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Investigation of Newly Isolated *Methylobacterium sp.* as Potential Biofertilizer.

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Abstract. *Methylobacterium sp.* are pink-pigmented facultative methylotrophs (PPFMs) that can promote plant's growth due to several physiological characteristics such as the ability to produce phytohormone namely indole acetic acid (IAA) and effective roles in phosphate solubilization and nitrogen fixation process. Members of this genus are ubiquitous in the environment and characterized by its ability to utilize single C compound such as methanol as its energy source. These characteristics made *Methylobacterium sp.* as potential candidates for biofertilizer. In this study, *Methylobacterium sp.* were isolated from the leaves of paddy and palm oil tree using Ammonia Mineral Salt (AMS) agar supplemented with methanol. Nine isolates were selected based on the appearance of pink colonies on AMS agar. Several analyses were conducted to evaluate plant growth-promoting activities of the selected isolates. The production of IAA was quantified spectrophotometrically using Salkowski's reagent, and solubilization of inorganic phosphate was determined using vanadate molybdate assay. The results showed that, all the isolates are capable to produce IAA and solubilize inorganic phosphate. As for nitrogen fixation, the lack/poor growth of the isolates on two nitrogen-free media, namely Burks's and Jensen media manifested the absence or weak nitrogen fixing activity. Findings from these qualitative and quantitative analyses are vital and it will serve as preliminary data for the future exploration of *Methylobacterium sp.* as biofertilizer.

1. Introduction

Methylotrophs is a group of microorganism that has the ability to assimilate carbon through RuBP and serine pathways. It can be bacteria, archaea, fungi or yeast. It comprises of obligate and facultative aerobic eubacteria, which possess the ability to use C1 compounds as the source of carbon and energy [1] (Mosin & Ignatov, 2014). Methylotrophs was 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 [2] [3]. *Methylobacterium sp.* is one of the well-known methylotrophs that had been extensively studied. The



member of *Methylobacterium* genus are classified as the member of class α -proteobacteria, order *Rhizobiales* and family of *Methylobacteriaceae*. This 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 [4] [5].

One of the features of *Methylobacterium sp.* is they promote plant growth. When these bacteria colonized a plant, utilization of plant exudates resulted in accumulation of secondary metabolites by bacteria which can influence plant growth and development. Phytohormone is the major secondary metabolites that stimulate growth in all plants [6][7]. Phytohormones such as cytokinin and auxin are the essential growth hormone for plants. Cytokinin regulates cell division and elongation in shoots and roots while 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 in plants can lead to stress condition, deleterious effect on plant growth, causes early anscission and senescence. IAA production by methltotrophs will increase if the ethylene synthesis increases. Thus, methylotrophs are important in modulating plant growth and development by balancing the IAA and ethylene level [8].

Besides oxidization of single carbon compound, it was reported that, *Methylobacterium sp.* that isolated from soil or mud are efficient in fixation of atmospheric nitrogen due to the presence of nitrogenase enzyme in their metabolic pathway. A study by Rekadwad (2014) found that, inoculation of *Methylobacterium organophilum* into soil had shown remarkable effect on seed germination and promote plant growth by providing nitrogenous compound [9]. Apart from nitrogen, another critical minerals elements which control soil and plant productivity is phosphate. Phosphate solubilisation by bacteria which can promote plant growth was reported in many studies. Even though phosphate is abundant in the soil (Lim *et al.*, 2007) [10][11][12] but the concentration of soluble phosphorus in the form of $H_2PO_4^{-1}$, which can be used by the plants are limited. Most of it is in the form of inorganic compounds bound to calcium, iron, or aluminium or immobilized in organic matter such as phytate (phytic acid, myo-inositol hexakisphosphate). Therefore, the transformation of insoluble phosphate by phosphate-solubilizing bacteria increase soil fertility and facilitate the uptake of phosphate by the plants [13][14].

Thus, in the development of biofertilizer, the manifestation of plant growth promoting activities such as phytohormones production, phosphate solubilization and nitrogen fixation is a major consideration. In this study, we reported the isolation of endophytic and epiphytic pink pigmented facultative methylotrophs from the phylosphere of palm oil and paddy. In order to explore the potential on these isolates for future application as biofertilizer, quantitative and qualitative analyses were carried out to determine their plant growth promoting (PGP) properties. PGP activities were evaluated based on the production of indol-3-acetic acid (IAA), phosphate solubilization and nitrogen fixation.

2. Material and Methods

2.1 Isolation of pink pigmented facultative methylotrophs (PPFMs).

In this study, two types of PPFMs were isolated from palm oil and paddy leaves which are endophytic PPFMs and ephyphitic PPFMs. Isolation both of types of PPFMs were carried on ammonia mineral salts (AMS) media supplemented with 0.5% (v/v) methanol. The composition of AMS media is shown in Table 2.1. Leaf imprint method was used to isolate endophytic PPFMs. Palm oil and paddy leaves were trimmed to 5cm in size and pressed firmly on AMS for 1-2 seconds. Then, the plates were incubated at 30°C until bacterial colonies were observed. As for epiphytic PPFMs, The leaves were disinfected with 0.1% sodium hypochlorite and 2 drops of Tween 20 for 20 minutes follow by rinsing with sterile distilled water. The disinfected leaves were then homogenized using pestle and mortar. 0.1ml aliquot of the homogenized leaves was spread on AMS medium with 0.5% (v/v) of methanol and incubated at 30°C for 5 to 10 days until

bacterial colonies can be observed.

Table 2.1 Composition of AMS medium plate

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 Evaluation of 3-indol-acetic acid (IAA) production

2.2.1 Preparation of Salkowski Reagent. The reagent was prepared by mixing 1 ml of 0.5M iron (III) chloride and 30 ml of concentrated sulfuric acid in 50 ml of distilled water.

2.2.2 Construction of IAA standard curve. Several concentration of IAA solutions were prepared (0.05, 0.1, 0.2, 0.3, 0.4, 0.5, 1.0, 5, 10, 20, 50, 100 and 1000 $\mu\text{g/ml}$) to construct the standard curve for IAA solution. 1 ml of IAA solution with different concentrations were mixed with 2ml of Salkowski reagents and incubated at room temperature in the dark for 25 minutes. Then, the absorbance of the solution were quantified spectrophotometrically at 530nm.

2.2.3 IAA assay. IAA assay was conducted based on method by Goswami [15]. Bacteria isolates were inoculated into AMS broth and incubated at 36°C for 7-10 days until they reached $\text{OD}_{600} = \pm 0.4$. 1000 $\mu\text{g/ml}$ of L-tryptophan was added to the culture respectively and the cultures were further incubated for 7 days. After 7 days, the cultures were centrifuged at 9000 rpm for 15 minutes. 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. Then, the absorbance of the mixtures solution were quantified spectrophotometrically at 530nm.

2.3 Determination of phosphate solubilisation activity

Newly isolated *Methylobacterium sp* were grown on two types of phosphate solubilisation media (PSM) namely National Botanical Research Institute's phosphate growth medium (NBRIP) and Pivovskaya's medium. Composition of both media are shown in Table 2.2 and Table 2.3. The ability of the bacterial isolates to solubilize inorganic phosphate in the media was assessed by the formation of halo zone surrounding the colonies. Then, the solubilisation of inorganic phosphate by the bacterial isolates were determined quantitatively by vanadate-molybdate assay.

Table 2.2: 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

Table 2.3: 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

2.3.1 Vanadate-molybdate assay. Bacterial isolates were inoculated into NBRIP and Pikovskaya's broth and incubated at 37°C for 7 to 10 days until the growth reached optical density (OD_{470}) ± 0.4 . The cultures then centrifuged at 9000 rpm for 15 minutes and the supernatant was filtered using a 0-22- μ m membrane filter. The supernatant was dissolved with 0.25 mL of vanadate-molybdate reagent and incubated for 10 minutes. After 10 minutes of incubation, colour changes started to develop. The absorbance of samples were quantified spectrophotometrically at 470nm.

2.4 Evaluation nitrogen fixing ability

Bacterial isolates were grown on two types of nitrogen free media to evaluate their ability to fix atmospheric nitrogen which are Jensen medium and Burk's medium. The composition of both medium are shown in Table 2.4 and Table 2.5 respectively. A single colony of bacterial isolates were streaked on both media and incubated at 30°C for 7 days on Burk's media and on Jensen media for 8 days. The presence of bacterial colonies on both media were observed as observed as qualitative evidence of atmospheric nitrogen.

Table 2.4: Composition of Jensen medium.

Composition of Jensen medium	Concentration (g/L)
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

Table 2.5: Composition of Burk medium.

Composition of Burk medium	Concentration (g/L)
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

3. Result and Discussion

3.1 Isolation of pink pigmented facultative methylotrophs (PPFMs)

Pink pigmented facultative methylotrophs (PPFMs) are generally characterized by their ability to utilize single carbon substances such as methanol as sole energy source. The pathway of methanol assimilations are almost similar in all methylotrophs and the key enzyme in pathway is methanol dehydrogenase encoded by *mxoF* [2]. Thus, the isolation of PPFMs especially *Methylobacterium sp.* is commonly conducted on AMS media supplemented with methanol to select the strain. *Methylobacterium sp.* can be detected with the presence of pink colonies after 5-7 days of incubation on AMS media. In this study, we had successfully isolated 6 endophytic and 3 epiphytic *Methylobacterium sp.* from the leaves of palm oil and paddy. The result is tabulated in Table 3.1

Table 3.1 *Methylobacterium sp.* isolated from palm oil and paddy leaves

Isolates	Source	Endophyte /epiphyte
ENPM1	Palm oil	Endophyte
ENPM2	Palm oil	Endophyte
ENPM3	Palm oil	Endophyte
ENPD1	Paddy	Endophyte
ENPD2	Paddy	Endophyte
ENPD3	Paddy	Endophyte
EPPM1	Palm oil	Epiphyte
EPPD1	Paddy	Epiphyte
EPPD4	Paddy	Epiphyte

3.2 Evaluation of plant growth promoting activity.

Plant growth promoting bacteria utilize a combination of biochemical and genetic traits to facilitate plant growth. Among the major characteristics are the production of phytohormones, solubilization of inorganic phosphate and fixation of atmospheric nitrogen to ammonia [16] [17]. Several *Methylobacterium sp.* were reported to promote the growth of tomato, paddy and crambe [7][6][18]. Thus, *in vitro* analyses on the for the newly isolated *Methylobacterium sp.* were carried out to investigate their plant growth promoting properties. One of the most influential mechanism is the production of plant indole-3-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 [19][20][21]. Figure 3.1 shows the production IAA by the isolates with the addition of 1000 $\mu\text{g/ml}$ L-tryptophan. From the results, it was shown that most of the *Methylobacterium sp.* isolated from palm oil leaves demonstrated higher IAA production compared to isolates from paddy leaves. The highest IAA production was recorded by epiphytic *Methylobacterium sp.* from palm oil leaves, EPPM1 which produced approximately 2 $\mu\text{g/ml}$ IAA. All of the isolates from palm oil leaves produced more than 1.5 $\mu\text{g/ml}$ of IAA while only one isolates from paddy leaves produced 1.5 $\mu\text{g/ml}$ IAA.

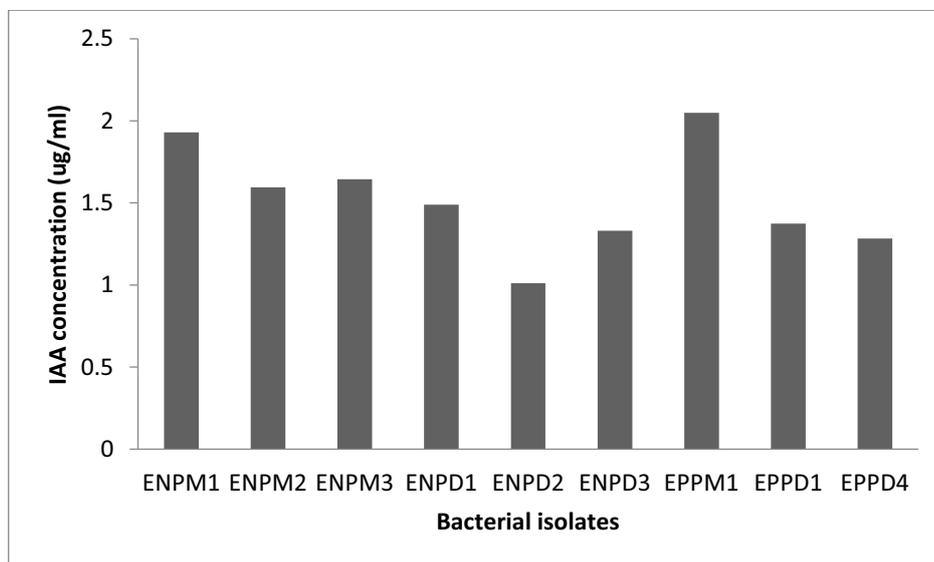


Figure 3.1 Production of IAA by bacterial isolates with the alteration of 1000 $\mu\text{g/ml}$ L-tryptophan

Phosphorus one of the major macronutrien for plant growth and development. However, the availability of soluble phosphorus for plant uptake was limited due it fixation as insoluble phosphate in iron, aluminium and calcium. Mineralization of soil P by phosphate solubilizing bacteria was attained by the production of phosphatase like phytase thus facilitate the mobilization of soluble inorganic phosphorus for plant uptake[22]. Phosphate solubilization assay was also conducted to determine the ability of the newly isolated *Methylobacterium sp.* to solubilize insoluble phosphate. Bacterial isolates were grown in NBRIP and Pikovskaya media which contain insoluble tricalcium phosphate (TCP). The solubilization of TCP in both media was quantitatively measured by vanadium-molybdate assay and the result is summarized in Figure 3.2. Based on the result observed, solubilization of TCP in Pikovskaya media is relatively higher than in NBRIP media. The highest phosphate solubilization activity was recorded by EPPD1 which solubilized 4.12 g/ml of inorganic phosphate in Pikovskaya broth. Isolate ENPD2 also demonstrated almost similar phosphate solubilization activity with 3.97 mg/ml and 3.3 mg/ml of inorganic phosphate were solubilized in Pikovskaya broth and NBRIP broth respectively.

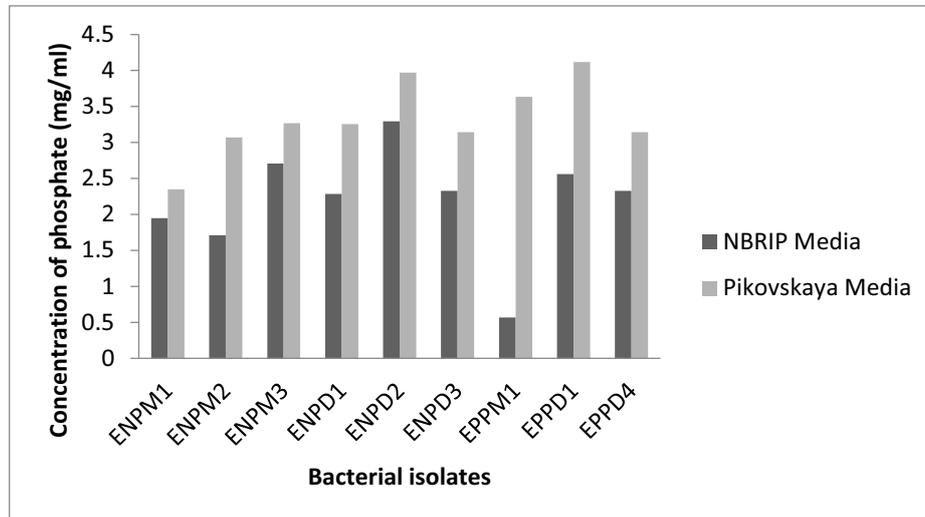


Figure 3.2: Solubilization of inorganic phosphate by newly isolated *Methylobacterium sp.*

Nitrogen is an important element in plant metabolism. In plant, nitrogen is needed for the synthesis of chlorophyll, amino acids, nucleic acids, and ATP. Although nitrogen happened to be the most abundant element on earth, plants however unable to directly utilize the atmospheric nitrogen. The fixation of atmospheric nitrogen to ammonia is carried out by a group of microorganism known as diazotroph. The role of diazotrophs to improve soil fertility and their application as biofertilizer had extensively studied by many researchers [23][24][25]. In this study, the ability of the newly isolated *Methylobacterium sp.* were tested by growing them on nitrogen free media. Two types of nitrogen free media were used namely Jensen media and Burk's media. The growth of the bacterial isolates on both media were evaluated qualitatively after 7-8 days of incubation and the result is tabulated in Table 3.1. However, all of the isolates either unable to grow or demonstrated poor growth on both of the nitrogen free media. This observation suggesting lack of nitrogen fixation ability or very weak of nitrogen fixation activities.

Table 3.2: Growth of bacterial isolates on nitrogen free media

Isolates	Jensen Media	Burk's Media
ENPM1	Poor growth	Poor growth
ENPM2	Poor growth	Poor growth
ENPM3	Poor growth	Poor growth
ENPD1	Poor growth	Poor growth
ENPD2	Poor growth	Poor growth
ENPD3	No growth	No growth
EPPM1	No growth	No growth
EPPD1	No growth	No growth
EPPD4	Poor growth	Poor growth

4. Conclusion

As the conclusion, the growth promoting traits for nine newly isolated *Methylobacterium sp.* were evaluated based on their ability to produce IAA, solubilizing inorganic phosphate and fixing atmospheric nitrogen. It

was shown that most of the *Methylobacterium sp* isolated from palm oil leaves demonstrated higher IAA production compared to isolates from paddy leaves. The highest IAA production was recorded by epiphytic *Methylobacterium sp* from palm oil leaves, EPPM1 which produced approximately 2ug/ml IAA. As for phosphate solubilization, isolates from both samples are able to solubilized inorganic phosphate in phosphate solubilization media. The highest phosphate solubilization activity was recorded by EPPD1 which solubilized 4.12 g/ml of inorganic phosphate in Pikovskaya broth. Isolate ENPD2 also demonstrated almost similar phosphate solubilization activity with 3.97 mg/ml and 3.3 mg/ml of inorganic phosphate were solubilized in Pikovskaya broth and NBRIP broth respectively. In contrast, all the isolates did not show significant nitrogen fixing activity when grown on nitrogen free media. Based on the quantitative and qualitative analyses conducted, these newly isolated *Methylobacterium sp* demonstrated promising potential to be developed as biofertilizer.

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