Available online at www.ijpab.com

DOI: http://dx.doi.org/10.18782/2582-2845.8647

ISSN: 2582 – 2845

Ind. J. Pure App. Biosci. (2019) *7*(4), 535-542

Indian Journal of Pure & Applied Biosciences

Peer-Reviewed, Refereed, Open Access Journal

Research Article

Isolation and Identification of PPFMs Isolates and their Potentiality as Biofertilizers

Y.R. Govekar*, A.M. Navale, S.B. Raut

Department of Plant Pathology, Dr. Balasaheb Sawant Konkan Krishi Vidyapeeth, Dapoli-415712

*Corresponding Author E-mail: govekaryr@gmail.com

Received: 20.06.2019 | Revised: 26.07.2019 | Accepted: 4.08.2019

ABSTRACT

The present investigation was carried out at Department of Plant Pathology and Agricultural Microbiology, PGI MPKV, Rahuri. The 24 pink pigmented facultative methylobacteria (PPFMs) were isolated from surfaces of leaf samples, rhizosphere soils of different crops such as Tomato, Chilli, Brinjal, Soybean, Gram, Sorghum, Cowpea, Sunflower, Maize and Sugarcane from different locations of Ahamadnagar District. Out of twenty four isolates, seven isolates along with reference strain were found positive to produce IAA. The efficient PPFMs are subjected for morphological and biochemical characterization .The isolates were further screened for their beneficial growth characters. MT_{10}, MT_{23} and MT_4 were found superior in producing IAA, GA, nitrogen fixation and sidrophore production as compared to the other isolate which clearly shows that the selected strain having a good potentiality as a biofertilizer.

Key word: *PPFMs bacteria, Indole acetic acid, Gibberlic acid, Nitrogen fixation and sidrophore production.*

INTRODUCTION

The Pink-pigmented facultative methylotrophs (PPFMs) are ubiquitous inhabitants of *phyllosphere* and *rhizosphere* of plants. These are physiologically an interesting group of bacteria able to grow on methanol, methylamine as well as on a variety of C_2, C_3 and C_4 compounds as sole sources of carbon and energy (Lidstrom, 1992), potentially dominating the *phyllosphere* population. They are commonly found in soil as well as on the surfaces of leaves, seeds and in the *rhizosphere* of a wide variety of plants (Shepelyakovskaya et al., 1999).

The association of PPFMs with plant possesses an associative symbiotic relationship in which PPFMs utilizes the methanol emitted from leaves of plants as sole carbon and energy source.

In response, PPFMs produces plant growth promoting substance such as Indole acetic acid (IAA), Gibberlic acid (GA) etc which are known to stimulate plant growth (Koenig et al., 2002); fix the atmospheric nitrogen (Sy et al., 2001); solubilise mineral phosphate (Jones et al., 2007); and chelation of inorganic compound such as iron.

Cite this article: Govekar, Y.R., Navale, A.M., & Raut, S. B. (2019). Isolation and Identification of PPFMs Isolates and their Potentiality as Biofertilizers, *Ind. J. Pure App. Biosci. 7*(4), 535-542. doi: http://dx.doi.org/10.18782/2582-2845.8647

Due to these importance, the PPFMs have received a great deal of attention as bio inoculants for use in agriculture. In the present study, the potential of PPFMs to produce hormones like Indole acetic acid, Gibberllic acid, nitrogen fixation and sidrophore production were evaluated.

MATERIALS AND METHODS

1. Media used:-It is worthy to note that every media is used for isolation and study the characteristic of PPFMs bacteria: a. Isolation of Pink-pigmented facultative methylotrophes by ammonium mineral salt medium (AMS), (Whittenbury et al., 1970). **b.** Estimation of casein hydrolysis test by skim milk agar medium (Smibert & Kreig, 1981). c. Estimation of citrate utilization test by simmon's citrate agar medium (Seeley & Vandemark, 1981). d. Estimation of cellulose degradation test by czapadox mineral salt agar medium (Seeley & Vandemark, 1981). e. Estimation of indole production test by tryptone glucose broth medium (Seeley & Vandemark, 1981). f. Estimation of fixed nitrogen by N-free malate medium.

2- Isolation and purification of methylotrophs (PPFMs):

a. Leaf impregnated technique:-On the solidified AMS agar medium, upper and lower surface of leaf samples were placed separately, in such a way to make impression of it. Then the leaves were lifted away and plates were incubated at 30^0 C for 5 to 7 days (Corpe, 1985).

B. Serial dilution technique: One gram of sample (soil) was grinded using pestle and mortar, serially diluted up to 10^{-6} dilutions and one ml of the aliquot from 10^{-4}, 10^{-5} and 10^{-6} dilutions was transferred to the sterile petri dishes. After plating with AMS medium, the plates were incubated for 3-5 days at 30°C. colonies of methylotrophs were picked, purified by the streak plate method and the well isolated colonies were preserved in AMS agar slants. Isolates were maintained on AMS slants at 4°C in a refrigerator for further use. All isolates were examined for their cell shape, motility and gram reaction. Also, all isolates were examined for oxidase, urease, catalase tests; casein hydrolysis; starch hydrolysis, cellulose degradation, citrate utilization and indole production.

3. Screening the efficiency of PPFMs isolates based on the production of some beneficial growth properties:

1. Indole acetic acid production: The IAA production by PPFM isolates under in vitro condition was determined following the method of Ivanova et al. (2001). One hundred ml quantities of AMS liquid medium were dispensed in 250 ml Erlenmeyer flasks and sterilized at 1.5 atmosphere for 15 min. Freshly prepared, filter sterilized solution of L-tryptophan was added to a final concentration of 100 mg 1^{-1}, one ml of the standard inoculum (10^{-9} cells ml-1) of PPFM isolate was added to each flask and incubated at 30°C. in a shaker. In order to avoid photo inactivation of the biologically active compounds, the flasks were wrapped with black paper during incubation. After 7 days of incubation period, 25 ml of the sample was withdrawn and the cells were centrifuged at 5000 rpm for 15 min for quantitative estimation of IAA. Quantitative estimation of IAA production in PPFM cell free extract were done by spectrophotometric method (Sy et al., 2001). A quantity of 0.5 ml of the sample was taken in a test tube and 1.5 ml of distilled water was added followed by a 4 ml of Salper's reagent (1.0 ml of 0.5 M FeCl3 and 50 ml of 35% $HClO_4$) and incubated in darkness for 1 hr at 28°C. The intensity of the pink colour developed was read in UV/Visible Spectrophotometer at 540 nm. By referring to a standard graph prepared with chemical grade indole-3-acetic acid, the quantity of IAA in the sample was determined and expressed as µg $ml^{-1}$of culture filtrate.

2. Gibberellic acid (GA) production: Extraction of gibberellins was done as described by Tien et al., 1979. Three to four days old PPFM culture of AMS liquid medium was centrifuged for 15 min at 10,000 rpm and the supernatant was taken. The cell pellet was re-extracted with phosphate buffer (pH 8.0) and again centrifuged. Both supernatants were pooled, acidified at pH 2.5 using 5 N

hydrochloric acid and partitioned (1:5) of ethyl acetate then was dried at 32°C and the residue redissolved in 2 ml of distilled water containing 0.05 percent of Tween 80. Fifteen ml of ethyl acetate fraction was taken and 2 ml of zinc acetate solution was added (21.9 g of zinc acetate was dissolved in 80 ml of distilled water then one ml of glacial acetic acid was added and the volume made up to 100 ml with distilled water) After 2 min, 2 ml of potassium ferrocyanide solution was added (1.6 g of potassium ferrocyanide solution in 100 ml of distilled water) and the mixture was centrifuged at 10,000 rpm for 10 min. Five ml of supernatant was added to 5 ml of 30 percent hydrochloric acid and the mixture was incubated at 20°C for 75 minutes. The blank was prepared with 50 percent hydrochloric acid. The absorbance was measured at 254 nm in UV/Visible Spectrophotometer (Model 6705). From the standard graph using standard gibberellic acid solution the amount of GA produced by the PPFM isolates was calculated and expressed as µg ml-1 broth (Mahadevan & Sridhar, 1982).

3. Nitrogen estimation (MicroKjeldahl method): To 250 ml conical flask 100 ml of the N-free malate medium was dispensed and autoclaved. Later one ml of 24 h old culture inoculum was added to each flask. The flasks were incubated at 37°C for seven days. After 7 days of incubation the culture was homogenized and 10 ml was digested with 5 ml of concentrated H2SO4 along with 0.2 g digestion catalyst mixture K2SO4: CuSO4: selenium (100:10:1). After cooling, volume was made up to 10 ml with distilled water. Later ten ml of aliquot were transferred to microkjeldhal distillation unit. The sample was mixed with 20 ml of 40 % NaOH Ammonia evolved was trapped in four percent boric acid mixed indicator (bromocresol green, 0.066 g and methyl red, 0.033 g in 100 ml methanol) till the solution turned from pink to green. It was titrated against 0.05 N H2SO4 and total nitrogen content of the culture was determined and results were expressed as mg N fixed per g of malate.

$$(\%)\ N = \frac{\text{Titrate value} \times 0.014 \times N \text{ of } H_2SO_4 \times \text{Vol made}}{\text{Volume of sample used}} \times 100$$

4. Siderophores production: The PPFM isolates were grown in AMS liquid medium for 7 days in shaking inocubator at 250 rpm at 28°C. The cultures were centrifuged at 12000 rpm for 30 min and 20 ml of the supernatant filtrate was extracted twice with equal amount of ethyl acetate after adjusting the pH to 2.0 with 0.1 N HCl then evaporated to dryness and dissolved in 5 ml distilled water. Five ml of Hathway reagent (added 1 ml of 0.1 M ferric chloride and 1 ml of 0.1 N HCl to 100 ml of distilled water followed by 1 ml of 0.1 M potassium ferricyanide) were added to the assay solution and allowed to stand for the colour to develop. To estimate of siderophores, the absorbance was read in UV/Visible Spectrophotometer at 700 nm with 2, 3-dihydroxy benzoic acid as standard according to Reeves et al. 1983.

RESULT AND DISCUSSION

1. Isolation of Pink Pigmented Facultative Methylotrophs (PPFMs).

The pink pigmented facultative methylobacteria (PPFMs) were isolated from surfaces of leaf samples, *rhizosphere* soils of different crops such as Tomato, Chilli, Brinjal, Soybean, Gram, Sorghum, Cowpea, Sunflower, Maize and Sugarcane from different locations of Ahamadnagar District.

As many as 24 isolates were obtained, purified and given code number as PPFM series.They were tentatively identified as PPFMs based on the characteristic pink pigmented colonies on ammonium mineral salts (AMS) agar media with methanol as sole source of carbon and energy .

2. Assessment of Pink Pigmented Facultative Methylotroph as a plant growth Promoters, Nitrogen fixation and Sidrophore production.

A) Indole acetic acid production

Out of 24 Pink Pigmented Facultative Methylotrophs (PPFMs), 7 isolates along with reference strain were found positive to produce IAA. PPFMs producing IAA were identified by the formation of a characteristic red halo within the filter paper immediately surrounding the colony on the AMS agar plates supplemented with L- tryptophan. Those isolates which gave positive result in rapid screening were considered as efficient PPFM isolates for growth promotion.

Table 1: Assessment of PPFMs as a plant growth promoter

Isolate	Indole acetic acid Production. (IAA)
MT_1	-
MT_3	+
MT_4	+
MT_5	-
MT_7	-
MT_8	+
MT_9	-
MT_{10}	+
MT_{12}	-
MT_{14}	+
MT_{15}	+
MT_{16}	-
MT_{18}	-
MT_{19}	-
MT_{21}	-
MT_{22}	-
MT_{23}	+
MT_{25}	-
MT_{26}	-
MT_{29}	-
MT_{30}	-
MT_{32}	-
MT_{34}	-
MT_{35}	-
Reference strain	+

All the 24 isolates were tested for the production of IAA in which only 07 PPFMs isolates were found positive to produce IAA. However there was clear variation in IAA production among the isolates was noticed. The 3 isolates namely $MT_{10,}$ MT_{23} and MT_4 were superior in producing IAA (22.43µg/ml, 27.65 µg/ml and 25.43µg/ml respectively (Table 2).

The production of IAA by *Methylobcterium* is due to the presence of serine pathway of C1 metabolism. The C1 metabolism is responsible for the synthesis of IAA. This has been supported by Costacurta and Vanderleden (1995) mentioned more details about the biosynthesis of indole acetic acid. They concluded that several IAA biothynesis with the serine pathway of C1 metabolism (*Methylobacteriummesophilicum* and *Aminobacter aminovorans*), the ribulose monophosphate pathway (*Methylobacterium mays*) and the ribulose biphosphate pathway (*Paracoccus kondratievae*) synthesize IAA. The serine pathway bacteria, indole-3 acetamide was also detected IAA is synthesized from tryptophan through tryptamine in three steps of which only the first step (the decarboxylation of tryptophan) is specific to this biosynthetic pathway. Soumya and sundaram 2018 reported PPFMs were able to synthesize IAA in presence or absence of tryptophan ranged from 0.14 to 4.69 µg/ml from PPFM strain isolated from rhizosphere and *phyllosphere* of cowpea crop.

The results are in line with the Pesal savitha (2013) who reported that out of 200

PPFMs isolates 30 isolates showed positive results for qualitative assay for the production of IAA ranging from 0.17 µg ml^{-1} to 19.77 µg ml^{-1}. Hence these efficient strain were selected for further characterization.

A) Gibberlic acid acid production

In the present study, all the 7 selected PPFMs isolates produced GA in variable amount ranging from 51.63 to 70.54 µg /ml, out of which 3 isolates namely $MT_{4,}$ MT_{10} and MT_{23} were superior in producing gibberlic acid 70.54µg/ml 67.25 µg/ml and 64.58 µg/ml respectively (Table 2). These are in consistent with the results of Pesal Savitha (2013) (88.45 to 128.28 µg /ml).

Table 2: Production of Phytoharmones by PPFM isolates

Sr No	Isolates	Production of Indole acetic acid (µg/ml)	Production of Gibberlic acid (µg/ml)
1	MT_3	15.58	58.50
2	MT_4	22.43	70.54
3	MT_8	19.33	53.87
4	MT_{10}	27.65	67.25
5	MT_{14}	21.83	51.63
6	MT_{15}	11.36	42.58
7	MT_{23}	25.43	64.58
8	Ref.strain	13.66	52.56
	S.Em _+	0.13	0.21
	CD at 1%	0.54	0.89

A) Sidrophore Production

Iron plays a crucial role in plant-microbe interactions and known as one of the factors limiting the bacterial growth in plants. It is biologically made available by iron-chelating compounds that are synthesized and secreted by many bacteria and fungi under the conditions of iron limitation in order to scavenge iron from the environment. Bacterial sidrophores are low-molecular weight compounds with high Fe^{3+} chelating affinity. It is responsible for the solubilization and transport of this element into bacterial cells. The production of siderophores by microorganisms is beneficial to plants, because it can inhibit the growth of plant pathogens (Sharma & Johri, 2003).

In the present study, these 7 PPFM isolates tested produced siderophore ranging from 0.32 µmoles of a-2,3, dihydroxybenzoic acid (DHBA)to 0.62 µmoles of a-2,3,dihydroxybenzoic acid(DHBA).The 3 isolates namely $MT_{4,}$ MT_{10} and MT_{23} shows higher sidrophore production 0.57 µmoles, 0.62 µmoles and 0.52 µmoles respectively (Table 3).

The present observations correlates the findings of Radha (2007), Pesal savitha (2013) and Kassem (2013) reported sidrophore production ranging from 0.24 to 0.60, 0.24 to 0.61,0.24 to0.58 µmoles of a-2,3, dihydroxybenzoic acid (DHBA) respectively by various PPFMs isolates.

B) Nitrogen fixation

In the present study, all the 7 selected PPFMs isolates are involved in nitrogen fixation ranging from 0.38 to 1.28 mg /g of malate, out of which 3 isolates namely MT_4, MT_{10} and $MT_{23,}$ were higher in amount of nitrogen fixed 1.17 mg/g of malate 1.03 mg/g of malate and 1.28 mg/g of malate (Table 3) respectively.

The nitrogen fixation is due to the presence of nitrogenase enzyme in PPFMs without any formation of nodule (Raja et al., 2006). He for the first time reported the presence of a functional nifH gene in a non-nodulating nitrogen-fixing, *phyllosphere*-colonizing, *Methylobacterium sp* which does not belongs to the *Methylobacterium nodulans* (Strain that nodulates plants of *Crotolaria sp*) and hence is very significant.

The results are in line with Radha (2007) reported 40 PPFMs isolates isolated from *rhizosphere* and *phyllosphere* involved in nitrogen fixation ranging from0.11 to 1.32 mg /g of malate. Similar finding also shown by Kassem (2013) who reported 12 PPFM

isolates involved in nitrogen fixation ranging from 0.38 to 1.21mg /g of malate.

Table 3: Estimation of nitrogen fixation and sidrophore by PPFM isolates

Sr No	Isolates	Nitrogen Fixed (mg/g of malate)	Sidrophore production (µmoles)
1	MT_3	0.38	0.47
2	MT_4	1.17	0.57
3	MT_8	0.56	0.32
4	MT_{10}	1.03	0.62
5	MT_{14}	0.51	0.44
6	MT_{15}	0.69	0.54
7	MT_{23}	1.28	0.52
8	Ref.strain	0.61	0.41
	S.Em _+	0.02	0.01
	CD at 1%	0.07	0.03

1. Morphological and cultural characterization of Pink Pigmented Facultative Methylotrophs (PPFMs)

The selected 7 PPFMs isolates were subjected for morphological characterization and the results are presented in (Table 4). The results revealed that all these isolates were rod shaped, stained Gram negative and exhibited motility with accumulation of PHB in their cell. The pink colonies with different color intensities were observed on AMS medium. Out of seven efficient isolates, five isolates showed medium pink colored colonies, and remaining two isolates were found to be of dark pink color.

Table 4: Morphological and cultural characteristics of Pink pigmented facultative Methylotrophs (PPFMs)

Sr No	Isolates	Cell □□m)	Cell Shape	Motility	Gram reaction	Colony colour	Accumulation of PHB.
1	MT_3	0.5x1.2	Rod	Positive	Negative	Medium	Positive
2	MT_4	0.7x1.3	Rod	Positive	Negative	Medium	Positive
3	MT_8	0.6x1.3	Rod	Positive	Negative	Dark	Positive
4	MT_{10}	0.9x1.3	Rod	Positive	Negative	Medium	Positive
5	MT_{14}	0.6x1.3	Rod	Positive	Negative	Medium	Positive
6	MT_{15}	0.9x1.1	Rod	Positive	Negative	Dark	Positive
7	MT_{23}	0.7x1.3	Rod	Positive	Negative	Medium	Positive
8	Ref.strain	0.7x1.2	Rod	Positive	Negative	Medium	Positive

4. Biochemical characterization of Pink Pigmented Facultative Methylotrophs

The isolates were tested for a selective biochemical tests, and the results are presented in (Table 5). All the 7 isolates showed positive results for oxidase, urease, catalase activity, citrate utilization and indole production and. None of the isolates showed positive results for starch hydrolysis, casein hydrolysis, MR and VP test, cellulose degradation and nitrate reduction test.

Table 5: Biochemical characteristics of Pink pigmented facultative Methylotrophs

Sr No	Characteristics	MT_3	MT_4	MT_8	MT_{10}	MT_{14}	MT_{15}	MT_{23}	Ref. strain
1	Oxidase	+	+	+	+	+	+	+	+
2	Catalase	+	+	+	+	+	+	+	+
3	Urease	+	+	+	+	+	+	+	+
4	Nitrate Reduction test	-	-	-	-	-	-	-	-
5	MR and VP test	-	-	-	-	-	-	-	-
6	Casein hydrolysis test	-	-	-	-	-	-	-	-
7	Starch hydrolysis test	-	-	-	-	-	-	-	-
8	Cellulose degradation test	-	-	-	-	-	-	-	-
9	Citrate utilization test	+	+	+	+	+	+	+	+
10	Indole production	+	+	+	+	+	+	+	+
11	Probable genus	*Methylobacterium spp*	*Methylobacterium spp.*	*Methylobacterium spp*	*Methylobacteriumspp.*	*Methylobacterium spp*	*Methylobacterium spp.*	*Methylobacterium spp*	*Methylobacterium spp.*

5. Carbon utilization test

The PPFMs having capability to grow on different C_1 compounds as sole source of carbon and energy, can also grow on wide range of multi carbon growth substrates making them facultatively methylotrophic. All the isolates were tested for the utilization of the five different carbon compounds viz. Glycerol, fructose, arabinose, glucose and methanol. The utilization pattern of different carbon substrates by PPFM isolates are presented in (Table 6). Most of the PPFMs isolates showed positivity in carbon utilization pattern. This has been supported by Lidstrom (1992) who differentiated *Methylobacterium* species, concluded that they are capable of growing on C_1 compounds as sole source of carbon and energy and can also grow on wide range of multicarbon substrates making them facultatively methylotrophic. Carbon utilization by PPFMs also supported by the onother worker Anthony (1982) who demonstrated that facultative methylotrophs could utilize different carbon sources and assimilate C1 compound via serine pathway. However, when they are grown on complex organic substrates they have complete Tri carboxlic acid cycle, TCA.

The present observations correlates with the finding of Radha (2007) and Pesal Savitha (2013). Radha reported the PPFMs isolates utilize Glycerol while Pesal Savitha and Kassem et al. (2013) reported the utilization of Fructose, Arabinose, Glucose and methanol related compound by PPFMs isolates.

Table 6: Utilization of different carbon substrates by PPFMs isolates

Sr No	Isolates	Glycerol	Fructose	Arabinose	Glucose	Methanol
1	MT_3	-	+	+	+	+
2	MT_4	+	+	+	+	+
3	MT_8	+	-	-	+	+
4	MT_{10}	+	+	+	+	+
5	MT_{14}	+	+	+	+	+
6	MT_{15}	+	+	+	+	+
7	MT_{23}	+	+	+	+	+
8	Ref strain	+	+	+	+	+

CONCLUSION

From above data it is confirmed that the three isolates namely MT_4 MT_{10} and MT_{23} are the pink pigmented facultative methylotrophs with a probable genus *Methylobacterium* based on their morphological and biochemical characteristics. It was also confirmed that these isolates having good ability of producing Indole acetic acid production, Gibberlic acid production, nitrogen fixation and sidrophore production which indicate that they are having a high potential of producing bio fertilizer.

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