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Characterization of methanol metabolism genes and plant growth-promoting traits in bacteria from the rice (*Oryza sativa*) rhizosphere

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ABSTRACT

Rice (*Oryza sativa* L.), a dominant cereal crop is a major contributor of greenhouse gas methane emissions. The present study was carried out during 2023–2024 at ICAR-Indian Agricultural Research Institute, New Delhi to evaluate six methylotrophic bacteria isolated from the rice rhizosphere for their methanol carbon use efficiency and plant growth-promoting characteristics. Amplification of three methane metabolism genes, namely mmoX, pmoA and *mxaF*; and 16S rRNA gene sequencing, were identified as methylotrophs *Bacillus rugosus* strain MB1, *Bacillus subtilis* strain MB2, *Priestia aryabhattai* strain MB3, *Rhizobium pusense* strain MB4, *Priestia megaterium* strain MB5, and *Sphingobium* spp. strain MB6. Their methanol use efficiency expressed as specific respiration was in the range from from 0.009–0.063 mg of CO_2/µg of protein. Their plant growth-promoting (PGP) traits, including N-fixation, solubilisation of P, K, and Zn, as well as IAA and exopolysaccharide production, revealed significant differences. *Rhizobium pusense* strain MB4 and *Sphingobium* spp. strain MB6 were best in terms of both PGP traits and methanol use efficiency. This study suggests that these methylotrophs serve dual purpose of mitigation of methane carbon emission and can be used as promising candidates for enhancing crop growth.

Keywords: Carbon use efficiency, Exopolysaccharide, Methylotrophs, PGP traits

Global mean annual area-scaled and yield-scaled GHG emissions are ~7,870 kg CO_2e/ha and 0.9 kg CO_2e/kg, respectively, with 94% from CH_4 (Qian *et al.* 2023). Rice (*Oryza sativa* L.) is the world's most important stable crop globally, feeding about half of the world's population (Asma 2023), but also contribute ~48% of greenhouse gas (GHG) emissions from croplands. Paddy fields emit substantial amounts of methane (CH_4), primarily as a result of prolonged flooding and post-harvest practices such as straw incorporation or burning. These activities have been found to increase methane emissions by 3–11 times in straw-contaminated soils compared to control conditions (Ma *et al.* 2007, Zhang *et al.* 2016). Hence, there is a pressing need to devise efficient strategies for lowering greenhouse gas emissions linked to straw management in paddy fields. Utilizing beneficial micro-organisms that enhance plant growth and concurrently reduce greenhouse gas emissions presents a sustainable and eco-friendly solution. Recent studies reflect a special group of bacteria i.e. methylotrophs which can utilize a single carbon compound like methane/methanol as their carbon source for their growth and have the ability to reduce CH_4 from atmosphere (Visscher *et al.* 2007, Malyan *et al.* 2016). These methylotrophs possess two important iso-enzymes which helps in conversion of atmosphere methane to methanol i.e. soluble methane monooxygenase (sMMO), found only as subset of known methanotrophs, and particle methane monooxygenase (pMMO) which is located in specialized internal membrane structures (Aimen *et al.* 2018). And other enzymes, include methanol dehydrogenase (MDH), formaldehyde dehydrogenase (FADH), formate dehydrogenase (FDH) and related oxidases that sequentially oxidize methanol to formaldehyde, formate and finally to carbon dioxide.

Previous research has shown that methylotrophic bacteria in the rhizosphere effectively colonize plant roots, engaging in various interactions with the host that can enhance plant growth and suppress pathogenic infections. These interactions occur through various mechanisms, including atmospheric dinitrogen (N_2) fixation, mineral phosphorus (P) solubilization, potassium (K) solubilization, and the production of phytohormones such as indole-3-acetic acid (IAA), cytokinins, gibberellic acid, ammonia, siderophores, and ACC deaminase activity (Chawngthu *et al.* 2020). P solubilization activity has been reported in the methylotrophs genera *Methylophilus, Methylobacillus, Methylovorus, Methylopila, Methylobacterium* and

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Methylobacterium radiotolerans (Agafonova *et al.* 2013, Mondal *et al.* 2024), Nitrogen fixation by *Methylobacterium* spp. 4-46 and *Methylobacterium* spp. L2-4, (Menna *et al.* 2006), K-solubilisation by *Methylobacterium oryzae* MNL7, Indole-3-acetic acid (IAA) production has been observed in *Methylobacterium extorquens* MP1 and *Bacillus amyloliquefaciens* RWL-1 (Pattnaik *et al.* 2017, Shahzad *et al.* 2019). Additionally, *Methylobacterium oryzae* CBMB20 exhibits ACC deaminase activity and possesses two *miaA* genes, which are essential for the biosynthesis of zeatin, a key cytokinin (Madhaiyan *et al.* 2007, Kwak *et al.* 2014). Methylotrophic bacteria are also known to release osmoprotective compounds, such as sugars and alcohols, onto the surfaces of host plants. These exudates play a protective role by enhancing plant tolerance to desiccation and extreme temperature conditions (Gamit and Amaresan 2024). Thus, from above mentioned studies it is evident that methylotrophs play a dual role of greenhouse gas emission reduction and plant growth promotion. However, there is a limited information regarding the conversion efficiency of CH_4OH to CO_2 or its accumulation in cell biomass by the methylotrophs. Therefore, present research work was undertaken with the aim of understanding the conversion efficiency of methylotrophs present in the rice rhizosphere and the plant growth promoting traits processed by methylotrophs.

MATERIALS AND METHODS

Source of bacteria: The present study was carried out during 2023–2024 at ICAR-Indian Agricultural Research Institute (28.08°N and 77.12°E; with an altitude of 228.61 m amsl), New Delhi. This study utilized bacterial isolates previously obtained from rhizosphere soil of rice-growing fields. Six isolates were chosen for detailed analysis and further experimentation and details of isolates is given in Table 1.

Screening of selected bacteria for their growth in different concentration of methanol: The growth potential of selected bacterial isolates in varying concentrations of methanol was evaluated using Nitrate Mineral Salt (NMS) medium as described by Jhala *et al.* (2014). The NMS medium was supplemented with methanol at concentrations of 1%, 2%, 3%, 4%, and 5% (v/v), serving as the sole carbon source. A loopful of individual pure bacterial colonies, previously isolated from rice rhizosphere soil, was inoculated into the respective tubes. All cultures were incubated in a shaking incubator at 30°C for 10 days. Following incubation, bacterial growth and survival were assessed by recording the optical density (OD) at 600 nm using a spectrophotometer. The obtained OD values were further converted into cell biomass for comparative analysis.

DNA isolation and molecular identification of methylotrophic bacteria: Methanol-utilizing bacterial isolates were cultured in nutrient broth and incubated at 30°C for three days. Cell pellets were obtained by centrifugation at 10,000 rpm for 10 min. Genomic DNA was extracted from the pellets using the QIAGEN DNA isolation kit, and the DNA concentration was measured using a NanoDrop spectrophotometer. PCR amplification of 16SrRNA was performed using universal primers pairs 8F (5' AGAGTTTGATCCTTGGCTCAG 3') and 1492R (5' TACGGYTACCTTGTTACGACTT 3') (Lopez-Fernandez *et al.* 2015) and PCR product was separated by electrophoresis on 1.2% agarose gel stained with ethidium bromide and gel was observed under Alphaimager EC for bands at 1500 bp. Purified product of PCR were sequenced using sanger sequencing. The sequence data from the bacterial isolates were analyzed using the BLAST tool to determine their closest phylogenetic relatives in the NCBI GenBank database. The sequences of all selected methylotrophic strains were submitted to NCBI, and accession numbers were assigned for each submitted sequence (Supplementary Table 1).

Detection of methane and methanol metabolism genes in selected bacteria: The presence of the 3 methylotrophic metabolism genes such as *mmoX* encoding for soluble methane monooxygenase, *pmoA* encoding for particle methane monooxygenase and *mxaF* encoding for methanol dehydrogenase. These genes were amplified by using specific primer pairs A189f (5′GGNGACTGGGACTTCTGG3′) and A682r (5′GAASGCNGAGAAGAASGC3′) for *pmoA*, 534f (5′CCGCTGTGGAAGGGCATGAA3′) and 1393r (5′CACTCGTAGCGCTCCGGCTC3′) for *mmoX* and 1003f (5′GCGGCACCAACTGGGGCTGGT3′) and 1561r (5′GGGCAGCATGAAGGGCTCCC3′) for *mxaF* respectively (Mcdonald and Murrell 1997, Horz *et al.* 2001, Jhala *et al.* 2014). The 25 µl PCR reaction mixture containing 1 µl DNA template, 5 µl of 5X Taq buffer, 2.5 µl of 1mM dNTPs mixture, 0.5 µl of each primer, 2.5 µl of 25 mM $MgCl_2$, and 0.2 µl of 2 U Taq DNA polymerase.

The PCR amplification was performed which consisted of initial denaturation at 95ºC for 1 min and 30 cycles

Table 1 Morphological characters of selected methylotrophic bacteria on NMS media supplemented

Cultures	Surface texture	Shape	Thickness	Margin	Colony colour
Isolate 1	Smooth	Point	Convex	Irregular	Beige
Isolate 2	Smooth	Circular	Flat	Corrugated	Lightish brown
Isolate 3	Smooth	Irregular	Convex	Eroded	Brown
Isolate 4	Mucus	Round	Flat	Regular	White
Isolate 5	Smooth	Round	Raised	Regular	Muddy white
Isolate 6	Smooth	Circular	Convex	Regular	Dark yellow

program consisting of denaturation at 95ºC for 1 min, annealing at 62–52ºC touch down for 1 min was used for amplifying *mmoX* gene, while 52ºC and 55ºC annealing temperature for 1 min is used amplification of *pmoA* and *mxaF* gene, respectively, 72°C for 1 min, followed by final extension 72ºC for 5 min. The electrophoresis was performed using 1.2% agarose gel to separate the PCR products and presence of bands at corresponding base pairs for each gene were observed under Alphaimager EC.

Estimation of carbon use efficiency of methylotrophic bacteria: Bacterial cultures were inoculated into screw cap bottle containing 50 ml of NMS broth supplemented with 1% of methanol as sole carbon source and one uninoculated tube served as control. To each screw capped bottle, a test tube containing 10 ml 0.1 N NaOH was suspended into the screw cap bottle and tighten the mouth with lid. The setup was incubated at 30ºC for 4 days. After incubation, periodically the amount of carbon respired by the test organisms in each screw capped bottle system was quantified by alkali entrapment method (Stotzky 2016). Briefly the 0.1 N NaOH suspended in the screw capped bottle was removed under aseptic conditions in fresh 100 mL conical flask and 2–3 drops of saturated barium chloride was added and titrated with 0.1 N HCl using phenolphthalein indicator. For time course observation of respiration each screw capped inoculated bottle were replenished with fresh 0.1N NaOH solution. The same procedure was repeated at subsequent intervals of 10 days upto 30 days. Cumulative CO_2 evolution for 30 days was calculated as a sum of the value of CO_2 evolution value at each interval of incubation period. CO_2 evolution and C mineralization/day was calculated by dividing cumulative CO_2 evolution and C mineralization by 30 and expressed in terms of mg of CO_2 evolution/day and mg of C mineralization/day.

$$CO_2 \text{ or C (mg)} = (B-V) \times N \times E$$

Where B, Volume of sulphuric acid (mL) used to titrate the blank (without sample); V, Volume of sulphuric acid (mL) used to titrate sample after CO_2 absorption; N, Normality of HCL.

To express data in terms of CO_2 use E=22 and to express data in terms of C, use E=6.

Screening of methylotrophic isolates for plant growth promotion traits

Nitrogen fixation: Nitrogen fixation potential of the methylotrophic bacterial isolates was qualitatively assessed by culturing these bacteria in a nitrogen free Jensen's medium (Jensen 1958). The Jensen's media was modified for methylotrophic bacteria by replacing 2% sucrose with 1% methanol. The bacteria were streaked on this media to verify their ability to fix nitrogen. After 5 days of incubation at 30°C, we observed the growth of the isolates to ascertain their nitrogen-fixing activity.

Phosphorous solubilisation: P-solubilisation ability of methylotrophic bacterial isolates was qualitatively estimated by using modified Pikovskaya agar media (Pikovskaya 1948) where methanol at 0.5% was the sole carbon source. The methylotrophic bacteria were spot-inoculated onto this medium and incubated at 30ºC for five days. Formation of a clear halo zone around the inoculation spots indicated the phosphate-solubilizing potential of the isolates.

K, ZnO and $ZnCO_3$ solubilisation: The potassium (K) and zinc (Zn) solubilisation abilities of the isolates were qualitatively assessed by observing clear zones around the spot-inoculated colonies on their specific growth media. For potassium solubilisation, a modified Aleksandrov medium was prepared by replacing 5.0 g of glucose with 5.0 ml of methanol (Singh *et al.* 2010). Zn solubilisation activity of the isolates was screened using NMS medium supplemented with 1% methanol, where 100 µg of $ZnSO_4.7H_2O$ was replaced with different zinc salts, including ZnO and $ZnCO_3$ at 1 g/L, to evaluate the isolates ability to solubilize various forms of zinc.

Indole acetic acid (IAA) production: IAA production was evaluated by growing isolates in nutrient broth with or without 10 mg/mL L-tryptophan (Gordon and Weber 1951, Gong *et al.* 2018), at 30ºC for 5 days under shaking. After centrifugation (5000 g, 10 min), 2 ml of supernatant was mixed with orthophosphoric acid and 4 ml Salkowsky reagent (50 mL of 35% perchloric acid, 7.5 ml of 0.5 M iron (III) chloride hexahydrate, and 250 ml distilled water) incubated in the dark for 30 min, and absorbance was recorded at 530 nm.

Exopolysaccharide production (EPS): All methylotrophic bacterial isolates were tested for EPS production following Shahnavaz *et al.* (2015) with minor modifications. Cultures were grown in nutrient agar medium (25 ml) supplemented with 2% sucrose at 30°C for 72 h in 100 ml flasks under shaking (120 rpm). After centrifugation (10,000 rpm, 15 min), the cell-free supernatant was treated with chloroform:butanol (4:1, v/v) to remove proteins and kept overnight on a shaker. EPS was precipitated by adding two volumes of cold ethanol and incubating at 4ºC overnight, followed by centrifugation (15,000 rpm, 20 min, 4ºC). The pellet was dried to obtain EPS dry weight, and carbohydrate content was determined by the Anthrone method (Bala Subramanian *et al.* 2010).

Statistical analysis: The experiments were conducted in triplicate, and the data were analyzed using analysis of variance (ANOVA), followed by Duncan's multiple range test (DMRT) to determine statistical significance at $p \leq 0.05$, using WASP 2.0 software (Web Agri Stat Package, Indian Council of Agricultural Research, India). Mean comparisons were performed using the completely randomised design (CRD) at a significance level of $p \leq 0.05$. Graphs were plotted using Microsoft excel and Origin Pro software 2024.

RESULTS AND DISCUSSION

Isolation and screening of efficient methylotrophic bacteria from rice rhizosphere: Six methanol-utilizing bacterial isolates were obtained from rice rhizosphere soil and distinguished based on colony morphology (Table 1, Fig. 1). Their methanol utilization efficiency was tested on

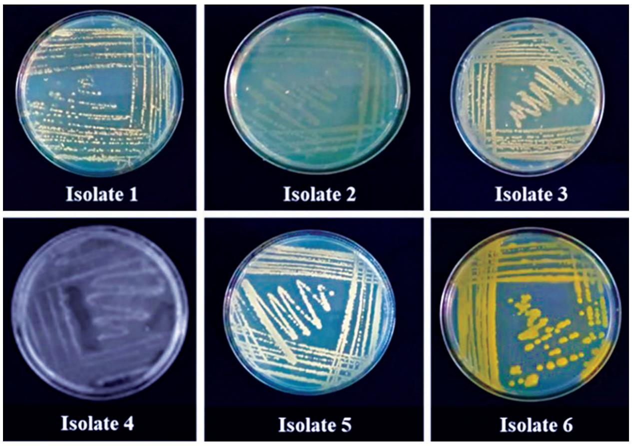

Fig. 1 Morphological and cultural characters of selected methylotrophic bacteria on NMS agar plates supplemented with 1% methanol.

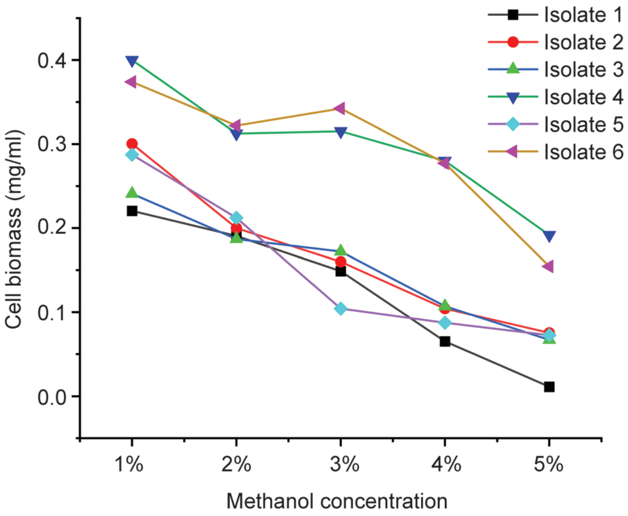

Fig. 2 Cell biomass of selected methylotrophic bacterial isolates by utilizing 1-5 % methanol after 10 days of inoculation.

NMS medium supplemented with 1–5% methanol. Among the isolates, isolate 4 and isolate 6 demonstrated superior growth at 5% methanol, while isolate 1 exhibited the lowest tolerance. Similar reports indicating differences in utilizing methanol by bacterial isolates from rice ecosystem has been reported by Jhala *et al.* (2014).

At 1% methanol, isolate 2, isolate 3, isolate 4, isolate 5, and isolate 6 exhibited biomass increases of 36.3%, 9.3%, 81.4%, 30.4%, and 69.6%, respectively, compared to isolate 1. At 2% methanol, isolate 6 and isolate 4 showed the highest biomass accumulation, with 72.1% and 67.2% increase relative to isolate 3. At 3% methanol, isolate 6 showed nearly 99% more biomass compared to isolate 3. At 5% methanol concentration, the anabolic potential reflected by the conversion of methanol to cell biomass was highest in isolate 4, followed by isolate 6 (Fig. 2). These variations may be attributed to differences in carbon metabolism pathways among the bacterial isolates, which influence their ability to assimilate methanol into biomass (Wu *et al.* 2024).

Molecular characterization and screening of mmoX, pmoA, *and* mxaF *genes involved in methane and methanol metabolism*: The BLAST result of partial sequence of 16S rRNA gene of these selected six bacterial isolates revealed their identity as *Bacillus rugosus* strain MB1, *Bacillus subtilis* strain MB2, *Priestia aryabhattai* strain MB3, *Rhizobium pusense* strain MB4, *Priestia megaterium* strain MB5, and *Sphingobium* spp. strain MB6 (Supplementary Table 1). To further confirm the methylotrophic traits of the isolates, they were tested for the presence of key enzymes involved in methane and methanol metabolism: particulate methane monooxygenase (pMMO), soluble methane monooxygenase (sMMO), and methanol dehydrogenase (MDH) (Hanson and Hanson 1996). Screening of functional genes clearly indicated that *B. subtilis* strain MB2 and *Sphingobium* spp. strain MB6 possessed all three target genes of methane and methanol metabolism (*mmoX*, *pmoA*, and *mxaF*) (Fig. 3). Similar findings were reported by Rusmana *et al.* (2009), who detected *mmoX* and *pmoA* in the BGM 9 strain from Indonesian rice fields. In contrast, *P. aryabhattai* strain MB3 exhibited a distinct band at ~520–550 bp corresponding to the *pmoA* gene, while no amplification was observed at ~800–900 bp for the *mmoX* gene (Fig. 3B, 3A). The result suggested that the co-occurrence of these genes is not universal among methylotrophs, as also emphasized by Stainthorpe *et al.* (2006). A similar observation was made by Madhaiyan *et al.* (2010), who reported variability in the distribution of methylotrophy-associated genes among rice rhizosphere bacteria, indicating differential metabolic potential and ecological adaptation across species. Although

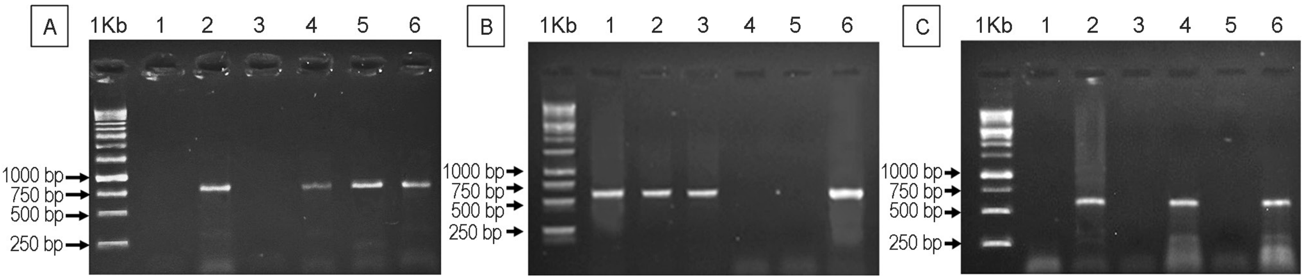

Fig. 3 (A) *mmoX* (soluble methane monooxygenase), (B) *pmoA* (particle methane monooxygenase), (C) *mxaF* (methanol dehydrogenase) gene amplification profile of 6 methylotrophic bacteria isolates. Well number (1) represents *Bacillus rugosus* strain MB1, (2) *Bacillus subtilis* strain MB2, (3) *Priestia aryabhattai* strain MB3, (4) *Rhizobium pusense* strain MB4, (5) *Priestia megaterium* strain MB5, (6) *Sphingobium* spp. strain MB6.

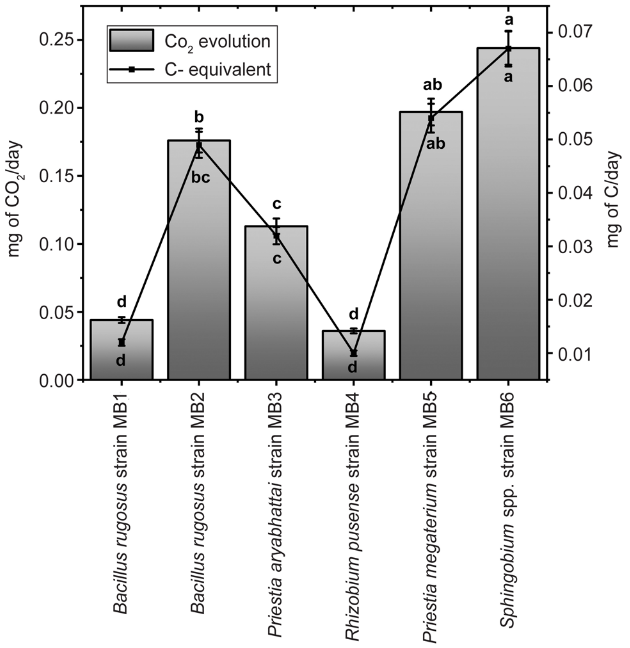

Fig. 4 A comparison of carbon respired as CO_2 and its equivalent loss as C of six selected methylotrophic bacterial.

previous studies have reported absence of the methanol dehydrogenase gene in *Rhizobium* (Jhala *et al.* 2014), the present investigation found *Rhizobium pusense* strain MB4 tested positive for methanol dehydrogenase gene amplification. This indicated that species of methylotrophic genus may differ with respect to the genes responsible for this functional trait. Overall, the results indicated that *Bacillus rugosus* strain MB1, *Priestia aryabhattai* strain MB3, and *Priestia megaterium* strain MB5, although capable of utilizing methanol, did not showed amplification of all the target genes involved in methanol metabolism possibly due to the absence of suitable primers for amplifying the *mxaF* gene.

Methanol use efficiency and specific respiration of methylotrophic bacteria: The methylotrophic bacterial isolates were evaluated with respect to their efficiency in conversion of C1 compounds eg. methanol to their C biomass and its respiration to carbon dioxide is conventionally referred as methanol use efficiency. The cumulative carbon dioxide evolution of the isolates was estimated by alkali entrapment method for a duration of 30 days. The carbon dioxide evolution per day and its equivalent loss of C/day varied significantly among the 6 methylotrophic bacteria and ranged from 0.036 to 0.244 mg of CO_2/day and 0.010 to 0.067 mg of C/day, respectively (Fig. 4). Growth under 1% methanol as the sole carbon source was assessed by measuring total protein content, which also varied significantly among the bacteria, ranging from 63.02–146.51 µg protein/ml (Supplementary Fig. 1A). The highest protein content was recorded for *Sphingobium* spp. strain MB6 (146.51 µg/ml), which also showed the maximum CO_2 evolution (0.24 mg of CO_2/day) while *R. pusense* strain MB4 exhibited the lowest (0.030 mg/24 h) but accumulated high protein biomass (138.73 µg/mL) (Fig. 4, Supplementary Fig. 1A). These variations in respiratory carbon losses among the test organisms confirm that methylotrophs differ significantly in their ability to convert methanol to CO_2. Notably, the lowest specific respiration (0.009 mg of CO_2/µg of protein) was recorded in *R. pusense* strain MB4, followed by *Bacillus subtilis* strain MB2 (0.022 mg of CO_2/µg of protein), indicating that *R. pusense* strain MB4 was the most efficient organism in terms of methanol use efficiency (Supplementary Fig. 1B). The difference in methanol use efficiency among the bacterial isolates are likely influenced by a combination of physiological, environmental, and ecological factors that affect microbial metabolic pathways, ultimately determining the proportion of assimilated carbon converted into biomass versus that lost as CO_2 through respiration (Wu *et al.* 2024).

Plant growth-promoting (PGP) traits of methylotrophic isolates: Plant-associated methylotrophic bacteria with plant growth-promoting traits have been widely reported from diverse ecological niches, including the phyllosphere, rhizosphere, and endosphere, across various plant species (Raja *et al.* 2008, Alibrandi *et al.* 2018, Yadav *et al.* 2019, Yousaf *et al.* 2024). All six isolates tested positive for nitrogen fixation on Jensen's medium supplemented with methanol, although with varying intensity. *B. rugosus* MB1 and *R. pusense* strain MB4 showed the highest nitrogen fixation ability, while *B. subtilis* strain MB2 was the least (Table 2). This aligned with earlier studies reporting nitrogen-fixing methylotrophs such as *Methylosinus, Methylocystis*, and *Rhizobium* spp. from paddy ecosystems (Cao *et al.* 2022, Yu *et al.* 2024). Nitrogen-fixing methylotrophs are widely prevalent; for instance, root-associated diazotrophic bacteria such as *Methylosinus* and *Rhizobium* have been found to dominate the root microbiomes of *Scirpus triqueter* and *Typha angustifolia*, respectively (Cui *et al.* 2022). Another study *Methylobacterium* spp. SEMIA 658 showed amplification of gene responsible for biological nitrogen fixation (BNF) such as *nifH* and *nodC*, which enable them to fix atmospheric nitrogen (Menna *et al.* 2006).

Phosphorus solubilization was observed in 3 methylotrophs highest in *R. pusense* strain MB4 followed by *B. subtilis* strain MB2 and *B. rugosus* strain MB1, while potassium solubilization was exhibited by *R. pusense* strain MB4, *P. megaterium* MB5 and *Sphingobium* spp. strain MB6. Zinc solubilization was widespread, with *P. megaterium* MB5 and *B. rugosus* MB1 showing strong activity for ZnO and *R. pusense* strain MB4 for $ZnCO_3$. Previous studies have similarly demonstrated the role of methylotroph *Paenibacillus polymyxa* MaAL70, isolated from the phyllosphere of rice plants in potassium (K) and zinc (Zn) solubilization activity (Rani *et al.* 2021). Similarly, *Priestia megaterium* A25 and *Sphingobium chungbukense* Sx8-8 demonstrated dual solubilization of phosphorus (P) and Zn. These findings collectively highlight the potential of methylotrophic bacteria in enhancing nutrient availability and promoting plant growth through solubilization of essential minerals. IAA production also varied considerably, ranging from 0.1–15.34 µg/mL (without tryptophan) and

Table 2 Overview of PGPR traits of selected methylotrophic bacteria isolated from rice-rhizosphere

Isolates	N-fixation	P-solubilization	K-solubilization	ZnO-solubilization	$ZnCO_3$-solubilization	IAA production (µg/mL)	
						Without tryptophan[a]	With tryptophan[a]
Bacillus rugosus strain MB1	+++	+	-	++	++	14.10±0.42	26.31±1.23
Bacillus subtilis strain MB2	+	+	-	-	-	0.1±2.41	0.4±0.97
Priestia aryabhattai strain MB3	++	-	-	+	-	--	--
Rhizobium pusense strain MB4	+++	++	+	+	+++	11.62±1.65	42.41±0.45
Priestia megaterium strain MB5	++	-	+	++	++	--	--
Sphingobium spp. strain MB6	++	-	+	+	+	15.34±0.78	36.12±0.36

+, low level of a specific trait; ++, Moderate level of the trait; +++, High level or strong expression of the trait; [a]Values of IAA production are average of 3 replicates ± SD (n = 3).

0.4–42.41 µg/mL (with tryptophan). The highest IAA concentration was observed in *R. pusense* strain MB4 (42.41 µg/mL) under tryptophan enrichment, followed by *Sphingobium* spp. strain MB6 (36.12 µg/mL) and *B. rugosus* strain MB1 (26.31 µg/mL). By contrast, *B. subtilis* strain MB2 produced negligible amounts under both conditions. In the absence of tryptophan maximum production reported by *Sphingobium* spp. strain MB6 (15.34 µg/mL) followed by *Bacillus rugosus* (14.10 µg/mL). The observed levels of IAA production were higher than earlier reports for methylotrophs (Krishnamoorthy *et al.* 2020, Gamit and Amaresan 2024), suggesting strong auxin-producing potential of the isolates. The observed variation in quantity of IAA produced, particularly when the growth medium was supplemented with tryptophan, a known precursor for IAA biosynthesis suggests differential metabolic capacities among the isolates for IAA production under tryptophan-enriched conditions. This finding underscores the enhanced IAA-producing capacity of the isolates evaluated in this study, particularly *R. pusense* MB4, which may have implications for their potential application in plant growth promotion under field conditions.

Exopolysaccharide (EPS) production by methylotrophic bacteria is recognized as a key plant growth-promoting trait, primarily due to its role in enhancing soil aggregation in the rhizosphere, thereby improving moisture retention and nutrient availability to plants (Ghosh and Maiti 2016). Additionally, EPS contributes to increased tolerance of plants to both biotic and abiotic stresses. EPS dry weight and their corresponding total carbohydrate content of the selected methylotrophic bacterial isolates were varied between 0.28–1.08 mg/mL and 0.034–0.592 mg/mL, respectively (Supplementary Fig. 2). The highest EPS production was observed in *Sphingobium* spp. (1.08 mg/mL), while the lowest was recorded in *B. rugosus* (0.28 mg/mL). These differences in EPS production are indicative of varying carbon utilization efficiencies among the isolates. Interestingly, *R. pusense* strain MB4, which exhibited the lowest specific respiration rate, appears to demonstrate the highest carbon use efficiency. This is likely due to its effective allocation of carbon and energy towards the synthesis of functional biomolecules and enzymes associated with key plant growth-promoting activities. Such metabolic efficiency suggested that *R. pusense* MB4 may serve as a particularly effective bioinoculant for enhancing plant productivity under diverse environmental conditions.

The present study characterized rice rhizosphere methylotrophic bacteria and were found to efficiently metabolize methanol. This trait was attributed to the presence of key functional genes associated with metabolism of single carbon compounds. This metabolic trait underscores their ecological role in carbon cycling and potential for sustainable agriculture. In addition, plant growth promoting attributes present in these organisms qualify them as promising candidates for bio-inoculant development aimed for enhancing crop productivity. All together these organisms are promising candidates for bioinoculant formulations with dual capacity of mitigating greenhouse gas emissions as well as enhancing the rice crop growth. To harness their optimal potential, multi-location field studies needs to be undertaken to develop climate-resilient low nutrient input rice cultivation systems.

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