce the use of chemical fertilizers. Recent study found that inoculation of *Methylobacterium organophilum* into soil had shown remarkable effect on seed germination and promote plant growth by providing nitrogenous compound (Rekadwad, 2014). This isolated bacteria strain from hotspring mud grew well at high temperature in nitrogen free medium. It indicated this bacteria strain could fix atmospheric nitrogen to survive and grow meanwhile release nitrogenous compound for the plant as nitrogen source. This study prove that some species of *Methylobacterium* suitable to produce biofertilizer. Plant hormone production on plant phyllosphere by methylotrophic *Methylobacterium* along with their biocontrol activity

against pathogens made them an important component for efficient and sustained agriculture (Kumar *et al.*, 2017)

2.7 Bacterial identification method by 16S rRNA analysis

16S rRNA is the RNA encoded in ribosome small subunit in all prokaryotes and archaea. The gene encoded in it is highly conserved which is crucial for survival of every organisms (Lane, 1991), so it is commonly used for phylogenetic marker in taxonomic studies on prokaryotes (Kim & Chun, 2014). To classify and identify the bacteria, it depends on the comparisons of 16S rRNA samples against the database of known sequence. Nowadays, the sequence of the prokaryotic species is now available in public databases with its published name. In general, 16S rRNA sequence are used in two ways in microbial systematics, namely for calculating pairwise sequence similarities and for phylogenetic analyses following multiple sequence alignments. The 16S rRNA gene sequence similarity between two strains provides a simple yet very robust criterion for the identification of newly isolated strains, whereas phylogenetic analyses can be used to elucidate overall evolutionary relationships between related taxa (Kim & Chun, 2014). The identification of 16S rRNA gene is now well-developed as molecular identification. 16S rRNA gene sequence analysis is more precise than biochemical test. Thus, for the bacteria species that cannot be recognized by biochemical properties, 16S rRNA gene sequence information is able to provide genus and species identification (Janda & Abbott, 2007).

CHAPTER 3

METHODOLOGY

3.1 Apparatus & Materials

3.1.1 Apparatus

The equipment that was used in this research are autoclave machine, Bunsen burner, electrophoresis equipments, laminar flow chamber, incubator, electronic pH meter, orbital shaker, PCR thermocycler, micro centrifuge, conical flask, electronic balance, micro pipette and tips, microcentrifuge tubes, PCR tubes, inoculating loop, test tube with cap, petri dish, spatula, medium bottles, beakers, measuring cylinders, aluminium foil and parafilm, microscopic glass slide, cover slip dropper, DNA extraction kit.

3.1.2 Materials

The material that was used in this research are 70% ethanol, steriled distilled water, agar powder, component for AMS medium: ammonium chloride, dipotassium phosphate, monopotassium phosphate, magnesium sulphate, calcium chloride, iron sulphate, zinc sulphate, magnesium chloride, borane, cobalt dichloride, nickel chloride, sodium molybdate dihydrate, 0.5% methanol, sodium hydroxide, chemicals used in DNA extraction: GTE buffer, TE buffer, RNAse, lysozyme, proteinase K, 25% SDS, sodium acetate, isopropanol, and phenol:chloroform:isoamyl alcohol, chemicals use in Gram staining: Crystal violet, iodine, ethanol-acetone and safranin, chemicals use in agarose gel electrophoresis: agarose powder, ethidium bromide (EtBr), 6x loading dye, DNA ladder and TAE buffer, materials for PCR: PCR master mix (PCR buffer, dNTPs, *Taq* polymerase), forward primer (10pmol, reverse primer (10pmol), and QIAquick PCR Purification Kit (Qiagen).

3.2 Methods

3.2.1 Sample Collection

i. Collection of Paddy Leaves.

Paddy (*Oryza sativa*) leaves were collected from paddy field in Bachok, Kelantan. The whole plant is pulled out and kept in container together with paddy field soil and stored at room temperature.

ii. Collection of Oil Palm Leaves.

Oil palm leaves were collected from oil palm plantation field in Jeli, Kelantan. The leaves were cut from a leaf bundle, the leaves were stored at room temperature.

3.2.2 Media Preparation

Ammonium mineral salt media was prepared by mixing 1L of distilled water with the compositions as shown in the Table 3.1 below with the pH adjusted to 6.8 with NaOH. Then 15g of agar powder was added into the solution. The media was sterilized by autoclaving at 121℃ for 15 minutes. Next, the media was let cooled to 50℃ , 0.5% (v/v) of methanol was added to the media and mixed well before poured into sterile petri dish.

Table 3.1 Composition of ammonium mineral salt medium

Composition	Concentration (g/L)
Ammonium Chloride, NH_4Cl	0.5
Dipotassium Phosphate, K_2HPO_4	0.7
Monopotassium Phosphate, KH_2PO_4	0.54
Magnesium sulphate, $MgSO_4 \cdot 7H_2O$	1.0
Calcium Chloride, $CaCl_2 \cdot 2H_2O$	0.2
Iron Sulphate, $FeSO_4 \cdot 7H_2O$	0.004
Zinc Sulphate, $ZnSO_4 \cdot 7H_2O$	0.0001
Magnesium Chloride, $MnCl_2 \cdot 4H_2O$	0.00003
Boric acid, H_3BO_4	0.0003
Cobalt Dichloride, $CoCl_2 \cdot 6H_2O$	0.0002
Copper Chloride, $CuCl_2 \cdot 2H_2O$	0.00001
Nickel Chloride, $NiCl_2 \cdot 6H_2O$	0.00002
Sodium Molybdate Dihydrate, $Na_2MoO_4 \cdot 2H_2O$	0.00006

3.2.3 ISOLATION AND SCREENING OF BACTERIA

i. Isolation of Epiphytic PPFMs by Leaf Imprinting

The paddy leaves was trimmed into 5cm, both side of leaf surface was pressed firmly onto the surface of AMS Medium supplemented with 0.5% (v/v) of methanol for 1-2 seconds (Chanprame, Todd, & Widholm, 1996) , then the leaves were removed and the plates were incubated at 30℃ for 5 to 10 days until bacterial colonies can be observed.

ii. Isolation of Endophytic PPFMs by Spread Plate

The leaves were disinfected with 0.1% sodium hypochlorite and 2 drops of Tween 20 per 150ml sterile for 20 minutes follow by rinsing with sterile distilled water (Chanprame *et al.*, 1996). The disinfected leaves were cut into smaller pieces and transferred into a sterile mortar and 5ml of sterile distilled water was added to mortar. The leaves were homogenized with sterile pestle. 0.1ml of the homogenized leaves was aliquotted and spread on AMS medium with 0.5% (v/v) of methanol and incubated at 30℃ 5 to 10 days until bacterial colonies can be observed.

Each bacteria colony is identified by observing its morphology. The colonies that are pink in color were subcultured onto a new AMS media with 0.5% (v/v) of methanol by streaking method. A loopful of single colony was took and streaked across the medium surface. Then the plates were incubated at 30℃ for 5 to 10 days. Subculturing of bacteria was repeated until pure culture was obtained.

3.2.4 Cellular Morphology Identification and Characterization by Gram Staining

Gram staining was performed to identify the Gram positive and Gram negative bacteria before proceeded with DNA extraction. A sterile inoculating loop was used to take a colony of isolates and smeared on the glass slide. The smear was heated to fix the isolates on the slide by passing slowly through the flame. Staining of the smear was performed by using Gram stain kit. The smear was stained with crystal violet for 60 seconds and washed off with water. The smear then flooded with iodine for 60 seconds and washed off with water. Then, few drops of decoloriser (ethanol-acetone) were added. The ethanol-acetone was rinse off with water after 5 seconds or when the solvent no longer colored as it flows over the slide. Lastly, the smear was counterstained with safranin for 60 seconds and washed off with water. The smear was blotted to remove the excess water, air dried and observed under light microscope.

3.2.5 Molecular Identification by 16S rRNA Sequence Analysis

A. Genomic DNA Extraction

i. Phenol: Chlroform: Isoamyl Alcohol Extraction

A loopful of pure bacteria colony was taken and resuspended into a microcentrifuge tube with Glucose-Tris-HCL-EDTA (GTE) buffer. The buffer solution with bacteria culture was centrifuged at 2000 rpm at 4℃ for 5 minutes. This step was repeated for 3 to 4 times until the pellet reach desired size. The supernatant was discarded. The pellet was washed with 1mL of Glucose-Tris-HCL-EDTA (GTE) buffer twice and centrifuged again at

10,000rpm for 5 minutes. The supernatant was discarded and 200µl of GTE was added to resuspend the pellet then kept the mixture on ice for 5 minutes. 100µl of lysozyme was added and incubated at 37℃ for 2 hours. 50µl of proteinase K and 50 µl of 25% sodium dodecyl sulphate (SDS) was added then incubated at 50˚C for 30 minutes. After incubation, 200 µl Tris-HCl-EDTA (TE) buffer, 500 µl Phenol: Chloroform: Isoamyl Alcohol (25:24:1) was added and inverted for several times. The mixture was then centrifuged at 10000 rpm for 15 minutes at 4˚C and two layers were formed. The upper layer was transferred into a new tube. 400 µl sodium acetate and 800 µl isopropanol was added to precipitate DNA and then incubated at room temperature for 10 minutes followed by centrifuged at 10000 rpm for 5 minutes at 4˚C. The DNA pellet was washed with 500µl of 80% ice-cold ethanol by centrifuged at 10000 rpm for 5 minutes at 4˚C. After centrifugation, the pellet was dried and dissolved in 50 µl distilled water and kept at -20˚C.

ii. G-spin™ Total DNA Extraction Kit

A loopful of pure bacteria culture was taken and inoculated into a microcentrifuge tube with Glucose-Tris-HCL-EDTA (GTE) buffer. The buffer solution with bacteria culture was centrifuged at 2000 rpm at 4℃ for 5 minutes. This step was repeated for 3 to 4 times until the pellet reach desired size. The supernatant was discarded. 300µl of G-buffer was added to each sample and mixed well. The mixtures were incubated at 65℃ for 15 minutes. The mixtures were inverted every 5 minutes to help cell lysis. 250µl of binding buffer was added into the tubes and completely mixed well by pipetting. The cell lysates were loaded onto the column and centrifuged at 13,000rpm for 1 minute. Next, 500µl of washing buffer A was added to the column and centrifuged at 13,000rpm for 1 minute. The flow through

was discarded, and 500µl of Washing Buffer B was added to the columns and centrifuged again at 13,000 rpm for 1 minute. The flow through was discarded, the columns were centrifuged again at 13,000 rpm for 1 minute to dry the column. Then, transfer the column into a sterile microcentrifuge tube and 100µl of Elution Buffer directly onto the membrane, the columns with Elution Buffer were incubated at room temperature for 1 minute and centrifuge for 1 minute at 13, 000 rpm. The flow through was store at -20℃.

B. Agarose Gel Electrophoresis of DNA Sample and PCR Product

1% of agarose gel was prepared by mixing 30ml of 1x TAE buffer with 0.3g of agarose powder. The mixture was heated with microwave for 1-3 minutes until the agarose was completely dissolved. The agarose solution was left to cool and then 2µl of ethidium bromide (EtBr) was added into the agarose solution. The agarose solution was poured into a gel tray with the well comb in place and left for 20 minutes to solidify. After that, the garose gel was placed into the gel box at the direction of black (-ve) to red (+ve) and the gel box was filled with 1xTAE buffer until the gel was covered. 5µl of DNA sample or PCR product was mixed with 1µl of 6x loading dye. 2µl 1k bp DNA marker and DNA sample or PCR product with loading dye was loaded carefully into the gel well and the gel was run with 75V for 40 minutes. The power supply was turned off before removing the gel for visualization of DNA fragment using UV transilluminator.

C. Amplification of 16S rRNA

A 25µl of PCR reaction mixture was prepared which was containing the following composition and volume as shown in Table 3.2.

Table 3.2 PCR master mix

Composition	Volume (µl)
dNTPs	0.5
10x *Taq* Buffer	2.5
25mM $MgCl_2$	2
Nuclease free water	17.75
Taq polymerase (5unit/µl)	0.25
68F forward primer (10pmol)	0.5
1392R reverse primer (10pmol)	0.5
DNA templete	1

The PCR reaction mixture was inserted into PCR thermocycler. The reaction condition of the PCR thermocycler was shown in Table 3.3. The PCR products was then kept at -20˚C. The sequences of both forward and reverse primer used are stated as follows:

68F forward primer: 5‘- TNA NAC ATG CAA GTC GAR -3‘

1392R reverse primer: 5‘- ACG GGC GGT GTG TRC -3

Table 3.3: PCR conditions in the thermocycler

Stages	Temperature (^{o}C)	Time (minute)	Number of cycle
Initialization	94	4	1
Denaturation	94	1	30
Annealing	53.8	1	30
Extension	72	1	30
Final extension	72	7	1

D. Purification of PCR Product

PCR product was purified with MEGAquick-spin™ Total Fragment DNA Purification Kit. 5 volumes of BNL buffer was mixed with 1 volume of PCR sample. For example, 500µl of buffer PB added to 100µl PCR sample. A column was assembled with 2ml collection tube. The sample was added to the column and centrifuged at 13,000 rpm for 1 minute. The flow-through was discarded and the column was assembled back to the collection tube. 700µl of washing buffer was added to the column and centrifuged for 1 minute. The flow through was discarded and the column was assembled back to the collection tube. Next, the column was centrifuged for 1 minute to dry the column. After that, the column was placed in a clean 1.5mL microcentrifuge tube and 50µl of the elution buffer directly to the center of the column without touching the membrane. The column was incubateed at room temperature for 1 minute. After 1 minute, the column was centrifuged for 1 minute, the flow-through was kept and store at -20 ^{o}C.

E. DNA Sequencing

The purified PCR products were sent to First BASE Laboratories Sdn Bhd for DNA sequencing.

F. BLAST Analysis

The sequenced result was analyzed by comparing with the database in Nucleotide BLAST (https;//www.ncbi.nlm.nih.gov/).

CHAPTER 4

RESULT AND DISCUSSION

4.1 Isolation of *Methylobacterium* sp. (PPFM) Bacteria

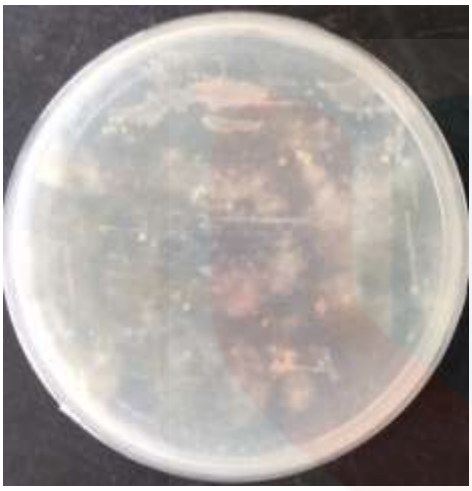
Isolation of bacteria is the first step in identifying the bacteria and to obtain pure bacterial culture. *Methylobacterium* sp. is one of the pink pigmented facultatively methylotrophic bacteria, thus, only pink color colonies are selected. In this study, *Methylobacterium* sp. (PPFM) bacteria were isolated from paddy leaves and palm oil leaves.

Ammonium mineral salt (AMS) media supplemented with 0.5% (v/v) of methanol was used to isolate epiphytic and endophytic *Methylobacterium* sp. . Leaf imprinting method is commonly applied as standard procedure to isolate the endophytic *Methylobacterium* sp. on the plant leaf surface (Madhaiyan, Poonguzhali, Kwon, & Sa, 2009; Omer *et al.*, 2004; Zhao *et al.*, 2009), however, homogenized plant leaf was used to isolate the endophytic bacteria (Chanprame *et al.*, 1996). Combination of both methods can

recover and increase the detection of PPFMs. The plant sample was imprinted and spread on AMS media and incubated at 30℃. After 5 to 7 days, about 20 pink colonies were observed on the selective media. There were about 20 pink colonies grown on the media from the surface of paddy leaf and oil palm leaf respectively. While there was few big spread colonies formed from the homogenized paddy and oil palm leaf. The colonies from imprinted method comparatively easier to be observed as there were different colonies are overlapped. The colonies from imprinted method are easier to be observed and isolated as 10 pink colonies were selected and subcultured onto new AMS media with 0.5% (v/v) of methanol until pure colonies were obtained. The selected isolates are shown in Figure 4.2. From Figure 4.2, the isolates were pink in color but with different intensity.

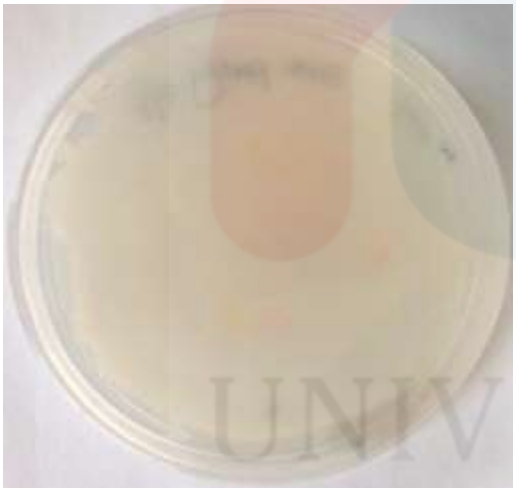
The media used in this study was AMS media. The elements such as iron, calcium, and magnesium are essential for all types of strains. However, the composition of the media was chosen as selective media was because of the ammonium chloride concentration was 0.05% and phosphate concentration was greater than 0.2% which effectively inhibits many strains to grow (Whittenbury, Phillips, & Wilkinson, 1970). Moreover, the addition of methanol is the only carbon source provided that favors the growth of pink pigmented facultatively methylotrophic bacteria (PPFMs) (Chanprame *et al.*, 1996; KIST, 2012) meanwhile inhibited the growth of the undesired bacteria. The result of isolation of epiphytic and endophytic bacteria is shown in Figure 4.1. The growth of colonies indicated the bacteria were able to utilize methanol.

A.



B.

C.



D.

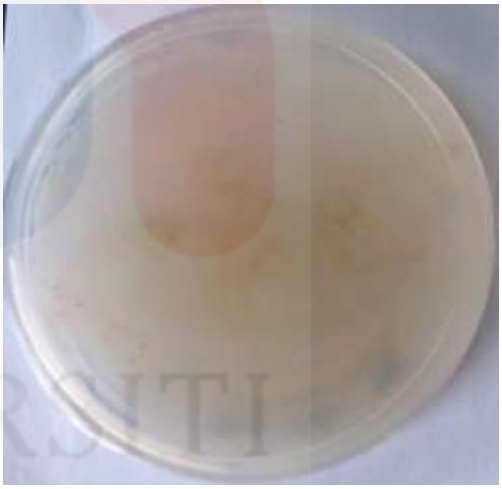


Figure 4.1 Isolation of bacteria from paddy leaves and oil palm leaves on AMS media supplemented with 0.5% methanol. A.: Epiphytic bacteria isolation from paddy leaf; B.: Epiphytic bacteria isolation from oil palm leaf; C.: Endophytic bacteria isolation from paddy leaf; D.: Endophytic bacteria isolation from oil palm leaf.

(Note: Epiphytic bacteria isolated by leaf imprinting method. Endophytic bacteria isolated by spread method.)

4.2 Characterization and identification of Bacteria

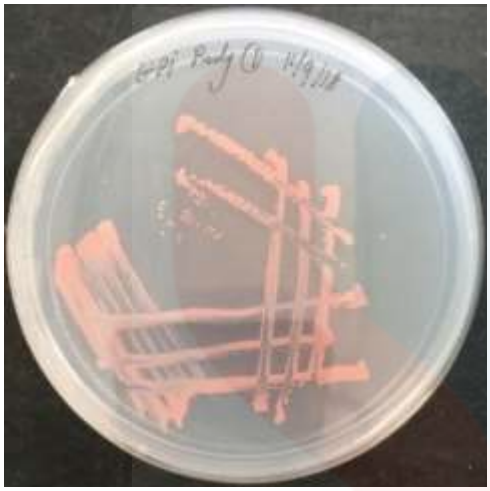
4.2.1 Colony Morphology and Identification of Bacteria

10 pink colonies from preliminary screening were selected and subcultured until pure colonies were obtained. The isolates were then selected by observing the colony morphology then further identification by performing Gram stain and 16S rRNA sequence analysis to identify the species of the isolated PPFM. All the isolates were observed after 7 days of incubation. The isolates from different part of leaf were had similar colony morphology which exhibited circular, and raise opaque characteristics but in different color intensity. The expression of the pink pigmentation range from reddish pink to orange-pink which indicated the bacteria have the ability to synthesis carotenoids. The presence of carotenoids protects the bacteria form intense light stress (Kwak *et al.*, 2014). The isolates are slow growing bacteria. The colonies took at least 5 days to form visible colonies and took 7 days to reach their maximum size. The size of the bacterial colonies from oil palm leaves was smaller than that from paddy leaves. The colonies morphology is tabulated in Table 4.1.

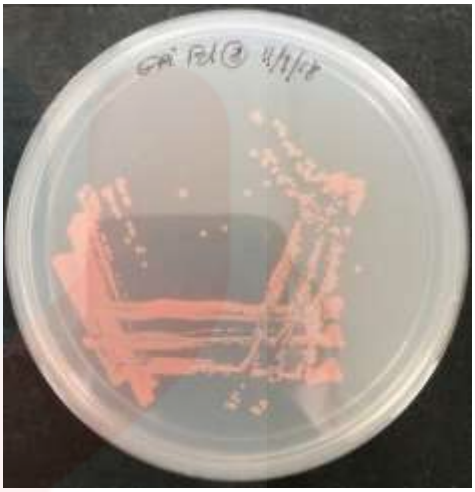
Table 4.1 Colony morphology of each isolate

Isolates	Colony shape	Pigmentation	Elevation	Margin edge	Colony size (mm)
EPPD1	Circular	Light pink	Raise	Entire	1
EPPD3	Circular	Orange- pink	Raise	Entire	1
EPPD4	Circular	Light pink	Raise	Entire	1
ENPD1	Circular	Light pink	Raise	Entire	1
ENPD2	Circular	Light pink	Raise	Entire	<1
ENPD3	Circular	Light pink	Raise	Entire	<1
EPPM1	Circular	Reddish pink	Raise	Entire	<1
ENPM1	Circular	Light pink	Raise	Entire	<1
ENPM2	Circular	Reddish pink	Raise	Entire	<1
ENPM3	Circular	Light pink	Raise	Entire	<1

(a)



(b)



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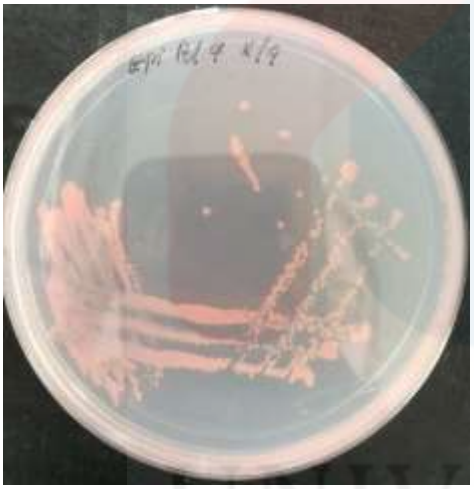
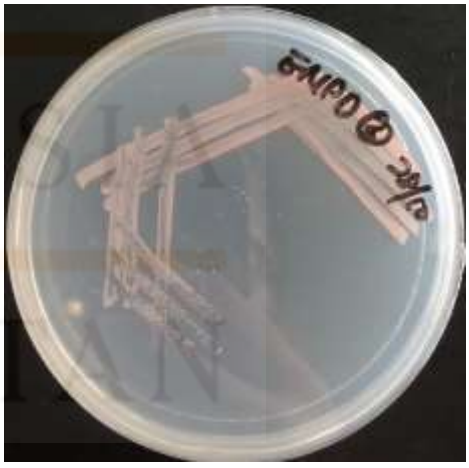


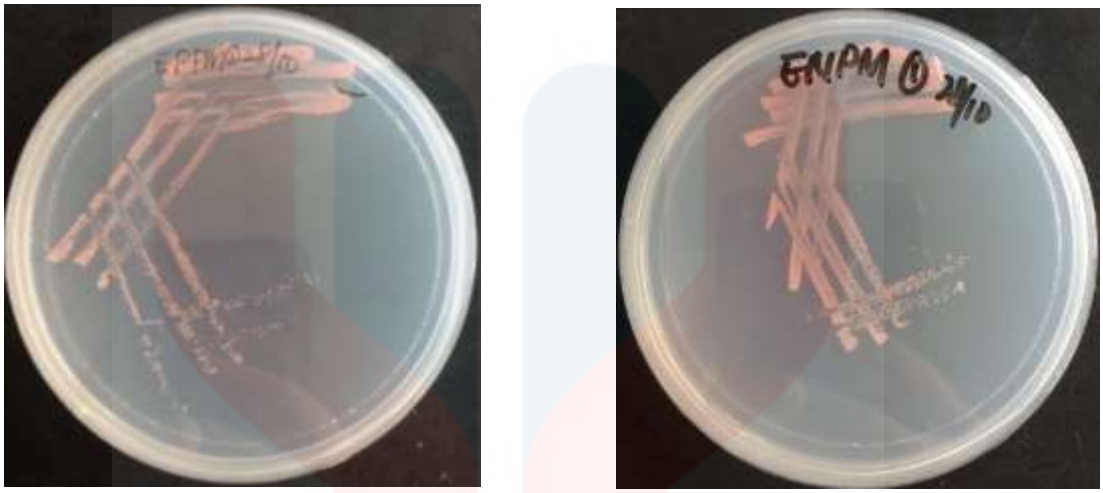
(d)

(e)

(f)



(g) (h)



(i) (j)

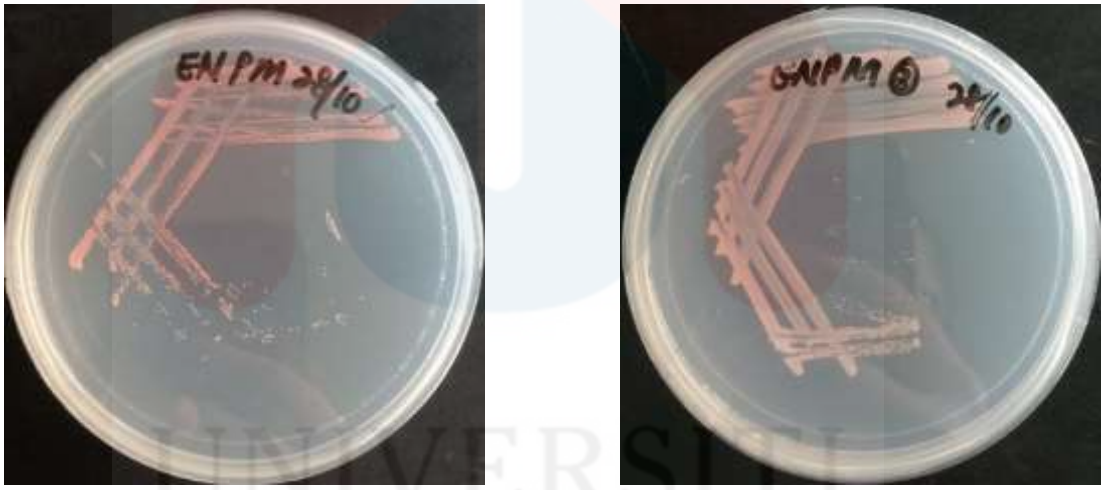


Figure 4.2 Colony morphology of the bacteria isolates on AMS media supplemented with 0.5% methanol. (a) to (c): EPPD1, 3, 4 (epiphytic PPFM from paddy leaf); (d) to (f): ENPD1, 2, 3 (endophytic PPFM from paddy leaf; (g): EPPM1 (epiphytic PPFM from oil palm leaf); (h) to (j): ENPM1, 2, 3 (endophytic PPFM from oil palm leaf).

4.2.2 Cellular Morphology Characterization by Gram Staining

Gram stain was performed on the isolates to identify the bacteria are belongs to Gram positive or Gram negative group and to observe the cell morphology. After staining, the bacteria were observed under 100x magnifications to identify the bacteria physical morphology. Gram positive bacteria stained in purple color while Gram negative bacteria stained in pink. This is due to the different composition and structure of the cell walls. Peptidoglycan is a type of polymer that builds the cell wall of bacteria. Gram positive bacteria have thicker peptidoglycan on the cell walls thus it retains dye during decoloration while Gram negative bacteria lose dye during decoloration. All of the isolates were Gram negative and the cellular morphology were in rod shape.

Gram stain was the basic staining method that allows visualization of bacteria under microscope. There are four important steps in performing Gram stain technique. First, primary staining dye crystal violet was added on the heat fixed bacteria smear. Since crystal violet is water soluble, it dissociates into CV^+ and CV^- ions. Positive ion penetrates through the outer membrane and interacts with negatively charged cell components. Next, iodine act as a mordant as iodine ions I^- or I_3^- interact with CV^+ ions to form crystal violet complexes. Next, decolorizer, a mixture of acetone and ethanol applies onto the smear. Ethanol dehydrates the poor lipid layer of Gram positive cells resulting the large CV-I complexes traps within the cell, thus the cells stain purple. While in Gram negative cell, acetone will wash of liposaccharide layer of the cell wall leaving peptidoglycan layer exposed to acetone, the large CV-I complexes will wash away by acetone resulting the cells colourless. Lastly, when the smear is counterstain with weakly water soluble dye such as safranin, the

decolorized Gram negative cells stained red, while Gram positive cells does not interrupt by safranin as safranin is lighter than crystal violet (Smith & Hussey, 2015).

Methylobacterium sp. bacteria are Gram's negative and in rod shape (Madhaiyan *et al.*, 2011; Mosin & Ignatov, 2014). Figure 4.3 is the results of Gram stain for the 10 bacterial isolates. Both isolates from paddy leaves and oil palm leaves have same cellular morphology. They were Gram's negative and the cellular morphology were in rod shape which matched with the morphology as stated as Dourado *et al.* (2015), Madhaiyan *et al.* (2011) and Mosin & Ignatov (2014).

Table 4.2 Gram's reaction and cellular morphology of bacterial isolates

Plant	Isolate	Gram's reaction	Color	Cellular morphology
Paddy	EPPD1	Negative	Pink	Rod shape
	EPPD3	Negative	Pink	Rod shape
	EPPD4	Negative	Pink	Rod shape
	ENPD1	Negative	Pink	Rod shape
	ENPD2	Negative	Pink	Rod shape
	ENPD3	Negative	Pink	Rod shape
Oil Palm	EPPM1	Negative	Pink	Rod shape
	ENPM1	Negative	Pink	Rod shape
	ENPM2	Negative	Pink	Rod shape
	ENPM3	Negative	Pink	Rod shape

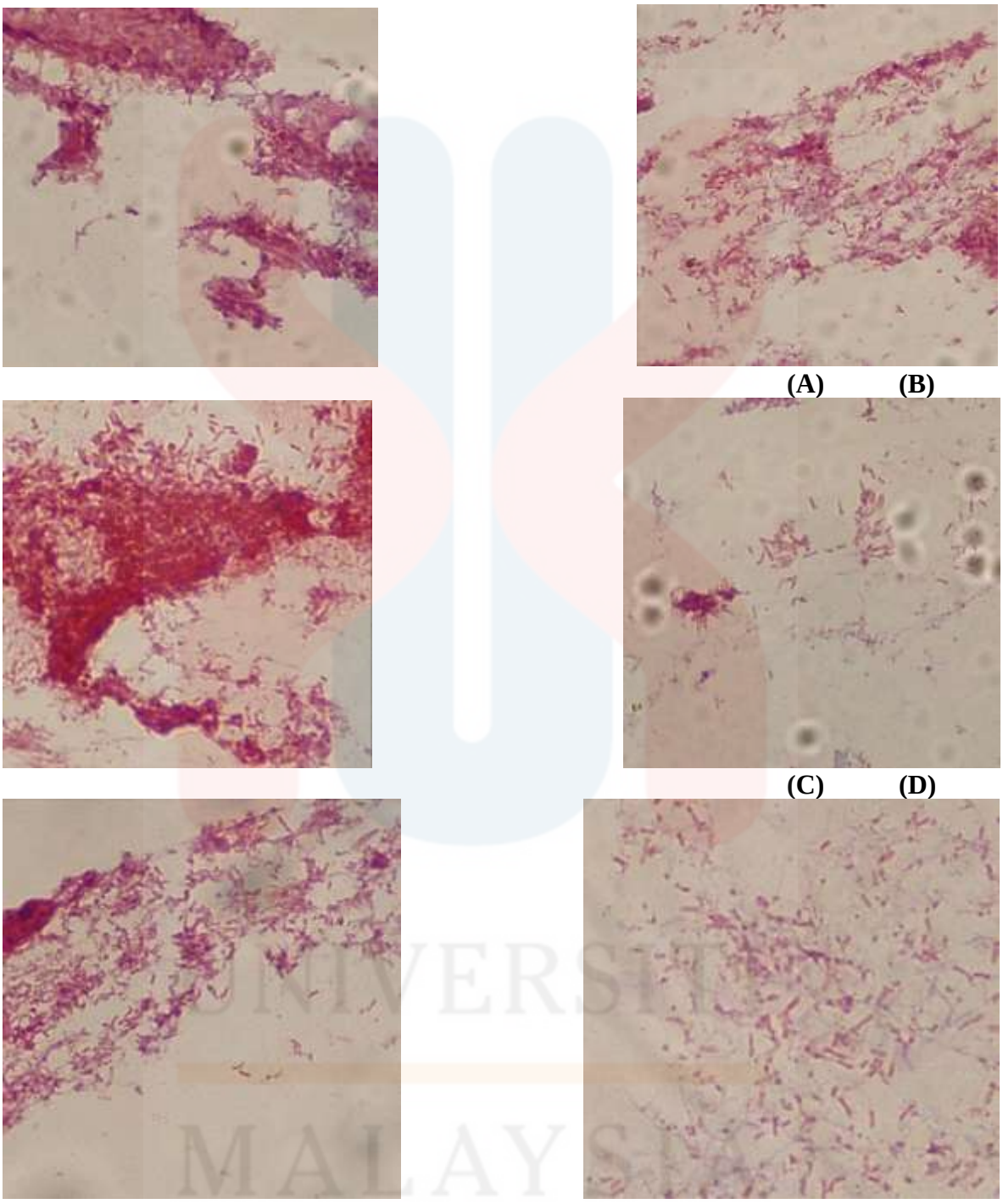
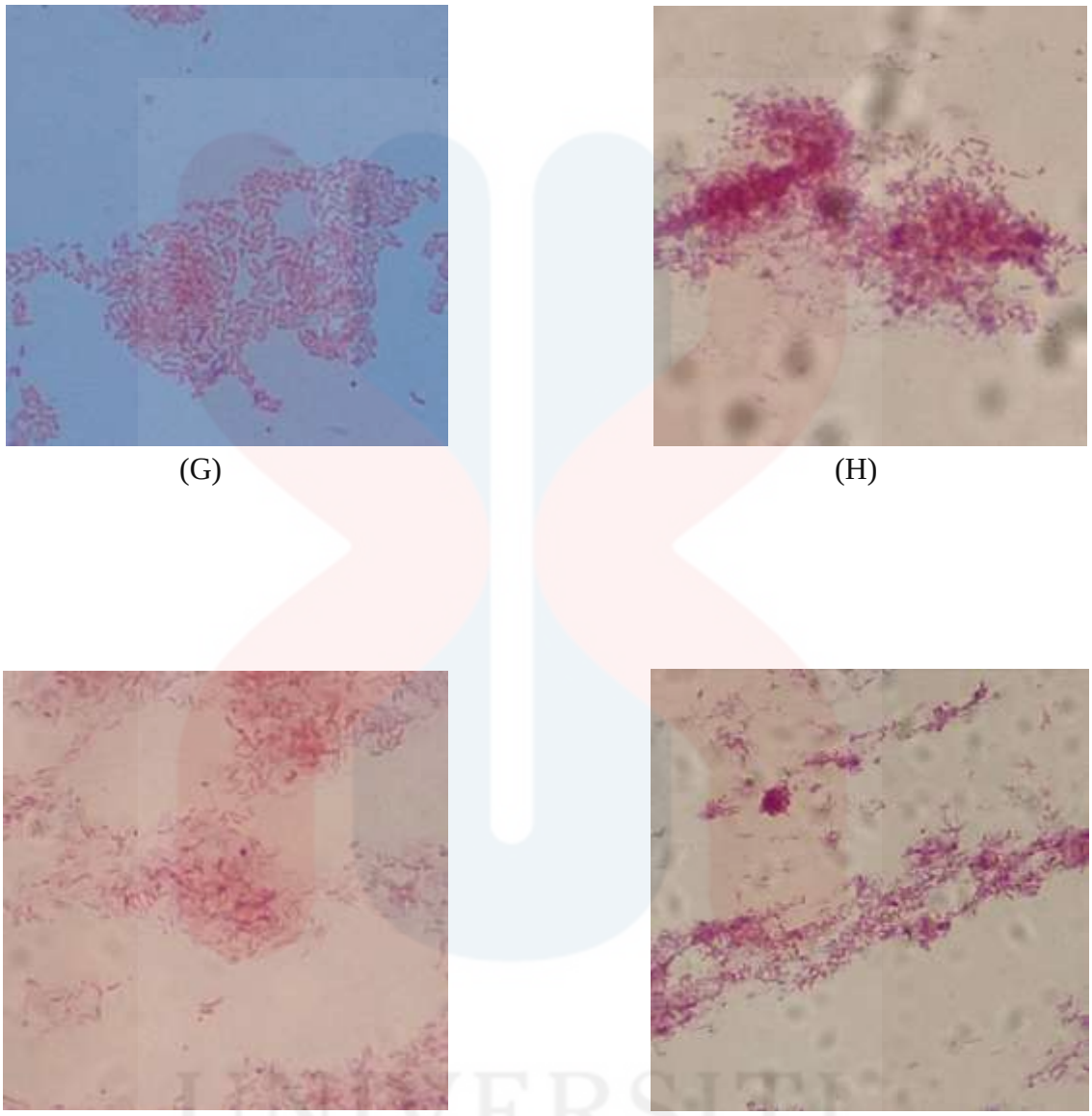


Figure 4.3 Cellular morphology of bacteria isolated from paddy leaf observed under microscope with 100x magnification. (A) to (C): EPPD1, 3, 4 (epiphytic PPFM); (D) to (F): ENPD1, 2, 3 (endophytic PPFM)



(G) (H)

(I) (J)

Figure 4.4 Cellular morphology of bacteria isolated from oil palm leaf observed under microscope with 100x magnification. (G): EPPM1 (epiphytic PPFM); (H) to (J): ENPM1, 2, 3 (endophytic PPFM)

4.2.3 Molecular Identification by 16S rRNA Sequence Analysis

i. Genomic DNA Extraction

The isolates were then proceeded to molecular identification. There are few protocols needed to be done in order to identify the bacteria species using 16S rRNA sequence including DNA extraction, amplifying 16S rRNA gene, gene sequencing and BLAST analysis.

The genomic DNA were extracted from the bacterial isolates to obtain their genetic information, the desired gene sequence can use for further analysis. The method used in this study was phenol: chloroform: isoamyl alcohol extraction and G-spin^{TM} Total DNA Extraction Kit. First, lysozyme was added to degrade the cell walls, Proteinase K and 25% SDS were added to purify the DNA. The proteinase K help in inactivates nucleases that contain in the bacteria, this nucleases may degrade the DNA during purifying due to the bacterial DNA is no longer protected after cell lysed (Helmuth *et al.*, 1975). SDS is one of the cleaning procedure that denaturing protein in the preparation for electrophoresis (Kimberley, 2012). The SDS help in unfolding enzyme and protein that make it susceptible for the proteinase K to digest or inhibit. After further purification of DNA was proceed with the addition of phenol, chloroform and isoamyl alcohol. It formed two layers when these solvent were added. The upper layer was known as aqueous layer, and the lower layer known as phenol layer. The role phenol is to denature the excess cellular protein and dissolve into the phenol layer while the DNA remains in the aqueous phase and protected by TE buffer. Next, it proceed by precipitation of DNA using sodium acetate, ethanol and

isopropanol. The precipitation of DNA can be separated easily with the solution. Besides, ethanol and isopropanol were effectively washed away the remaining salt residue.

After extracting DNA, gel electrophoresis was carried out to determine the DNA is successfully extracted. From result shown in Figure 4.5 all of the DNA bands can be observed. The bands showed are indicates the DNA of the isolates were successfully extracted. The thickness of the bands was different among the isolates. This is due to the extracted DNA concentration are different. The band show brighter indicates the DNA concentration higher. Furthermore, there were smear band occur in L4, L7, L9 and L10, it might due to the presence of large genomic DNA fragment and protein in the sample. These large molecules affected the samples mobilities on the gel (Mayer, 2018). Purification of extracted genomic DNA is necessary in order to improve the resolution and sample quality.

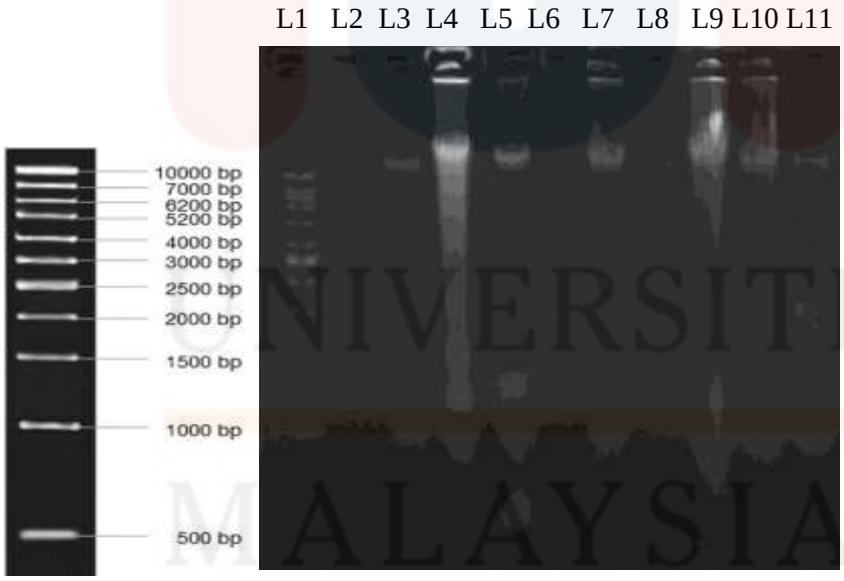


Figure 4.5 Gel electrophoresis of extracted genomic DNA. L1: VC1kb DNA ladder (Vivantis); L2: gDNA from isolate EPPD1; L3: gDNA from isolate EPPD3; L4: gDNA from isolate EPPD4; L5: gDNA from isolate ENPD1; L6: gDNA from isolate ENPD2; L7: gDNA from isolate ENPD3; L8: gDNA from isolate EPPM1; L9: gDNA from isolate ENPM1; L10: gDNA from isolate ENPM2; L11: gDNA from isolate ENPM3.

ii. Amplification of 16S rRNA

The extracted DNA was proceeded to Polymerase Chain Reaction (PCR). PCR was carried out to amplify the targeted gene copies from the extracted DNA for further sequencing analysis. The PCR components such as dNTPs, primers, *Taq* polymerase and 10x buffer and $MgCl_2$ were mixed with DNA template and carried out in repeated cycles to carry out the amplification of the 16S rRNA gene. The repeated cycles consist of denaturation, annealing and extension that play critical role in amplifies the desired gene sequence into millions copies.

In denaturation, the temperature was increased to heat up the double stranded DNA template and caused it to unwind and separated into single stranded form. Then, temperature was cooled to annealing optimum temperature to allow primer anneal to it complimentary parts. Next, the *Taq* DNA polymerase synthesized new copies of DNA strand using dNTP as building blocks at the location of the annealed primers. The addition of $MgCl_2$ was added as a cofactor of polymerase to increase the specificity(Schmidt, Stiverson, Angen, & Yu, 2014). These three steps were repeated and each cycle double up the copies of new DNA strands (Harris, Innis, McPherson , Moller, O'Connell *et al.*, 2011).

The PCR products were undergone gel electrophoresis to view and access the PCR product yield and purity. The result is shown in Figure 4.5. The targeted product was successfully amplified from all of the bacterial isolates except for isolates ENPD2 (L6). The reason of the failure of PCR might due to the DNA template concentration was too low to proceed for PCR. Another reason that causes to failure is the impurity presence in the DNA sample. The remaining impurities from DNA extraction step like SDS, phenol, ethanol, isopropanol and proteinase K will inhibit the polymerase activity and thus the

impurities have great inhibitory effect on PCR (Rossen, Nørskov, Holmstrøm, & Rasmussen, 1992).

The size of the amplified gene is expected to be about 1324bp by using 68F and 1392R primers. However, there are some non-specific bands presence in L7, L8, L9 and L11. For L8, the non-specific band occurred at around 1000 bp. The presence of non-specific bands is due to the temperature of annealing was too low that cause primers bind nonspecifically to the template. For L7, L9, L11, the non-specific band were showed at 10,000 bp which indicated the presence of excessive DNA template. Besides, there are low molecular weight smears occurred in all of the samples, this may due to the DNA template contains exonuclease and the DNA template maybe sheared during DNA extraction step (Breu, Guggenbichler, & Wollmann, 2008) .

Next, purification of amplified DNA was carried out by using MEGAquick-spin™ Total Fragment DNA Purification Kit. Purification of PCR product is necessary to eliminate the excess primers, nucleotides, non-specific fragments and DNA templates. These impurities will causes disturbance while sequencing the gene resulting to poor DNA sequence analysis.

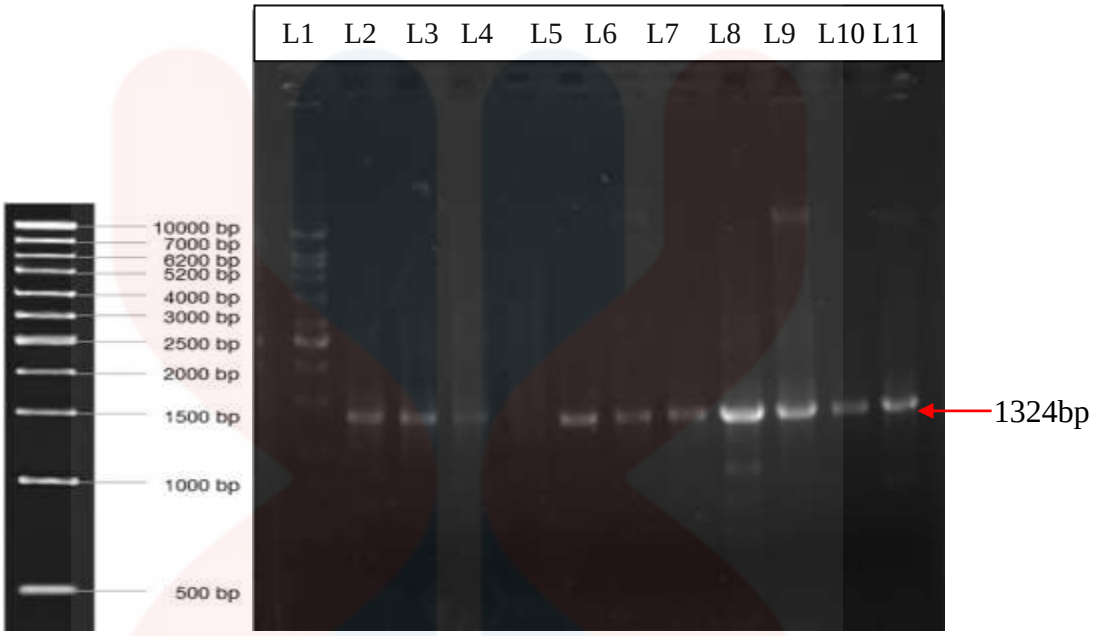


Figure 4.6 Gel electrophoresis of amplified PCR product from isolates. L1: VC1kb DNA ladder (Vivantis); L2: PCR product from isolate EPPD1; L3: PCR product from isolate EPPD3; L4: PCR product from isolate EPPD4; L5: PCR product from isolate ENPD1; L6: PCR product from isolate ENPD2; L7: PCR product from isolate ENPD3; L8: PCR product from isolate EPPM1; L9: PCR product from isolate ENPM1; L10: PCR product from isolate ENPM2; L11: PCR product from isolate ENPM3.

iii. BLAST Analysis

The amplified and purified DNA samples had been sequenced and BLAST analysis had been carried out to compare the nucleotides sequence of the sample to data bases and calculate the statistical significance of similarity. Sequencing of 16S rRNA gene analysis generally used for determining the speices and phylogenetic relationship between bacteria. The sequenced gene result from First Base Laboratories Sdn. Bhd. was analyse using BLAST at https://blast.ncbi.nlm.nih.gov/ to obtain percentage of similarity with the data base.

Both reverse and forward sequence of the isolates was analyzed. In order to obtain more reliable results, reverse and forward sequence of the isolate were compared after BLAST analysis in terms of sequence length and sequencing chromatogram to determine the species of the isolates. The sequence that is longer and shaper peak in sequencing chromatogram was chosen. Based on the BLAST analysis, there are two isolates, EPPD3 and EPPM1are failed to identifiy a match species from the data base. This might due to low concentration of DNA, resulting most nucleotides unreadable. Next, species of the rest of isolates were identified. ENPM2 was identified as *Methylobacterium aerolatum* and EPPD1, EPPD4, ENPD1, ENPD3, ENPM1, ENPM2 and ENPM3 identified as *Methylobacterium radiotolerans*. There is less information of both *M. aerolatum* and *M. radiotolerence*. Weon *et al.* (2015) had discovered *M. aerolatum*, from air samples and did not state any relationship between this species and plant. Eevers *et al*. (2015) had discovered the abilities of *M. radiotolerence* which are promoting plant growth and degrading DDT (2,2-bis(p-chlorophenyl)-1,1,1-trichloroethane) that use as pesticide. Thus,

it has potential to be studied as an inoculant for plant growth and bioremediation of DDT contaminated soils.

Table 4.3 Bacteria species identification by BLAST

Isolates	Species	Similarity, %
EPPD1	*Methylobacterium radiotolerans*	88
EPPD3	Unidentified	-
EPPD4	*Methylobacterium radiotolerans*	97
ENPD1	*Methylobacterium radiotolerans*	97
ENPD3	*Methylobacterium radiotolerans*	98
EPPM1	Unidentified	-
ENPM1	*Methylobacterium radiotolerans*	98
ENPM2	*Methylobacterium aerolatum*	97
ENPM3	*Methylobacterium radiotolerans*	98

CHAPTER 5

CONCLUSION & RECOMMENDATION

5.1 Conclusions

In this study, endophytic and epiphytic *Methylobacterium* sp. bacteria were successfully isolated from paddy leaves and oil palm leaves on AMS media supplemented with 0.5% (v/v) methanol. There are about 20 pink colonies were observed on each media, and in total ten colonies were selected for morphological characterization and molecular identification. The isolates were Gram's negative and rod shape. Moleclar identification was done by DNA extraction, amplification of 16SrRNA and BLAST analysis. Amplification of 16SrRNA was done and send for sequencing. The targeted product of all selected bacterial isolates was successfully amplified except for isolates ENPD2. Out of nine isolates, 2 isolates (EPPD3 and EPPM1) were failed to be identified their species, six isolates were identified as a same species. EPPD1, EPPD4, ENPD1, ENPD3, ENPM1,

ENPM2 and ENPM3 were identified as *Methylobacterium radiotolerans* and ENPM2 was identified as *Methylobacterium aerolatum.*

5.2 Recommendations

There are few recommendations are suggested for further study of *Methylobacterium* sp. and to obtain more reliable and accurate results.

1. Different C1 substrate such as methylamine and formaldehyde are suggested to use as carbon source in isolating different species of *Methylobacterium* bacteria.
2. The study on isolation of *Methylobacterium* sp. from different source such as soil and water.
3. The isolates that failed to identify species are suggested to resubmit for DNA sequencing with better quality DNA.
4. Evaluation of the *Methylobacterium* sp for their agriculturally important properties to study the potential of *Methylobacterium* sp.

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APPENDIX A

Preparation of Buffer

Preparation of TE Buffer:

i. 1M Tris-HCL (1L), pH 8.0
   1. 121.1g of Tris base dissolve in 700mL of distilled water.
   2. Adjust pH to 8.0 using 12M HCL.
   3. Make up the volume to 1000ml using distilled water.
   4. Autoclave

ii. 0.5M EDTA (1L), pH 8.0
   1. 186.12g of EDTA and 20g of NaOH pallet dissolve in distilled water.
   2. Slowly add more NaOH pallet until pH 8.0 and EDTA complete dissolved.
   3. Make up the volume to 1000ml using distilled water.
   4. Autoclave

iii. TE Buffer (1L)
   1. 10ml of 1M Tris-HCL + 2ml 0.5M EDTA
   2. Make up to 1000ml
   3. Autoclave.

Preparation of GTE Buffer (100ml)

i. 1M Glucose stock solution
   1. Dissolve 180g of glucose in 1L of distilled water

ii. 5ml 1M Glucose solution + 2.5ml 1M Tris-HCL + 2ml 0.5M EDTA
iii. Make up to 100ml
iv. Autoclave

Preparation of 50x TAE Buffer (100ml)

i. 24.2g Tris base + 5.71ml glacial acetic acid
ii. Add 10ml of 0.5M EDTA
iii. Make up to 100ml with distilled water
iv. Autoclave

Preparation of 1x TAE Buffer (1000ml)

i. 20ml of 50x TAE+ 980ml of distilled water
ii. Autoclave

Preparation of Reagent

1. 25% SDS: 25g of SDS + 100ml distilled water
2. 20mg/ml proteinase K: 0.02g of proteinase K powder + 1ml sterile distilled water
3. 50mg/ml lysozyme: 0.05g of lysozyme powder + 1ml sterile distilled water
4. 80% ethanol: 8ml absolute ethanol + 2 ml sterile distilled water
5. 10mM sodium acetate: 0.41g of sodium acetate + 50ml sterile distilled water

Appendix B

Nucleotide BLAST result for sequence of isolate

Description	Max score	Total score	Query cover	E value	Ident
Methylobacterium radiotolerans strain DSM 1819 16S ribosomal RNA gene, partial sequence	1777	1777	88%	0.0	96%
Methylobacterium radiotolerans strain JCM 2831 16S ribosomal RNA, partial sequence	1775	1775	88%	0.0	96%
Methylobacterium radiotolerans strain 0-1 16S ribosomal RNA gene, partial sequence	1762	1762	88%	0.0	96%
Methylobacterium tardum strain RB677 16S ribosomal RNA gene, partial sequence	1760	1760	88%	0.0	96%
Methylobacterium fujisawaense strain DSM 5686 16S ribosomal RNA gene, partial sequence	1753	1753	88%	0.0	96%

Forward sequence of EPPD1

Description	Max score	Total score	Query cover	E value	Ident
Methylobacterium radiotolerans strain DSM 1819 16S ribosomal RNA gene, partial sequence	1044	1044	55%	0.0	91%
Methylobacterium radiotolerans strain JCM 2831 16S ribosomal RNA, partial sequence	1042	1042	55%	0.0	91%
Methylobacterium tardum strain RB677 16S ribosomal RNA gene, partial sequence	1033	1033	55%	0.0	91%
Methylobacterium radiotolerans strain 0-1 16S ribosomal RNA gene, partial sequence	1024	1024	55%	0.0	91%
Methylobacterium oryzae strain CBMB20 16S ribosomal RNA gene, partial sequence	1022	1022	55%	0.0	91%

Reverse sequence of EPPD1

RID 0V3GURTJ015 (Expires on 12-11 04:50 am)

Query ID lcl|Query_154159
Description 1st_BASE_3362181_16S-PD3a_Reverse_1392R.ab1
Molecule type nucleic acid
Query Length 108

Database Name rRNA_typestrains/prokaryotic_16S_ribosor
Description 16S ribosomal RNA (Bacteria and Archaea)
Program BLASTN 2.8.1+ ▷Citation

Forward sequence of EPPD3

RID 0V3G5JNM014 (Expires on 12-11 04:49 am)

Query ID lcl|Query_7065
Description 1st_BASE_3362180_16S-PD3a_Forward_68F.ab1
Molecule type nucleic acid
Query Length 5

Database Name
Description ▷See details
Program BLASTN 2.8.1+ ▷Citation

Reverse sequence of EPPD3

RID	0VKR6G84015 (Expires on 12-11 09:26 am)		
Query ID	lcl|Query_198131	**Database Name**	rRNA_typestrains/prokaryotic_16S_ribosomal_RNA
Description	None	**Description**	16S ribosomal RNA (Bacteria and Archaea)
Molecule type	nucleic acid	**Program**	BLASTN 2.8.1+ ▷Citation
Query Length	684		

No significant similarity found. For reasons why,click here

Forward sequence of EPPD4

Description	Max score	Total score	Query cover	E value	Ident
Methylobacterium radiotolerans strain DSM 1819 16S ribosomal RNA gene, partial sequence	1284	1284	83%	0.0	97%
Methylobacterium radiotolerans strain JCM 2831 16S ribosomal RNA, partial sequence	1282	1282	83%	0.0	97%
Methylobacterium tardum strain RB677 16S ribosomal RNA gene, partial sequence	1279	1279	83%	0.0	97%
Methylobacterium fujisawaense strain DSM 5686 16S ribosomal RNA gene, partial sequence	1271	1271	83%	0.0	97%
Methylobacterium oryzae strain CBMB20 16S ribosomal RNA gene, partial sequence	1267	1267	83%	0.0	97%

Reverse sequence of EPPD4

Description	Max score	Total score	Query cover	E value	Ident
Uncultured bacterium clone MD2_aap38g04 16S ribosomal RNA gene, partial sequence	1993	1993	90%	0.0	97%
Methylobacterium radiotolerans strain RT98 16S ribosomal RNA gene, partial sequence	1991	1991	89%	0.0	97%
Methylobacterium sp. strain CI087 16S ribosomal RNA gene, partial sequence	1991	1991	89%	0.0	97%
Methylobacterium radiotolerans strain VRI8-1 16S ribosomal RNA gene, partial sequence	1991	1991	89%	0.0	97%
Methylobacterium radiotolerans strain VRI7-A5 16S ribosomal RNA gene, partial sequence	1991	1991	89%	0.0	97%

Forward sequence of ENPD1

Description	Max score	Total score	Query cover	E value	Ident
Methylobacterium radiotolerans strain DSM 1819 16S ribosomal RNA gene, partial sequence	1419	1419	85%	0.0	91%
Methylobacterium radiotolerans strain JCM 2831 16S ribosomal RNA, partial sequence	1417	1417	85%	0.0	91%
Methylobacterium tardum strain RB677 16S ribosomal RNA gene, partial sequence	1413	1413	85%	0.0	91%
Methylobacterium fujisawaense strain DSM 5686 16S ribosomal RNA gene, partial sequence	1402	1402	81%	0.0	92%
Methylobacterium oryzae strain CBMB20 16S ribosomal RNA gene, partial sequence	1399	1399	81%	0.0	92%

Reverse sequence of ENPD1

Description	Max score	Total score	Query cover	E value	Ident
Methylobacterium radiotolerans strain RT98 16S ribosomal RNA gene, partial sequence	2119	2119	95%	0.0	98%
Methylobacterium sp. strain CI087 16S ribosomal RNA gene, partial sequence	2119	2119	95%	0.0	98%
Methylobacterium radiotolerans strain VRI8-1 16S ribosomal RNA gene, partial sequence	2119	2119	95%	0.0	98%
Methylobacterium radiotolerans strain VRI7-A5 16S ribosomal RNA gene, partial sequence	2119	2119	95%	0.0	98%
Methylobacterium radiotolerans strain VRI6-A2 16S ribosomal RNA gene, partial sequence	2119	2119	95%	0.0	98%

Forward sequence of ENPD3

Description	Max score	Total score	Query cover	E value	Ident
Methylobacterium radiotolerans strain DSM 1819 16S ribosomal RNA gene, partial sequence	2130	2130	95%	0.0	98%
Methylobacterium radiotolerans strain JCM 2831 16S ribosomal RNA, partial sequence	2128	2128	95%	0.0	98%
Methylobacterium tardum strain RB677 16S ribosomal RNA gene, partial sequence	2117	2117	95%	0.0	98%
Methylobacterium radiotolerans strain 0-1 16S ribosomal RNA gene, partial sequence	2102	2102	95%	0.0	98%
Methylobacterium fujisawaense strain DSM 5686 16S ribosomal RNA gene, partial sequence	2095	2095	95%	0.0	98%

Reverse sequence of ENPD3

RID 0VM66PW501R (Expires on 12-11 09:34 am)
Query ID lcl|Query_39271
Description None
Molecule type nucleic acid
Query Length 276

Database Name rRNA_typestrains/prokaryotic_16S_ribosomal_RNA
Description 16S ribosomal RNA (Bacteria and Archaea)
Program BLASTN 2.8.1+ ▷Citation

No significant similarity found. For reasons why,click here

Other reports: ▷Search Summary

Forward sequence of EPPM1

RID 0VM6SF6601R (Expires on 12-11 09:35 am)
Query ID lcl|Query_66079
Description None
Molecule type nucleic acid
Query Length 206

Database Name rRNA_typestrains/prokaryotic_16S_ribosomal_RNA
Description 16S ribosomal RNA (Bacteria and Archaea)
Program BLASTN 2.8.1+ ▷Citation

No significant similarity found. For reasons why,click here

Reverse sequence of EPPM1

Description	Max score	Total score	Query cover	E value	Ident
Methylobacterium radiotolerans strain JCM 2831 16S ribosomal RNA, partial sequence	2036	2036	89%	0.0	98%
Methylobacterium radiotolerans strain DSM 1819 16S ribosomal RNA gene, partial sequence	2026	2026	89%	0.0	98%
Methylobacterium tardum strain RB677 16S ribosomal RNA gene, partial sequence	2013	2013	89%	0.0	98%
Methylobacterium radiotolerans strain 0-1 16S ribosomal RNA gene, partial sequence	1999	1999	89%	0.0	97%
Methylobacterium fujisawaense strain DSM 5686 16S ribosomal RNA gene, partial sequence	1991	1991	89%	0.0	97%

Forward sequence of ENPM1

Description	Max score	Total score	Query cover	E value	Ident
Methylobacterium radiotolerans strain JCM 2831 16S ribosomal RNA, partial sequence	2128	2128	94%	0.0	98%
Methylobacterium radiotolerans strain DSM 1819 16S ribosomal RNA gene, partial sequence	2119	2119	94%	0.0	97%
Methylobacterium tardum strain RB677 16S ribosomal RNA gene, partial sequence	2095	2095	94%	0.0	97%
Methylobacterium fujisawaense strain DSM 5686 16S ribosomal RNA gene, partial sequence	2089	2089	94%	0.0	97%
Methylobacterium oryzae strain CBMB20 16S ribosomal RNA gene, partial sequence	2089	2089	94%	0.0	97%

Reverse sequence of ENPM1

Description	Max score	Total score	Query cover	E value	Ident
Methylobacterium aerolatum strain 5413S-11 16S ribosomal RNA gene, partial sequence	1694	1694	83%	0.0	94%
Methylobacterium persicinum strain 002-165 16S ribosomal RNA gene, partial sequence	1650	1650	83%	0.0	93%
Methylobacterium komagatae strain 002-079 16S ribosomal RNA gene, partial sequence	1576	1576	92%	0.0	90%
Methylobacterium radiotolerans strain JCM 2831 16S ribosomal RNA, partial sequence	1572	1572	83%	0.0	92%
Methylobacterium radiotolerans strain DSM 1819 16S ribosomal RNA gene, partial sequence	1563	1563	83%	0.0	92%

Forward sequence of ENPM2

Description	Max score	Total score	Query cover	E value	Ident
Methylobacterium aerolatum strain 5413S-11 16S ribosomal RNA gene, partial sequence	1971	1971	88%	0.0	97%
Methylobacterium persicinum strain 002-165 16S ribosomal RNA gene, partial sequence	1932	1932	89%	0.0	96%
Methylobacterium komagatae strain 002-079 16S ribosomal RNA gene, partial sequence	1845	1845	89%	0.0	95%
Methylobacterium radiotolerans strain JCM 2831 16S ribosomal RNA, partial sequence	1842	1842	89%	0.0	95%
Methylobacterium tardum strain RB677 16S ribosomal RNA gene, partial sequence	1832	1832	89%	0.0	95%

Reverse sequence of ENPM2

Description	Max score	Total score	Query cover	E value	Ident
Methylobacterium radiotolerans strain DSM 1819 16S ribosomal RNA gene, partial sequence	2041	2041	91%	0.0	98%
Methylobacterium radiotolerans strain JCM 2831 16S ribosomal RNA, partial sequence	2039	2039	91%	0.0	98%
Methylobacterium tardum strain RB677 16S ribosomal RNA gene, partial sequence	2039	2039	91%	0.0	98%
Methylobacterium fujisawaense strain DSM 5686 16S ribosomal RNA gene, partial sequence	2023	2023	91%	0.0	98%
Methylobacterium oryzae strain CBMB20 16S ribosomal RNA gene, partial sequence	2023	2023	91%	0.0	98%

Forward sequence of ENPM3

Description	Max score	Total score	Query cover	E value	Ident
Methylobacterium radiotolerans strain DSM 1819 16S ribosomal RNA gene, partial sequence	2183	2183	97%	0.0	98%
Methylobacterium radiotolerans strain JCM 2831 16S ribosomal RNA, partial sequence	2182	2182	97%	0.0	98%
Methylobacterium tardum strain RB677 16S ribosomal RNA gene, partial sequence	2169	2169	96%	0.0	98%
Methylobacterium radiotolerans strain 0-1 16S ribosomal RNA gene, partial sequence	2161	2161	97%	0.0	98%
Methylobacterium oryzae strain CBMB20 16S ribosomal RNA gene, partial sequence	2158	2158	96%	0.0	98%

Reverse sequence of ENPM3

Research Flow Chart

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