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Isolation, Characterization and Evaluation of Pink Pigmented Facultative Methylotrophs (PPFMS) Associated with Paddy

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A B S T R A C T

Methylobacterium spp. are a group of bacteria known as pink-pigmented facultative methylotrophs (PPFMs) capable of growing on single carbon compounds such as formate, formaldehyde, and methanol as well as on a variety of multicarbon compounds having no carbon-carbon bonds. They are distributed ubiquitously in the plant phyllosphere and rhizosphere and have been isolated from many species of plants. Methylotrophs are known to play an important role in increasing crop yield and soil fertility. *Methylobacterium* is able to produce indole-3-acetic acid (IAA), suggesting that inoculation of PPFM bacteria could increase plant IAA concentrations and promote plant growth. The present programme envisages isolation, characterization and evaluation of Pink Pigmented Facultative Methylotrophs (PPFMs) associated with paddy. As part of the study conducted in the Department of Agricultural Microbiology, College of Agriculture, Vellayani, Thiruvananthapuram, Kerala, during 2015-2017, Pink Pigmented Facultative Methylotrophs (PPFMs) were isolated from the phyllosphere of paddy collected from different agro climatic conditions of Kerala by leaf imprint method using Ammonium Mineral Salt (AMS) agar media supplemented with 0.5% methanol and cycloheximide. In all, 46 isolates were obtained. The isolates were obtained from different districts of Kerala such as Thiruvananthapuram, Alappuzha and Palakkad including Attappadi hill tract. They were tentatively identified as PPFMs based on the characteristic pink pigmented colonies on AMS agar media with methanol as sole source of carbon and energy. All the 47 PPFM isolates including reference culture were found to produce IAA under *in vitro* conditions. However, it showed wide variations ranging from 6.74 to 33.35 µg mL^{-1} of culture filtrate. Maximum IAA production of 33.35µg mL^{-1} of culture was recorded by PPFM35. Paddy seeds [var. Jyothi (Ptb-39)] treated with PPFMs improved seed germination, biomass and seedling vigor index of paddy seedlings. Maximum germination percentage of 100 was recorded in seeds treated with PPFM35. The isolate PPFM22 treated seedlings recorded the highest seedling vigour index of 4756.36 whereas PPFM35 recorded seedling vigour index of 4250.00 over the control (3037.91). The root shoot ratio of seedlings showed significant increase when seeds were treated with PPFM isolates. Maximum root shoot ratio of 0.62 was observed when seeds were treated with PPFM26 and PPFM35 compared to control (0.33). The isolate PPFM35 was adjudged as superior isolate based on indole-3-acetic acid (IAA) production, maximum germination percentage, seedling vigour index and root shoot ratio. This isolate was identified as *Methylobacterium populi* based on morphological, biochemical and molecular characteristics.

Introduction

The living space on the leaf surface, known as the phyllosphere, harbours a wide variety of organisms having beneficial, harmful or neutral effects on the plant. The interaction between such microorganisms and higher plants affect the physiological activities of the plant. Pink pigmented facultative methylotrophs (PPFMs) of the genus *Methylobacterium* are commonly found in association with plants. It is hypothesized that they potentially dominate the phyllosphere bacterial population. The degree of the plant *Methylobacterium* association varies from strong, or symbiotic to loose, or epiphytic; a range that also includes the intermediate endophytic association (Lacava *et al*., 2004). The *Methylobacterium* spp. is characterized by a distinctive pink pigmentation which is due to the presence of carotenoid pigment (Jyothilaxmi *et al.,* 2012).

PPFMs are aerobic, Gram-negative, methylotrophic rod shaped bacteria, capable of growing on a wide range of multicarbon substrates and also on single carbon compounds such as formate, formaldehyde and methanol as their sole carbon and energy source. It was assumed that significant quantity of methanol is emitted from the plant parts as a by-product of pectin metabolism during cell wall synthesis (McDonald and Fall, 1993; Nemecek- Marshall *et al*., 1995).Numerous species and strains of *Methylobacterium* have been isolated from plants (Knief *et al*., 2010). PPFMs have been isolated from more than 100 species of plants ranging from liverworts and mosses to angiosperms and gymnosperms (Corpe and Basile, 1982). They are isolated on a methanol based mineral medium, Ammonium Mineral Salt (AMS) agar medium supplemented with 0.5% of methanol and cycloheximide at 100 mg L^{-1} (to inhibit fungal growth) by leaf impression method.

Many reports suggest that PPFMs can act as potential agents as plant growth promoters and also help in surviving plants from pathogenic attack (Madhayan *et al.,* 2004). They have been reported to produce plant growth regulators like zeatin and related cytokinins and auxins, which have significant effect on seed germination and seedling growth. Production of gibberellic acid (GA) by Methylobacteria has already been reported (Thangamani, 2005; Radha, 2007; Jones, 2010). Additionally, *Methylobacterium* have been reported for the production of urease enzyme (Holland and Polacco 1992), vitamin B_{12} production (Basile *et al*., 1985), nitrogen fixation and nodule formation (Raja *et al*., 2006), phosphate solubilization (Jones, 2007), synthesis of siderophores (Simionato *et al.,* 2006) and for the existence and prevalence of ACC deaminase enzyme (Madhaiyan *et al*., 2006). The first report on the production of IAA in significant amount by methylotrophs was by Ivanova *et al.* (2001) who detected various indole compounds in the culture liquids of 37 methylotrophic bacteria belonging to different taxa and different strains of *Methylobacterium.* Auxins produced by theses strains were found to range between 3-100 µg mL^{-1}. Omer *et al.,* (2004) unambiguously confirmed by high performance liquid chromatography in combination with nuclear magnetic resonance chromatography (NMR) that PPFM produced plant hormone IAA. Thangamani and Sundaram (2005) and Radha (2007) have documented production of IAA by PPFM ranging from 3.44 µg mL^{-1} to 25.51 µg mL^{-1} and 9.04 µg mL^{-1} to 28.15 µg mL^{-1} respectively. Madhaiyan *et al.,* (2004) observed higher photosynthetic activity in rice cultivar Co-47 that received *Methylobacterium* and attributed the effect due to enhancement of chlorophyll concentration, maleic acid content and increased number of stomata. Several workers reported growth promotional ability of PPFMs in several crops including

cotton (Madhaiyan *et al.*, 2005), rice (Senthilkumar, 2003), groundnut (Reddy *et al*., 2002), tomato (Thangamani and Sundaram, 2005), soybean, blackgram and sugarcane (Madhaiyan *et. al*., 2005).

Considering the importance of PPFM as plant growth promoting bacteria, an attempt was made to isolate, characterize and to select efficient PPFM strains from paddy based on indole-3-acetic acid (IAA) production and effect on paddy seed germination and seedling growth.

Materials and Methods

Collection of leaf samples

The leaf samples of paddy were collected from different agro climatic conditions of Kerala. The samples were brought to the laboratory in sterile polythene bags and stored at 4^0C.

Isolation of pink pigmented facultative methylotrophs (PPFMs)

Ammonium Mineral Salts (AMS) medium (Whittenburry *et al.*, 1970) is a selective medium for isolation of methylotrophs. The AMS medium was sterilized by autoclaving at 121°C for 15 min and cooled to 45°C. Filter sterilized vitamin solution (Colby and Zatman, 1973) along with 0.5 per cent (v/v) methanol was added after sterilization and before pouring media on to petriplates. The pH of the medium was adjusted to pH 7.0.

On the solidified AMS agar medium upper and lower surface of leaf samples were placed separately, in such a way as to make impression of it. Then the leaves were lifted away and plates were incubated at 30^0C for 7 days (Corpe, 1985). Based on characteristic pink pigmentation of colonies they were tentatively identified as PPFMs. The isolate obtained from the commercial product of Tamil Nadu Agricultural University was taken as reference culture.

Purification of Pink Pigmented Facultative Methylotrophs (PPFMs).

PPFMs obtained by leaf imprint technique were purified by the streak plate method and well isolated colonies on the plates were preserved on Peptone Glycerol Agar (enrichment medium) slants at 4°C in a refrigerator for further use.

Estimation of indole acetic acid production by the different isolates obtained

Indole Acetic Acid was estimated as per the procedure described by Gordon and Weber (1951).

100 ml of AMS broth supplemented with 0.5 per cent methanol and cycloheximide was prepared in 250 ml flasks. To this medium, 0.1 per cent tryptophan was added. Using sterile technique, the medium was inoculated with one ml of PPFM inoculum (10^7 cfu/ ml). Flasks were kept for incubation at 30^0C for 7 days. After incubation, culture was centrifuged at 10000 rpm for 10 minutes. To the 10 ml of culture supernatant 2 ml of the Salkowski reagent was added. Incubated at room temperature for 25 minutes and then read at OD_{530}.

Using the standard curve for IAA, the amount of IAA was calculated.

Effect of isolates of PPFM on paddy seed germination

Seeds of variety Jyothi (Ptb-39) were surface sterilized before treatment with the bacterial suspension. The seeds were first washed with sterile distilled water twice and then treated with 70% ethanol for 1 min. This was followed by treatment with 2% sodium hypochlorite solution for 30 seconds. Finally,

the treated seeds were thoroughly rinsed for more than five times with sterile distilled water. Surface sterilized seeds were soaked overnight in 1 per cent of 7 days old liquid culture (10^7 cfu/ ml) of the respective isolates. After decanting the liquid culture, the dried seeds were placed on filter paper in a petri dish. Plates were incubated at 30^0 C for 72 hrs. The untreated seeds were kept as control. The germination percent was calculated after 72 hrs. After taking the number of germinated seeds, percentage seed germination was calculated using the formula,

$$\text{Seed germination (\%)} = \frac{\text{Number of germinated seeds}}{\text{Total number of seeds}} \times 100$$

Effect of isolates of PPFM on paddy seedling growth

To calculate the effect of PPFM inoculation on seedling vigor index of paddy, the seeds were surface sterilized with 70% alcohol and 0.1% mercuric chloride, which was followed by a series of washings with sterile distilled water. Surface sterilized seeds were soaked overnight in 1 per cent of 7 days old liquid culture ((10^7 cfu/ ml) of the respective isolates and sown in plastic pots filled with wetland soil.

The untreated seeds were taken as control. Seedling vigour index was calculated using the formula,

Seedling Vigour Index = Germination Percent x (Shoot length + Root length) (Baki and Anderson, 1973).

After taking the dry weight of shoot and root (g), Root Shoot ratio was calculated using the formula,

$$\text{Root Shoot Ratio} = \frac{\text{Dry weight of root (g)}}{\text{Dry weight of shoot (g)}} \times 100$$

Morphological, biochemical and molecular characterization of isolates of PPFM

Morphological tests *viz*., cell shape, gram reaction and motility were carried out to characterize the tentatively identified PPFM isolate. Biochemical characterization of selected bacterial isolate were done by performing various biochemical tests and carbohydrate utilization tests by using readymade Himedia© kits (HiCarbo™, Part A, Band C, Hi25™ Enterobacteriaceae). Colour change observed on the biochemical amended media of the kit after spot inoculating culture suspensions of selected isolates followed by incubation for 72 h indicated the reaction with respect to different biochemicals or carbohydrates as positive or negative. The results of biochemical tests were utilized to arrive at a tentative genus level identification of isolate. Bergey's manual of determinative of bacteriology was used as a reference to identify the isolate. Molecular characterization of selected isolate was done by 16S rRNA cataloging using universal primers.

Results and Discussion

The pink pigmented facultative methylobacteria (PPFM) were isolated from the phyllosphere of paddy, collected from different locations of Kerala. Isolations were made following leaf imprint method using Ammonium Mineral Salt (AMS) agar media supplemented with 0.5% methanol and cycloheximide (Lindstrom and Chistoserdova, 2002). Forty six isolates were obtained from different locations and allotted code numbers for each of the isolate. They were tentatively identified as PPFMs based on the characteristic pink pigmented colonies on

AMS agar media with methanol as sole source of carbon and energy (Plate 1and Plate 2). The isolate obtained from the commercial product of Tamil Nadu Agricultural University was taken as reference culture.

The first report on the production of IAA in significant amount by methylotrophs was by Ivanova *et al.* (2001) who detected various indole compounds in the culture liquids of 37 methylotrophic bacteria belonging to different taxa and different strains of *Methylobacterium.* Auxins produced by these strains were found to range between 3-100 µg mL^{-1}. Omer *et al.*, (2004) unambiguously confirmed by high performance liquid chromatography in combination with nuclear magnetic resonance chromatography (NMR) that PPFM produced plant hormone IAA. Thangamani and Sundaram (2005) and Radha (2007) have documented production of IAA by PPFM ranging from 3.44 µg mL^{-1} to 25.51 µg mL^{-1} and 9.04 µg mL^{-1} to 28.15 µg mL^{-1} respectively. *Methylobacterium* is able to produce IAA, suggesting that inoculation of these bacteria could increase plant IAA concentrations and promote plant growth (Lee *et al*., 2006). The presence of IAA was reported in supernatants of PPFM cultures (Omer *et al.*, 2004). There are numerous reports available on indole-3-acetic acid (IAA) production by PPFMs (Omer *et al*., 2004; Anitha, 2010). In the present investigation, all the 47 PPFM isolates were found to produce IAA under *in vitro* conditions. However, it showed wide variations ranging from 6.74 to 33.35 µg mL^{-1} of culture filtrate. Maximum IAA production of 33.35 µg $mL^{-1}$of culture was recorded by PPFM35. The reference culture produced 18.01 µg mL^{-1} of IAA (Table 1).

PPFMs have been reported to influence seed germination and seedling growth by producing plant growth regulators like zeatin and related cytokinins and auxins. Seeds treated with the methylotrophic strains improved seed germination, seedling vigor index (SVI) and biomass of rice seedlings. The methylotrophic population in the treated seedlings increased in the vegetative stages when compared to seeding stages. Treated seedlings showed a higher accumulation of plant hormones viz trans-zeatin riboside, isopentenyladenosine, and indole-3-acetic acid than untreated seedlings (Lee *et al*., 2006). Based on these findings, effect of PPFM isolates on paddy seed germination and seedling growth was tested and the results revealed that the germination percentage of inoculated seeds showed a significant increase compared to uninoculated control. Maximum germination percentage of 100 was recorded in seeds treated with PPFM35. This treatment was found to be significantly superior to the uninoculated control which recorded a germination percentage of 86 per cent (Table 2).

The present investigation conclusively proved that, PPFM inoculation in paddy seeds had significant effect on biomass and seedling vigor index (Plate 3). Observations on shoot length and root length of PPFM isolates inoculated seeds, after 14 days of sowing showed a significant increase compared to uninoculated control. Inoculation with PPFM6 recorded the maximum shoot length of 26.38 cm and was found to be significantly superior to the uninoculated control which recorded a shoot length of 17.84 cm. The reference culture treated seedlings recorded a shoot length of 23.03 cm. Maximum root length of 24.20 cm was obtained in seeds treated with PPFM22 and this treatment was significantly superior to the uninoculated control and reference strain which recorded a root length of 17.50 and 18.90 cm respectively. Paddy seeds treated with PPFM22 recorded the highest seedling vigour index of 4756.36, whereas, PPFM35 recorded seedling vigour index of 4250.00 and this was significantly

superior compared to the control which recorded a vigour index of 3037.91. The reference culture recorded a seedling vigour index of 3943.45 (Table 3)

Significant increase in seedling shoot fresh weight, root fresh weight, shoot dry weight, root dry weight and root shoot ratio compared to control was observed when seeds were treated with PPFM isolates. In the present investigation, PPFM26 and PPFM35 strains gave the best performance of seedling root shoot ratio and increased significantly with value of 0.62. These treatments showed 87.88 per cent increase in root shoot ratio over uninoculated control (Table 4 and 5).

The isolate PPFM35 was adjudged as superior isolate based on indole-3-acetic acid (IAA) production, maximum germination percentage, seedling vigour index and root shoot ratio of paddy seedlings. This selected isolate was characterized based on morphological, biochemical and molecular characteristics.

Microscopic studies revealed that the PPFM isolates were rod shaped, motile, gram negative and produced poly β-hydroxy butyrate granules (Green and Bousifield, 1982). In the present investigation, the superior isolate selected was subjected to morphological characterization. The results revealed that the isolate was rod shaped, stained Gram negative and exhibited motility (Plate 4). The expression of pink pigmentation with varied level of intensity in PPFM indicates the presence of carotenoids (Fasim, 2003) which is known to protect these bacteria from intense light and UV radiation (Liu *et al*., 1993). In the present study, medium pink coloured colonies of PPFM35 were observed after one week of incubation (Table 6).

All isolates were aerobes producing catalase and oxidase as already demonstrated by Bellin and Spain (1976) and positive for urease test and indole production (Thangamani, 2005). However, hydrolysis of casein, starch, cellulose degradation, MR and VP test and nitrate reduction test was not recorded in any of the isolates.

In the present investigation, for further characterization, the isolate PPFM35 was subjected to a series of biochemical tests (Table 7).

The methylotrophic bacteria having capability to grow on different single carbon compounds as sole source of carbon and energy, can also grow on wide range of multi carbon growth substrates making them facultatively methylotrophic. The selected isolate was tested for the utilization of the 29 different carbon compounds. Using the results of various biochemical tests, a tentative genus level identification was done. Bergey's manual of determinative of bacteriology was used as a reference to identify the isolate and the isolate PPFM35 was identified to belong to genus *Methylobacterium.* The results are presented in Table 8.

The present investigation demonstrated that it is possible to distinguish and classify the methylotrophic bacteria using 16S rRNA sequence analysis. Our results also indicated that phylogenetic relationships based on 16S rRNA sequences reflect the classical taxonomic classification systems based on phenotypic characteristics for methylotrophs. Thus, 16S rRNA sequence analysis could be a useful tool for detailed classification of methylotrophs. 16S rRNA gene phylogenetic analysis performed clearly showed the position of the isolate within the genus *Methylobacterium*. The 16S rRNA gene sequencing analysis showed 100% homology with that of *Methylobacterium populi* in the existing database of National Center of Bioinformatics.

Table.1 Indole-acetic acid (IAA) production by the PPFM isolates

Sl. No.	Isolate code No.	IAA ($\mu g\ mL^{-1}$)*
1	PPFM1	7.12
2	PPFM2	19.52
3	PPFM3	25.67
4	PPFM4	19.82
5	PPFM5	11.09
6	PPFM6	15.77
7	PPFM7	19.44
8	PPFM8	16.24
9	PPFM9	17.40
10	PPFM10	9.33
11	PPFM11	22.60
12	PPFM12	8.09
13	PPFM13	7.28
14	PPFM14	8.26
15	PPFM15	10.47
16	PPFM16	9.78
17	PPFM17	9.55
18	PPFM18	6.74
19	PPFM19	19.67
20	PPFM20	29.19
21	PPFM21	22.74
22	PPFM22	16.55
23	PPFM23	9.92
24	PPFM24	22.88
25	PPFM25	19.19
26	PPFM26	24.73

27	PPFM27	8.97
28	PPFM28	10.64
29	PPFM29	6.89
30	PPFM30	25.16
31	PPFM31	9.61
32	PPFM32	10.98
33	PPFM33	7.30
34	PPFM34	11.27
35	PPFM35	33.35
36	PPFM36	18.39
37	PPFM37	29.72
38	PPFM38	22.43
39	PPFM39	19.74
40	PPFM40	7.74
41	PPFM41	13.01
42	PPFM42	20.25
43	PPFM43	13.23
44	PPFM44	8.58
45	PPFM45	12.93
46	PPFM46	15.79
47	PPFM47 (Reference strain)	18.01
	CD (0.05)	6.14
	SEm (±)	2.15

* Mean of 2 independent replications

Table.2 Effect of PPFM isolates on paddy seed germination

Sl. No.	Isolate code No.	Seed germination* (%)
1	PPFM1	93.00
2	PPFM2	93.00
3	PPFM3	94.00
4	PPFM4	87.67
5	PPFM5	84.33
6	PPFM6	89.67
7	PPFM7	89.67
8	PPFM8	96.00
9	PPFM9	95.00
10	PPFM10	96.00
11	PPFM11	98.00
12	PPFM12	93.00
13	PPFM13	92.33
14	PPFM14	90.67
15	PPFM15	87.67
16	PPFM16	94.00
17	PPFM17	75.33
18	PPFM18	90.67
19	PPFM19	95.00
20	PPFM20	79.33
21	PPFM21	86.33
22	PPFM22	96.00
23	PPFM23	79.67
24	PPFM24	95.00
25	PPFM25	93.00
26	PPFM26	94.00

27	PPFM27	98.00
28	PPFM28	84.67
29	PPFM29	97.00
30	PPFM30	98.00
31	PPFM31	95.00
32	PPFM32	93.00
33	PPFM33	91.00
34	PPFM34	94.00
35	PPFM35	100.00
36	PPFM36	92.00
37	PPFM37	92.00
38	PPFM38	93.00
39	PPFM39	77.67
40	PPFM40	97.67
41	PPFM41	90.67
42	PPFM42	96.00
43	PPFM43	86.00
44	PPFM44	90.00
45	PPFM45	84.67
46	PPFM46	88.00
47	PPFM47 (Reference strain)	94.00
48	Control	86.00
	CD (0.05)	6.92
	SEm (±)	2.46

*Mean of 3 independent replications

Table.3 Effect of PPFM isolates on shoot length, root length and seedling vigour index of paddy seedlings

Sl. No.	Isolate code No.	Shoot length (cm)/ seedling*	Root length (cm)/ seedling*	Seedling Vigour Index
1	PPFM1	19.58	17.93	3,489.06
2	PPFM2	24.62	19.70	4,121.48
3	PPFM3	25.02	17.90	4,039.46
4	PPFM4	25.00	22.40	4,155.06
5	PPFM5	25.25	18.80	3,716.88
6	PPFM6	26.38	17.57	3,939.10
7	PPFM7	20.03	17.70	3,380.53
8	PPFM8	25.72	20.17	4,402.82
9	PPFM9	23.38	24.10	4,515.58
10	PPFM10	19.77	19.67	3,784.00
11	PPFM11	19.75	22.80	4,173.17
12	PPFM12	21.25	22.60	4,073.94
13	PPFM13	21.25	18.57	3,656.82
14	PPFM14	24.40	18.40	3,885.80
15	PPFM15	23.80	21.50	3,963.71
16	PPFM16	21.95	20.87	4,021.87
17	PPFM17	20.12	17.97	2,849.70
18	PPFM18	23.17	17.70	3,707.40
19	PPFM19	23.50	18.87	4,020.85
20	PPFM20	23.61	20.03	3,465.24
21	PPFM21	24.82	21.30	3,978.15
22	PPFM22	25.38	24.20	4,756.36
23	PPFM23	24.07	17.93	3,345.03
24	PPFM24	25.38	20.03	4,313.27
25	PPFM25	22.60	19.23	3,889.83
26	PPFM26	24.75	18.70	4,090.04

27	PPFM27	22.08	15.87	3,719.78
28	PPFM28	23.50	18.97	3,570.58
29	PPFM29	20.55	19.73	3,904.60
30	PPFM30	23.60	20.13	4,285.96
31	PPFM31	25.85	14.20	3,813.18
32	PPFM32	23.68	18.27	3,900.67
33	PPFM33	21.37	18.23	3,604.07
34	PPFM34	24.97	18.17	4,054.37
35	PPFM35	23.37	19.13	4,250.00
36	PPFM36	24.33	19.60	4,037.78
37	PPFM37	23.20	18.97	3,877.02
38	PPFM38	22.35	19.70	3,912.22
39	PPFM39	22.02	19.60	3,221.33
40	PPFM40	21.55	17.57	3,817.84
41	PPFM41	23.65	15.47	3,545.07
42	PPFM42	24.34	21.33	4,385.67
43	PPFM43	19.17	22.00	3,537.80
44	PPFM44	20.95	21.17	3,786.82
45	PPFM45	23.67	21.97	3,867.93
46	PPFM46	26.25	19.07	3,986.13
47	PPFM47 (Reference strain)	23.03	18.90	3,943.45
48	Control	17.84	17.50	3,037.91
	CD (0.05)	2.91	1.34	365.25
	SEm (±)	1.03	0.48	129.91

*Mean of 3 replications

Table.4 Effect of PPFM isolates on shoot and root fresh weight of paddy seedlings

Sl. No.	Isolate code No.	Shoot fresh weight (g)/ seedling*	Root fresh weight (g)/ seedling*
1	PPFM1	0.51	0.25
2	PPFM2	0.45	0.21
3	PPFM3	0.54	0.30
4	PPFM4	0.54	0.28
5	PPFM5	0.53	0.26
6	PPFM6	0.65	0.33
7	PPFM7	0.57	0.28
8	PPFM8	0.67	0.30
9	PPFM9	0.63	0.29
10	PPFM10	0.56	0.25
11	PPFM11	0.54	0.25
12	PPFM12	0.52	0.26
13	PPFM13	0.54	0.23
14	PPFM14	0.62	0.27
15	PPFM15	0.60	0.27
16	PPFM16	0.60	0.35
17	PPFM17	0.57	0.22
18	PPFM18	0.55	0.25
19	PPFM19	0.58	0.30
20	PPFM20	0.60	0.29
21	PPFM21	0.61	0.30
22	PPFM22	0.58	0.29
23	PPFM23	0.55	0.24
24	PPFM24	0.66	0.30
25	PPFM25	0.57	0.31
26	PPFM26	0.57	0.33
27	PPFM27	0.56	0.27

28	PPFM28	0.52	0.25
29	PPFM29	0.55	0.29
30	PPFM30	0.60	0.31
31	PPFM31	0.50	0.24
32	PPFM32	0.57	0.28
33	PPFM33	0.65	0.30
34	PPFM34	0.54	0.27
35	PPFM35	0.58	0.34
36	PPFM36	0.54	0.24
37	PPFM37	0.57	0.30
38	PPFM38	0.46	0.23
39	PPFM39	0.61	0.29
40	PPFM40	0.59	0.25
41	PPFM41	0.61	0.27
42	PPFM42	0.67	0.32
43	PPFM43	0.49	0.23
44	PPFM44	0.42	0.26
45	PPFM45	0.65	0.34
46	PPFM46	0.56	0.26
47	PPFM47 (Reference strain)	0.54	0.28
48	Control	0.57	0.24
	CD (0.05)	0.096	0.050
	SEm (±)	0.03	0.02

*Mean of 3 replications

Table.5 Effect of PPFM isolates on shoot dry weight, root dry weight and root shoot ratio of paddy seedlings

Sl. No.	Isolate code No.	Shoot dry weight (g)/ seedling*	Root dry weight (g)/ seedling*	RS Ratio
1	PPFM1	0.21	0.09	0.41
2	PPFM2	0.16	0.05	0.32
3	PPFM3	0.24	0.14	0.58
4	PPFM4	0.28	0.12	0.43
5	PPFM5	0.23	0.10	0.42
6	PPFM6	0.35	0.17	0.48
7	PPFM7	0.26	0.12	0.45
8	PPFM8	0.34	0.14	0.43
9	PPFM9	0.33	0.13	0.39
10	PPFM10	0.26	0.09	0.36
11	PPFM11	0.24	0.09	0.36
12	PPFM12	0.22	0.10	0.45
13	PPFM13	0.24	0.07	0.29
14	PPFM14	0.32	0.11	0.35
15	PPFM15	0.30	0.11	0.35
16	PPFM16	0.33	0.19	0.57
17	PPFM17	0.27	0.06	0.24
18	PPFM18	0.25	0.09	0.36
19	PPFM19	0.28	0.14	0.51
20	PPFM20	0.30	0.13	0.45
21	PPFM21	0.27	0.14	0.54
22	PPFM22	0.28	0.13	0.46
23	PPFM23	0.25	0.08	0.31
24	PPFM24	0.36	0.14	0.40
25	PPFM25	0.27	0.15	0.54
26	PPFM26	0.27	0.17	0.62
27	PPFM27	0.26	0.11	0.43

28	PPFM28	0.22	0.09	0.40
29	PPFM29	0.25	0.13	0.54
30	PPFM30	0.30	0.15	0.49
31	PPFM31	0.20	0.08	0.41
32	PPFM32	0.27	0.12	0.47
33	PPFM33	0.28	0.14	0.48
34	PPFM34	0.24	0.11	0.47
35	PPFM35	0.28	0.18	0.62
36	PPFM36	0.24	0.08	0.35
37	PPFM37	0.27	0.14	0.53
38	PPFM38	0.16	0.07	0.43
39	PPFM39	0.31	0.13	0.43
40	PPFM40	0.29	0.09	0.33
41	PPFM41	0.31	0.11	0.34
42	PPFM42	0.37	0.20	0.52
43	PPFM43	0.19	0.07	0.38
44	PPFM44	0.26	0.10	0.39
45	PPFM45	0.35	0.17	0.48
46	PPFM46	0.26	0.10	0.37
47	PPFM47 (Reference strain)	0.24	0.12	0.48
48	Control	0.27	0.09	0.33
	CD (0.05)	0.078	0.039	0.054
	SEm (±)	0.03	0.01	0.02

*Mean of 3 replications

Table.6 Morphological characterization of selected PPFM isolate

Sl. No.	Isolate code No.	Cell shape	Motility	Gram reaction	Pigmentation
1	PPFM35	Rod	Positive	Negative	Medium pink

Table.7 Biochemical characterization of selected PPFM isolate

Sl. No	Biochemical Tests	PPFM35
1	Citrate utilization	Positive
2	Lysine utilization	Positive
3	Ornithine utilization	Positive
4	Urease	Positive
5	Phenylalanine deamination	Negative
6	H_2S production	Negative
7	Nitrate reduction	Negative
8	Catalase	Positive
9	Arginine lyase	Negative
10	Malonate utilization	Positive
11	VogesProskauer	Negative
12	Indole	Positive
13	Oxidase	Positive
14	Methyl red	Negative

Table.8 Utilization of different carbon substrates by selected PPFM isolate

Sl. No	Carbon Substrate	PPFM35
1	D- Glucose	Positive
2	D- Fucose	Negative
3	D- Xylose	Negative
4	L- Arabinose	Negative
5	D- Fructose	Positive
6	L- Aspartate/ L- Glutamate	Negative
7	Sebacate	Negative
8	Acetate	Positive
9	Betaine	Positive
10	Tartarate	Positive
11	Ethanol	Positive
12	Methylamine	Negative
13	Dimethylamine	Positive
14	Formaldehyde	Positive
15	Glycerol	Positive
16	Methanol	Positive
17	Formate	Positive
18	Succinate	Negative
19	Lactate	Positive
20	Pyruvate	Positive
21	Salicylate	Positive
22	Nutrient agar	Positive
23	Fumarate	Positive
24	Rhamnose	Negative
25	Raffinose	Negative
26	Esculine	Negative
27	Cellobiose	Negative
28	Melibiose	Negative
29	Saccharose	Negative

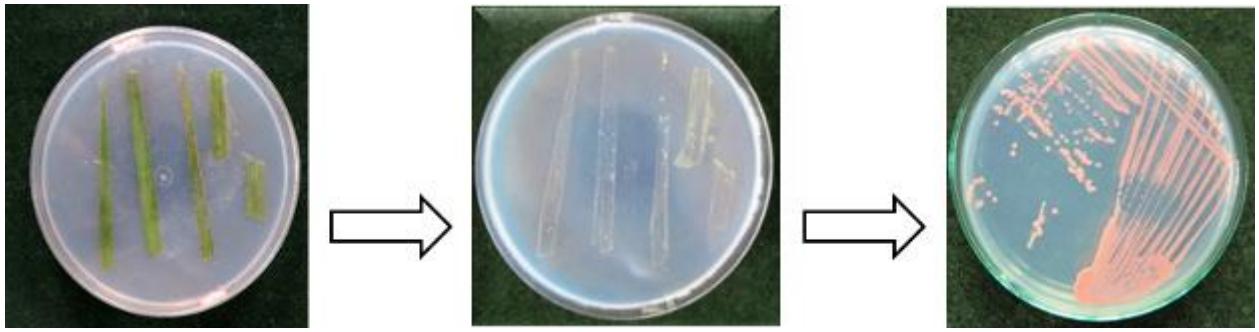
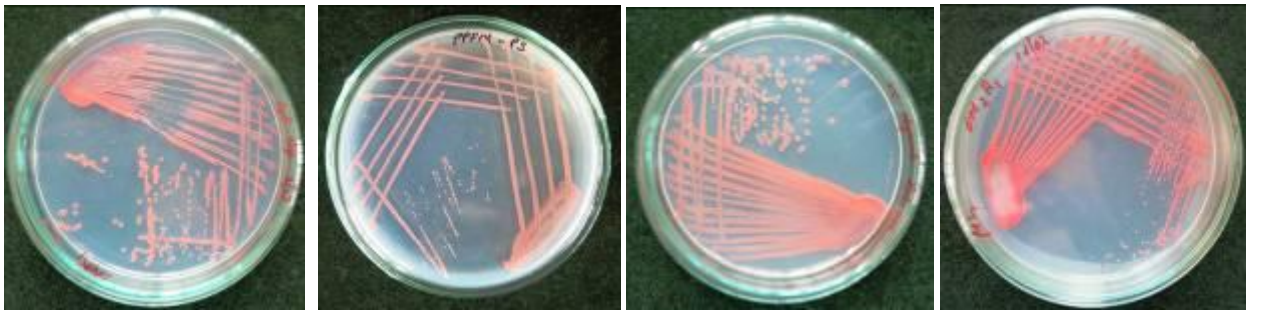


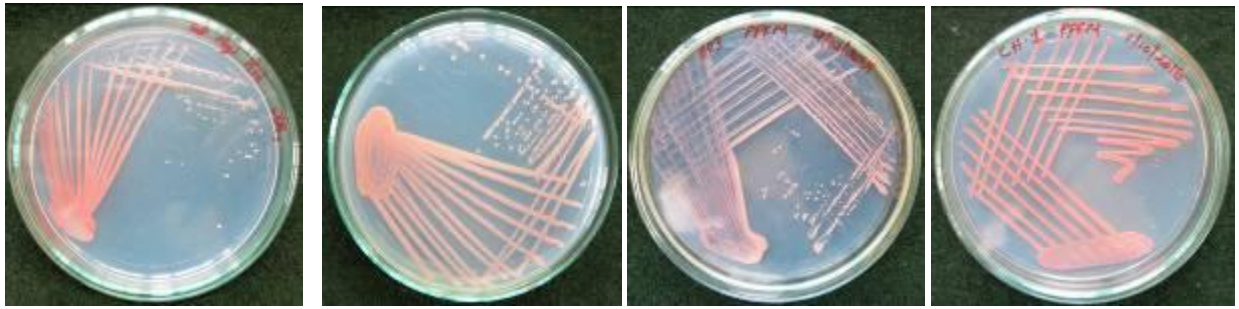
Plate 1(A). Isolation of Pink Pigmented Facultative Methylotrophs (PPFMs) by leaf impression method on AMS agar medium

(B) (C)

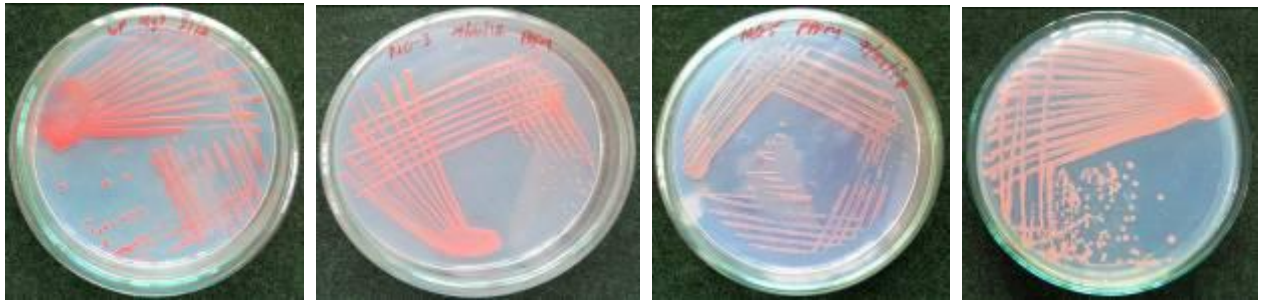
(B) Liquid culture of PPFM isolates C) Maintenance of PPFM culture in slants



PPFM1 PPFM6 PPFM8 PPFM11



PPFM12 PPFM16 PPFM19 PPFM22



PPFM25 PPFM35 PPFM38 PPFM42

Plate.2 Different PPFM isolates obtained by leaf impression method

PPFM22 Control PPFM35 Control PPFM9 Control

PPFM30 Control PPFM8 Control PPFM24 Control

Plate.3 Effect of PPFM isolates on shoot and root growth of paddy seedlings

Plate.4 Colony morphology of the selected PPFM isolate and Gram reaction

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