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# Isolation and Characterisation of Pink Pigmented Facultative Methylotrophs (PPFM) from Malaysian *Ulam* Leaves

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## Abstract

This study aims to isolate and characterise the Pink Pigmented Facultative Methylotrophs (PPFM) bacteria from the leaves of common Malaysian table salad (*ulam*). The colonies of PPFM bacteria were obtained using selectively modified *Pseudomonas* agar based on the appearance of the pink pigment colony. The three selected isolates labelled OJ4, OJ154 [*Oenanthe javanica* (Blume) DC.] and ML8 [from *Melicope lunu-ankenda* (Gaertn.) T. G. Hartley] were chosen for characterisation. The result showed the PPFM bacteria colonies (log CFU/g) at the leaf surface of *C. caudatus* are  $4.4 \pm 0.1$ , significantly higher than *O. javanica*,  $3.8 \pm 0.2$ , and *M. lunu-ankenda*,  $3.2 \pm 0.1$ . The selected isolates belong to the Gram-negative group, motile with rod shape with size [length (*l*) × width (*w*)] in  $\mu\text{m}$  unit  $4.3 \pm 1.1 \times 1.6 \pm 0.6$ ,  $5.4 \pm 0.2 \times 1.2 \pm 0.1$ , and  $3.5 \pm 0.7 \times 1.0 \pm 0.0$ , respectively. They show positive urease, catalase, and oxidase activities, while none of them can degrade starch, gelatine, or cellulose, as well as glucose fermentation (MR test) and metabolism actions (VP test), producing indole and hydrogen sulphide gaseous. Only isolate OJ154 demonstrates positive casein hydrolysis and nitrate reduction activities, while only isolate ML8 can utilise citrate but not in lipid degradation. Their sequence analysis of the 16S rRNA indicated that OJ4 and ML8 are *Methylobacterium radiotolerans* with a similarity of 99%, whereas, OJ154 is *Methylorubrum salsuginis* with a similarity of 99%. To conclude, PPFM bacteria from the leaves of *C. caudatus*, *O. javanica*, and *M. lunu-ankenda* have been isolated and characterised in particular.

**Keywords:** *Ulam*, Pink Pigmented Facultative Methylotrophs, Bacteria Isolation, Bacteria Characterisation, Biochemical Assay, Molecular Identification

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## INTRODUCTION

There are numerous vegetable kinds available on the market that can be consumed in cooked form, like broccoli, or in raw form, such as salad. Their nutritional value may be variable depending on how they are prepared to eat. Previous studies have shown that some vegetables reduce their phytochemical contents, for example, polyphenols, carotenoids, and ascorbic acid, after cooking compared to raw.<sup>1</sup> Another study revealed that raw vegetables and salad cause an increase in serum levels of vitamins, including vitamin C, vitamin E, folic acid,  $\beta$ -carotene, and lycopene, in their consumers' bodies.<sup>2</sup>

Hence, consuming fresh and raw vegetables like *ulam* can be one of the best choices for a healthy diet, as its nutritional value, including phytochemical contents remains unchanged due to no heat applied. Malaysian table salad also known as *ulam*, is commonly eaten freshly (raw) with rice as a side dish.<sup>3</sup> Throughout generations, *ulam* has been consumed by mankind due to its believed possession of various therapeutic benefits, including the prevention of degenerative diseases, the delay of ageing, and the improvement of overall health.<sup>2,4</sup>

*Ulam* may consist of young leaves, shoots, flowers, fruits, roots, and rhizomes as part of vegetables.<sup>5</sup> Several examples of common *ulam* consumed by Malaysians are *pegaga* (*Centella asiatica*), *petai* (*Parkia speciosa*), *ulam raja* (*C. caudatus*), and *selom* (*O. javanica*).<sup>6</sup> Fascinatingly, much scientific research has shown the benefits of the phytochemicals contained in *ulam* to its consumers. For example, *C. caudatus*, *O. javanica*, and *M. lunu-ankenda* have nutritional values like carbohydrates, proteins, minerals, and vitamins.<sup>4,7,8</sup> Their structure also contains phytochemicals such as phenolic compounds of caffeoylquinic acids and proanthocyanidins (*C. caudatus*); flavonoids, phenolic, and volatile oils that contribute to its aromatic smell (*O. javanica*); and isorhamnetin, skimmianine, and leptanol (*M. lunu-ankenda*).<sup>4,8,10</sup> The chemical compound content of the 3 types of *ulam* are responsible for giving similar benefits to health, such as anti-inflammatory and antioxidant effects.<sup>4,8,10</sup>

Besides, the raw condition of vegetables also preserves symbiotic microorganisms that

are present in their structure. These symbiotic microorganisms may add value to the quality of the raw vegetables themselves. Typically, the inhabitants of symbiotic microorganisms who occupy the leaf surface of *ulam* are identified as PPFM bacteria. It is the methylotrophs that inhabit the phyllosphere, mostly on the leaf surfaces of plants, including *ulam* leaves. The group is named methylotrophs and is known as PPFM bacteria, classified into the genus *Methylobacterium*.<sup>11,12</sup>

Uniquely, Pink Pigmented Facultative Methylotrophs (PPFM) bacteria could use single-carbon compounds as well as multi-carbon growth substrates for their energy and carbon source solely,<sup>13</sup> allowing them to consume 'food' such as methanol released by plants and, in return, providing essential compounds for the plant's development.<sup>14</sup> A valuable compound identified as pyrroloquinoline quinone (PQQ) was formed by them during the oxidation of the single compound. This compound also has anti-diabetic and anti-oxidant activities. Additionally, this kind of symbiotic bacteria does no harm to their host plants, making their bioactive compounds likely to be less toxic to humans, which is important for application.<sup>15,16</sup>

Previous studies have stated the same or similarity in a few symbiotic microbes' cell structures with their host plants.<sup>17,18</sup> For example, compounds, namely camptothecin and its structural analogues produced by endophytic fungi are also synthesised by their host.<sup>19-22</sup> Moreover, Sarjono *et al.*<sup>23</sup> reported that both the extracted isolated bacteria's compound and their host plant's leaf (*Carica papaya* L.) contain alkaloids, flavonoids, and tannins. Hence, the knowledge discovered from this study regarding the properties of PPFM bacteria obtained from *ulam* may improve the quality of food in general. In other words, people who consume *ulam* may get both benefits from the phytochemicals of *ulam* and bioactive compounds from the PPFM bacteria residing in its structure.

As mentioned previously, these bacteria are a type of microorganism that can utilise one-carbon compounds as a sole source of carbon and energy, as well as other multicarbon substrates present ubiquitously in nature, such as plant leaves, in symbiotic relationships.<sup>24</sup> To date, numerous studies have been conducted to identify

the benefits of the pigments extracted from this bacterial species, but none of them focused on the same bacteria that originated from Malaysian *ulam* leaf as well as its potential application as a food colourant. Hence, this study emphasised the PPFM bacteria properties (number of colonies, biochemical and molecular characterisation) together with their extracted pigments for bioactivity (antioxidant) performance evaluation from selected *ulam* leaf species for further study.

Previous studies have shown bacteria cells or their extracted compounds have been implemented as food, such as a supplement, not only for animals but also for humans. For example, Jones *et al.*<sup>25</sup> reported that several microorganism species, including PPFM, have been genetically modified to produce carotenoids and taurine or its precursors in their aquaculture stock.<sup>26</sup> Moreover, the biomass of PPFM, specifically from *M. extorquens*, has been applied in trout feeds as an alternative protein source, replacing soybean meal.<sup>27</sup>

In addition, one example of a bacteria compound (PQQ) has been extracted from *Hyphomicrobium denitrificans* CK-275 fermentation with the trade name BioPQQ™ and is considered safe to consume at the intended conditions approved by the European Food Safety Authority (EFSA).<sup>28</sup> However, the further objective of this study concerns the application of pigment extracted from PPFM to food. Therefore, the pigment's toxicity level has been tested and can be considered safe for consumption under certain concentrations (manuscript publication in process).

## MATERIALS AND METHODS

### Materials

The 3 samples of fresh *ulam* leaves, namely *C. caudatus*, *O. javanica*, and *Melicope lunu-ankenda* (Gaertn.) T. G. Hartley leaves, were obtained from both wet and night markets (each approximately 150-200 g per species) at Sri Serdang, Selangor, Malaysia. For chemicals, phosphate buffer solution (PBS) and sulfanilic acid were obtained from Sigma (Missouri, United States), while Pseudomonas agar F, peptone was bought from Difco™, BD (New Jersey, US). Agar-agar types I, nutrient gelatin, Simmons

citrate agar (SCA), sulphide indole motility (SIM) medium, and nitrate broth were purchased from HiMedia (Mumbai, India). Glycerol (anhydrous), skim milk powder, potassium chloride, sodium nitrate, carboxymethyl cellulose (CMC), N-cetyl-N,N,N-trimethyl ammonium bromide (CTAB), potassium dihydrogen phosphate, phenol red, methylene blue, 1-naphthol, methyl red, Kovac's (indole reagent) solution, D(+)-glucose (anhydrous), potassium sulphate, and 1-naphthylamine were purchased from R & M Chemicals (Essex, UK). N,N-Dimethyl-1,4-phenylenediamine oxalate (compound) was obtained from Sigma-Aldrich (Missouri, United States).

Furthermore, crystal violet, safranin O, and zinc powder were brought from Bendosen (Johor, Malaysia), while ethanol (95%), magnesium sulphate, di-potassium hydrogen phosphate (phosphate buffer), and glacial acetic acid were obtained from System (Selangor, Malaysia). Ammonium oxalate, soluble starch, urea, potassium hydroxide, and hydrogen peroxide were bought from Chemiz (Selangor, Malaysia). Sodium chloride and iodine pearl were purchased from QRëC™ (Bangkok, Thailand). Nutrient agar, tributyrin agar, yeast extract, and potassium iodide were purchased from Fisher Scientific (New Hampshire, US). In addition, trypticase soy agar (TSA) and disodium phosphate were bought from Merck (Darmstadt, Germany), cellulose nitrate membrane filter was purchased from LabServ® (Massachusetts, United States), and BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) was from Thermo Fisher Scientific, Massachusetts, United States. For the experiment, all chemical reagents were carefully selected to be of analytical grade, and all aqueous solutions required for the study were meticulously prepared using distilled water.

### Methods

#### Isolation of PPFM bacteria

Firstly, according to Madhaiyan<sup>29</sup>, Mizuno *et al.*<sup>30</sup>, Sanjee *et al.*<sup>31</sup>, and Kaparullina *et al.*<sup>32</sup> an amount of 1 g of the fresh *ulam* leaves were weighed, washed with sterilised distilled water, and mashed using a mortar and pestle, serially diluted, and 100 µl of the aliquot from each dilution were transferred on the sterile modified *Pseudomonas* agar (MPA) medium using the

spread plate method.<sup>33</sup> The preparation of 1 L of MPA is done by mixing 3.8 g of *Pseudomonas* agar, glycerol 1.5 ml, and agar Type I 18.9 g.

*Pseudomonas* agar F was used in this study rather than others because *Methylobacterium* species have sufficient nutrients to grow on the media. Its basic composition, which contains glycerol, proteose peptone no. 3, pancreatic digest of casein, dipotassium hydrogen phosphate, and magnesium sulphate heptahydrate, resembles King's B agar medium composition except for the addition of pancreatic digest of casein. Both of these media were used to isolate, cultivate, and differentiate *Pseudomonas* species generally and have been applied to PPFM species since these bacteria have similar characteristics to *Pseudomonas* species.<sup>34,35</sup> Moreover, the pigmentation of PPFM bacteria on *Pseudomonas* agar is deep, similar to when growing on glycerol-peptone agar, making it suitable for subculture but not for culture enrichment.<sup>36</sup>

Next, the plates were incubated in aerobic conditions at 30°C for 3-7 days. This assay was performed 3 times with duplicates (n = 3×2). The presence of pink-coloured colonies that appear on the MPA medium was counted for CFU value. Afterwards, the streak plate method was employed to isolate and purify a total of 100 pink-coloured bacterial colonies from each type of *ulam*, with each colony displaying distinct characteristics. Once purified, the colonies were carefully stored at a temperature of 4°C for future use.<sup>33</sup>

### Morphological and biochemical characterisation

Among the 100 PPFM colonies that have been isolated and purified randomly from each type of *ulam* leaf (*ulam raja*, *selom*, and *tenggek burung*), 40 pure cultures from them (each *ulam* type) were selected based on their colour intensity (from pale pink to deep pink) and colony size differences (big and small). Then, they proceeded with further examination steps involving microscopic observation as well as several biochemical assays, and those experiments were done in triplicate. All the procedures described for morphology and biochemical activity analysis except lipolytic and cellulose activities were referred to by Barrow and Feltham<sup>37</sup> in general.

### Gram staining

To differentiate the main class of bacteria, the Gram staining method with a slight modification from Engelkirk *et al.*<sup>38</sup> and the U.S. Food and Drug Administration<sup>39</sup> was used. The slide containing Gram-stained bacteria cells was examined under a compound microscope for Gram identification. The bacteria cells that retained the crystal violet-iodine complex during the decolorization step are considered as Gram-positive and vice-versa. The Gram-negative bacteria cell was stained pink to red due to the safranin (a red dye).<sup>38</sup>

### Motility and size observation

Initially, by referring to Laxmi *et al.*<sup>40</sup> and Pattanashetti,<sup>41</sup> a loopful of isolated bacteria was placed on a clean glass slide, and a small drop of distilled water was added. The mixture was then evenly smeared and covered with a glass cover slip. After that, the prepared slide was labelled, observed, and measured microscopically, including body size, and the data was recorded.

### Cell shape examination

For cell shape examination based on Pattanashetti,<sup>41</sup> the same isolate and steps from the previous experiment (motility) were used except for the application of a methylene blue solution for staining purposes. The staining procedure begins with placing a drop of methylene blue solution on a glass slide containing a thin film of isolate and leaving it for a few seconds. Then, the slide was covered with a glass cover slip, and the shape was examined. Finally, the data was recorded.

### Starch hydrolysis

Starch agar medium was prepared according to Madhaiyan.<sup>29</sup> The assay started with streaking a linear line of isolate culture on the medium, followed by incubation at 35°C for 48 hours. Then, the medium was flooded with Gram's iodine, and the reaction was observed. The presence of a clear zone surrounding the colonies indicates the hydrolysis of starch and is considered a positive reaction.

### **Casein hydrolysis**

The agar preparation method was modified from Atlas<sup>42</sup> and Madhaiyan.<sup>29</sup> The isolate culture was streaked on the prepared medium and incubated at 35°C for 48 hours. Clear zone formation around the colonies gives positive results for casein hydrolysis.

### **Gelatin hydrolysis**

The gelatin medium was ready, as stated by Madhaiyan<sup>29</sup> and the isolate culture was stabbed deep into it. The medium was then incubated at 35°C for 48 hours. After that, the medium was put back into the chiller for 48 hours to allow the gelatin to solidify for a second time. Then, the result was observed directly. In detail, a gelatin medium with no solidification is a positive result as the process of gelatin liquefaction activity occurs.

### **Cellulose degradation**

According to Aneja,<sup>43</sup> the Czapek mineral salt agar medium was prepared to perform the cellulose degradation test. The isolate culture was streaked on the plates and incubated at 35°C for 48 hours. As a result, the presence of clear zones surrounding the colonies after flooding with 1% of CTAB solution onto the agar surface showed a positive reaction.

### **Citrate utilisation test**

To observe citrate utilisation, SCA was prepared based on Madhaiyan.<sup>29</sup> The isolate culture was inoculated into the medium and incubated for 48 hours at 35°C. The positive result was illustrated by the changing colour of the medium, from green to blue.

### **Lipolytic activity**

The ready-made tributyrin agar medium that was used for lipolytic activity assessment, as referred to by Madhaiyan.<sup>29</sup> The isolate culture was streaked on the medium and incubated at 37°C for 48 hours. The formation of a clear zone surrounding the colonies is taken as a positive reaction.

### **Urease test**

Based on Atlas,<sup>41</sup> the urea broth medium was prepared as described. The isolate culture was

inoculated in the broth medium and incubated for 24 hours at 35°C. Changing the colour of the urea broth medium from orange to pink/red colour indicated a positive reaction.<sup>29</sup>

### **Methyl red (MR) test**

The broth medium for the MR test was also used for the Voges-Proskauer (VP) test. Therefore, the name is Methyl Red-Voges-Proskauer (MRVP) medium. The MRVP medium was prepared according to Atlas<sup>41</sup> with a slight modification. In a separate process, the MR reagent was prepared, following the guidelines outlined by FDA.<sup>39</sup> Following a procedure similar to the previous step, the isolated culture was inoculated into the MRVP broth medium and allowed to incubate for 48 hours at a temperature of 35°C. The result was determined by adding a few drops of the MR reagent into the broth, and the presence of a red colour indicated a positive reaction.<sup>29</sup>

### **Voges-Proskauer (VP) test**

As per the guidelines provided by FDA,<sup>39</sup> the VP reagents were prepared. Utilising the same broth culture prepared for the MR test as described earlier in the section *Methyl Red (MR) Test*, 0.6 ml of the first reagent was added to 1 ml of the broth culture, followed by the addition of 0.2 ml of the second reagent. The broth culture was shaken during each reagent addition. The reaction was observed after 5 minutes, and the presence of a red colour indicated a positive reaction.<sup>44</sup>

### **Nitrate reduction test**

The preparation of the nitrate reduction test in this study was based on Faddin's<sup>45</sup> method. As for the reagents, a 0.5% solution of 1-naphthylamine (reagent A) was prepared together with reagent B (0.8% sulfanilic acid). In the test, the isolate culture was inoculated into the nitrate broth and incubated at 35°C for 24 hours. The nitrate reduction reaction of the culture was determined by adding an equal amount of both reagents to the broth and gently shaking to mix them. After 1 to 2 minutes, the appearance of a red colour indicated a positive reaction. If no colour changes were observed (a negative reaction), a further technique was employed by adding a small amount (approximately 20 mg) of zinc dust to the

broth, and the colour development was observed after 5 to 10 minutes. The presence of a red colour indicated a negative reaction.

#### **Catalase activity**

A modified method based on the Madhaiyan<sup>29</sup> protocol was used, where a small number of isolate cultures from an MPA plate were transferred onto a glass slide using a sterile cotton bud. This was followed by the addition of 3% hydrogen peroxide (a drop) to the cultures. The presence of effervescence (bubble formation) indicates a positive reaction.

#### **Hydrogen sulphide production**

By referring to Madhaiyan<sup>2,9</sup> SIM agar medium was used for the hydrogen sulphide production test. The isolate culture was carefully stab-inoculated into the medium, ensuring proper placement along the line, and incubated for 48 hours at 35°C. Subsequently, the formation of black colouration along the stab inoculation line was observed. The positive reaction was identified by the presence of the black line.

#### **Indole production**

Differently, the medium used for indole production is not glucose tryptone broth, as done by Madhaiyan,<sup>29</sup> but using the same medium in a hydrogen sulphide production test based on Faddin.<sup>45</sup> Then, the result was examined by placing 0.3 ml of Kovacs reagent into the medium, and the change in colour was documented. The reddening of the Kovacs reagent's layer within a few minutes was considered a positive reaction.

#### **Oxidase test**

To examine the oxidation reaction, a TSA medium was prepared for isolate culture growth.<sup>29</sup> Separately, reagents named Gaby and Hadley were prepared for the test, according to Faddin.<sup>45</sup> In the experimental procedure, the isolate culture was streaked onto a plate and incubated at 37°C for 48 hours. Following incubation, the oxidation reaction was tested using a direct plate procedure. This involved adding 2 to 3 drops of the reagents directly onto the colonies and observing any colour changes within 10 seconds, as per Public Health England<sup>46</sup> guidelines. The appearance of a blue colour shows a positive reaction.<sup>47</sup>

#### **Molecular characterisation**

For these assays, only 3 selected pure cultures of PPFM bacteria colonies from the isolate collection (OJ4, OJ154, and ML8) have proceeded for further examination steps and discussion. Those 3 isolates mentioned were selected due to their highest cell number production at 3 days of growth culture (preliminary study) instead of their pigment colour intensity. The assays involved are 16S rRNA gene amplification and DNA sequencing analysis based on Reysenbach *et al.*<sup>48</sup> The full-length 1.5 kb 16S rDNA of the PPFM bacteria was amplified by using universal primers 27F and 1492R. The amplification reaction was performed in a total volume of 25 µl, which included gDNA purified using an in-house extraction method, 0.3 pmol of each primer, deoxynucleotides triphosphates (dNTPs) at a concentration of 400 µM each, 0.5 units (U) of DNA polymerase, supplied PCR buffer, and water.

The PCR amplification procedure was directed as follows: an initial denaturation step at 94°C for 2 minutes was followed by 25 cycles of annealing and extension of the amplified DNA. Each cycle consisted of denaturation at 98°C for 10 seconds, annealing at 53°C for 30 seconds, and extension at 68°C for 1 minute. Subsequently, the PCR products were purified using a standard method and subjected to direct sequencing with primers 785F and 907R, employing the BigDye<sup>®</sup> Terminator v3.1 Cycle Sequencing Kit from Applied Biosystems. The resulting 16S rRNA gene sequences were assembled using SnapGene<sup>®</sup> software and analysed using the Basic Local Alignment Search Tool (BLAST) against PPFM bacteria strains available in the National Centre for Biotechnology Information (NCBI) database library to identify the specific gene sequence. The individual gene sequences for all strains were aligned using MUSCLE, and then the neighbor-joining (NJ) phylogenetic tree was generated using Mega 10.2.6 software.<sup>49</sup>

NJ can be defined as two taxa that are neighbors in a tree if the path between them contains only one node.<sup>50</sup> The algorithms for the construction of the phylogenetic tree in this study use NJ due to its characteristics: few assumptions, fast operation, and high accuracy, based on the distance between taxa. They can be used with

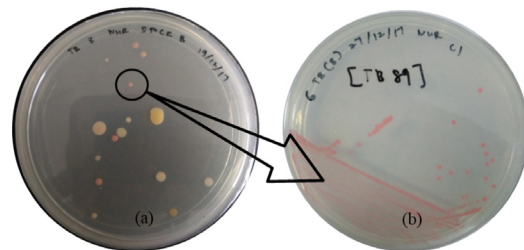
a variety of models.<sup>51,52</sup> This method usually constructs different phylogenetic trees for the same dataset with differences in input order, also known as ‘tied trees’.<sup>52</sup> In other words, it provides an unrooted tree and a single resultant tree.<sup>51</sup>

**Table 1.** The number of PPFM bacteria colonies (log CFU/g) at the leaf surface of *ulam*

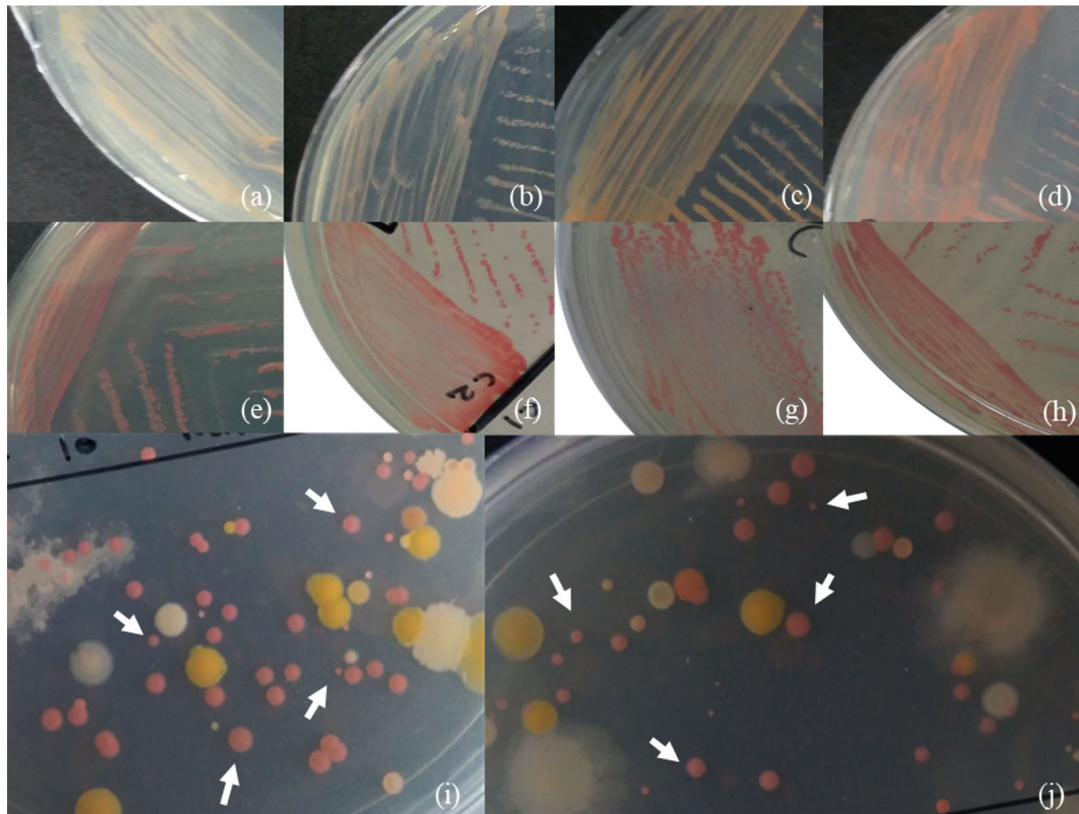
No.	Sample	Total colony (CFU/g)	Log CFU/g	Mean log CFU/g
1	<i>C. caudatus</i>	$3.2 \times 10^4$	4.5	$4.4 \pm 0.1a$
		$2.7 \times 10^4$	4.4	
		$2.4 \times 10^4$	4.4	
2	<i>O. javanica</i>	$9.4 \times 10^3$	4.0	$3.8 \pm 0.2b$
		$5.0 \times 10^3$	3.7	
		$3.9 \times 10^3$	3.6	
3	<i>M. lunu-ankenda</i>	$1.8 \times 10^3$	3.2	$3.2 \pm 0.1c$
		$1.9 \times 10^3$	3.3	
		$1.4 \times 10^3$	3.1	

Note: Mean values  $\pm$  SD with different letters in the same column have significance different ( $p < 0.05$ )

In detail, the individual taxa are iteratively grouped together, forming larger and larger clusters of taxa. Furthermore, the NJ does not assume a molecular clock, but it assumes that observed distances are near an additive metric, differently from the Unweighted Pair Group Method with Arithmetic Mean (UPGMA). Given an additive metric, the NJ method identifies the



**Figure 1.** (a) The microbes including PPFM colonies (in black circle) on the surface of modified *Pseudomonas* agar (MPA) and (b) single PPFM colony culture



**Figure 2.** Examples of the pink colour of the PPFM colony – (a,b) light or pale pink; (c, d) orange-pink; (e, f) pink; (g, h) dark or deep pink; (i, j) and their variety of sizes (white arrow)

correct tree, and it also correctly reconstructs trees if additivity only holds approximately. As neighbor relationships of nodes in a binary uniquely define the tree topology, successively identifying neighbors is a way to reconstruct the tree. In each iteration of the NJ algorithm, every pair of taxa is evaluated for being neighbors, and if so, they are grouped together to form a new taxon for the next iteration.<sup>50</sup>

Based on all the described methods, the experimental procedures to isolate and characterise PPFM bacteria from *ulam* leaf in this study involve isolation and purification using semi-selective media, characterization through physical and biochemical tests, followed by partial 16S rRNA gene sequence analysis of randomly picked up pink pigmented colonies (the first isolation step) and only the data of the 3 isolates was discussed.

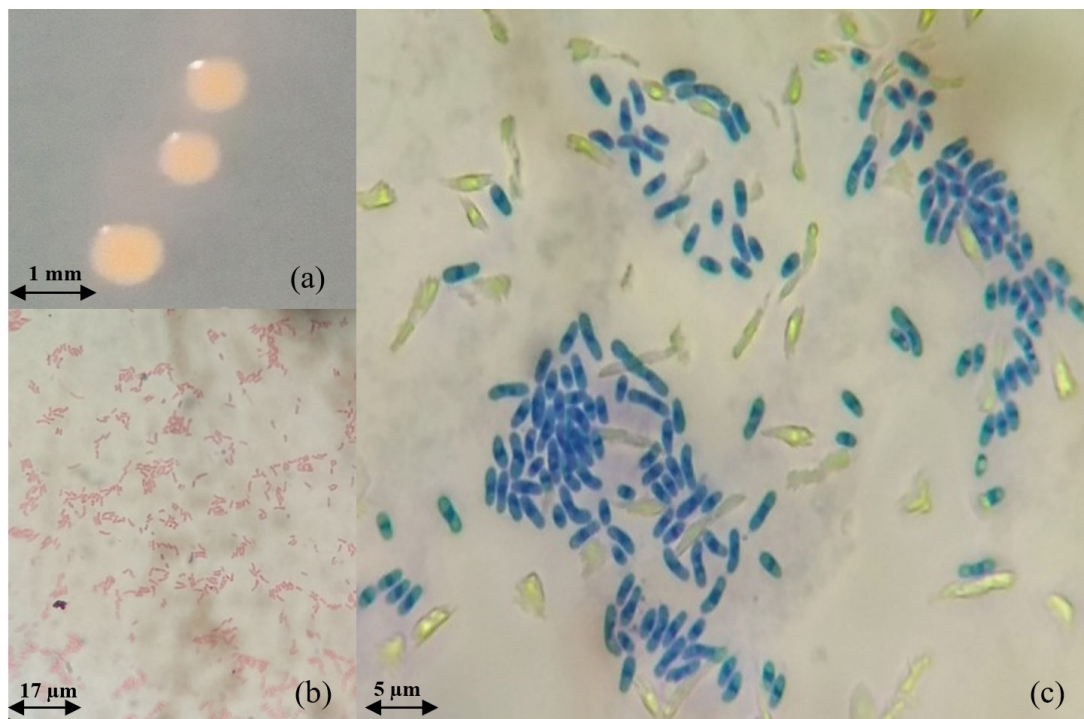
## RESULTS AND DISCUSSION

The PPFM bacteria from *ulam* leaves were isolated, followed by their mean CFU per

gram calculation. Several physical characteristics of the selected PPFM bacteria were identified through their Gram staining properties, motility, cell size, and shape. Also, its 14 biochemical activities, which are catalase, oxidase, MR, VP, indole, hydrogen sulphide, urea, starch, gelatin, cellulose, citrate, reduction of nitrate, casein, and lipolytic reactions, were analysed. The 16S rRNA gene amplification and DNA sequencing analysis were also completed for molecular identification purposes.

### Isolation of PPFM bacteria

PPFM is commonly found in plant structures, especially in leaves. Even though PPFM colonised the surface leaves of a plant, their population size may be different, as can be seen in Table 1, which shows significant differences in PPFM population between 3 types of *ulam* leaves. Based on the table, the total number mean log CFU/g of PPFM colonies at the leaf surface of *C. caudatus* is  $4.44 \pm 0.07$ , followed by *O. javanica*,  $3.75 \pm 0.20$ , and *M. lunu-ankenda*,  $3.22 \pm 0.07$ . *C. caudatus* leaves have the highest number of PPFM,



**Figure 3.** (a) PPFM colonies on the surface of selective agar after 6 days incubation (b) Gram staining (mag.: 400×) (c) Methylene blue cell staining (mag.: 1000×)



**Table 2.** The characteristic description of *Methylobacterium* and *Methylobrubrum* genera and a few species of them from previous studies

No.	Characteristic	<i>Methylobacterium</i> sp. <sup>36</sup>	<i>M. radiotolerans</i> <sup>76</sup>	OJ4	ML8	<i>M. ajmalii</i> <sup>79</sup>	<i>Methylobrubrum</i> sp. <sup>78</sup>	<i>M. salsuginis</i> <sup>77</sup>	OJ154
1.	GS	Negative	Negative	Negative	Negative	Negative	Negative/ variable	Negative	Negative
2.	Size (µm)	1.0 – 8.0 ×	1.4 – 2.5 ×	4.3 ± 1.1 ×	3.5 ± 0.7 ×	2.2 – 3.2 ×	1.0 – 10.0 ×	4.0 – 8.0 ×	5.4 ± 0.2 ×
	(l×w)	0.8 – 1.0 µm	0.6 – 0.8 µm	1.6 ± 0.6 µm	1.0 ± 0.0 µm	1.6 – 1.8 µm	1.0 – 1.5 µm	1.0 – 1.5 µm	1.2 ± 0.1 µm
3.	Shape	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod
4.	Motility	Motile	Motile	Motile	Motile	Motile	Motile	Motile	Motile
5.	SH	- (most strain)	+ (slight)	-	-	ND	- (most strain)	+	-
6.	CH	- (most strain)	-	-	-	ND	- (most strain)	ND	+
7.	GH	- (most strain)	-	-	-	ND	- (most strain)	-	-
8.	CD	- (most strain)	ND	-	-	ND	- (most strain)	ND	-
9.	CUT	+ (some strain)	+	-	+	ND	- (most strain)	+	-
10.	LA	+ (some strain)	ND	+	-	ND	+ (some strain)	ND	+
11.	UT	+	+	+	+	ND	+	+	+
12.	MRT	-	ND	-	-	ND	-	-	-
13.	VPT	-	ND	-	-	ND	-	-	-
14.	NRT	+ (some strain)	+	-	-	ND	+ (some strain)	+	+
15.	CA	+	+	+	+	+	+	+	+
16.	IP	- (except <i>M. thiocyanatum</i> )	-	-	-	ND	+ (some strain)	-	-
17.	HSP	-	+	-	-	ND	- (most strain)	-	-
18.	OT	+ (often weak)	+	+	+	+	+ (often weak)	ND	+

Note. (+) positive reaction; (-) negative reaction; ND = no data available, GS: Gram staining, l: Length, w: Width, MO: Microscopic observation, SH: Starch hydrolysis, CH: Casein hydrolysis, GH: Gelatin hydrolysis, CD: Cellulose degradation, CUT: Citrate utilization test, LA: Lipolytic activity, UT: Urease test, MRT: Methyl red test, VPT: Voges-Proskauer test, NRT: Nitrate reduction test, CA: Catalase activity, IP: Indole production, HSP: Hydrogen sulphide production, OT: Oxidase test

followed by *O. javanica* leaves, and the lowest is on *M. lunu-ankenda* leaves. The mean number of PPFM from *O. javanica* ( $3.75 \pm 0.20$  log CFU/g) leaves was similar to the PPFM at red clover leaves ( $3.82$  log CFU/g), while the mean number of PPFM at *M. lunu-ankenda* leaves ( $3.22 \pm 0.07$  log CFU/g) was similar to the same leaves ( $3.18$  log CFU/g), as reported by Omer *et al.*<sup>53</sup>. However, the mean number of PPFM at *C. caudatus* leaves ( $4.44 \pm 0.07$  log CFU/g) agrees with Dourado *et al.*,<sup>54</sup> who claim that the population of PPFM bacteria is in the range of 4.00-6.00 log CFU/g of plant tissue.

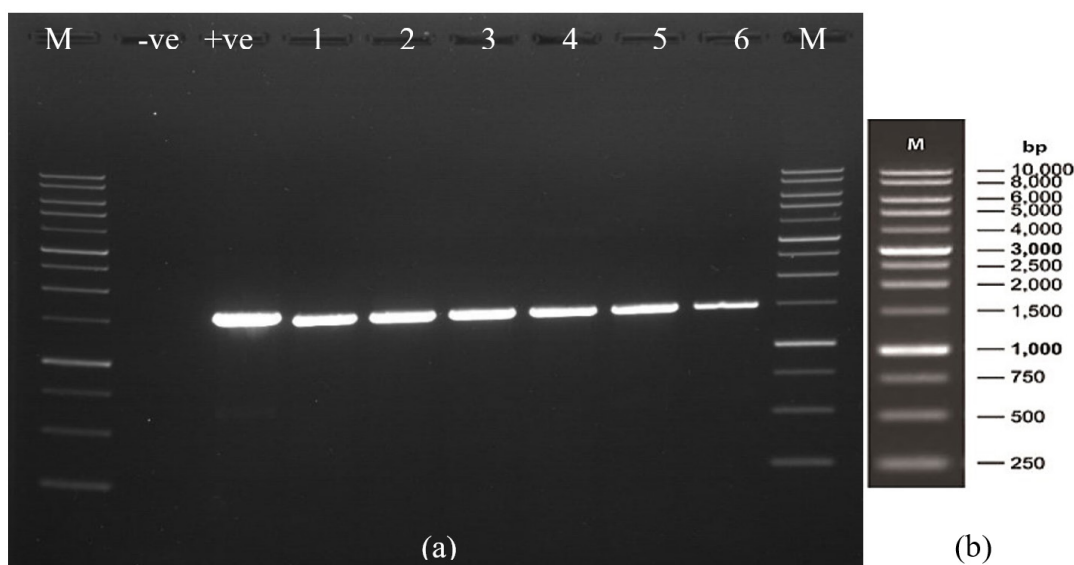
The diverse CFU/g number of PPFM on plant leaves may differ due to several factors, for example, different species of plants as well as seasonal change.<sup>30,53</sup> Further details reported by Hunter *et al.*<sup>55</sup> are the plant species, gross plant morphology, the position and height of leaves, leaf age, the distribution of stomata, trichomes, and other leaf surface appendages on the waxes surface, together with distinct epidermis and layer,<sup>56</sup> which also play roles for PPFM to colonise the leaf surfaces. For instance, the number of culturable bacteria obtained from broad-leaf plants is higher as compared to grasses or waxy broad-leaf plants.<sup>57</sup>

In other words, the morphology of the leaf surface may influence the ability of PPFM to make the leaf surface their resident place in the

fluctuating environment.<sup>57</sup> This is because the thick waxy cuticles limit the diffusion of nutrients and inhibit the wetting of the leaf surface.<sup>57</sup> If water molecules fall on the thick waxy leaves, they will stay in tightly formed droplets rather than spreading over the surface,<sup>58</sup> giving the PPFM a low chance of obtaining the water source. Hence, those explanations likely confirm the reason for different population numbers of PPFM at different types of *ulam* leaves. This information may aid in obtaining various species of PPFM or providing *ulam* selection for consumption preference.

During the isolation step, Figure 1 (a) shows the various microbes that are present at the surfaces of *ulam* leaves, including PPFM bacteria colonies (in a black circle) that grow on the MPA, while Figure 1 (b) shows the pure culture streak of a single PPFM colony that has been transferred from the mixture culture plate. PPFM is expected to be isolated from the *ulam* leaves based on the fact that they exist in the plant's leaves abundantly<sup>53</sup> since leaves can support a large and complex population of bacteria.<sup>30</sup>

The distinctive pink colour of PPFM colonies can be present in different ranges or intensities. Based on previous studies, they described their PPFM colonies' colour as light or pale pink, dark or deep pink, pink, and orange-pink.<sup>59,60</sup> Therefore, a few examples of different



**Figure 4.** (a) The gel photo of the band of the 16S rRNA gene of the isolate after amplification at 1500 base pairs – band: 5 (OJ4), 6 (OJ154), 3 (ML8) (b) base pair indicator

**Table 3.** The 16S rRNA sequence of isolates based on the BLAST database

Isolate name	Length of 16S rRNA gene sequenced (bp)	Most closely related organism			
		Species	Accession description	% Gene identity	% Query coverage
OJ4	1418	<i>M. radiotolerans</i> JCM 2831 strain DSM 1819	NR_112235.1	99%	100%
OJ154	1411	<i>M. salsuginis</i> strain MR	NR_044038.1	99%	100%
ML8	1411	<i>M. radiotolerans</i> JCM 2831	NR_074244.1	99%	100%

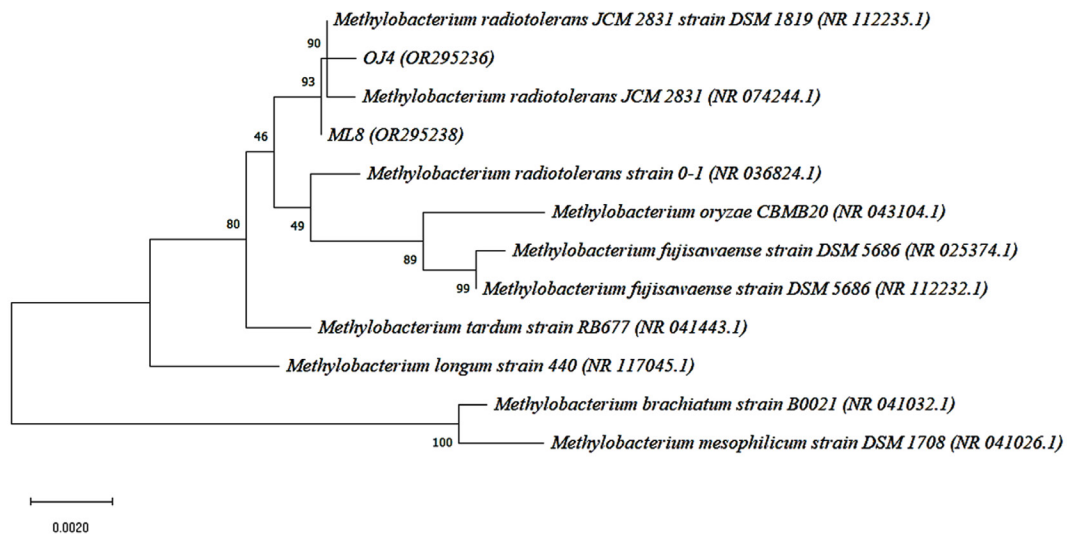
pink variations of PPFM colonies collected from the *ulam* have been presented in Figure 2, including their size differences, similar to findings reported by Kumar & Lee,<sup>35</sup> Vadivukkarasi,<sup>59</sup> and Kassem *et al.*<sup>60</sup>

PPFM is a member of the genus *Methylobacterium* that belongs to the proteobacterial subgroup (class) Alpha-proteobacteria, order Rhizobiales, and family Methylobacteriaceae. The important characteristic of identifying PPFM is their ability to oxidise methanol due to the presence of the methanol dehydrogenase (*mdhA*) gene in their gene's code.<sup>24,61</sup> Physically, their pink colony feature is another unique characteristic of PPFM because of

carotenoid's existence in their cell structure. This carotenoid element is important to allow them to survive in extreme conditions such as excessive light exposure and radiation.<sup>35</sup>

**Morphology and biochemical activities**

Generally, the PPFM colonies' diameter is approximately 1.03 ± 0.06 mm with a reddish-pink colour, circular form, entire margin, raised elevation, butyrous consistency surface, and opaque colonies, as shown in Figure 3 (a). Those morphologies are identical to the morphology of PPFM described by Hirano and Upper<sup>62</sup> and Uy *et al.*<sup>63</sup> In detail, the Gram staining type (positive or negative), size, shape, mobility, and 14 biochemical



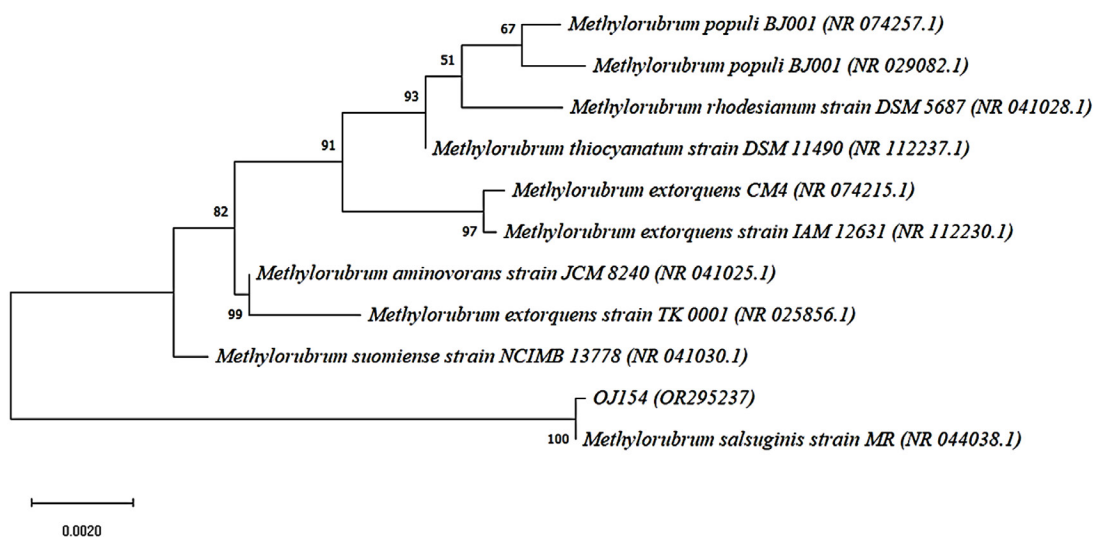
**Figure 5.** Neighbor-joining phylogenetic tree based on 16S rRNA gene sequence shows the relationship of the samples [OJ4 (OR295236)] and [ML8 (OR295238)] with members of the genus *Methylobacterium*. Bootstrap values from 1000 replications are shown at branch points. Bar, 0.002 substitution per site.

tests of the selected isolates (OJ4, OJ154, and ML8) were recorded. Based on those tables, all 3 isolates belong to the Gram-negative group and are motile with rod shapes, as can be seen similarly in Figures 3 (b) and (c). Moreover, in Figure 3 (c), the isolated cell structure was stained by methylene blue solution on a glass slide before being covered with a glass cover slip. In this study, instead of relying on Gram staining, simple methylene blue staining was added for morphology examination because this assay shows the brilliant colour of bacteria cells, making them stand out against their background as this alkaline cationic dye attracted to slightly negatively charged bacteria cells for a more presentable image.<sup>64-66</sup> Therefore, only the live cell was stained, and the unstained thing shown in the image was due to the glass reflection during the microscopy observation and photography process. Moreover, only one photograph was shown as a representative among the 3 selected strains, OJ4, OJ154, and ML8, since they have the same or similar physical structure. The size ( $l \times w$ ) in  $\mu\text{m}$  units of OJ4 is  $4.3 \pm 1.1 \times 1.6 \pm 0.6$ , while OJ154 is  $5.4 \pm 0.2 \times 1.2 \pm 0.1$ , and ML8 isolates are  $3.5 \pm 0.7 \times 1.0 \pm 0.0$ .

All isolates show positive urease, catalase, and oxidase activities, while none of the isolates can degrade starch, gelatine, or cellulose, as well as glucose fermentation (MR test) and

metabolism actions (VP test), producing indole and hydrogen sulphide gaseous. In addition, only OJ154 demonstrates positive casein hydrolysis and nitrate reduction activities. However, only ML8 can utilise citrate, but not in lipid degradation. Particularly, Gram stains were done on all isolates to confirm their groups since bacteria have 2 large groups – Gram-positive and Gram-negative. These 2 groups refer to the peptidoglycan layer in the cell wall of bacteria, where the Gram-positive group has a thick peptidoglycan layer and the Gram-negative group has a thin peptidoglycan layer with an additional outer membrane composed of lipopolysaccharide. The gram-positive group shows a purple colour as a result of crystal violet stains that remain in their thick peptidoglycan layer, while the Gram-negative group shows a red colour from counterstaining safranin in their thin peptidoglycan layer during the Gram staining procedure.<sup>67</sup> Thus, all the collected isolates belong to Gram-negative, as all of them show a red colour after the Gram staining process.

From a biochemical perspective, all 3 isolates produce the urease enzyme to breakdown urea substances into ammonia, carbon dioxide, and water, as well as the catalase enzyme to breakdown hydrogen peroxide into water and oxygen (bubble formation) during the catalase test and the oxidase enzyme for cell respiration,



**Figure 6.** Neighbor-joining phylogenetic tree based on 16S rRNA gene sequence shows the relationship of the sample [OJ154 (OR295237)] with members of the genus *Methylobacterium*. Bootstrap values from 1000 replications are shown at branch points. Bar, 0.002 substitution per site.

according to Faddin.<sup>45</sup> Besides, most of the isolates have a lipase enzyme to change the tributyrin (lipid) structure into glycerol and butyric acid (a fatty acid), showing transparent, clear zone formation around colonies on tributyrin-based (glycerol tributyrate) agar.<sup>68,69</sup>

On the other hand, none of them expresses the  $\alpha$ -amylase enzyme to hydrolyse starch, including the gelatinase and cellulase enzymes, since there is no gelatine liquefaction activity or cellulose compound degradation.<sup>45,70</sup> Also, they can't ferment glucose because there is no production of hydrogen ions and acetoin, no indole production from the activity of tryptophan breakdown, indicating no tryptophanase enzyme present, and no production of hydrogen sulphide gaseous, showing no reduction of sulphur-containing amino acids or through the reduction of inorganic sulphur compounds protein degradation by the enzyme cysteine desulfurase/desulphydrase.<sup>45,71,72</sup>

In addition, only OJ154 is capable of hydrolysing casein, demonstrating proteinase (caseinase) and nitrate reductase enzymes present in its cell for nitrate reduction.<sup>45,73,74</sup> Only ML8 has the citritase or citrase demolase enzyme to utilise citrate.<sup>45</sup> From all the biochemical activity data, various enzymes produced by isolates are essential to digest macromolecules into smaller molecules that can be used as nutrients for their development and to maintain their survival.<sup>75</sup>

In summary, the compilation of microscopic observation and biochemical activity characteristics of all isolates from *ulam* leaves shows similar PPFM characteristics as defined by Ito and Izuka,<sup>76</sup> Green,<sup>36</sup> Wang *et al.*,<sup>77</sup> Green and Ardley,<sup>78</sup> and Bijlani *et al.*<sup>79</sup> in Table 2. Hence, those PPFM isolates in this study most probably belong to the *Methylobacterium* genus.

Further tests, which are molecular identifications, were done to identify the species of the isolates. The 16S rRNA sequence analysis of the selected isolates acted as a complement to verify their closest related species.

### Molecular identification

For species confirmation, the 3 selected isolates undergo a deep gene profile. Figures 4 (a) and (b) demonstrate the band of the 16S rRNA gene after being amplified from the 3 selected

isolates' genes at 1500 base pairs. Then, the 16S rRNA partial gene sequences of the 3 isolates were matched with BLAST from the NCBI database for species identification. According to the 16S rRNA sequence (Table 3), the most closely related (99% similarity) bacteria species to the isolate OJ4 is *M. radiotolerans* JCM 2831 strain DSM 1819, while OJ154 is *M. salsuginis* strain MR. Similar to isolate OJ4 with different strains, the most closely related (99% similarity) bacteria species of isolate ML8 is *M. radiotolerans* JCM 2831. Additionally, the phylogenetic tree based on the 16S rRNA sequence of members of the genus *Methylobacterium* shows the location of each isolate (Figures 5 and 6). Those phylogenetic trees display the close relationship of OJ4 with *M. radiotolerans* JCM 2831 strain DSM 1819, as well as ML8 with *M. radiotolerans* JCM 2831. Differently, OJ154 has a close relationship with *M. salsuginis* strain MR.

As discussed by Green and Ardley,<sup>78</sup> some genera of *Methylobacterium* have been reclassified into a new genus, namely *Methylorubrum*, due to a few dissimilarities. For example, most species of *Methylorubrum* gen. nov. can utilise methylamine as the sole source of carbon and energy, whereas most species belonging to *Methylobacterium* cannot. Therefore, OJ154 falls into this newly classified genus, *Methylorubrum*. As mentioned previously, all the isolates are considered members of the *Methylobacterium* genus via microscopic examination and a few biochemical tests. However, a recent study discovered a new genus (*Methylorubrum*), which is similar to the *Methylobacterium* characteristic, raising the possibility that some of the isolates also belong to the *Methylorubrum* genus. Based on Table 3, OJ4 and ML8 show similar characteristics of *M. radiotolerans*, whereas OJ154 resembles *M. salsuginis*. Though they are closely related to the same species, each of them has a few different biochemical activities. This phenomenon may occur due to the different strains of the same species.<sup>80</sup>

Additionally, further chemotaxonomic characterisation of the species was not performed because the main objective of this study was to focus on the food product application (edible bacteria pigment). The species of *Methylobacterium* have been known ubiquitously everywhere, including at international space stations.<sup>37,79</sup> In most terrestrial

plants, of which there are more than 70 species, they can be found actively colonising the roots, branches, seeds, and leaves, as reported by Irvine *et al.*,<sup>14</sup> Mizuno *et al.*,<sup>30</sup> Iguchi *et al.*,<sup>81</sup> and Dourado *et al.*<sup>54</sup> and live dominantly on the leaf surface.<sup>57</sup> They obtain their carbon source from various carbon sources released by plants that leach from the cuticle and the volatile carbon substrates, mainly byproduct methanol released from cell wall metabolism via stomata.<sup>11</sup> Therefore, the taxonomic characterisation procedures that have been performed are to prove their presence on the surface of selected *ulam* leaves even after washing them with sterile distilled water during the isolation process.

## CONCLUSION

The PPFM bacteria have been isolated from *ulam*, and the highest population of them can be found in *C. caudatus* leaves, followed by *O. javanica* and *M. lunu-ankenda*. Selected isolate, namely ML8, can be considered the species of *M. radiotolerans* JCM 2831, similar to isolate OJ4 species but a different strain, *M. radiotolerans* JCM 2831 strain DSM 1819, while isolate OJ154 was characterised by the species of *M. salsuginis* strain MR based on their morphology, biochemical activities, and 16S rRNA gene sequence characteristics.

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## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

## AUTHORS' CONTRIBUTION

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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## DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

## ETHICS STATEMENT

This article does not contain any studies on human participants or animals performed by any of the authors.

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