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# Preliminary analysis of Endophytic Plant Growth Promoting (PGP) *Methylobacterium* sp. Isolated from Palm Oil (*Elaeis guineensis*) Leaves

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**Abstract.** The genus *Methylobacterium* is known for its pink-pigmented facultative methylotrophs (PPFMs). They are able to utilize methanol emitted from plants and in return produce plant growth promoting substances. The genus *Methylobacterium* can be found in diverse habitats and they are known particularly for their close association with plants. The aims of this study are to isolate and characterize endophytic *Methylobacterium* sp. from palm oil leaves. Isolation of *Methylobacterium* sp was conducted using Ammonia Mineral Salts (AMS) agar supplemented with methanol. Three isolates were selected namely ENPM1, ENPM2 and ENPM3. Production of IAA by the selected isolates was evaluated under different concentration of L-tryptophan as an inducer. It was observed that the amount of IAA produced increased when higher concentration of tryptophan was used and ENPM1 recorded the highest amount of IAA production (1.930 µg/mL) at the 1000 µg/mL concentration of L-tryptophan. The ability to solubilized inorganic phosphate was investigated using Solubilisation Index (SI) assay on phosphate solubilisation media (PSM). The largest clear zone formation on PSM agar was shown by ENPM3 with the SI 1.5. As for nitrogen fixation, the growth of the selected isolates on two nitrogen free media namely Burk's and Jensen media indicates potential nitrogen fixing activity.

## 1. Introduction

Patt et al. [1] identified the first *Methylobacterium* sp. as a new genus of methylotrophic facultative bacteria in 1976, also known as pink-pigmented facultative methylotrophs (PPFMs). It could be found ubiquitous inhabiting various habitats, including the phyllosphere, root nodules, soil, dust, freshwater, drinking water, and lake sediment [2-3]. PPFMs are aerobic, gram-negative, methylotrophic rod-shaped bacteria that grow either on multi-carbon substrates or on single carbon compounds such as formaldehyde, formate, and methanol as sole carbon and energy source [4]. It also can produce several plant growth promoting substances such as indole acetic acid (IAA), auxin, cytokinin, and Vitamin B<sub>12</sub> and promote plant growth through metabolic associations in endophytic and endosymbiotic relationships with host plants. Apart from that, they also help plants survive from pathogenic attacks [5].



Several studies reported the application of *Methylobacterium sp.* in promoting the growth of red pepper, tomato, rice, and canola by colonizing the host plant, and produce phytohormones to boost plant growth and yield [6]. These organisms are reported to influence seed germination and seedling growth or interacting with the microbial community (endophytes and pathogens) [7 8].

In order to improve the productivity of the host plant, biofertilizer will be the greatest alternative to replace chemical fertilizer containing generally effective strains of bacteria with multiple advantage characteristics [9]. *Methylobacterium sp.* is one of the potential bacteria that can play a role as biofertilizers and decrease chemical fertilizer usage. Biofertilizer are biological agents that can promote the growth and yield of crop by several processes such as nitrogen fixation, mobilization of nutrients in the soil and production of plant growth-promoting immunoanalysis [10]

In this study, three different methods were used to characterize endophytic *Methylobacterium sp.* isolated from the leaves of palm oil (*Elaeis guineensis*): (1) the production of phytohormone IAA with varying concentrations of L-tryptophan, (2) the ability of the isolates to fix atmospheric nitrogen and (3) the ability of the bacterial isolates to solubilise inorganic phosphate.

## 2. Material and Methods

### 2.1 Bacterial Strain

Three endophytic *Methylobacterium sp.* strains used in this study were isolated from oil palm (*Elaeis guineensis*) leaves by a previous study [11]. The bacteria strains were maintained in AMS agar media supplemented with 0.5% methanol. The strains were named as ENPM1, ENPM2, and ENPM3.

### 2.2 Preparation of media

#### 2.2.1. Preparation of Ammonium mineral salt (AMS)

Ammonium mineral salt (AMS) media was prepared by mixing 1 L of distilled water with the composition, as shown in Table 1, with the pH adjusted to 6.8 with NaOH. Then 15 g of agar powder was added into the solution. The media was sterilized by autoclaving at 121°C for 15 minutes. Next, media was cooled to 50°C and 0.5% (v/v) methanol was added.

**Table 1.** Composition of AMS medium.

Composition of AMS medium	Concentration (g/L)
Ammonium chloride	0.5
Dipotassium phosphate	0.7
Monopotassium phosphate	0.54
Magnesium sulphate	1.0
Calcium chloride	0.2
Iron sulphate	0.004
Zinc sulphate	0.0001
Magnesium chloride	0.00003
Boric acid	0.0003
Cobalt dichloride	0.0002
Copper chloride	0.00001

Nickel chloride	0.00002
Sodium Molybdate Dihydrate	0.00006

### 2.2.2 *Pikovskaya media*

Pikovskaya media was prepared by mixing 1 L of distilled water with the composition, as shown in Table 2 with the pH adjusted to 6.8 with NaOH. Then 15 g of agar was added into the solution and later was sterilized by autoclaving at 121°C for 15 minutes.

**Table 2.** Composition of Pikovskaya medium.

Composition of Pikovskaya Plate Method	Concentration (g/L)
Yeast Extract	0.5
Dextrose	10.0
Calcium Phosphate	5.0
Ammonium Sulphate	0.5
Potassium Chloride	0.2
Magnesium Sulphate	0.1
Manganese Sulphate	0.002
Ferrous Sulphate	0.002

### 2.2.3 *NBRIP media*

NBRIP media was prepared by mixing 1 L of distilled water with the composition as shown in Table 3 with the pH adjusted to 6.8 with NaOH. Then 15 g of agar was added into the solution and later was sterilized by autoclaving at 121°C for 15 minutes.

**Table 3.** Composition of NBRIP medium.

Composition of NBRIP medium	Concentration (g/L)
Glucose	10.00
Calcium Phosphate	5.00
Magnesium Chloride Hexahydrate	5.00
Magnesium Sulphate	0.25
Potassium Chloride	0.20
Ammonium Sulphate	0.10

### 2.2.4 *Jensen's Nitrogen-free Medium*

Jensen medium were prepared by mixing 1 L of distilled water with the composition as shown in Table 4 with pH adjusted to 6.8 with NaOH. Agar weighing 15 g was added into the solution and later was sterilized by autoclaving at 121°C for 15 minutes.

**Table 4.** Composition of Jensen medium.

<b>Composition of Jensen medium</b>	<b>Concentration (g/L)</b>
Sucrose	20.0
Dipotassium phosphate	1.0
Magnesium sulphate	0.5
Sodium chloride	0.5
Ferrous sulphate	0.1
Sodium molybdate	0.005
Calcium carbonate	2.0

### 2.2.5 Burk's Nitrogen-free media

Burk's nitrogen-free media was prepared by mixing 1 L of distilled water with the composition as shown in Table 5 with the pH adjusted to 6.8 with NaOH. Then 15 g of agar was added into the solution and later was sterilized by autoclaving at 121°C for 15 minutes.

**Table 5.** Composition of Burk's medium.

<b>Composition of Burk medium</b>	<b>Concentration (g/L)</b>
Magnesium sulphate	0.2
Dipotassium phosphate	0.8
Monopotassium phosphate	0.2
Calcium sulphate	0.13
Ferric chloride	0.00145
Sodium molybdate	0.000253
Sucrose	20

## 2.3 Characterisation of Plant Growth Promoting Activities

### 2.3.1 Evaluation of Indole Acetic Acid Production

#### a) Preparation of Salkowski Reagent

The reagent was prepared by mixing 1 ml of 0.5M iron (III) chloride, 50 ml of distilled water and 30 ml of concentrated sulfuric acid, as shown in Table 6.

**Table 6.** Composition of Salkowski reagent.

<b>Composition of Salkowski Reagent</b>	<b>Concentration (g/L)</b>
0.5 M Iron (III) chloride	1
Distilled water	50
Concentrated sulfuric acid	30

*b) Preparation of Standard Curve/Graph*

IAA powder weighing 100 mg was dissolved in 10 ml of acetone by swirling. Several concentrations of IAA were prepared which are 0, 0.05, 0.1, 0.2, 0.3, 0.4, 0.5, 1.0, 5, 10, 20, 50, 100 and 1000 µg/ml. One ml of IAA solution with different concentrations were mixed with 2 ml of Salkowski reagent. They were incubated at room temperature in the dark for 25 minutes. Blank was prepared by mixing 1 ml of distilled water with 2 ml of Salkowski reagent. Next, the optical density (OD) of different concentrations of was taken at 530 nm and a graph was plot for the concentration of IAA against absorbance value.

*c) IAA Assay*

Bacteria isolates were inoculated into AMS broth and incubated at 37°C for 7-10 days until they reached  $OD_{600} = \pm 0.4$ . Varying concentrations of tryptophan 50, 100, 200, 500, 1000 and 10000 µg/ml, was added to the culture respectively. After an incubation period of 7 days, the bacterial culture was centrifuged at 9000 rpm for 15 minutes. The supernatant was filtered using a 0.22-µm membrane filter and mixed with 2ml of Salkowski reagent and incubated in the dark at room temperature for 25 minutes. Absorbance was measure at  $OD_{530}$ .

*2.3.2. Determination of Nitrogen-fixing Ability*

To determine the ability of nitrogen fixation activity by the bacteria strain, the bacteria isolates were streaked on Burk's and Jensen's nitrogen-free medium agar plate and incubated at 30°C. The bacteria isolates were incubated on Burk's media for 7 days while on Jensen media for 8 days. The growth of the isolates were then observed.

*2.3.3 Determination of Phosphate Solubilising (SI) Activity*

Solubilising Index (SI) was determined by growing isolates on NBRIP and Pikovskaya's media. A single colony of bacteria isolates was stabbed into both agar and incubated at 30°C for 7 days. After 7 days, the diameter of the bacteria colony and halo zone around the colony was measured and recorded on 1, 3, 5 and 7 days of incubation. Phosphate Solubilising Index (SI) was calculated using the formula below [12].

$$\text{Solubilisation Index (SI)} = \frac{\text{Colony diameter} + \text{Halo zone diameter}}{\text{Colony diameter}}$$

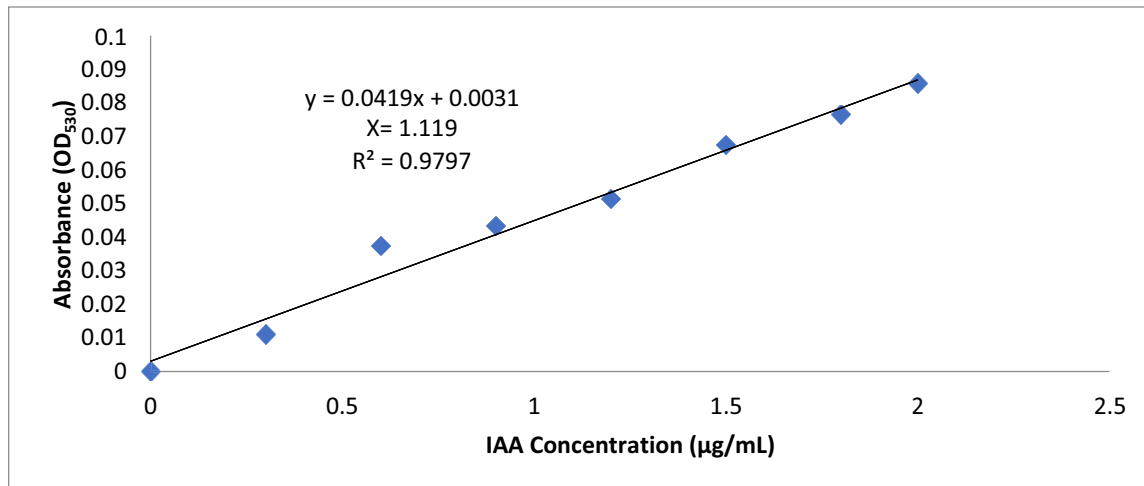
**Equation 2.1****3. Result and Discussion***3.1 Evaluation of Indole Acetic Acid (IAA) Production*

Plant growth-bacteria utilize a combination of biochemical and genetic techniques to facilitate plant growth. One of the most influential and relevant of these mechanisms is producing the plant hormone indole acetic acid (IAA). IAA is a phytohormone that acts as critical plant growth and development modulator. Almost every aspect of plant growth and architecture, including cell division, elongation, fruit development, root initiation, leaves and flowers, cambial growth, vascular development and senescence, is regulated by this significant phytohormone [13 14 15 16].

*Methylobacterium sp.* strains have been reported to produce auxin-derived indole acetic acid in stems, seeds and young leaves from the transmission and decarboxylation reaction L-tryptophan [17]. L-tryptophan is a distinctive amino acid forming an indole ring [18]. It is an auxin precursor that is biologically active [19 20 21 22] and its exogenous application determines the effectiveness of auxin in plant tissue [23]. L-

tryptophan is defined in many ways to plants including soil application [24 25], foliar spray [26 27], and seed priming [19 28].

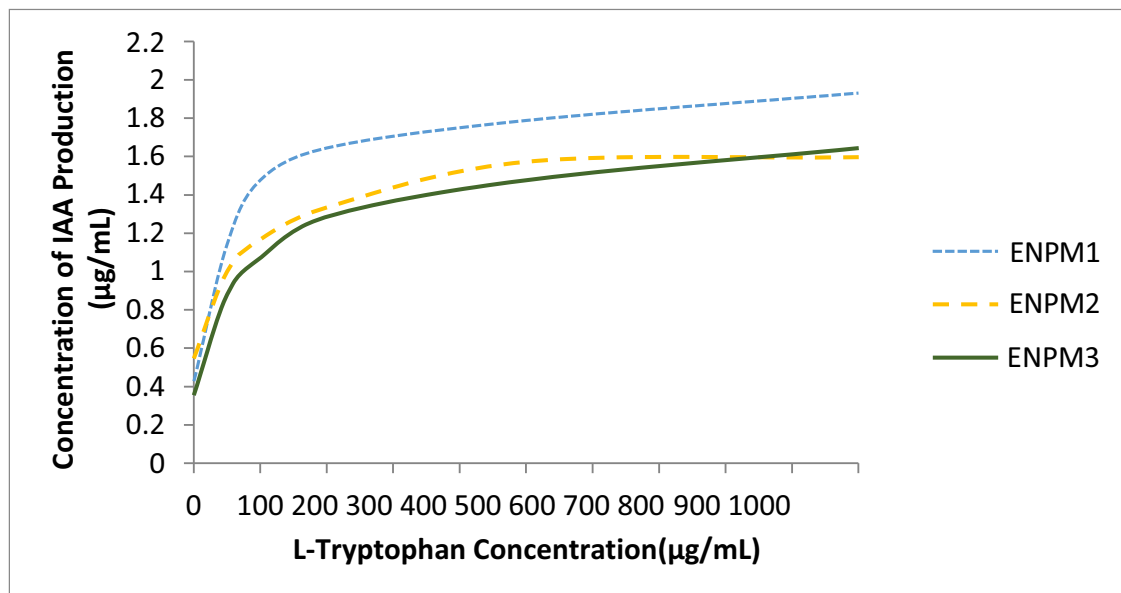
Production of IAA by newly isolated endophytic *Methylobacterium sp.* were conducted using different concentrations of L-Tryptophan. Figure 1 shows the standard curve that was constructed to quantify the amount of IAA. The standard curve is light absorption graphs versus solution concentration used in unidentified samples to know about the solute concentration.



**Figure 1.** Indole Acetic Acid Standard Curve

The production of IAA by ENPM1, ENPM2 and ENPM3 with different L-tryptophan concentrations is shown in Figure 2. Following 7 days incubation, the IAA production for all isolated bacteria shows increasing trends reciprocal with the L-tryptophan amount. The higher the concentration of L-tryptophan, the greater the IAA yields from the samples. The ENPM1 strains recorded maximum levels of IAA production (1.930µg/mL), followed by ENPM 3 (1.644µg/mL) and ENPM2 (1.596µg/mL). For isolate ENPM1 and ENPM3 the amount of IAA produced are steadily increased until the L-tryptophan concentration is 1000 µg/mL. In contrast, for isolate ENPM2; it shows on the graph the production of IAA slowed down after an initial surge at the concentration of 800 µg/mL.

Similar reports have previously been reported by Ahmad et al. [29] higher concentration of L-Tryptophan enhanced the production of IAA *Pseudomonas sp.*, *Enterobacter sp* and fluorescent *Azotobacter sp.*, respectively. This shows that L-Tryptophan plays a role as precursor of IAA production in *Methylobacterium sp.* As IAA production increased with the increased L-tryptophan concentration, the production began to remain constant until a certain point, representing the limit of the mechanism of IAA production.



**Figure 2.** IAA Production by three strains of endophytic *Methylobacterium sp.*

The mechanism of IAA production are categorized into two types: the tryptophan independent-pathway and the tryptophan dependent-pathway. Both pathways able to synthesize IAA but without L- Tryptophan unknown enzymes generate tryptophan to IAA in the tryptophan-independent pathway. In contrast, a tryptophan-dependent pathway consists of known bacterial pathways including tryptamine, indole-3-acetaldehyde with tryptophan side chain oxidase (TSO) and indole-3-pyruvic acid (IPyA), indole-3-acetamide (IAM) [30]. L-Tryptophan's transmission and decarboxylation reaction are occurred to produce the IAA [17]. Based on Figure 3.2, it was observed that IAA was produced even without the addition of L-tryptophan. However, the amount of IAA was increases dramatically after the addition L-tryptophan. It shows that the production IAA in the newly isolated *Methylobacterium sp.* are dominantly involved the L-tryptophan dependent-pathway.

In general, the methylotrophic strains produced IAA regardless of the presence or absence of the precursor L-tryptophan. Based on previous report, indicated that even strains belonging to the same genus had different IAA levels because it was affected by the culture condition and the substrate's growth stage [29].

### 3.2 Determination of Nitrogen-fixing Ability

Nitrogen is needed for the synthesis of chlorophyll, amino acids, nucleic acids, and ATP which are required for plant growth and survival. Plants cannot directly utilize the atmospheric nitrogen gas, though nitrogen happened to be the most abundant element on earth [31]. The reduced form of nitrogen can be used by plants in the form of either ammonia or nitrate. Nitrogen ( $N_2$ ) can be converted to ammonia ( $NH_3$ ) by a specialized group of bacteria called "diazotrophs" which the ability to fix nitrogen is conferred by the "nitrogenase" enzyme complex [32 33]. As plants do not have the enzyme nitrogenase, they are unable to synthesize ammonia on their own from aerial nitrogen [31]. Therefore, plants use microbe for fixing ammonia to produce the nitrogenous bio-molecules for their growth and survival.

A qualitative technique was used to evaluate the nitrogen fixation capability of endophytic *Methylobacterium sp.* isolated from palm oil leaves. It was tested by growing the isolates on the Burk's nitrogen-free medium and Jensen's nitrogen free medium for 7 days and 8 days respectively. The growth of the isolates on the both medium indicated that *Methylobacterium sp.* were able to fix the atmospheric



nitrogen. The growth of bacterial isolates on Burk's media and Jensen's media were observed and recorded as shown in Table 7. All the three isolates demonstrated poor growth on both media indicated weak nitrogen-fixing activity.

**Table 7.** Growth of bacterial isolates Burk's Medium and Jensen's Medium

<i>Methylobacterium sp.</i>	Burk's Medium	Jensen's Medium
ENPM1	Poor growth	Poor growth
ENPM2	Poor growth	Poor growth
ENPM3	Poor growth	Poor growth

Both media was recommended to determine the ability of bacteria in nitrogen-fixation. The difference between those two media was the composition used in Burk's Medium were Monopotassium Phosphate, Calcium sulphate and Ferric chloride. Burk's media has inorganic salts along with carbohydrate source, but lacks of nitrogen source. The calcium sulphate on the media is to stimulate nodulation when present as chloride and sulphate. Jensen's Medium are consists of dipotassium phosphate, sodium chloride and ferrous sulphate. The purpose of sodium molybdate is to increase the nitrogen-fixation activity [34], while sodium chloride is to maintain the osmotic equilibrium of the media.

### 3.3 Determination of phosphate solubilising activity

Preliminary screening for phosphate solubilization was carried out using NBRIP's medium and Pikovskaya medium. Pikovskaya Agar was modified by Sundara Rao and Sinha [35] for detection of phosphate-solubilizing bacteria from soil. Yeast extract in the medium provides nitrogen and other nutrients necessary to support bacterial growth. Dextrose acts as an energy source. Different salts and yeast extract support the growth of organisms. For NBRIP media, it was modified by removing yeast extract and improved the concentration of magnesium sulphate ( $MgSO_4$ ) because the magnesium chloride provides for a better synergistic effect on phosphate solubilization activity in the presence of  $MgSO_4$  compared to manganese sulphate ( $MnSO_4$ ) [36]

The strains were inoculated into Pikovskaya and NBRIP and incubated at 30° C for 10 days. Solubilisation of phosphate was indicated by the presence of a clear zone around the bacterial colonies. The solubilization index (SI) for all the three isolates were recorded for day 1, 3, 5, and 7 days after the cultivation period and tabulated in Table 8.

**Table 8.** Phosphate Solubilizing Index (SI) for bacterial isolates using NBRIP Medium and Pikovskaya's Medium

meth	NBRIP				Pikovskaya's			
	1 <sup>st</sup>	3 <sup>rd</sup>	5 <sup>rd</sup>	7 <sup>th</sup>	1 <sup>st</sup>	3 <sup>rd</sup>	5 <sup>rd</sup>	7 <sup>th</sup>
ENPM1	0.0	0	1.4	1.4	0	0	0	0
ENPM2	0	0	0	0	0	0	0	1.15
ENPM3	0	1.3	1.3	1.3	0	1.4	1.5	1.5

For NBRIP Medium, only ENPM1 and ENPM3 have the phosphate solubilising activity while ENPM2 does not have the phosphate solubilizing activity. For Pikovskaya's Medium, only ENPM2 and ENPM3 have the phosphate solubilizing activity while ENPM1 does not have the phosphate solubilizing activity. It is possible to determine from isolates ENPM1, ENPM2 and ENPM3 that the strains able to perform phosphate solubilization activities and are potentially used in biofertilizer to promote plant growth.

According to some studies [37 38], not all phosphate solubilizing bacteria can produce a halo zone phosphate solubilization media (PSM). Thus, phosphate solubilization assay was recommended for further study. A quantitative assay using vanadate molybdate reagent is recommended to evaluate the amount of

phosphate solubilizing activity produced by each isolate. The assay was described as in a dilute orthophosphate solution, ammonium molybdate responds under acid conditions to form a heteropoly acid, molybdate phosphoric acid. Yellow vanadate molybdate phosphoric acid is formed in the presence of vanadium. The yellow colour intensity was relative to the phosphate concentration [39].

#### 4. Conclusion

In this study, the plant growth-promoting traits for 3 strains of endophytic *Methylobacterium sp.* isolated from palm leaves were characterised by IAA production, nitrogen-fixing ability and phosphate solubilising ability. With the various alteration of L-tryptophan concentrations in AMS broth, all the isolates demonstrated increased in IAA production with increasing L-tryptophan concentration. The highest IAA production was shown by the isolates ENPM1 (1.930 $\mu$ g/mL), followed by ENPM 3 (1.644 $\mu$ g/mL) and ENPM2 (1.596 $\mu$ g/mL). In nitrogen-fixing assessment, all isolates are able to grow on Burk's nitrogen-free medium and Jensen's nitrogen-free medium. As for phosphate solubilization, three strains showed different results when using two phosphate solubilization media (NBRIP and Pivkovskaya). However, isolate ENPM3 showed the most consistent phosphate solubilization activity with SI 1.3 and 1.5 on NBRIP and Pivkovskaya respectively.

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