Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2019; 8(4): 2245-2247
Received: 01-05-2019
Accepted: 03-06-2019

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Utilization of different carbon substrates by native pink pigmented facultative methylotrophs isolated from direct seeded rice

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Abstract
Investigations were carried to study the pink pigmented facultative methylotrophs and their beneficial effects on direct seeded rice. Samples of phyllosphere leaves and rhizosphere soil were collected from major DSR growing districts of Hyderabad-Karnataka; about fifty isolates were isolated on selective AMS medium. Further the isolates were characterized based on their morphological and biochemical characteristics, based on the observed characteristics, the isolates obtained belong to the genus *Methylobacterium*. There after, all the isolates carried out for carbon source utilization for the purpose of tentatively identification of PPFM isolates. The twenty isolates selected on the bases of pigmentation were tested for the utilization of the different carbon compounds for the purpose of tentatively identification of PPFM isolates. The obtained results based on carbon source utilization all the isolates could be classified as follows: PPFM-8, PPFM-31, PPFM-32 *Methylobacterium phyllosphaerae*, PPFM-1, PPFM-5, PPFM-18 and PPFM-42 *M. oryzae* PPFM-3, PPFM-15, PPFM-16, PPFM-19 and PPFM-44 *M. mesophilicum*, PPFM-28, PPFM-29 and reference strain *M. extorquens*, PPFM-4, PPFM-17, PPFM-22, PPFM-30, PPFM-43 and PPFM-47 *Methylobacterium* sp.

Keywords: *Methylobacterium sp*, PPFM, phyllosphere, rhizosphere

Introduction
Members of the genus *Methylobacterium* are pink-pigmented facultative methylotrophs (PPFMs). The pink pigments are carotenoids mainly xanthophylls (Konovalova *et al*., 2007) [5]. They utilize one-carbon compounds, including methanol (CH_3OH), methylamine (CH_3NH_2) and formaldehyde (HCHO) and multi-carbon compounds containing no carbon-carbon bonds, as well as organic substrates with carbon-carbon bonds as sole source of carbon and energy (Green, 2006; Patt *et al*., 1976; Patt *et al*., 1974) [10, 11]. 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.
Carbon source utilization for the purpose of tentatively identification of PPFM isolates. Based on the pattern of compounds they utilize as carbon and energy source, Lidstrom (1992) 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.
The compounds that were used by more than 95 per cent of *Methylobacterium* strains include, methylamine, trimethylamine, acetate, citrate, L-glutamate, D-glucose, D-xylose, fructose and betaine and carbon source utilization pattern by 12 known species of the genus *Methylobacterium* revealed that none of the strains appear to use any of the disaccharides or sugar alcohols examined but most of *Methylobacterium* strains used glycerol, malonate, succinate, fumarate, α-ketoglutarate, DL-lactate, DL-malate, acetate, pyruvate, propylene glycol, ethanol, methanol and formate as carbon and energy sources (Green and Bousifield, 1982) [3].

Methodology
Carbon source utilization test
The pink pigmented facultative methylotrophic isolates isolated from direct seeded rice along with reference strain *M. extorquens* were tested for the utilization of various carbon sources as mentioned below for the purpose of tentative identification. All carbon utilization tests were carried out using AMS broth. The carbon compounds *viz.*, glucose, fructose, chloroform,

acetate, tartarate, ethanol, methylamine, methane, lactose, sucrose, maltose, galactose, methanol, glycerol and dichloromethane were substituted at 0.5 percent level for methanol in AMS broth medium. The growth was observed after 14 days of incubation at 30ºC and growth was compared to a negative control containing no added carbon source. This long incubation time was needed because of the slow growing nature of cultures on certain carbon compounds. Based on the utilization of carbon compounds, 20 PPFM isolates were classified as given below (Green and Bousifield, 1982; Green and Bousifield, 1988; Madhaiyan, 2003; Madhaiyan *et al.*, 2007; Madhaiyan *et al.*, 2009) [3, 4, 7, 8, 9].

Based on the utilization of carbon compounds, 20 PPFM isolates were classified as given below

Sl. No.	Carbon source utilization	*Methylobacterium* sp.
1.	Glucose, Fructose, Chloroform, Acetate, Ethanol, Methylamine, Lactose, Sucrose, Galactose, Methanol, Glycerol	*Methylobacterium phyllosphaerae*
2.	Chloroform, Ethanol, Sucrose, Methanol, Glycerol	*Methylobacterium oryzae*
3.	Glucose, Chloroform, Ethanol, Methanol and Dichloromethane	*Methylobacterium mesophilicum*
4.	Acetate, Chloroform, Tartarate, Ethanol, Methylamine, Sucrose, Galactose, Methanol and Dichloromethane	*Methylobacterium extorquens*
5.	Showed wide variations in carbon utilization	*Methylobacterium* sp.

Results and Discussion

Isolation and identification of pink pigmented facultative methylotrophs (PPFMs) 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. As many as 50 isolates were obtained, purified and designated by code numbers serially from PPFM-1 to PPFM-50. Out of fifty isolates, 25 were isolated from rhizosphere soil while remaining 25 isolates were isolated from phyllosphere of direct seeded rice. They were tentatively identified as PPFM's based on the characteristic pink pigmented colonies on ammonium mineral salts (AMS) agar media with methanol as sole source of carbon and energy. 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) [6]. Several authors have already reported the natural association of PPFM's with several plants. Basile *et al.* (1969) [2] 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.

Carbon utilization test

The genus *Methylobacterium* is composed of a variety of facultative methylotrophic bacteria which are capable of growing on C_1 compounds such as formate, formaldehyde, and methanol as sole source of carbon and energy. They also grow on a wide range of multi carbon growth substrates. Out of fifty PPFM isolates, twenty isolates were selected for carbon utilization test on the basis of dark pink pigmentation. These isolates were tested for the utilization of the carbon compounds *viz*., glucose, fructose, chloroform, acetate, tartarate, ethanol, methylamine, methane, lactose, sucrose, maltose, galactose, methanol, glycerol and dichloromethane for the purpose of tentatively identification of PPFM isolates.

Based on the carbon utilization, the isolates were classified into five groups. PPFM-8, PPFM-31, PPFM-32 were classified under Group I utilizing glucose, fructose, chloroform, acetate, ethanol, methylamine, lactose, sucrose, galactose, methanol, glycerol and were tentatively identified as *Methylobacterium phyllosphaerae.* Four isolates, PPFM-1, PPFM-5, PPFM-18, PPFM-42 came under Group II utilizing chloroform, ethanol, sucrose, methanol, glycerol and were tentatively identified as *Methylobacterium oryzae.* Five isolates *viz*., PPFM-3, PPFM-15, PPFM-16, PPFM-19 and PPFM-44 came under Group III utilizing glucose, chloroform, ethanol, methanol and dichloromethane which were tentatively identified as *Methylobacterium mesophilicum.* Strains PPFM-28, PPFM-29 and reference strain came under Group IV utilizing acetate, chloroform, tartarate, ethanol, methylamine, sucrose, galactose, methanol, dichloromethane and were tentatively identified as *Methylobacterium extorquens.* While, strains PPFM-4, PPFM-17, PPFM-22, PPFM-30, PPFM-43 and PPFM-47 showed wide variations in carbon utilization and were grouped under Group V and designated as *Methylobacterium* sp.

The results were clearly compared to those cited in Bergey's manual of systematic bacteriology (Green and Bousifield, 1982; Madhaiyan *et al*., 2007 and Raja *et al*., 2008) [3, 8, 12]. The observations were also consistent with the reports of Green and Bousifield (1988) [4], Madhaiyan, (2003) [7] and Madhaiyan *et al.* (2007) [8]. Anthony (1982) [1] also demonstrated that facultative methylotrophs could utilize different carbon compounds and assimilate C_1 compound via serine pathway. For the purpose of classification of *Methylobacterium* genera at species level or tentatively identification of *Methylobacterium*, an attempt was made to find out variability of selected isolates to utilize various carbon sources including C_1 and C_2. Based on their carbon utilization pattern, classification of methylotrophic bacteria at the species level was carried out as established by Green and Bousifield (1982) [3].

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Isolate code	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	Tentative identification
PPFM-1	-	-	+	-	-	+	-	-	-	+	-	-	+	+	-	*M. oryzae*
PPFM-3	+	-	+	-	-	+	-	-	-	-	-	-	+	+	+	*M. mesophilicum*
PPFM-4	+	-	+	+	+	-	-	-	-	-	-	+	+	+	+	*Methylobacterium* sp.
PPFM-5	-	-	+	-	-	+	-	-	-	+	-	-	+	+	-	*M. oryzae*
PPFM-8	+	+	+	+	-	+	+	-	+	+	-	+	+	+	-	*M. phyllosphaerae*
PPFM-15	+	-	+	-	-	+	-	-	-	-	-	-	+	+	+	*M. mesophilicum*
PPFM-16	+	-	+	-	-	+	-	-	-	-	-	-	+	+	+	*M. mesophilicum*
PPFM-17	+	-	+	-	-	+	-	-	-	-	-	-	+	+	+	*Methylobacterium* sp.

PPFM-18	-	-	+	-	-	+	-	-	-	+	-	-	+	+	-	*M. oryzae*
PPFM-19	+	-	+	-	-	+	-	-	-	-	-	-	+	+	+	*M. mesophilicum*
PPFM-22	-	-	+	-	-	-	-	-	-	-	-	+	+	+	+	*Methylobacterium* sp.
PPFM-28	-	-	+	+	+	+	+	-	-	-	-	+	+	+	+	*M. extorquens*
PPFM-29	-	-	+	+	+	+	+	-	-	+	-	+	+	-	+	*M. extorquens*
PPFM-30	-	+	+	+	-	+	+	-	-	+	-	+	+	+	+	*Methylobacterium* sp.
PPFM-31	+	+	+	+	-	+	+	-	+	+	-	+	+	+	-	*M. phyllosphaerae*
PPFM-32	+	+	+	+	-	+	+	-	+	+	-	+	+	+	-	*M. phyllosphaerae*
PPFM-42	-	-	+	-	-	+	-	-	-	+	-	-	+	+	-	*M. oryzae*
PPFM-43	-	+	+	+	-	+	+	-	-	+	-	+	+	+	+	*Methylobacterium* sp.
PPFM-44	+	-	+	-	-	+	-	-	-	-	-	-	+	+	+	*M. mesophilicum*
PPFM-47	-	-	+	-	-	-	-	-	-	+	-	+	+	+	+	*Methylobacterium* sp.
Reference strain	-	-	+	+	+	+	-	-	+	-	+	+	+	+	+	*M. extorquens*

+ Growth, – No growth

1) Glucose 2) Fructose 3) Chloroform 4) Acetate 5) Tartarate 6) Ethanol 7) Methylamine 8) Methane 9) Lactose 10) Sucrose 11) Maltose 12) Galactose 13) Methanol 14) Glycerol 15) Dichloromethane

Conclusion

The study demonstrated the occurrence of PPFM in the leaves of the Direct seeded rice. 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). Utilization of various carbon sources for the purpose of tentative identification may be employed to further identify the isolates up to the species level. Variations on the morphological and biochemical properties of the different isolates underscore the diversity of PPFM bacterial strains residing the leaves of the plant. The ability of these bacteria to thrive in this plant therefore necessitates further investigation. More studies are required to explain the association of PPFM bacteria with the DSR leaves.

The twenty isolates including reference strain were tested for the utilization of the carbon compounds for the purpose of tentative identification. Twenty isolates were classified into four groups and tentatively identified as *M. extorquens*, *M. phyllospherea*, *M. mesophilicum*, *M. oryzae* and unidentified groups (*Methylobacterium* sp.).

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