

# Isolation and characterization of methane utilizing bacteria from wetland paddy ecosystem

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**Abstract** Methylophilic bacteria which are known to utilize C1 compounds including methane. Research during past few decades increased the interest in finding out novel genera of methane degrading bacteria to efficiently utilize methane to decrease global warming effect. Moreover, evaluation of certain known plant growth promoting strains for their methane degrading potential may open up a new direction for multiple utility of such cultures. In this study, efficient methylophilic cultures were isolated from wetland paddy fields of Gujarat. From the overall morphological, biochemical and molecular characterization studies, the isolates were identified and designated as *Bacillus aerius* AAU M 8; *Rhizobium* sp. AAU M 10; *B. subtilis* AAU M 14; *Paenibacillus illinoisensis* AAU M 17 and *B. megaterium* AAU M 29. Gene specific PCR analysis of the isolates, *P. illinoisensis*, *B. aerius*, *Rhizobium* sp. and *B. subtilis* showed presence of *pmoA* gene encoding  $\alpha$  subunit particulate methane monooxygenase cluster. *B. megaterium*, *P. illinoisensis*, *Rhizobium* sp. and *Methylobacterium extrorquens* showed presence of *mmoX* gene encoding  $\alpha$  subunit of the hydroxylase component of the soluble methane monooxygenase cluster. *P. illinoisensis* and *Rhizobium* sp. showed presence *mxoF* gene encoding  $\alpha$  subunit region of methanol dehydrogenase gene cluster showing that both isolates are efficient utilizers of methane. To the best of our knowledge, this is the first time report showing presence of methane degradation enzymes and genes within the known PGPB group of organisms from wet land paddy agro-ecosystem, which is considered as one of the leading methane producer.

**Keywords** Methylophilic · Methane monooxygenase · *mmoX* · *pmoA* · Methanol dehydrogenase · *mxoF*

## Introduction

The atmospheric trace gas methane (CH<sub>4</sub>) is a prominent “greenhouse” gas. Methane accounts for 15–20 % of global warming (IPCC 2007). Up to 70–80 % of atmospheric CH<sub>4</sub> is biogenic (Wahlen et al. 1989). Major sources of this input include natural wetlands, rice fields, enteric fermentation in animals, termites and landfills. The contribution from rice cultivation is estimated to range from 39 to 112 Tg CH<sub>4</sub> y<sup>-1</sup> (Khosa et al. 2011). About 80 % of the rice harvest is grown under the more productive flooded conditions (wetland paddy) and thereby major source of methane emission. The only known biological sink for atmospheric methane is its oxidation in aerobic soils by “methanotrophic (=methylophilic) bacteria”, this may contribute up to 10–20 % to the total methane destruction (Reeburgh et al. 1993). Methanotrophs are classified as type I (*γ-proteobacteria*) and type II (*α-proteobacteria*), based on several characteristics including phylogeny, guanine and cytosine content of their DNA, intracellular membrane arrangement, carbon assimilation pathways and phospholipid fatty acids (PLFAs) composition (Hanson and Hanson 1996; Whittenbury et al. 1970). At present, there are 13 recognized genera of methanotrophs viz. *Methylomonas*, *Methylosphaera*, *Methylomicrobium*, *Methylosarcina*, *Methylobacter*, *Methylocaldum*, *Methylococcus*, *Methylohalobius*, *Methylosoma* *Methylocystis*, *Methylocella*, *Methylocapsa* and *Methylosinus* (Hanson and Hanson 1996; Bowman et al. 1997; Bodrossy et al. 1997; Dedys et al. 2000, 2002; Wise et al. 2001; Heyer et al. 2005; Tsubota et al. 2005) consisting of both type I and type II methanotrophs.

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All methanotrophs are having highly conserved key enzymes viz. particulate methane monooxygenase (pMMO), soluble methane monooxygenase (sMMO) and methanol dehydrogenase (MDH), so that detection of enzymatic activity may offer the possibility of detecting all known methanotrophs (Hanson and Hanson 1996). So application of methanotrophic bacteria having PGPR activity can present a great opportunity to reduce methane emission from agriculture sector and easily adoptable technology by farmers. Present research first time uncover five methane degrading bacterial strains within the known PGPB group of organisms from wet land paddy agro-ecosystem which are not previously reported.

## Materials and methods

### Isolation and screening of methane utilizing bacteria

For isolation of methylotrophic bacteria from wet land paddy, total 15 soil samples were collected at flowering stage representing major traditionally rice growing areas of Middle and South Gujarat, India. Out of fifteen soil samples, 7 samples were collected from Main Rice Research Station, Nawagam, 4 samples from Agricultural Research Station for Irrigated Crops, Thasra, 2 each from farmer's field at Tarapur and Vyara Taluka, respectively. For bacterial isolation method suggested by Hoppe et al. (2011) was followed with some modifications as follows. Instead of Ammonium Mineral Salt (AMS) medium, Nitrate Mineral Salt (NMS) broth (Sodium nitrate—2.0 g l<sup>-1</sup>, MgSO<sub>4</sub>·7H<sub>2</sub>O—0.2 g/l, KCl—0.04 g l<sup>-1</sup>, Calcium chloride—0.015 g l<sup>-1</sup>, Na<sub>2</sub>HPO<sub>4</sub>—0.21 g l<sup>-1</sup>, NaH<sub>2</sub>PO<sub>4</sub>—0.09 g l<sup>-1</sup>, FeSO<sub>4</sub>·7H<sub>2</sub>O—0.01 mg l<sup>-1</sup>, CuSO<sub>4</sub>·5H<sub>2</sub>O—5 µg l<sup>-1</sup>, H<sub>3</sub>BO<sub>4</sub>—10 µg l<sup>-1</sup>, MnSO<sub>4</sub>·5H<sub>2</sub>O—10 µg l<sup>-1</sup>, ZnSO<sub>4</sub>·7H<sub>2</sub>O—70 µg l<sup>-1</sup>, MoO<sub>3</sub>—10 g l<sup>-1</sup>) supplemented with 1 % methane gas was inoculated with soil samples and enriched for 15 days. After enrichment bacterial growth was purified on NMS agar supplemented with 1 % methanol. Additionally, all the suspected isolates were screened for growth in the evacuated tubes containing water as the basal media with 1 % methane in the head space of the tube and in second set water containing 1 % methanol as sole source of carbon was used. Growth of each isolates has been observed by colorimeter to screen their methylotrophic property.

### In vitro conformation of methylotrophic activity

Suspected isolates (after preliminary screening) were tested for utilization of methane gas at the rate of 1 % and methanol at the rate of 1–5 % as sole source of carbon in evacuated tubes containing water (basal medium) + methane (head space of the tube) or methanol separately to confirm methylotrophic

metabolism. Growth and survival of isolates were measured by recording colony counts from inoculated tubes at 10 days after inoculation on NMS agar and recorded as colony forming units (CFU) ml<sup>-1</sup> after 10 days of inoculation.

### Characterization and identification of potential methylotrophic isolates

Selected isolates after in vitro methylotrophic activity were characterized on the basis of morphological, cultural and biochemical characteristics (The prokaryotes 2006; Halt et al. 1994). Molecular characterization was carried out using PCR detection of genes representing methylotrophic metabolism and amplified ribosomal DNA restriction analysis (ARDRA) technique (Waturangi et al. 2011 and Horz et al. 2001). For identification and phylogenetic relationship of the potential isolates, 16S rDNA sequencing was carried out as described by Waturangi et al. 2011.

### Genomic DNA extraction

Five methylotrophic isolates were grown in Luria broth for 24 h, and genomic DNA was extracted by C-TAB method. The integrity and concentration of purified DNA was determined by agarose gel electrophoresis. The total genomic DNA extracted was dissolved in sterile distilled water and stored at 4 °C.

### Detection of bacterial methylotrophic metabolism genes

The presence of *mmoX*, *pmoA* and *mxoF* gene in the isolates, encoding sMMO, pMMO and methanol dehydrogenase which are key enzymes in bacterial methane metabolism pathway, was used for authentication of the isolates. The presence of genes in the isolates was detected by partial amplification of the genes using specific primers (Horz et al. 2001). Following primer pairs used for *mmoX* (534f—ccgctgtggaagggcatgaa & 1393r—cactcgtagcgtccggctc); *pmoA* (A189f—ggngactgggacttctgg & A682r—gaasgcngagaagaasc)<sup>1</sup> and *mxoF* (1003f—gcggcaccactgggctggt & 1561r—gggcagcatgaagggtccc), respectively. The 40 µl PCR reaction mixture contains DNA template 50 ng, 1X Taq buffer, 0.2 mM of each of dNTPs mixture, 1 µM of each primers, 1.5 mM·MgCl<sub>2</sub>, and 2 U of Taq DNA polymerase (Bangalore Genei, India). PCR amplification was performed in a thermal cycler (Eppendorf Master cycler, Germany) using the following conditions: initial denaturation at 95 °C for 1 min, 30 cycles consisting of 95 °C for 1 min (denaturation), annealing 62–52 °C touchdown—1 min for *mmoX* and *pmoA*, while 55 °C—1 min for *mxoF* gene, 72 °C for 1 min (primer extension), and final extension

<sup>1</sup> N: bases A, C, T, or G; S: bases G or C.

72 °C for 5 min. PCR products were separated by electrophoresis on 1.5 % agarose gels stained with ethidium bromide and documented in AlphaImager TM1200 documentation and analysis system.

#### *Amplified ribosomal DNA restriction analysis*

16S *rDNA* amplification was performed in a thermal cycler (Eppendorf Master cycler, Germany) with a 25 µl reaction mixture containing 50 ng of genomic DNA, 0.2 mM of each dNTPs, 1 µM of each primer (Waturangi et al. 2011), 2.5 mM of MgCl<sub>2</sub>, and 1 U of Taq DNA polymerase (Bangalore Genei, India) and the buffer supplied with the enzyme. Approximately 1 µg of PCR-amplified 16S *rDNA* fragments were restricted with endonucleases *TaqI*, *HinfI*, and *HaeIII* (Fermentas, USA) separately at 37 °C for 3 h and resolved by electrophoresis in 3 % agarose gels. Banding patterns were visualized by ethidium bromide staining and documented in AlphaImager TM1200 documentation and analysis system. Strong and clear bands were scored for similarity and clustering analysis using the software, NTSYS-PC2 package (Numerical taxonomy analysis program package, Exeter software, USA). Similarity among the strains was calculated by Jaccard's coefficient (Jaccard 1912), and dendrogram was constructed using UPGMA method (Nei and Li 1979).

#### *DNA sequencing analysis*

DNA fragment from five isolates were chosen to be sequenced based on the dominant isolates that isolated from soil samples. PCR product was purified using DNA Gel Extraction Kit (Bangalore Genei, India). Purified products were sequenced using big dye terminator with ABI PRISM™ model 3130 Genetic Analyzer. The sequence data of bacterial isolates were compared to sequence from GenBank in National Center for Biotechnology Information (NCBI, [www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)) using BLASTN program for identification and phylogenetic analysis of the isolates.

#### *In vitro enzyme activities for methane degradation*

Qualitative and quantitative determination of enzyme activities for two key enzymes viz. methane monoxygenase and methanol dehydrogenase involved in methane oxidation were carried out according to standard literature.

#### *Qualitative detection of soluble methane monoxygenase*

For qualitative detection of sMMO enzyme, isolates were grown on NMS media with and without CuSO<sub>4</sub> and

incubated for 72 h at 30 °C. After incubation, few naphthalene crystals were sprinkled in the lid of the plate and the plates were stored in inverted position at 30 °C for 15 min in air followed by gentle spray of freshly prepared, ortho-dianisidine dye at the rate of 5 mg ml<sup>-1</sup>, for 2–3 s. The lids were replaced and the plates were stored for 15 min in the presence of the dye. If naphthol was produced by the colonies, a purple-red colour appeared upon contact with the dye. The colour once formed, remained stable for at least 24 h at room temperature (Graham et al. 1992).

#### *Quantitative detection of soluble methane monoxygenase and methanol dehydrogenase*

##### *Preparation of cell free extract*

Cell free extract was prepared by centrifugation of cell mass for 30 min at 10,000×g washed once by suspending in 0.05 M potassium phosphate buffer (pH 7.0), the washed cells were suspended in the same buffer, homogenized and disrupted by ultrasonic treatment of 50 Hz (10 s ml<sup>-1</sup>). The suspension was then centrifuged at 10,000×g for 30 min and the resulting supernatant was used as crude extract. Proteins were determined by Lowry's method using bovine serum albumin as standard and methanol dehydrogenase activity was assayed by the method suggested by Anthony (1971).

##### *Soluble methane monoxygenase activity*

For quantitative determination of soluble methane monoxygenase a slightly modified version of the naphthalene oxidation assay of Koh et al. (1993) was followed. Each culture was transferred in 1 ml aliquots to 10 ml screw-cap tubes and 1 ml of pre-filtered saturated naphthalene solution was added to each tube. The samples were prepared in triplicate keeping sterile medium control as blank. The reaction mixtures were incubated at 200 rpm on incubator shaker at 25 °C for 1–3 h. After incubation, 100 µl of freshly prepared 4.21 mM tetrazotized-o-dianisidine solution was added to each tube and the intensity of coloured diazo-dye complex was immediately monitored by recording the A<sub>525</sub> by spectrophotometry. The intensity of diazo-dye formation is proportional to the naphthol concentration (1-naphthol and 2-naphthol). The specific activity of sMMO was expressed as nanomoles of naphthol formed per milligram of cell protein per minute.

##### *Methanol dehydrogenase activity*

Qualitative detection of methanol dehydrogenase was carried out following method of Eggeling and Sahm (1980)

with some modifications. The enzyme assays were performed in triplicate and data obtained were subjected to statistical analysis by using completely randomized design (CRD) (Panse and Sukhatme 1978).

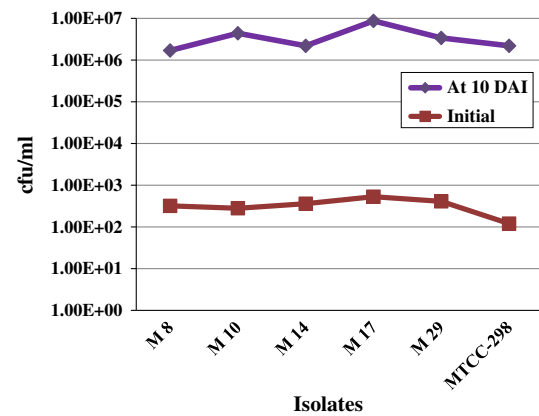
## Results

### Isolation of methylotrophic bacteria from wetland paddy

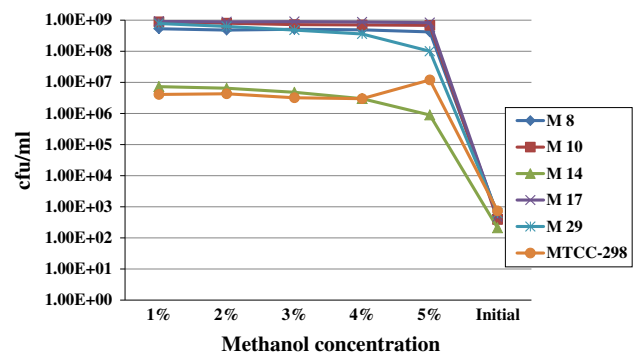
In all total 74 isolates were obtained and during screening of isolates' for their ability to survive by utilizing methane and methanol as sole carbon source isolate M 8 (from rhizospheric soil of organically grown rice cv. IR-64 at main rice research station, Nawagam, Gujarat, India), M 10 (from rhizospheric soil of rice grown at farmers' field of Vyara, Surat, Gujarat, India), M 17 (from rhizospheric soil of rice grown at farmers' field of Tarapur, Gujarat, India) and M 29 (from rhizospheric soil of organically grown rice cv. GR-11 at main rice research station, Nawagam, Gujarat, India) were found to multiply in 1 % methane + water as well as 1 % methanol + water. Whereas, isolate M 14 (from rhizospheric soil of rice grown at farmers' field of Vyara, Surat, Gujarat, India) was found to multiply in by utilizing 1 % methane and failed to multiply on methanol substrate. So inspired from the results, these five potent isolates viz. M 8, M 10, M14, M 17 and M 29 were chosen for further study. As the reference standard *Methylobacterium extrorquens* strain (Cat. No. MTCC 298) obtained from Microbial Type Culture Collection Center, IMTECH, Chandigarh was used as these strain is reported to possess soluble methane monooxygenase and methanol dehydrogenase activities which are involved in bacterial methane metabolism.

### Survival of isolates using methane gas and methanol as sole source of carbon

Results showed that five selected isolates were found capable of utilizing methane as sole source of carbon for their growth and survival. After 10 days of inoculation, there was fourfold increase in cell numbers confirming methanotrophic nature of isolates. (Figs. 1, 10). Results pertaining to survival of isolates on methanol in the concentration range of 1–5 % are presented in Table 4. Results showed that all the isolates were capable to survive and multiply on methanol concentration up to 5 % level and found to utilize them in their metabolism showing increase in cell numbers maximum after 10 days of inoculation. Isolate M 8, M 10, M 17 and M 29 were found to efficiently utilize methanol showing six fold increase in cell numbers from initial  $10^2$ – $10^8$  at 10 DAI. Whereas, isolate M 14 and



**Fig. 1** Growth and survival of methylotrophic isolates by utilizing 1 % methane after 10 days of inoculation



**Fig. 2** Growth and survival of methylotrophic isolates by utilizing 1-5 % methanol after 10 days of inoculation

standard strain *M. extrorquens* MTCC 298 showed four fold increase (from  $10^2$  to  $10^6$ ) (Fig. 2).

### Characterization of isolates

Colonial characteristics of all the isolates were noted in Table 1. Isolate M 8, M 14, M 17 and M 29 were Gram +ve medium sized rods occurring singly, whereas, isolate M 10 found to be Gram –ve short rod. All the isolates showed variable response during biochemical characterization as shown in Table 1 showing that they are different from each other.

Based on the biochemical characterization of selected methylotrophic isolates, two major groups were formed (Fig. 3). First cluster comprised three isolates viz. M 8, M 14 & standard strain MTCC 298 (*M. extrorquens*). While group II consisted of another three methylotrophic isolates viz. M 10, M 17 & M 29. Here the biochemical characters of the isolates distinguish them from each other and represent their different metabolism by utilizing variety of substrates.

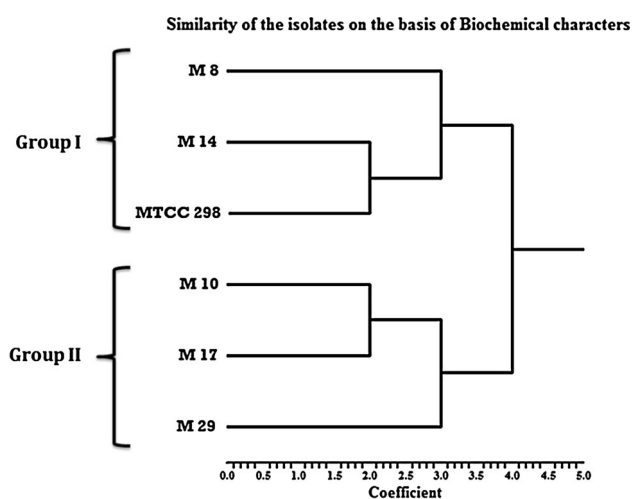
**Table 1** Characterization of methylotrophic isolates

Tests	Methylotrophic isolates				
	M 8	M 10	M 14	M 17	M 29
<b>Colonial characteristics</b>					
Shape	Round	Round	Irregular	Round	Round
Margin	Undulate	Entire	Fimbriated	Entire	Entire
Elevation	Flat	Convex	Flat	Slightly convex	Convex
Texture	Rough	Smooth	Rough	Smooth	Smooth
Opacity	Translucent	Translucent	Opaque	Translucent	Opaque
Pigmentation	Nil	Nil	Yellow	Pink	Off-white
<b>Morphological characteristics</b>					
Shape	Rod	Short rod	Rod	Rod	Rod
Arrangement	Single	Single	Single	Single	Single
Gram's reaction	G +ve	G –ve	G +ve	G +ve	G +ve
<b>Biochemical characteristics</b>					
ONPG	–	–	–	–	+
Lysine utilization	–	–	–	–	–
Ornithine utilization	–	–	–	–	–
Urease	–	–	–	–	–
Phenyl alanine deamination	–	–	–	–	+
Nitrate reductase	–	+	–	+	–
H <sub>2</sub> S production	–	–	–	–	–
Citrate utilization	+	+	+	+	+
Voges proskauer's	+	–	+	–	–
Methyl red	–	–	–	–	–
Indole	+	+	+	+	+
Malonate utilization	–	+	–	+	–
Esculine hydrolysis	+	+	+	+	+
Maltose	+	+	–	–	+
Arabinose	+	–	–	–	–
Xylose	+	+	–	+	+
Adonitol	–	+	–	–	–
Rhamnose	–	+	–	–	–
Cellobiose	+	+	–	–	–
Melibiose	–	+	–	+	+
Sachharose	+	+	+	–	+
Raffinose	–	+	–	–	+
Trehalose	+	+	+	–	+
Glucose	+	+	+	+	+
Fructose	+	+	–	+	+
Galactose	–	+	–	+	–
Sucrose	+	+	–	–	+
Mannose	+	+	+	+	–
Inulin	–	–	+	–	+
Sodium gluconate	–	–	–	–	–
Glycerol	+	–	+	–	+
Salicin	–	–	+	–	–
Dulcitol	–	–	–	–	–
Inositol	–	–	–	–	–
Sorbitol	–	–	–	–	–

**Table 1** continued

Tests	Methylotrophic isolates				
	M 8	M 10	M 14	M 17	M 29
Mannitol	–	–	–	–	+
Adonitol	–	–	–	–	–
Arabitol	–	–	–	–	–
Ethythretol	–	–	–	–	–
$\alpha$ -methyl D-gloside	–	–	–	–	–
Melezitol	–	–	–	–	+

Keys + positive test, – negative reaction



**Fig. 3** Relationship of the methylotrophic isolates on the bases of their biochemical characters

#### Utilization of other $C_1$ compounds

All the isolates showed good growth on 1 % formaldehyde which is the third product of methane metabolism after methane gas and methanol. Isolate M 17 and M 29 were found to utilize 1–5 % concentration of methyl acetate. Whereas, isolates M 8, M 10 and M 14 were not able to utilize methyl acetate. Isolate M 8 and M 29 were found to utilize 1 % concentration of trichloroethylene, whereas, isolate M 14 and M 17 tolerated 1–3 % level of trichloroethylene and isolate M 10 showed good growth up to 2 % trichloroethylene concentration (Table 2). These results support the presence of methane mono oxygenase enzyme in isolates which is required for oxidation of trichloroethylene like compounds.

#### Molecular characterization

An about 1,500 bp fragment of the 16S *rRNA* gene from all five selected isolates and standard strain was further amplified

using the universal primer U27f and U1492r for restriction analysis. The results of the virtual restriction, chosen three tetra cutter restriction enzymes based on better outcomes obtained, namely *TaqI*, *HinI* and *HaeIII* were employed for the characterization of methylotrophic isolates. The amplified products obtained from all isolates were subjected to restriction analysis yielding total of 60 fragments (Figs. 12, 13, 14). In order to assess the existence of species, specific restriction patterns were performed for each of the enzymes utilized and have showed different and distinguished pattern of native methylotrophic isolates. Pooled analysis of ARDRA for methylotrophic isolates is represented by dendrogram (Fig. 4). Coefficient of similarity for native methylotrophic isolates M 8 and M 14 showed some similarity (19 %) (Table 3).

Isolate M 17 showed 17 % similarity with *M. extorquens* (MTCC 298) strain, isolate M 17 showed 13 % similarity with Isolate M 29, isolate M 10 showed 10 % similarity with isolate M 14. Isolate M 8 and M 14 showed 4 and 5 % similarity with *M. extorquens* (MTCC 298) strain respectively. Isolate M 8 showed 5 % similarity with M 10, isolate M 14 showed 4 % similarity with isolate M 29.

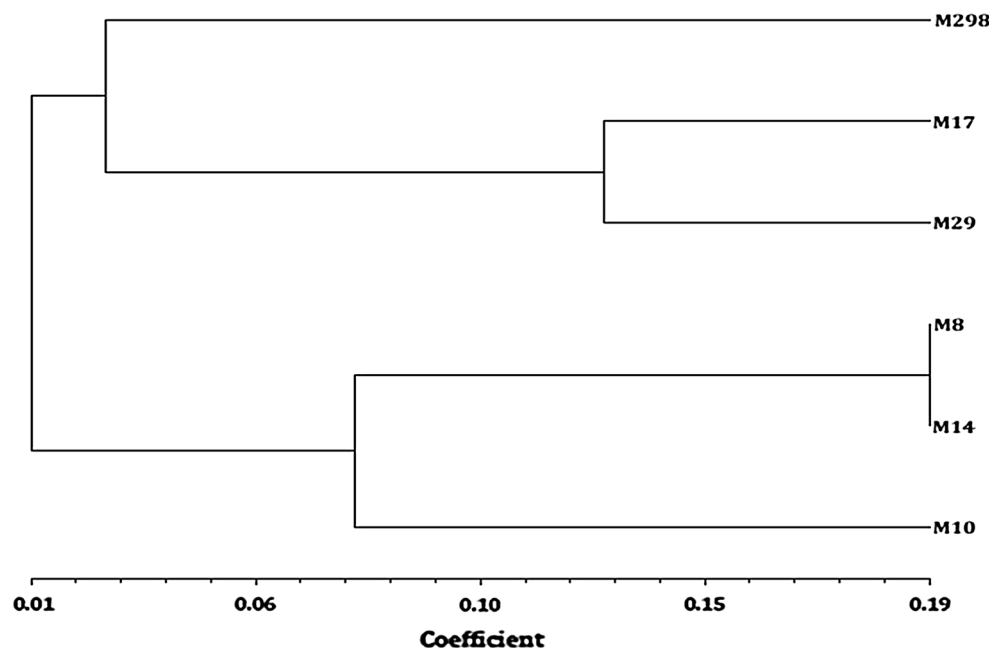
From 16S *rRNA* partial gene sequence isolate M 8 was identified as *Bacillus aerius* with 100 % similarity and 100 % query coverage to *B. aerius* strain 24 K (Fig. 5). Isolate M 10 was identified as *Rhizobium* sp. showing 96 % identity with *R. selenitireducens* strain B1 with 100 % query coverage which confirms the isolate M 10 belongs to *Rhizobium* genus but the species was not confirmed. (Figure 6). Isolate M 14 was also identified as *B. subtilis* with 99 % similarity and 100 % query coverage to *B. subtilis* strain DSM 10 (Fig. 7). The phylogenetic tree constructed showed one major clusters showing close similarity with *B. subtilis*. Isolate M 17 showed about 99 % similarity and 100 % query coverage with *Paenibacillus illinoisensis* strain JCM 9907 indicating M 17 may be the native species of *P. illinoisensis* (Fig. 8). Isolate M 29 showed about 99 % similarity and 100 % query coverage with *B. megaterium* QM B1551 (Fig. 9). The sequence analysis of partial 16S *rRNA* gene of isolate M 8, M 10, M 14, M 17 and M 29 have been deposited



**Table 2** Evaluation of isolates for C<sub>1</sub> compounds utilization

Isolates	Concentration (%)	M 8	M 10	M 14	M 17	M 29	<i>M. extorquens</i>
Formaldehyde	1	+	+	+	+	+	+
	2	–	–	–	–	–	–
	3	–	–	–	–	–	–
	4	–	–	–	–	–	–
	5	–	–	–	–	–	–
Methyl acetate	1	–	–	–	+	+	–
	2	–	–	–	+	+	–
	3	–	–	–	+	+	–
	4	–	–	–	+	+	–
	5	–	–	–	+	+	–
Trichloro ethylene	1	+	+	+	+	+	+
	2	–	+	+	+	–	–
	3	–	–	+	+	–	–
	4	–	–	–	–	–	–
	5	–	–	–	–	–	–

– no growth, + moderate growth

**Fig. 4** Pooled ARDRA dendrogram of methylotrophic isolates

in NCBI, GeneBank under accession numbers KC787582, KC787583, KC855269, KC787584 and KC787585 respectively. The analysis named isolate M 8 as *B. aerius* AAU M 8, Isolate M 10 as *Rhizobium* sp. AAU M 10, isolate M14 as *B. subtilis* AAU M 14, isolate M 17 as *P. illinoisensis* AAU M 17 and M 29 as *B. megaterium* AAU M 29.

In vitro detection of methane monooxygenase and methanol dehydrogenase enzyme activities

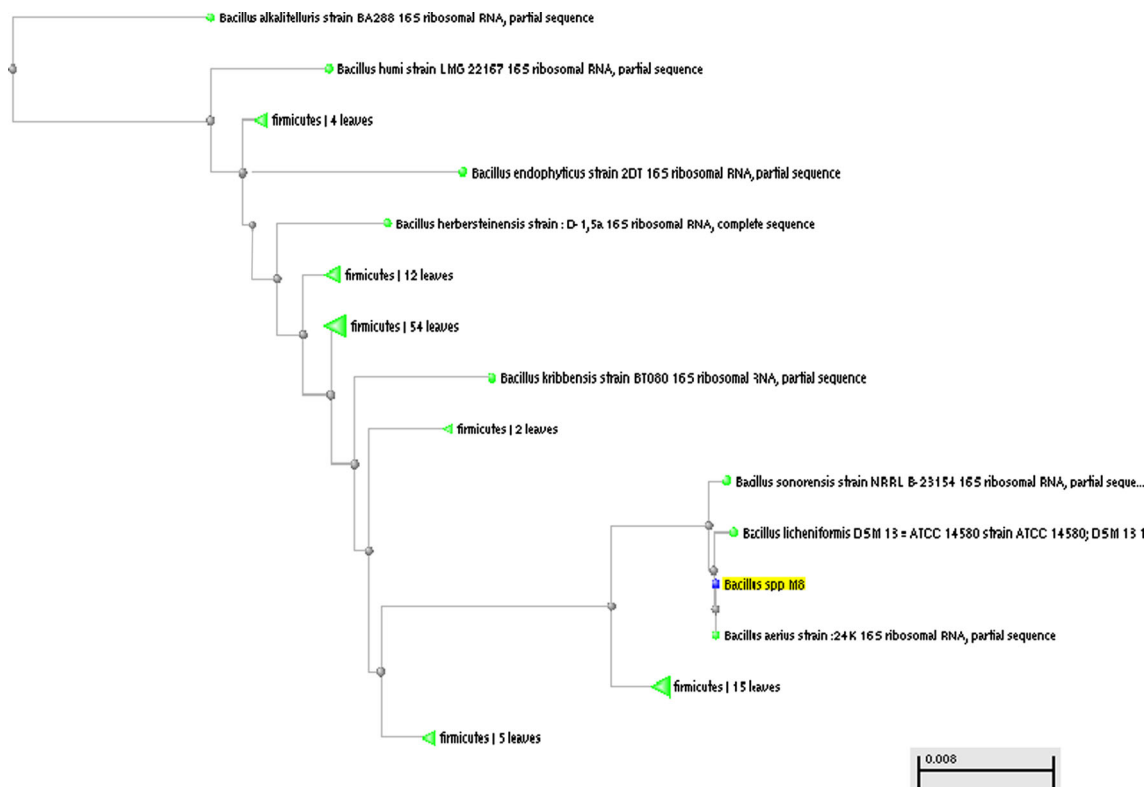
Chosen isolates were further subjected to qualitative and quantitative detection of two key enzymes of methane

degradation pathway viz. methane monooxygenase catalysing oxidation of methane to methanol and methanol dehydrogenase catalysing oxidation of methanol to formaldehyde (Murrell 1994).

Methanotrophic colonies expressing sMMO turned deep purple when exposed successively to naphthalene and o-dianisidine. Isolate M 10, M 17, M 29 and *M. extorquens* (MTCC 298) were found positive for sMMO activity in absence of copper ions in media. Moreover, when media was supplemented with CuSo<sub>4</sub>, sMMO activity was not detected (Fig. 11). The soluble methane monooxygenase activity of all the isolates ranged from 22.5 to 37.0 nmol min<sup>-1</sup> mg of protein<sup>-1</sup>

**Table 3** Pooled biometrics analysis

Isolate no.	<i>M. extrorquens</i> MTCC 298	M 8	M 10	M 14	M 17	M 29
<i>M. extrorquens</i> MTCC 298	1.00					
M 8	0.04	1.00				
M 10	0.00	0.05	1.00			
M 14	0.05	0.19	0.10	1.00		
M 17	0.17	0.00	0.00	0.00	1.00	
M 29	0.00	0.00	0.00	0.05	0.13	1.00

**Fig. 5** Phylogenetic tree based on 16S rDNA sequence of methylotrophic isolate M 8

(Table 4). Among all the isolates, *P. illinoisensis* showed higher soluble methane monooxygenase activity ( $37.0 \text{ nmol min}^{-1} \text{ mg of protein}^{-1}$ ) in cell free extract followed by *B. megaterium* ( $30.0 \text{ nmol min}^{-1} \text{ mg of protein}^{-1}$ ).

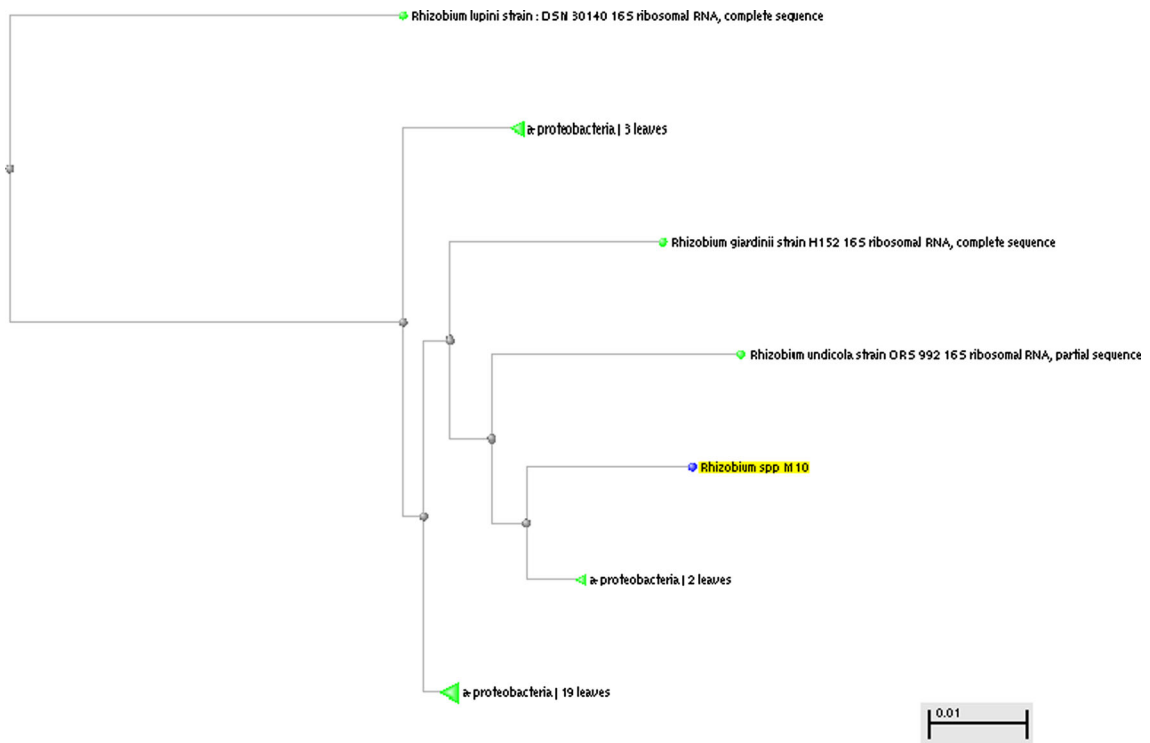
All the isolates were found positive for methanol dehydrogenase activity. Methanol dehydrogenase activity of all the isolates ranged from 35 to  $75 \text{ nmol min}^{-1} \text{ mg of protein}^{-1}$  (Table 4). Among all the isolates, *P. illinoisensis* showed the highest methanol dehydrogenase activity ( $75 \text{ nmol min}^{-1} \text{ mg of protein}^{-1}$ ) in cell free extract followed by *B. megaterium* ( $63 \text{ nmol min}^{-1} \text{ mg of protein}^{-1}$ ).

#### Detection of methane metabolism genes

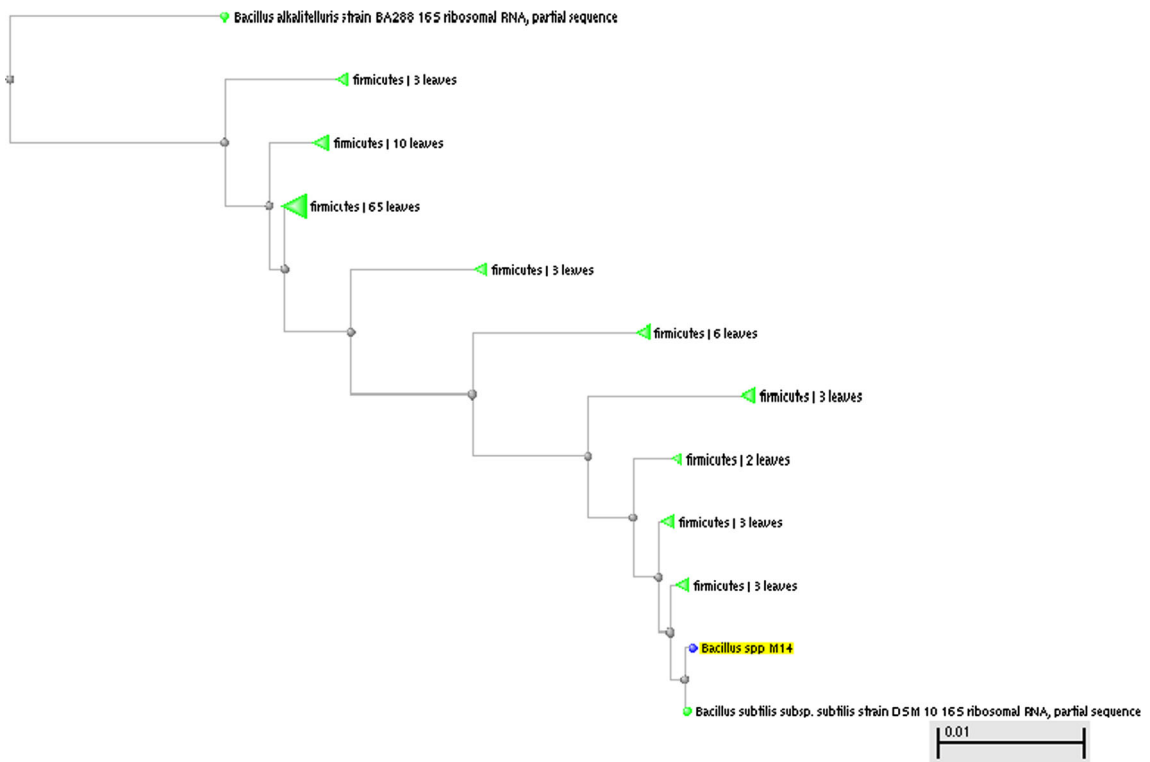
Among all the tested isolates *B. aerius*, *Rhizobium* sp., *B. subtilis* and *P. illinoisensis* showed  $\sim 518$ , 536, 518 and

527 bp respectively, indicating these isolates may possess pMMO enzyme responsible for capacity of methylotrophs to utilize methane (Fig. 15). Among the five tested isolates, *Rhizobium* sp., *P. illinoisensis*, *B. megaterium* and *M. extrorquens* strain gave single band of  $\sim 870$  bp indicating these isolates may also have capacity to utilize methane (Fig. 16) using soluble methane monooxygenase enzyme. Overall, from the above results it was clear that isolate *Rhizobium* sp. and *P. illinoisensis* possess both sMMO and pMMO genes indicating they are better cultures for methane degradation. Similarly, *Rhizobium* sp. and *P. illinoisensis* showed single band of  $\sim 507$  and  $\sim 579$  bp size indicating presence of *mxoF* gene (Fig. 17), whereas other isolates and standard strain failed to show amplification of specific gene may be due to non-availability of selective primers to amplify methanol dehydrogenase genes from *Bacillus* sp.





**Fig. 6** Phylogenetic tree based on 16S rDNA sequence of methylotrophic isolate M 10



**Fig. 7** Phylogenetic tree based on 16S rDNA sequence of methylotrophic isolate M 14

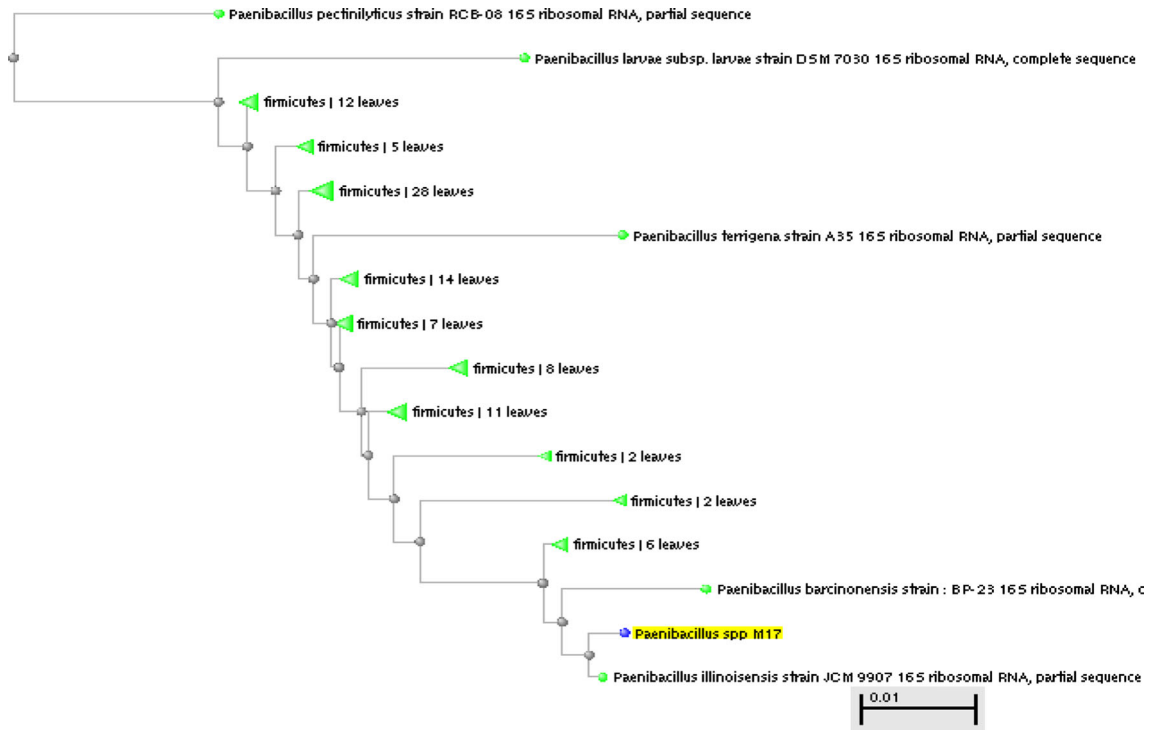


Fig. 8 Phylogenetic tree based on 16S rDNA sequence of methylotrophic isolate M 17

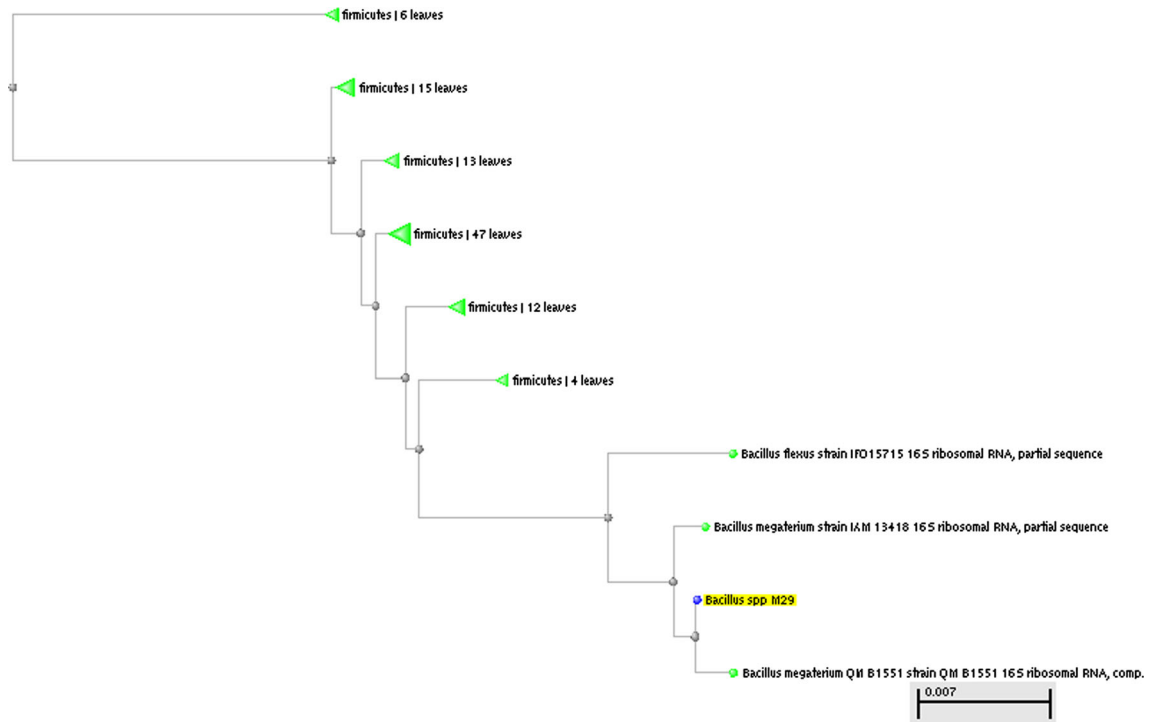
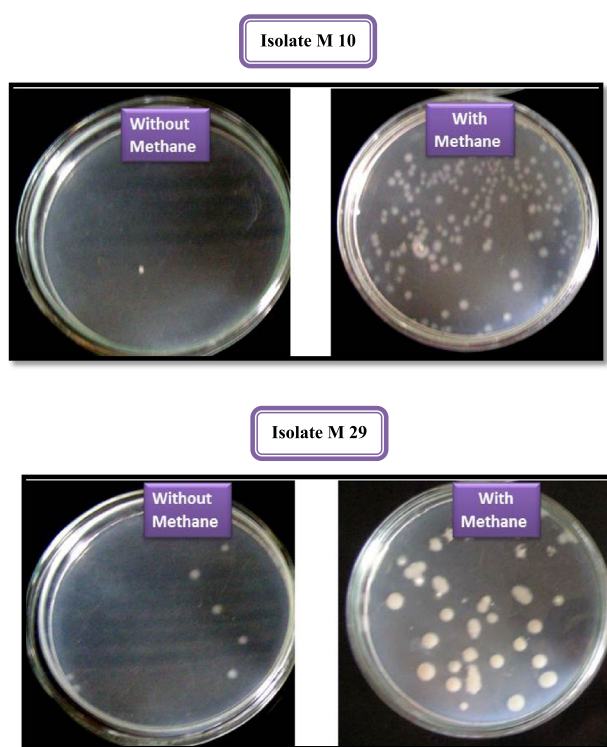


Fig. 9 Phylogenetic tree based on 16S rDNA sequence of methylotrophic isolate M 29

**Table 4** Methane degrading enzyme activity

Isolates	Soluble methane monooxygenase specific activity (Naphthalene oxidation nmol min <sup>-1</sup> mg of protein <sup>-1</sup> )	Methanol dehydrogenase specific activity <sup>a</sup> (nmol min <sup>-1</sup> mg of protein <sup>-1</sup> )
<i>B. aerius</i>	ND	41.0
<i>Rhizobium</i> sp.	22.5	49.0
<i>B. subtilis</i>	ND	37.0
<i>P. illinoisensis</i>	37.0	75.0
<i>B. megaterium</i>	30.0	63.0
<i>M. extrorquens</i>	26.7	35.0
S.Em.±	0.42	1.00
CD at 5 %	1.30	3.10
CV %	3.77	3.48

ND not detected

<sup>a</sup> nmol DCPIP reduced min<sup>-1</sup> mg of protein<sup>-1</sup>**Fig. 10** Growth and survival of methylotrophic isolates by utilizing 1 % methane after days of inoculation

## Discussion

### Isolation and screening of methylotrophic bacteria

As a source of isolation rhizospheric soils from wetland field at mid-season were selected as the chances of getting efficient methane degrading bacterial strains are maximum at this stage. Because of water logged conditions the methanogenic bacteria are producing methane at maximum rates during this time and due to abundance of favourable carbon source (methane) the methanotrophic bacteria are

flourishing. Till date most of the efforts pertaining to isolate methylotrophic bacteria are concentrated on phyllospheric population but in present investigation the isolation was attempted from rhizospheric samples of wetland paddy fields by using enrichment culture technique. Hoppe et al. (2011) isolated a novel, pink-pigmented aerobic, facultatively methylotrophic bacterial strain from the phyllosphere of *Funaria hygrometrica* using Ammonium mineral salt (AMS) media supplemented with 0.5 % methanol as sole carbon source. Van-Aken et al. 2004 isolated *Methylobacterium populi* sp. nov. a novel aerobic, pink-pigmented, facultatively methylotrophic, methane-utilizing bacterium from poplar trees by enrichment of tissue culture explants in Nitrate mineral salt media supplemented with 1 % methanol as carbon source.

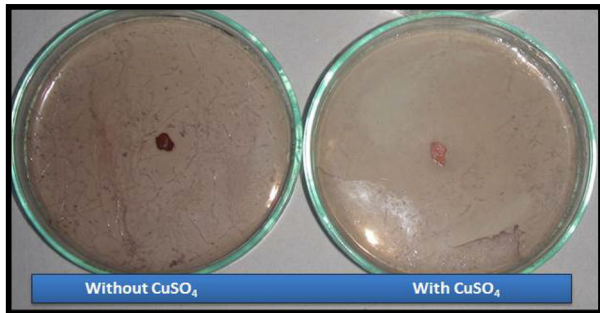
### Characterization of isolates

All the isolates showed variable morphological and biochemical characteristics showing that they are different from each other (Fig. 3). The grouping based on the biochemical characterization supports the initial methylotrophic bacterial screening for utilization and growth in media containing methane and methanol as a sole source of carbon wherein the group II isolates M 10, M 17 & M 29 showed comparatively higher growth than group I isolates. Urakami and Komagata (1986) reported that some strains of methylotrophic bacteria can also utilize Larabinose, D-xylose, D-fucose, D-glucose, D-galactose, D-fructose, L-Aspartate, L-glutamate, adipate, Sebacate, D-tartarate, citrate, saccharate, mono-methylamine, trimethylamine, trimethylamine N-oxide, ethanolamine, butylamine, dimethylglycine and betaine, ammonia, nitrate and urea as source of nitrogen.

To further confirm the results of morphological and biochemical studies technique that is widely used for microbial diversity analysis i.e. ARDRA having potential to discriminate the bacteria at the species level was



*Rhizobium* sp.

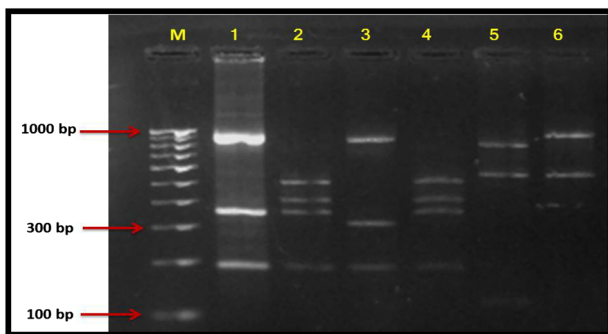


*P. illinoisensis*

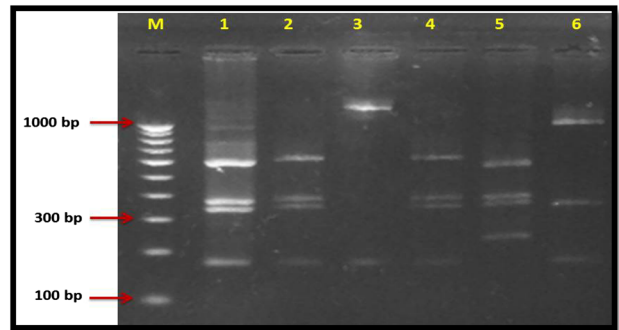


*B. megaterium*

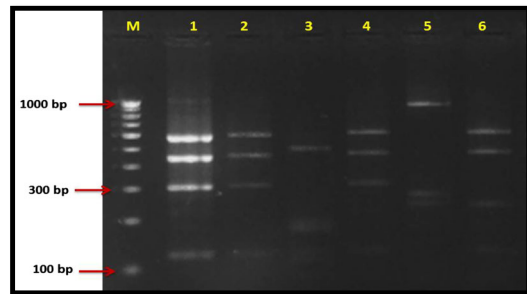
**Fig. 11** Detection of sMMO activity of isolates in presence and absence of copper



**Fig. 12** ARDRA pattern of methylotrophic isolates with *TaqI* enzyme

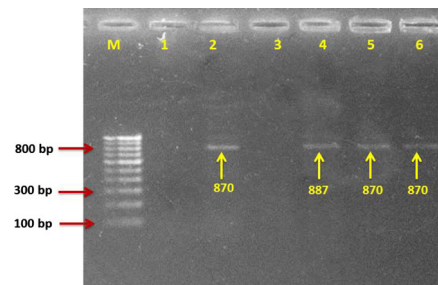


**Fig. 13** ARDRA pattern of methylotrophic isolates with *Hinfi* enzyme



Lane No	Isolate	Lane No.	Isolate
M	100 bp DNA ladder	4	M 14
1	MTCC 298	5	M 17
2	M 8	6	M 29
3	M 10		

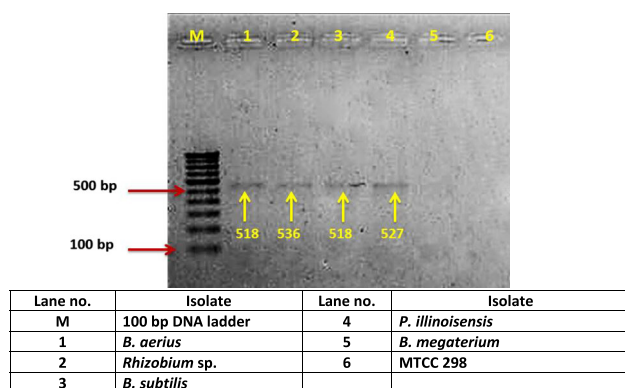
**Fig. 14** ARDRA pattern of methylotrophic isolates with *HaeIII* enzyme



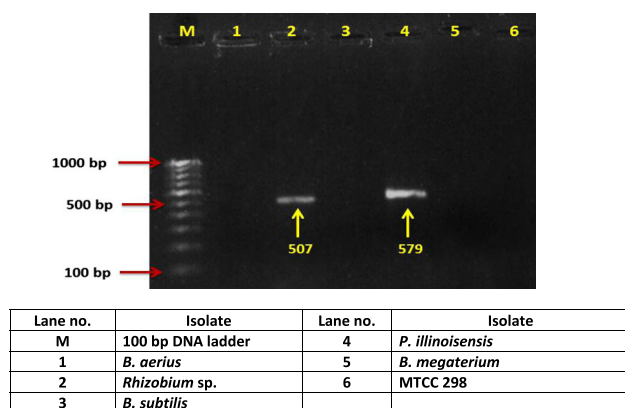
Lane no.	Isolate	Lane no.	Isolate
M	100 bp DNA ladder	4	<i>P. illinoisensis</i>
1	<i>B. aerius</i>	5	<i>B. megaterium</i>
2	<i>Rhizobium</i> sp.	6	MTCC 298
3	<i>B. subtilis</i>		

**Fig. 15** *mmoX* gene amplification profile of methylotrophic isolates

performed. Overall ARDRA results indicated that all the isolates have less than 50 % similarity and few have zero similarity showing all isolates are of different type and non-identical to each other, indicating good diversity in native methylotrophic isolates. These fact is supported by



**Fig. 16** *pmoA* gene amplification profile of methylotrophic isolates



**Fig. 17** *mxoF* gene amplification profile of methylotrophic isolates

morphological and biochemical characterization studies as discussed previously. ARDRA was proved to be useful for relatedness of bacterial strains at different taxonomic levels, depending on selection of conserved or variable regions in the ribosomal genes for the analysis. It is widely used to check the clonal diversity of the isolates. This method involves amplification of the 16S rDNA region followed by digestion with one or more selected restriction enzymes (Heyndrickx et al. 1996). The sequencing of the 16S rRNA gene is another such technique used for phylogenetic placement, identification and diversity analysis of bacteria. So in present investigation ARDRA analysis and 16S rRNA analysis was carried out. Similarly, Raja et al. 2008 have carried out ARDRA profiling of amplified 16S rRNA genes digested with *Hae*III enzyme, which discriminated 10 species of *Methylobacterium* strains isolated from phyllosphere, root nodules and internal tissues of selected plants into seven groups, but ARDRA could not easily discriminate more closely related *Methylobacterium* spp such as *M. suomiense* and *M. aminovorans*, *M. populi* and *M. thycocyanum*, *M. fugisawaense* and *M. mesophilicum*.

## Detection of methane metabolism enzymes

All the isolates showed utilization of methane and methanol as sole carbon source. To confirm methanotrophic nature of isolates detection of enzymatic activity of highly conserved key enzymes viz. particulate pMMO, sMMO and MDH, may offer the possibility of detecting known methanotrophs (Hanson and Hanson 1996). The ability of methanotrophs to oxidize methane is due to the possession of the enzyme called methane monooxygenase (MMO). This enzyme oxidizes methane to methanol. The MMO enzyme is the subject of extensive biochemical and molecular research. There are two distinct forms of this enzyme, a membrane-bound pMMO and cytoplasmic sMMO (Hanson and Hanson, 1996). Among all the isolates, Here three isolates viz. *Rhizobium* sp., *P. illinoisensis* and *B. megaterium* along with standard methylotrophic strain *M. extorquens* showed presence of soluble methane monooxygenase enzyme showing that the methane utilization showed by these isolates was due to presence of sMMO activity. Qualitative detection of sMMO activity was performed by naphthalene oxidation assay (Graham et al, 1992) on solid media as indicative from colonies turning to deep purple color due to development of a colored complex between 1-naphthol, formed when sMMO reacts with naphthalene and o-dianisidine (tetrazotized). Moreover, the isolates were found positive for sMMO activity in the absence of copper and in the presence of copper ions in media the isolates failed to show sMMO activity (Fig. 11). These results confirms the copper play vital role for expression of two forms of methane monooxygenase enzyme viz. sMMO present in the cytoplasm and membrane bound pMMO. In methanotrophs that have the genes for both pMMO and sMMO, their expression depends on the copper concentration in media. At high copper concentration, pMMO is expressed by incorporating  $\text{Cu}^{2+}$  into the active site of pMMO, while sMMO transcription in this condition is repressed by copper ions via an unknown mechanism and at low levels of copper ions in the growth medium sMMO is expressed (Kumaresan et al. 2009). Similar results were obtained by Koh et al. (1993) while working with the methylotrophic strain *M. methanica* 68-1 which showed higher rate of soluble methane monooxygenase activity in the absence of copper ions in media. Bowman et al. 1993 performed quantitative detection of sMMO activity by the naphthalene oxidation assay in NMS media for 25 methanotrophic bacteria obtained from groundwater samples contaminated with trichloroethylene and tetrachloroethylene and reported that most of the isolates have soluble methane monooxygenase activity in the range of  $<1\text{--}594 \text{ nmol h}^{-1} \text{ mg}^{-1}$  of protein.

Apart from methane and methanol, all the isolates showed utilization of formaldehyde which is the third important product in bacterial methane metabolism



pathway. The reducing power required for the oxidation of methane to methanol and also for bacterial growth further derived by oxidation of methanol, via formaldehyde (HCHO) and formate (HCOOH) to finally carbon dioxide (CO<sub>2</sub>). Approximately 50 % of the formaldehyde produced is assimilated into cell carbon and the remainder is oxidized to CO<sub>2</sub> and lost from the cells (Anthony 1982). All the isolates have also utilized trichloro ethylene which is used as an indicator molecule for detection of methane degrading enzymes within the microbial community as the first enzyme in methane metabolism pathway of bacteria i.e. methane monooxygenase is required for utilization of trichloroethylene. Aken et al. 2011 reported that *M. populi* sp. nov., a novel aerobic, pink-pigmented, facultatively methylotrophic, methane-utilizing bacterium isolated from poplar trees was able to utilize fructose, acetate, betadine, tartrate, ethanol methane and methylamine as carbon sources. Arfman et al. (1989) reported cells of *Bacillus* sp. C<sub>1</sub> grown on methanol in batch culture oxidized methanol at a high rate (1,100–1,500 nmol min<sup>-1</sup> mg of protein<sup>-1</sup>) only short chain (C<sub>1</sub>–C<sub>4</sub>) primary alcohols were oxidized, but compared to methanol (100 %) at lower relative rates (ethanol, 90 %; *n*-propanol, 57 %, *n*-butanol, 53 %). Secondary alcohols and formate were not oxidized. While the organism oxidized various alcohols but grew on only methanol. Rather than mere biochemical characterization studies, molecular techniques are most authenticated tools, providing significant information of species richness, diversity, and community analysis of microorganisms (Brusseau et al. 1994).

In all methylotrophic bacteria so far studied, methanol oxidation is catalyzed by MDH that catalyze the production of formaldehyde, the intermediate of both assimilative and dissimilative metabolism in methylotrophs. In methane-oxidizing bacteria (methanotrophs), MDH is the second enzyme in the methane oxidation pathway and it oxidizes the methanol produced from the oxidation of methane by methane monooxygenase (Murrell 1994). All the isolates showed methanol dehydrogenase enzyme activity which is the second key enzyme responsible for bacterial methane metabolism pathway which degrade second product of the pathway i.e. methanol. These results confirms the outcome of previous experiment wherein *P. illinoisensis* and *B. megaterium* were showing better survival at 1–5 % methanol concentration which may be due to higher rate of methanol dehydrogenase activity. Similarly, Arfman et al. (1989) reported that cell free extract of *Bacillus* sp. C<sub>1</sub> were found to possess NAD dependent methanol dehydrogenase activities ranging from 1,000 to 1,200 nmol min<sup>-1</sup> mg<sup>-1</sup> of protein and also reported that carbon assimilation was by way of RuMP cycle of formaldehyde fixation. Duine et al. (1984) reported dye-linked methanol dehydrogenase activity from cell-free extracts of methanol-grown *Nocardia* sp. 239 in presence of

NAD<sup>+</sup>. In the presence of NAD<sup>+</sup> dehydrogenase activity was 33 nmol DCIP reduced min<sup>-1</sup> mg<sup>-1</sup> protein.

#### Detection of methane metabolism genes

Functional genes unique to the physiology and metabolism of organisms of interest have been explored in molecular studies. Functional genes for enzymes, such as methane monooxygenase (pMMO and sMMO; genes *pmoA* and *mmoX*, respectively) and methanol dehydrogenase (MDH; gene *mxoF*), involved in the methane oxidation pathway, have been used for detection of methanotrophs (McDonald et al. 2008). Gene probes that target functional genes have been developed for the *pmoA* gene (Sambrook et al. 1989) coding for the  $\alpha$ -subunit of the pMMO and the *mxoF* gene (McDonald et al. 1996; McDonald and Murrell 1997) coding for the  $\alpha$ -subunit of the MDH present in all methylotrophs (Hanson and Hanson 1996; McDonald et al. 1995). In present investigation all the isolates showed either presence of *mmoX* gene encoding the  $\alpha$  subunit (mmoX) of the hydroxylase component of the sMMO cluster or *pmoA* gene encoding  $\alpha$ -subunit of particulate methane monooxygenase cluster which are the two forms of methane mono oxygenase enzyme responsible for methane degradation. The soluble MMO is not universal to all methanotrophs and is found predominantly in the genera *Methylosinus* and *Methylococcus* (Stainthorpe et al. 1990). *pmoA* gene encoding one of the subunits of the particulate MMO, has also been examined recently (Holmes et al. 1995). This gene has been found in all methanotrophs studied and reported so far. The use of a second functional gene probe, *mxoF*, which is found in all methanotrophs including gram negative strains was recommended to confirm methylotrophic nature of isolates. The advantage of using the *mxoF* gene is that it can indicate larger group of organisms which assimilate C<sub>1</sub> compounds for their role in environment. In present study, only two isolates showed presence of *mxoF* gene encoding  $\alpha$  subunit of the methanol dehydrogenase, present in all methylotrophs. Though all the isolates showed methanol dehydrogenase activity, except *Rhizobium* sp. and *P. illinoisensis*, other isolates and standard strain failed to show amplification of specific gene may be due to non-availability of selective primers to amplify methanol dehydrogenase genes from *Bacillus* sp. Till date decoding of methane metabolism pathway in *Paenibacillus* species was restricted up to discovery of alcohol dehydrogenase gene (*adh*) from *P. mucilaginosus* 3016 (KEGG pathway entry no. pmq00680). Whereas, discovery of methane metabolism pathway in *Bacillus* species i.e. *B. megaterium* WSH-002 (KEGG pathway entry No. bmh00680 and *B. subtilis* subsp. *subtilis* 168 (KEGG pathway entry No. bsu00680) as well as in *Rhizobium* species i. e. *R. etli* CFN 42 (KEGG pathway entry



No. ret 00680) was restricted up to the discovery of formaldehyde dehydrogenase gene which encodes enzyme responsible for oxidation of formaldehyde to CO<sub>2</sub> for energy and assimilation for biosynthesis in methanotrophs (<http://www.genome.jp/kegg/pathway/map/map01100.html>). Horz et al. (2001) detected methanotrophic diversity on roots of submerged paddy using primer sets A189/A682 encoding pMMO, 534f/1393r encoding sMMO and f1003/r1561 encoding methanol dehydrogenase yielding PCR products of 550, 863 and 525 bp size respectively.

## Conclusion

The results of present study clearly brought out that, all the five tested isolates from wetland paddy efficiently utilized methane and methanol as sole source of carbon and confirmed the presence of genes encoding enzymes responsible for methane degradation pathway of bacteria. Among all the isolates *Rhizobium* sp. and *P. illinoisensis* proved to be more efficient methane degraders followed by *B. megaterium*, *B. aerius* and *B. subtilis*. These novel indigenous methylophilic PGPR based indicated wide scope and prospects as agriculturally beneficial bioinput to reduce methane emission from rice ecosystem with additional advantage of plant growth promotion for sustainable agriculture in a long run.

## References

- Aken B, Tehrani R, Schnoor JL (2011) Endophyte-assisted phytoremediation of explosives in poplar trees by *Methylobacterium populi* BJ001T. For sci 80:217–234. doi:10.1007/978-94-007-1599-8\_14
- Anthony C (1971) Prosthetic group of an alcohol dehydrogenase of a *Pseudomonas*. In: McCormick DB and Wright LD (eds) Methods in enzymology, vol XVIII. Elsevier, pp 808–809
- Anthony C (1982) The biochemistry of methylophilic. Academic Press, London
- Arfman N, Watling EM, Clement W, Van Oosterwijk RJ, de Vries GE, Harder TW, Attwood MM, Dijkhuizen L (1989) Methanol metabolism in thermotolerant methylophilic *Bacillus* strains involving a novel catabolic NAD-dependent methanol dehydrogenase as a key enzyme. Arch Microbiol 152:280–288
- Bodrossy L, Holmes EM, Holmes AJ, Kovács KL, Murrell JC (1997) Analysis of 16S rRNA and methane monooxygenase gene sequences reveals a novel group of thermotolerant and thermophilic methanotrophs, *Methylocaldum* gen. nov. Arch Microbiol 168:493–503
- Bowman JP, Jiménez L, Rosario I, Hazen TC, Sayler GS (1993) Characterization of the methanotrophic bacterial community present in a Trichloroethylene-contaminated subsurface groundwater site. Arch Microbiol 59(8):2380–2387
- Bowman JP, McCammon SA, Skerratt JH (1997) *Methylosphaera hansonii* gen. nov., sp. nov., a psychrophilic, group I methanotroph from Antarctic marinesalinity, meromictic lakes. Microbiology 143:1451–1459
- Brusseau GA, Bulygina ES, Hanson RS (1994) Phylogenetic analysis and development of probes for differentiating methylophilic bacteria. Appl Environ Microbiol 60:626–636
- Dedysh SN, Liesack W, Khmelenina VN, Suzina NE, Trotsenko YA, Semrau JD, Bares AM, Panikov NS, Tiedje JM (2000) *Methylocella palustris* gen. nov., sp. nov., a new methane-oxidizing acidophilic bacterium from peat bogs, representing a novel subtype of serine-pathway methanotrophs. Int J Syst Evol Microbiol 50:955–969
- Dedysh SN, Khmelenina VN, Suzina NE, Trotsenko YA, Semrau JD, Liesack W, Tiedje JM (2002) *Methylocapsa acidiphila* gen. nov., sp. nov., a novel methane-oxidizing and dinitrogen-fixing acidophilic bacterium from Sphagnum bog. Int J Syst Evol Microbiol 52:251–261
- Duine JA, Frank J, Berkhout MPJ (1984) NAD-dependent, PQQ-containing methanol dehydrogenase: a bacterial dehydrogenase in a multienzyme complex. FEBS Lett 168:217–221
- Eggeling L, Sahn H (1980) Direct enzymatic assay for alcohol oxidase, alcohol dehydrogenase and formaldehyde dehydrogenase in colonies of *Hansenula polymorpha*. Appl Environ Microbiol 39(1):268–269
- Graham DW, Korich DG, Leblanc RP, Sinclair NA, Arnold RG (1992) Applications of a colorimetric plate assay for soluble methane monooxygenase activity. Appl Environ Microbiol 58(7):2231–2236
- Halt JG, Peter NR, Sneath HA, Staley JT, William ST (1994) Burgeys manual of detriminitave bacteriology, 9th edn. William and Wilkins, Baltimore, p 559
- Hanson RS, Hanson TE (1996) Methanotrophic bacteria. Microbiol Rev 62:439–471
- Heyer J, Berger U, Hardt M, Dunfield PF (2005) *Methylohalobius crimeensis* gen. nov., sp. nov., a moderately halophilic, methanotrophic bacterium isolated from hypersaline lakes of Crimea. Int J Syst Evol Microbiol 55:1817–1826
- Heyndrickx M, Vauterin L, Vandamme P, Kersters K, De Vos P (1996) Applicability of combined amplified 16S rDNA restriction analysis (ARDRA) patterns in bacterial phylogeny and taxonomy. J Microbiol Meth 26:247–259
- Holmes AJ, Costello A, Lidstrom ME, Murrell JC (1995) Evidence that particulate methane monooxygenase and ammonia monooxygenase may be evolutionarily related. FEMS Microbiol Lett 132:203–208
- Hoppe T, Peters K, Schmidt F (2011) *Methylobacterium bullatum* sp. nov., a methylophilic bacterium isolated from *Funaria hygrometrica*. Syst Appl Microbiol 34:482–486. doi:10.1016/j.syapm.2010.12.005
- Horz H, Yimga MT, Liesack W (2001) Detection of methanotroph diversity on roots of submerged rice plants by molecular retrieval of *pmoA*, *mmoX*, *mxoF*, and 16S rRNA and ribosomal DNA, including *pmoA*-based terminal restriction fragment length polymorphism profiling. Appl Environ Microbiol 67(9):4177–4185
- Intergovernmental Panel on Climate Change (IPCC) (2007) Climate change, Synthesis Report. An assessment of the intergovernmental panel on climate change. Working group contributions to the fourth assessment report
- Jaccard P (1912) The distribution of the flora in the alpine zone. New Phytol 11(2):37–50
- Khosa MK, Sidhu BS, Benbi DK (2011) Methane emission from rice fields in relation to management of irrigation water. J Environ Biol 32:169–172
- Koh S, Bowman JP, Sayler GS (1993) Soluble methane monooxygenase production and trichloroethylene degradation by a type I methanotroph, *Methylomonas methanica* 68-1. Appl Environ Microbiol 59(4):960–967
- Kumaresan D, Abell GCJ, Bodrossy L, Stralis-Pavese N, Murrell JC (2009) Spatial and temporal diversity of methanotrophs in a

- landfill cover soil are related to soil abiotic factors. *Environ Microbiol Rep* 1:398–407
- McDonald IR, Murrell JC (1997) The methanol dehydrogenase structural gene *mxoF* and its use as a functional gene probe for methanotrophs and methylotrophs. *Appl Environ Microbiol* 63:3218–3224
- McDonald IR, Kenna EM, Murrell JC (1995) Detection of methanotrophic bacteria in environmental samples with the PCR. *Appl Environ Microbiol* 61:116–121
- McDonald IR, Hall GH, Pickup RW, Murrell JC (1996) Methane oxidation potential and preliminary analysis of methanotrophs in blanket bog peat using molecular ecology techniques. *FEMS Microbiol Ecol* 21:197–211
- McDonald IR, Bodrossy L, Chen Y, Murrell JC (2008) Molecular ecology techniques for the study of aerobic methanotrophs. *Appl Environ Microbiol* 74:1305–1315
- Murrell JC (1994) Molecular genetics of methane oxidation. *Biodegradation* 5:145–159
- Nei M, Li WH (1979) Mathematical models for studying genetic variation in terms of restriction endonucleases. *Proc Natl Acad Sci USA* 76:5269–5273
- Panase VG and Sukhatme PV (1978) Statistical methods for agricultural workers, ICAR II Ed, New Delhi
- Raja P, Balachandar D, Sundaram SP (2008) PCR finger printing for identification and discrimination of plant associated facultative methylotrophs. *Ind J Biotechnol* 7:508–514
- Reeburgh WS, Whjalen SC, Alperin MJ (1993) The role of methylotrophy in the global methane budget. In: Murrell JC, Kelly DP (eds) *Microbial Growth on C-1 Compounds* Intercept, Ltd, Andover, pp 123–140
- Sambrook J, Fritsch EF, Maniatis T (1989) *Molecular cloning: a laboratory manual*, 2nd edn. Cold Spring Harbor Laboratory, Cold Spring Harbor
- Stainthorpe AC, Salmond GP, Dalton CH, Murrell JC (1990) Screening of obligate methanotrophs for soluble methane monoxygenase genes. *FEMS Microbiol Lett* 72:345–348
- Tsubota J, Eshinimaev BT, Khmelenina VN, Trotsenko YA (2005) *Methylothermus thermalis* gen. nov., sp. nov., a novel moderately thermophilic obligate methanotroph from a hot spring in Japan. *Int J Syst Evol Microbiol* 55:1877–1884
- Urakami T, Komagata K (1986) Emendation of *Methylobacillus Yordy* a genus for methanol-utilizing bacteria. *Int J Syst Bacteriol* 36:502–511
- Van-Aken B, Peres CM, Doty SL, Yoon JM, Schnoor JL (2004) *Methylobacterium populi* sp. nov., a novel aerobic, pink-pigmented, facultatively methylotrophic, methane-utilizing bacterium isolated from poplar trees (*Populus deltoides x nigra DN34*). *Int J Syst Evol Microbiol* 54:1191–1196. doi:10.1099/ijs.0.02796-0
- Wahlen M, Tanaka N, Henry R, Deck B, Zeglen J, Vogel JS, Southon J, Shemesh A, Fairbanks R, Broecker W (1989) Carbon-14 in methane sources and in atmospheric methane: the contribution from fossil carbon. *Science* 245:286–290
- Waturangi DE, Francisca I, Susanto CO (2011) Genetic diversity of methylotrophic bacteria from human mouth based on amplified ribosomal DNA restriction analysis (ARDRA). *Hay J of Biosci* 18(2):77–81. doi:10.4308/hjb.18.2.77
- Whittenbury R, Phillips KC, Wilkinson JF (1970) Enrichment, isolation and some properties of methane-utilizing bacteria. *J Gen Microbiol* 61:205–218
- Wise MG, McArthur JV, Shimkets LJ (2001) *Methylosarcina fibrata* gen. nov., sp. nov. and *Methylosarcina quisquiliarum* sp. nov., novel type I methanotrophs. *Int J Syst Evol Microbiol* 51:611–621