Int.J.Curr.Microbiol.App.Sci (2019) *8*(7): 152-163

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 8 Number 07 (2019)**
Journal homepage: http://www.ijcmas.com

Original Research Article https://doi.org/10.20546/ijcmas.2019.807.019

Isolation and Identification of PPFM Bacterial Isolates Isolated From Direct Seeded Rice Growing Areas of Hyderabad-Karnataka, India

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A B S T R A C T

A total of 50 PPFM's were isolated from rhizosphere soil and phyllosphere of direct seeded rice regions of Hyderabad-Karnataka. Samples of leaves and rhizosphere soils were collected from direct seeded rice grown in different location (Raichur district, Bellary district, Koppal district and Yadgir district). Thereafter, all the classical recommended methods used for identification of all the obtained isolates were carried out including, morphological, microscopical as well as biochemical characteristics. The results revealed that all the isolates were gram negative, rod shaped, motile. Colonies were pink, pale pink or dark pink in color due to pigmentation. Biochemical characteristics revealed that all the isolates including reference strain confirmed to be negative for Methyl red and Voges-Proskauer test while, positive for oxidase, urease, catalase activity, indole production and citrate utilization. None of the isolates could reduce nitrate to nitrite, whereas; the hydrolysis of casein and starch was not recorded in any of the isolates tested They can be cultivated on ammonium mineral salt (AMS) agar with methanol colonies were pink, pale pink or dark pink in color due to pigmentation and variations in colonial morphology. Based on the observed characteristics, the isolates obtained belong to the genus *Methylobacterium*.

Keywords

PPFM's, Direct seeded rice, Rhizosphere, Phyllosphere and *Methylobacterium*

Article Info

Accepted:
04 June 2019
Available Online:
10 July 2019

Introduction

Rice is a staple food grown in almost all states of India accounting for more than 40 per cent of the food grain production. It is manually transplanted in standing water, destroys soil structure, is labour intensive and cumbersome. Hence, direct seeded rice without puddling is a viable alternative for its establishment as it can save water and also requires less labour. Methylotrophic bacteria are successful example that can achieve the two purposes as PGPR with antifungal activity. Many of the microbes living on the phylloplane probably lead a saprophytic lifestyle, feeding on materials leached from the leaf. One such

example is *Methylobacterium* sp. a pink pigmented facultative methylotroph (PPFM's) which was first identified as covert contaminants from the tissue culture of liverwort, *scapania nemorosa* (Basile *et al.*, 1969). This bacterium provides a useful model for the unappreciated kinds of interactions between plants and bacteria that take place routinely on lab and in culture dishes (Green and Bousifield, 1982).

The genus *Methylobacterium* is composed of a variety of pink pigmented methylotroph (PPFM) bacteria which are capable of growing on C_1 compounds such as formate, formaldehyde, methanol and methylamine as well as on a wide range of multicarbon growth substates such as C_2, C_3 and C_4 compounds. PPFMs are ubiquitous in nature and frequently reported on various plant species, those are a substantial part of the aerobic, heterotrophic microflora of the surfaces of young leaves. These bacteria are commonly found in soils, as well as on the surfaces of leaves, seeds and in the rhizosphere of a wide variety of plants, with highest numbers on actively growing and meristamatic tissue (Holland, 1997) Methylotrophs have been reported to influence seed germination and seedling growth by producing plant growth regulators like zeatin and related cytokinins and auxins and to alter agronomic traits like branching, seedling vigour, rooting and heat/cold tolerance (Omer *et al.*, 2004).

Members of the genus *Methylobacterium* are pink-pigmented facultative methylotrophs (PPFMs). They belong to the proteobacterial sub group (class) Alpha-proteobacteria, order Rhizobiales, and family Methylobacteriaceae. They are strictly aerobic, Gram-negative and rod shaped. *Methylobacterium* are found worldwide on the leaves of many different plant species. The unique feature of PPFM's is their ability to oxidize methanol, a methylotrophic property based on the presence of methanol dehydrogenase (mxaF) gene.

Materials and Methods

Isolation and purification of PPFMs isolates

A detailed survey was conducted in different locations of Hyderabad- Karnataka for isolation of an efficient strain of PPFM. Samples of leaves and rhizosphere soils were collected from direct seeded rice grown in different villages such as UASR Agriculture farm, Kasbali camp, Kappagal, Nir Manvi and Sindhanur from Raichur district, Dadesuguru and Siriguppa from Bellary district, Gangavati and Karatagi from Koppal district, Hunasagi, Shahapur, and Balichakra from Yadgir district.

The samples were brought to the laboratory in sterile polythene bags and stored at 4^{o}C to isolate methylotrophs.

About 50 different PPFM isolates from the rhizosphere and phyllosphere of direct seeded rice. Ammonium Mineral Salts (AMS) medium (Whittenbury *et al.*, 1970) was used as a selective medium for isolation of PPFMs.

The AMS medium was sterilized by autoclaving at 121°C for 20 minutes and cooled to 50°C. Filter sterilized methanol was added @ 0.5 per cent (v/v) after sterilization and before pouring media on to Petri plates. To isolate the PPFM bacteria from Phyllosphere region cyclohexamide (20µg/ml) was added to the medium to avoid the fungal contamination. The pH of the medium was adjusted to 6.7.

Leaf imprinting technique

On the solidified AMS agar medium upper and lower surface of leaf samples were placed separately, in such a way to impression on it. Then the leaves were lifted away and plates were incubated at 30 ^{o}C for 5-7 days (Corpe *et al.*, 1985).

Serial dilution technique

One gram sample of rhizosphere soil and leaves was ground using a pestle and mortar, serially diluted up to 10^{-6} dilutions and one ml each of the aliquots from 10^{-4}, 10^{-5} and 10^{-6} dilutions was transferred to sterile Petri dishes. The AMS medium was sterilized by autoclaving at 121°C for 20 min and cooled to 50 °C. Filter sterilized cyclohexamide (30 µg ml^{-1}) antibiotic solution and 0.5 per cent (v/v) methanol were incorporated into the sterilized AMS medium before plating. After plating with AMS medium, the plates were incubated in an inverted position for 5-7 days at 30°C. Characteristic pink colonies growing over the medium were identified (Corpe and Rheem, 1989). Further, the methylotrophs were purified by the streak plate method and well isolated colonies on the plates were preserved on AMS agar slants. Isolates were maintained on AMS slants at 4°C in a refrigerator for further use.

Morphological and biochemical characterization

Morphological and biochemical characterization will be carried out as per the standard procedures for the selected rhizosphere and phyllosphere group.

Results and Discussion

Isolation of pink pigmented facultative methylotrophs from direct seeded rice

In the present study, 50 PPFM's were isolated from rhizosphere soil and phyllosphere of direct seeded rice regions of Hyderabad-Karnataka. Out of fifty isolates, 25 were isolated from rhizosphere soil while remaining 25 isolates were isolated from phyllosphere of direct seeded rice. All the isolates were isolated by leaf imprinting and serial dilution method using AMS medium which was a selective medium for isolating PPFM's (Lidstrom and Chistoserdova, 2002). Several authors have already reported the natural association of PPFM's with several plants. Basile *et al.,* (1969) for the first time reported these organisms as a contaminant of tissue cultures of the leafy liverwort, *Scapania nemorosa* which lead to conclude that these organisms as inhabitants of plant surface. They acted as phytosymbionts by associating with roots, leaves and seeds of most terrestrial plants (Trotsenko *et al.,* 2001; Radha *et al.,* 2007). The isolation of many PPFM's on the phyllosphere of plants has been reported by Kouno and Ozoki (1975). Among different crop plants, their relationships with the cereals have been reported by Balachandra *et al.,* (2008), Kim *et al.,* (2010) and Madhaiyan *et al.,* (2003).

Morphological and Biochemical characterization of PPFM isolates

The results on characterization of PPFM isolates isolated from direct seeded rice, revealed that all the fifty PPFM isolates including reference strain were rod shaped, stained Gram negative and exhibited motility with accumulation to produce poly β-hydroxy butyrate granules. The observations were consistent with the report of Green and Bousifield (1982).

All PPFM isolates, obtained in the present study, exhibited pink colored colonies on ammonium mineral salts medium (AMS) with varying intensities of pigmentation ranging from light to dark pink which makes them to differentiate from other distinct methylotrophic organisms which are generally existing on plant surfaces.

These observations were confirmative with the reports of Corpe and Basile (1982) who observed that PPFM's were distinctive pink pigmentation due to carotenoids.

Table.1 Sources of PPFM isolates from major direct seeded rice growing areas of Hyderabad-Karnataka region

Sl. No.	Isolate code	Habitat	Place
Raichur district			
1	PPFM-1	Rhizosphere	Kasbe Camp
2	PPFM-2	Rhizosphere	Kasbe Camp
3	PPFM-3	Phyllosphere	Kasbe Camp
4	PPFM -4	Phyllosphere	Kasbe Camp
5	PPFM-5	Rhizosphere	UASR Agriculture farm
6	PPFM-6	Rhizosphere	UASR Agriculture farm
7	PPFM-7	Phyllosphere	UASR Agriculture farm
8	PPFM-8	Phyllosphere	UASR Agriculture farm
9	PPFM-9	Rhizosphere	Kappagal
10	PPFM-10	Rhizosphere	Kappagal
11	PPFM-11	Phyllosphere	Kappagal
12	PPFM-12	Phyllosphere	Kappagal
13	PPFM-13	Rhizosphere	Sindhanur
14	PPFM-14	Rhizosphere	Sindhanur
15	PPFM-15	Phyllosphere	Sindhanur
16	PPFM-16	Phyllosphere	Sindhanur
17	PPFM-17	Rhizosphere	Nir manvi
18	PPFM-18	Rhizosphere	Nir manvi
19	PPFM-19	Phyllosphere	Nir manvi
20	PPFM-20	Phyllosphere	Nir manvi
Bellary district			
21	PPFM-21	Rhizosphere	Dadesuguru
22	PPFM-22	Rhizosphere	Dadesuguru
23	PPFM-23	Phyllosphere	Dadesuguru
24	PPFM-24	Phyllosphere	Dadesuguru
25	PPFM-25	Rhizosphere	Siriguppa
26	PPFM-26	Rhizosphere	Siriguppa
27	PPFM-27	Phyllosphere	Siriguppa
28	PPFM-28	Phyllosphere	Siriguppa
Koppal District			
29	PPFM-29	Rhizosphere	Gangavati ARS Farm
30	PPFM-30	Rhizosphere	Gangavati ARS Farm
31	PPFM-31	Phyllosphere	Gangavati ARS Farm
32	PPFM-32	Phyllosphere	Gangavati ARS Farm
33	PPFM-33	Rhizosphere	Karatagi
34	PPFM-34	Rhizosphere	Karatagi
35	PPFM-35	Phyllosphere	Karatagi
36	PPFM-36	Phyllosphere	Karatagi
37	PPFM-37	Rhizosphere	Gangavati
38	PPFM-38	Rhizosphere	Gangavati
39	PPFM-39	Phyllosphere	Gangavati
40	PPFM-40	Phyllosphere	Gangavati
Yadgiri district			
41	PPFM-41	Rhizosphere	Hunasagi
42	PPFM-42	Rhizosphere	Hunasagi
43	PPFM-43	Phyllosphere	Hunasagi
44	PPFM-44	Phyllosphere	Hunasagi
45	PPFM-45	Rhizosphere	Shahapur
46	PPFM-46	Rhizosphere	Shahapur
47	PPFM-47	Phyllosphere	Shahapur
48	PPFM-48	Phyllosphere	Shahapur
49	PPFM-49	Rhizosphere	Balichakra
50	PPFM-50	Phyllosphere	Balichakra

Table.2 Morphological characteristics of pink pigmented facultative methylotroph isolates

Sl. No.	Isolate code	Cell shape	Motility	Gram reaction	Pigmentation	Accumulation of polyhydroxybutyrate (PHB)
1	PPFM-1	Rod	Positive	Negative	+ + +	Positive
2	PPFM-2	Rod	Positive	Negative	+ +	Positive
3	PPFM-3	Rod	Positive	Negative	+ + +	Positive
4	PPFM -4	Rod	Positive	Negative	+ + +	Positive
5	PPFM-5	Rod	Positive	Negative	+ + +	Positive
6	PPFM-6	Rod	Positive	Negative	+ +	Positive
7	PPFM-7	Rod	Positive	Negative	+ +	Positive
8	PPFM-8	Rod	Positive	Negative	+ + +	Positive
9	PPFM-9	Rod	Positive	Negative	+	Positive
10	PPFM-10	Rod	Positive	Negative	+ +	Positive
11	PPFM-11	Rod	Positive	Negative	+	Positive
12	PPFM-12	Rod	Positive	Negative	+ +	Positive
13	PPFM-13	Rod	Positive	Negative	+ +	Positive
14	PPFM-14	Rod	Positive	Negative	+ +	Positive
15	PPFM-15	Rod	Positive	Negative	+ + +	Positive
16	PPFM-16	Rod	Positive	Negative	+ + +	Positive
17	PPFM-17	Rod	Positive	Negative	+ + +	Positive
18	PPFM-18	Rod	Positive	Negative	+ + +	Positive
19	PPFM-19	Rod	Positive	Negative	+ + +	Positive
20	PPFM-20	Rod	Positive	Negative	+	Positive
21	PPFM-21	Rod	Positive	Negative	+ +	Positive
22	PPFM-22	Rod	Positive	Negative	+ + +	Positive
23	PPFM-23	Rod	Positive	Negative	+	Positive
24	PPFM-24	Rod	Positive	Negative	+ +	Positive
25	PPFM-25	Rod	Positive	Negative	+ +	Positive
26	PPFM-26	Rod	Positive	Negative	+	Positive
27	PPFM-27	Rod	Positive	Negative	+ +	Positive
28	PPFM-28	Rod	Positive	Negative	+ + +	Positive
29	PPFM-29	Rod	Positive	Negative	+ + +	Positive
30	PPFM-30	Rod	Positive	Negative	+ + +	Positive
31	PPFM-31	Rod	Positive	Negative	+ + +	Positive
32	PPFM-32	Rod	Positive	Negative	+ + +	Positive
33	PPFM-33	Rod	Positive	Negative	+	Positive
34	PPFM-34	Rod	Positive	Negative	+ +	Positive
35	PPFM-35	Rod	Positive	Negative	+ +	Positive
36	PPFM-36	Rod	Positive	Negative	+	Positive
37	PPFM-37	Rod	Positive	Negative	+	Positive
38	PPFM-38	Rod	Positive	Negative	+ +	Positive
39	PPFM-39	Rod	Positive	Negative	+ +	Positive
40	PPFM-40	Rod	Positive	Negative	+ +	Positive
41	PPFM-41	Rod	Positive	Negative	+ +	Positive
42	PPFM-42	Rod	Positive	Negative	+ + +	Positive
43	PPFM-43	Rod	Positive	Negative	+ + +	Positive
44	PPFM-44	Rod	Positive	Negative	+ + +	Positive
45	PPFM-45	Rod	Positive	Negative	+ +	Positive
46	PPFM-46	Rod	Positive	Negative	+ +	Positive
47	PPFM-47	Rod	Positive	Negative	+ + +	Positive
48	PPFM-48	Rod	Positive	Negative	+	Positive
49	PPFM-49	Rod	Positive	Negative	+ +	Positive
50	PPFM-50	Rod	Positive	Negative	+ +	Positive
51	Reference strain *(M. extorquens)*	Rod	Positive	Negative	+ + +	Positive

Note: + Pale pink
+ + Light / Medium pink
+ + + Dark / Bright pink

Table.3 Biochemical characteristics of pink pigmented facultative methylotroph isolates

Sl. No.	Isolates code	Oxidase Test	Urease Test	Catalase Test	Indole production Test	MR and VP Test	Citrate utilization	Casein Hydrolysis	Starch Hydrolysis	Nitrate reduction Test
1	PPFM-1	+	+	+	+	-	+	-	-	-
2	PPFM-2	+	+	+	+	-	+	-	-	-
3	PPFM-3	+	+	+	+	-	+	-	-	-
4	PPFM -4	+	+	+	+	-	+	-	-	-
5	PPFM-5	+	+	+	+	-	+	-	-	-
6	PPFM-6	+	+	+	+	-	+	-	-	-
7	PPFM-7	+	+	+	+	-	+	-	-	-
8	PPFM-8	+	+	+	+	-	+	-	-	-
9	PPFM-9	+	+	+	+	-	+	-	-	-
10	PPFM-10	+	+	+	+	-	+	-	-	-
11	PPFM-11	+	+	+	+	-	+	-	-	-
12	PPFM-12	+	+	+	+	-	+	-	-	-
13	PPFM-13	+	+	+	+	-	+	-	-	-
14	PPFM-14	+	+	+	+	-	+	-	-	-
15	PPFM-15	+	+	+	+	-	+	-	-	-
16	PPFM-16	+	+	+	+	-	+	-	-	-
17	PPFM-17	+	+	+	+	-	+	-	-	-
18	PPFM-18	+	+	+	+	-	+	-	-	-
19	PPFM-19	+	+	+	+	-	+	-	-	-
20	PPFM-20	+	+	+	+	-	+	-	-	-
21	PPFM-21	+	+	+	+	-	+	-	-	-
22	PPFM-22	+	+	+	+	-	+	-	-	-
23	PPFM-23	+	+	+	+	-	+	-	-	-
24	PPFM-24	+	+	+	+	-	+	-	-	-
25	PPFM-25	+	+	+	+	-	+	-	-	-

26	PPFM-26	+	+	+	+	-	+	-	-	-
27	PPFM-27	+	+	+	+	-	+	-	-	-
28	PPFM-28	+	+	+	+	-	+	-	-	-
29	PPFM-29	+	+	+	+	-	+	-	-	-
30	PPFM-30	+	+	+	+	-	+	-	-	-
31	PPFM-31	+	+	+	+	-	+	-	-	-
32	PPFM-32	+	+	+	+	-	+	-	-	-
33	PPFM-33	+	+	+	+	-	+	-	-	-
34	PPFM-34	+	+	+	+	-	+	-	-	-
35	PPFM-35	+	+	+	+	-	+	-	-	-
36	PPFM-36	+	+	+	+	-	+	-	-	-
37	PPFM-37	+	+	+	+	-	+	-	-	-
38	PPFM-38	+	+	+	+	-	+	-	-	-
39	PPFM-39	+	+	+	+	-	+	-	-	-
40	PPFM-40	+	+	+	+	-	+	-	-	-
41	PPFM-41	+	+	+	+	-	+	-	-	-
42	PPFM-42	+	+	+	+	-	+	-	-	-
43	PPFM-43	+	+	+	+	-	+	-	-	-
44	PPFM-44	+	+	+	+	-	+	-	-	-
45	PPFM-45	+	+	+	+	-	+	-	-	-
46	PPFM-46	+	+	+	+	-	+	-	-	-
47	PPFM-47	+	+	+	+	-	+	-	-	-
48	PPFM-48	+	+	+	+	-	+	-	-	-
49	PPFM-49	+	+	+	+	-	+	-	-	-
50	PPFM-50	+	+	+	+	-	+	-	-	-
51	Reference strain *(M. extorquens)*	+	+	+	+	-	+	-	-	-

+ Growth, – No growth

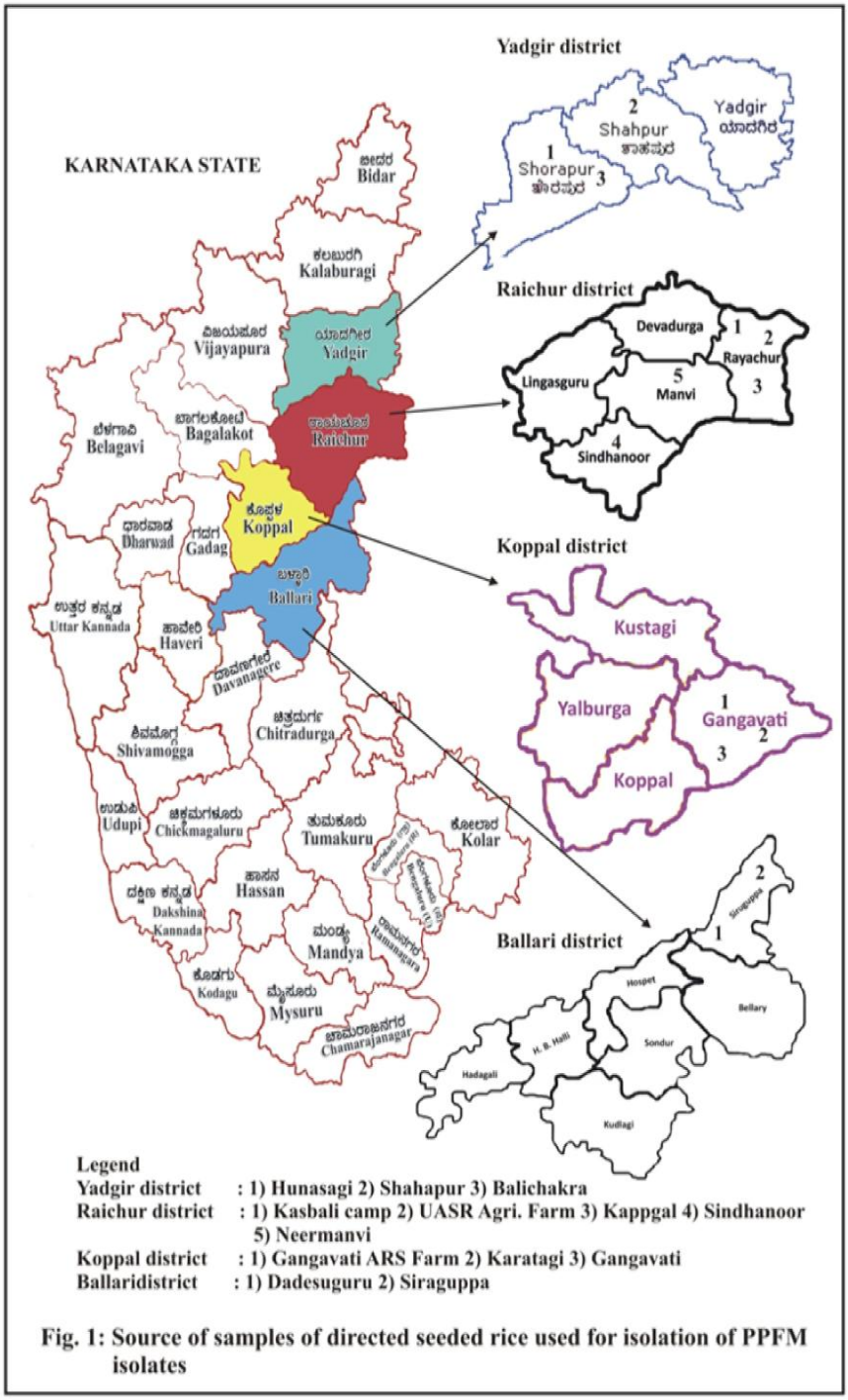


Fig. 1: Source of samples of directed seeded rice used for isolation of PPFM isolates

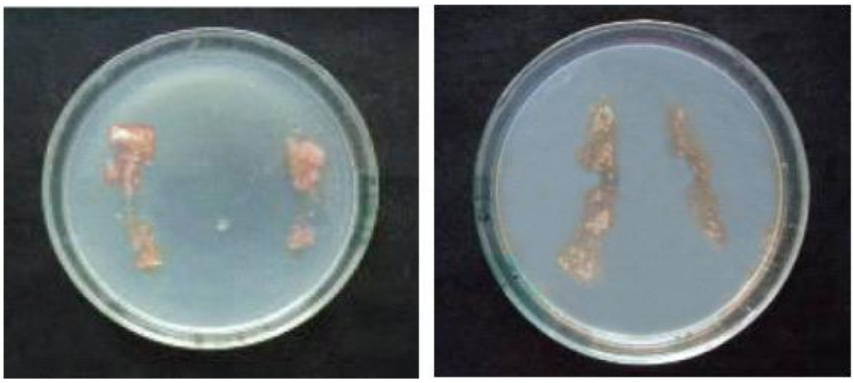


Plate 1: Isolation of PPFM by leaf impringment method

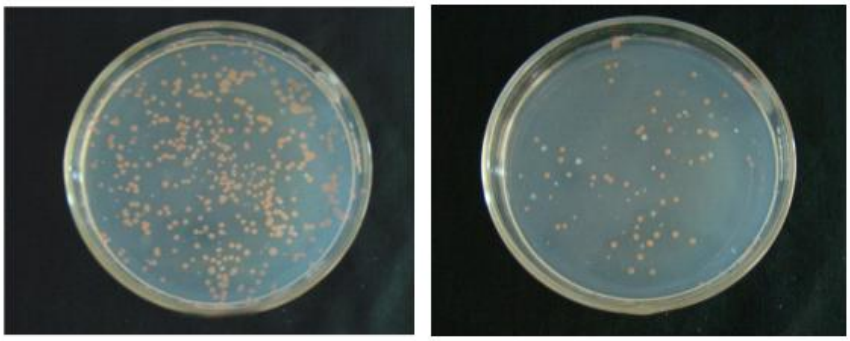
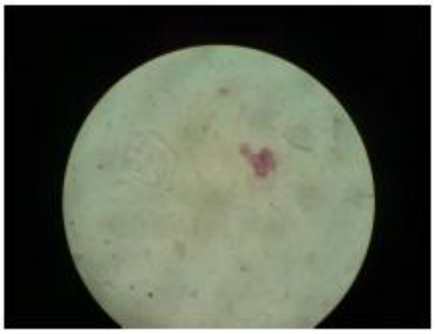


Plate 2: Isolation of PPFM by serial dilution plating technique



Gram staining

Biochemical characteristics of Pink pigmented facultative methylotrophs

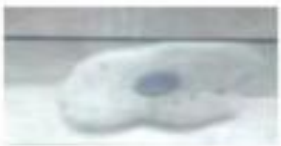
Indole Test

Methyl Red Test

Voges Proskauer Test

Citrate Test

Urease Test



Catalase Test

Oxidase Test

PPFM isolates were further characterized by a series of biochemical tests. All the isolates including reference strain were confirmed to be negative for Methyl red and Voges-Proskauer test while, positive for oxidase, urease, catalase activity, indole production and citrate utilization. None of the isolates could reduce nitrate to nitrite, whereas; the hydrolysis of casein and starch was not recorded in any of the isolates tested. Similar

observations were reported by Thangamani (2005) and Radha *et al.,* (2007).

Summary and Conclusion

The study demonstrated the occurrence of PPFM in the leaves AND rhizosphere region of the DSR. Based on the phenotypic characteristics of the isolates, they can be assigned to the genus *Methylobacterium* based on the minimum criteria set by Green (2001). Variations on the morphological and biochemical properties of the different isolates underscore the diversity of PPFM bacterial strains residing the leaves of the plant. it is interesting to note that this plant harbor PPFM bacteria. Fifty PPFMs were isolated from rhizosphere soil and phyllosphere of DSR. Out of fifty isolates, 25 were isolated from rhizosphere soil and remaining 25 isolates were isolated from phyllosphere, on AMS medium, which is a selective medium for PPFM's.

The isolates were characterized based on the morphological and biochemical characteristics. The results have clearly indicated the isolates to be positive for oxidase, urease, catalase and indole production. None of the isolates were found to be positive for casein hydrolysis, cellulose hydrolysis, nitrate reduction test, MR and VP test and starch hydrolysis. All the isolates including reference strain were Gram negative, rod shaped, exhibited motility, accumulated PHB granules with pink colonies of different color intensities.

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How to cite this article:

Raghavendra, J., G.P. Santhosh, Mahadevaswamy, S. Shubha and Ravi, M.V. 2019. Isolation and Identification of PPFM Bacterial Isolates Isolated From Direct Seeded Rice Growing Areas of Hyderabad-Karnataka, India. *Int.J.Curr.Microbiol.App.Sci.* 8(07): 152-163.
doi: https://doi.org/10.20546/ijcmas.2019.807.019