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Isolation, Screening and Characterization of Plant Growth Promoting Methylotrophic Bacteria from Paddy Ecosystem

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A B S T R A C T

Now a days there is a growing interest among the farmers for using naturally occurring microorganisms in agriculture to reduce the use of chemical fertilizers and pesticides in agriculture and increase the application of organic inputs like biofertilizers and biocontrol agents. Among different microorganisms the *Rhizobium, Azotobacter, Azospirillum*, *Mycorrhiza*, *Trichoderma* and *Pseudomonas fluorescens* have been explored and registered for commercial use. However, the use of methylotrophic microorganisms in agriculture have not been explored much, hence an experiment was conducted to isolate, screen and characterize the efficient plant growth promoting methylotrophic bacteria from rhizosphere soils of rice. As many as fifteen methylotrophic bacteria were isolated from the rhizosphere soil of paddy. Further, all the isolates were screened for their plant growth promotional traits *viz.,* phosphate, potassium, zinc and silicon solubilization. Out of 15 isolates tested, 4 bacterial isolates (SMB – 1, SMB – 5, SMB – 6 and SMB – 10) showed maximum phosphate solubilization index of 2.53, 3.10, 5.00 and 5.03 respectively. Further the same isolates also showed good potassium and zinc solubilization potentialities under *in vitro* conditions as they resulted potassium solubilization index of 4.77, 5.56, 4.03 and 4.13. Similarly, with respect to zinc solubilization the efficient isolates showed 4.56 by SMB - 5, 2.69 by SMB – 6 and 6.05 by SMB - 10 of zinc solubilization index. However, among four efficient methylotrophic bacterial isolates, the SMB – 10 showed the silicon solubilizing ability (0.72 silicon solubilizing index) in *in vitro* conditions. Further, based on morphological and biochemical characterization studies, the efficient isolates were identified as *Methylococcus* sp, *Methylobacterium* sp*, Methylosarcina* sp *and Methylorubrum* sp. Scale of studied are required to develop and evaluate the efficient methylotrophic bacteria consortia on growth and yield of rice under pot and field condition.

Introduction

As we enter the third millennium with more than six billion people, we are confronted with a herculean task of providing environmental and food security to the expanding population particularly in the developing countries. This calls for the reorientation of strategies to minimize the use of external inputs in agriculture and depend more on eco-friendly approaches to sustain food production without causing disruption to the fragile agroecosystem.

Soil microorganisms play a vital role in the field of agriculture by converting the unavailable form of nutrients to available form to the plants by various mechanisms like solubilization of insoluble nutrients and fixation of the nutrient element present in atmosphere. However, they also play an important role in controlling many of the plant pathogens and insects. The relationship between the microorganisms and plants is considered as symbiotic when both partners benefit from each other. So far considerable number of microbial species mostly associated with the plant rhizosphere have been tested and found to be beneficial to plant growth, yield and crop quality (Pyrlak and Kose, 2009).

There are many bacteria present in rhizosphere and phyllosphere, which probably lead a saprophytic lifestyle, feeding on materials leached from the roots and leaves, one of the such example of bacteria is methylotrophic bacteria.

These are physiologically an interesting group of bacteria able to grow on methanol, methylamine as well as on a variety of C2, C3 and C4 compounds (Lidstrom *et al.,* 1998) and presently, they are receiving more attention as plant growth promoting bacteria. Among methylotrophic bacteria the members of the genus *Methylobacterium* are versatile in nature and ubiquitous in rice rhizosphere. The association of methylotrophic bacteria with plants extends from free living to epiphytic, endophytic and symbiotic and their presence has been detected by Jackson *et al.,* (2006).

Methanotrophs are methane-utilizing bacteria which converts CH_4 into CO_2 by the sequential activity of enzymes. Methanotrophs are found in a various environment, including soil, water and rhizosphere of plants. They have been shown to have a significant impact on growth and yield of rice. Methanotrophs increase the availability of nitrogen to rice crop, which improve the chlorophyll content. They also involved in the photosynthetic process, (Taopan *et al*., 2018). Further, methanotrophs association with rice also influences the growth habits due to the release of phytohormones *viz.,* Cytokinins, Auxins, Indole Acetic acid, Gibberellic acid and other bioactive compounds (Doronina *et al*., 2004). Methanotrophs can help to break down organic matter in the soil, which can release nutrients such as nitrogen, phosphorus and potassium, but the plant growth promoting methylotrophic bacteria are not been explored by the farmers because of not much scientific work has been carried out for exploitation of methylotrophic microorganism in agriculture and horticulture crop production.

However, it is necessary to carefully coordinate the materials, environment and the technologies constituting the method. Hence, by considering the importance of methylotrophic bacteria as plant growth promoting bacteria an attempt was made to isolate, screen and characterize the efficient plant growth promoting methylotrophic bacterial isolates from the low land soil ecosystem.

Material and Methods

Sample collection

The leaf and rhizosphere soils were collected from paddy growing areas of Shivamogga district. The samples were brought to the laboratory aseptically in sterile polythene bags and stored at 4° C in refrigerator for further isolation of methylotrophic bacteria.

Isolation of methylotrophic bacteria

Methylotrophs were isolated using Ammonium Mineral Salts (AMS) medium supplemented with 0.5% (v/v) methanol as the sole carbon source (Whittenburry *et al.,* 1970). Leaf samples were processed using the leaf imprinting method (Corpe, 1985), while rhizosphere soil samples were serially diluted and plated on AMS agar. Colonies appearing after 3–7 days at 30°C were purified by streak plating and maintained on peptone glycerol agar slants at 4°C (Skinner *et al.,* 1952).

The representative colonies growing on the AMS media were purified by the streak plate method and were preserved on Peptone Glycerol Agar (Enrichment medium) slants at 4°C in a refrigerator for further use (Corpe and Rheem, 1989).

In vitro screening of methylotrophic bacterial isolates for plant growth promotional activities

The pure cultures of isolated native methylotrophic bacterial isolates were screened for the plant growth promotional traits *viz., phosphorus, potassium, silicon and zinc* solubilization using specific media like Sperber's, Alexandrove's, Bunt and Rovira's with $Mg_2Si_3O_8$ and mineral salt media supplemented with 1 per cent zinc oxide respectively. The nutrient solubilization efficiency was calculated based on percent solubilization index of P, K, Si and Zn using the formula given by Pande *et al.,* 2017.

$$Nutrient\ Solubilization\ Index = \frac{\text{Halo zone diameter} + \text{Colony diameter}}{\text{Colony diameter}}$$

The isolates showing the maximum zone of solubilization on the representative media were selected for further characterizations studies.

Selection and characterization of efficient methylotrophic bacterial isolates

The methylotrophic bacterial isolates showing superior performance in all the plant growth promotion traits were selected for further characterization studies.

The colony morphology (size, shape, pigmentation, elevation) (Anon (1957) and Gram staining (Rangaswami, 1975) and biochemical tests *viz.,* catalase, nitrate reduction, citrate utilisation, indole, Voges Proskauer's test, methyl red, urease, casein and starch hydrolysis, gelatin liquefaction, acid/gas production, oxidase were done using standard protocols and the efficient isolates were identified tentatively up to genus level (Barthalomew and Mittewer,1950).

Statistical analysis

The statistical analysis of the data was carried out for completely randomized design (Panse and Sukhatme, 1985) as well as for Dunkun's multiple range test (Steel and Torrie, 1960).

Results and Discussion

The present study focused on the isolation, screening and characterization of methylotrophic bacterial isolates from paddy ecosystems.

Collection of Samples

The leaf and rhizosphere soil samples were collected from different paddy growing regions of Shivamogga district (Table 1).

Isolation of Methylotrophic Bacteria

As many as fifteen methylotrophic bacteria were isolated from the soil and leaf samples collected from paddy ecosystem. The details of the isolates and locations from where the samples were collected are presented in Table 2. Further for easy identification all the bacterial isolates were designated Sahyadri Methylotrophic Bacteria (SMB), which were indicated with code number as SMB – 1, SMB – 2, SMB – 3, SMB – 4, SMB – 5, SMB – 6, SMB – 7, SMB – 8, SMB – 9, SMB – 10, SMB – 11, SMB – 12, SMB – 13, SMB – 14 and SMB –15 (Plate 1). The prefix SMB is the brand name of Keladi Shivappa Nayak University of Agricultural and Horticultural Sciences, Shivamogga.

The native methylotrophic bacteria were isolated from leaf and rhizosphere soil samples collected from paddy fields of Shivamogga district using AMS agar medium supplemented with 0.5% (v/v) methanol, following leaf imprinting (Corpe, 1985) and soil serial dilution (Skinner *et al*., 1952) techniques. Methanol served as the sole carbon source, ensuring the selective growth of methylotrophs (Whittenburry *et al*., 1970) and a total of fifteen isolates were obtained, consistent with earlier reports highlighting flooded rice ecosystems and phyllosphere as major habitats of methylotrophic bacteria (Kim *et al.,* 2010; Jahan and McDonald, 2023; Dhamodharan and Rajasekar, 2013).

In vitro screening of methylotrophic bacterial isolates for plant growth promoting activities

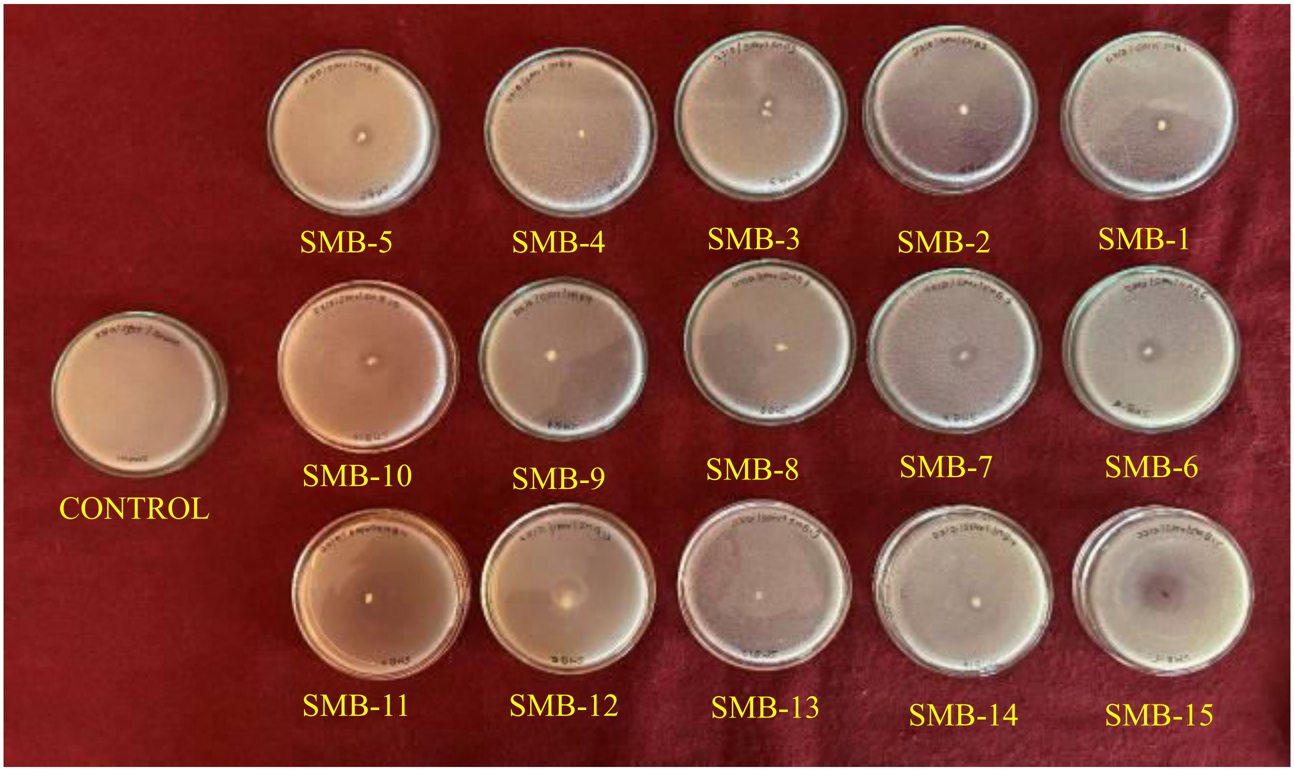
In the *In vitro* screening studies, out of the fifteen methylotrophic bacterial isolates tested for plant growth promotional activities revealed the potentiality of isolated methylotrophic bacterial isolates to solubilize nutrients such as phosphorous, potassium, zinc and silicon (Table 3).

Among the isolates, eight isolates exhibited phosphorus solubilization with phosphorus solubilization index (PSI) values ranging from 2.03 to 6.03, wherein SMB-15 recorded the highest PSI (6.03), followed by SMB-10 (5.03) (Table 4 and Plate 2).

Plate.1 Methylotrophic bacterial isolates isolated from paddy ecosystem

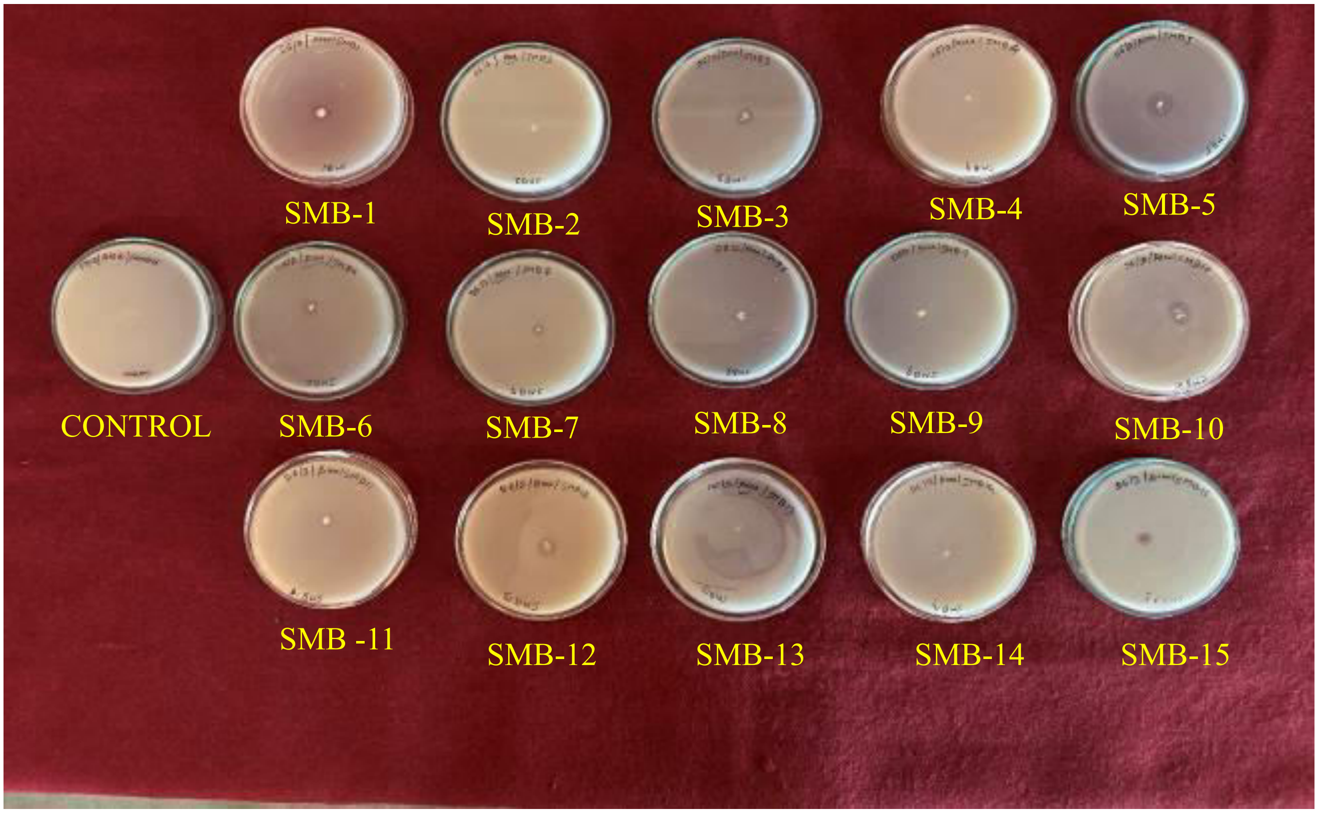
Note: SMB – Sahyadri Methylotrophic Bacteria

Plate.2 Phosphorous solubilization potentiality of methylotrophic bacterial isolates



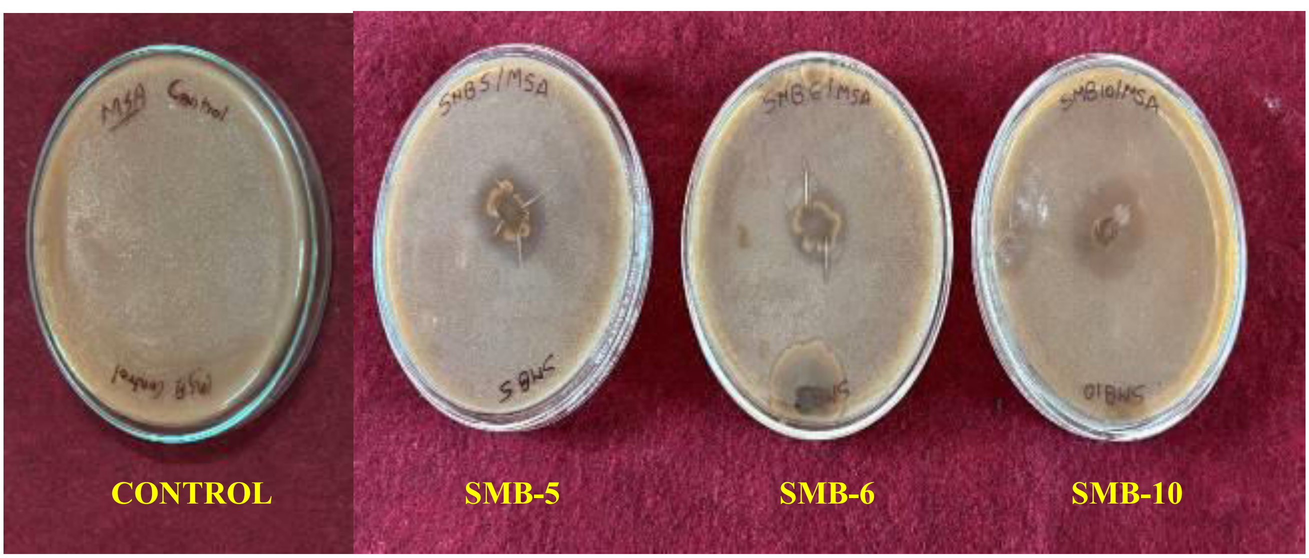
Note: SMB – Sahyadri Methylotrophic Bacteria

Plate.3 Potassium solubilization potentiality of methylotrophic bacterial isolates



Note: SMB – Sahyadri Methylotrophic Bacteria

Plate.4 Zinc solubilization potentiality of methylotrophic bacterial isolates



Note: SMB – Sahyadri Methylotrophic Bacteria

Plate.5 Silicon solubilization potentiality of methylotrophic bacterial isolates

Note: SMB – Sahyadri Methylotrophic Bacteria

Table.1 Locations of samples collected for isolation of methylotrophic bacteria

Sl. No.	Village	Taluk	Soil types	Sample type
1.	Kudaruru	Sagar	Lateritic soil	Paddy rhizosphere soil and leaves
2.	Belamakki	Tirthahalli	Red loamy soil	Paddy rhizosphere soil and leaves
3.	Sullali	Sagar	Lateritic soil	Paddy rhizosphere soil and leaves
4.	Mumbaru	Hosanagara	Lateritic soil	Paddy rhizosphere soil and leaves
5.	Chamundipura	Sagar	Red loamy soil	Paddy rhizosphere soil and leaves
6.	Mandagadde	Tirthahalli	Red loamy soil	Paddy rhizosphere soil and leaves
7.	Doddamane	Sagar	Lateritic soil	Paddy rhizosphere soil and leaves

Table.2 Methylotrophic bacterial isolates isolated from different locations of Shivamogga region

Sl. No.	Village	Isolates
1.	Kudaruru	SMB - 1, SMB - 2
2.	Belamakki	SMB - 3, SMB - 4
3.	Sullali	SMB - 5, SMB - 6
4.	Mumbaru	SMB - 7, SMB - 8, SMB - 9
5.	Chamundipura	SMB - 10, SMB - 11
6.	Mandagadde	SMB -1 2, SMB - 13
7.	Doddamane	SMB - 14, SMB - 15

Note: SMB = Sahyadri Methylotrophic Bacteria

Table.3 Plant growth promotional activity by methylotrophic bacterial isolates

Sl. No.	Isolate	Plant Growth Promotional activity			
		PSI	KSI	ZnSI	SiSI
1.	SMB - 1	++	++	-	-
2.	SMB - 2	-	-	-	-
3.	SMB - 3	+	+	-	-
4.	SMB - 4	-	-	-	-
5.	SMB - 5	++	++	++	-
6.	SMB - 6	++	++	++	-
7.	SMB - 7	+	+	-	-
8.	SMB - 8	-	-	-	-
9.	SMB - 9	-	-	-	-
10.	SMB - 10	++	++	++	++
11.	SMB - 11	-	+	-	-
12.	SMB - 12	-	+	-	-
13.	SMB - 13	-	-	-	-
14.	SMB - 14	+	+	-	-
15.	SMB - 15	+	+	-	-

Note:
1) SMB = Sahyadri Methylotrophic Bacteria, 2) Maximum halo zone produced indicated by "++"0
3) **T**he presences of activity indicated by "+" and the absence of activity indicated by "-"
4) PSI = Phosphorous solubilization index, 5) KSI = Potassium Solubilization Index
6) ZnSI = Zinc Solubilization Index, 7) SiSI = Silicon Solubilization Index

Table.4 Phosphorous solubilization potentiality of methylotrophic bacterial isolates

Sl. No.	Isolate	PSI
1.	SMB – 1	2.53[e]
2.	SMB - 3	2.53[e]
3.	SMB - 5	3.10[d]
4.	SMB - 6	5.00[b]
5.	SMB - 7	4.53[c]
6.	SMB - 10	5.03[b]
7.	SMB - 14	2.03[f]
8.	SMB - 15	6.03[a]
S. Em. ±		0.10
P		< 0.01
CV		4.49
CD at 1%		0.42

Note:
1) SMB = Sahyadri Methylotrophic Bacteria
2) PSI = Phosphorous Solubilization Index
3) Means followed by the same letters do not differ significantly

With respect to potassium solubilization on Aleksandrov's agar medium supplemented with mica, ten isolates showed potassium solubilization index (KSI) values between 2.15 and 5.56, of which SMB-5 showed the highest potassium solubilization (5.56), followed by SMB-1 of 4.77 (Table 5, Plate 3). Whereas only three isolates (SMB - 5, SMB - 6 and SMB - 10) showed zinc solubilization activity on mineral salt agar containing ZnO and SMB-10 recorded the highest zinc solubilization index (ZnSI) of 6.05 (Table 6, Plate 4) and finally with respect to silicon solubilization the SMB-10 showed measurable silicon solubilization on Bunt and Rovira medium supplemented with magnesium trisilicate, recording a silicon solubilization index (SiSI) of 0.72 (Plate 5). *In vitro* screening revealed that eight isolates solubilized phosphorus with PSI values ranging from 2.03 to 6.03, the highest by SMB-15 (6.03), in agreement with earlier findings of Jayashree *et al*. (2011) and Joel *et al.* (2023). Potassium solubilization was observed in ten isolates with SMB-5 recording the maximum (5.56), similar to the results of Rani *et al*. (2021).

Zinc solubilization was limited to SMB-5, SMB-6 and SMB-10, with SMB-10 showing the highest ZnSI (6.05), supporting previous observations that methylotrophs enhance zinc availability via organic acids and siderophores (Rani *et al*., 2021; Joel *et al*., 2023). Silicon solubilization was rare, with only SMB-10 exhibiting measurable activity (0.72 SiSI), corroborating the findings of Zhang (2024).

Selection of efficient methylotrophic bacterial isolates

Based on the cumulative evaluation of phosphorus, potassium, zinc and silicon solubilization abilities, four isolates (SMB-1, SMB-5, SMB-6 and SMB-10) were selected for further characterization studies.

Morphological and biochemical characterization

Morphological characteristics of the bacterial isolates were studied on AMS media at room temperature (27 ± 2 °C). When bacteria reached its maximal growth on AMS medium, the observations such as colony morphology, pigmentation, cell shape and Gram's reaction of the bacteria were recorded (Table 7). The results indicated that the isolate SMB-1 produced circular, convex and smooth margin colony with White/cream pigment, coccoid cell shape, and found negative for Gram's reaction. Similarly, the isolate SMB-5 produced circular, raised and smooth margin, light cream pigmented colony and were Gram negative rods. The isolate SMB-6 produced circular, entire with smooth margin, pigment as pale cream, sarcina like cocci in tetrads as cell shape, and showed negative for Gram reaction. However, with respect to isolate SMB-10, it was observed that isolate produced convex, smooth with entire margin, light pink colour pigment, and were Gram negative rods.

Table.5 Potassium solubilization potentiality of methylotrophic bacterial isolates

Sl. No.	Isolate	KSI
1.	SMB - 1	4.77^{b}
2.	SMB - 3	3.22^{d}
3.	SMB - 5	5.56^{a}
4.	SMB - 6	4.03^{c}
5.	SMB - 7	2.60^{de}
6.	SMB - 10	4.13^{bc}
7.	SMB - 11	2.47^{de}
8.	SMB - 12	2.83^{de}
9.	SMB - 14	2.15^{e}
10.	SMB - 15	2.50^{de}
S. Em. ±		0.24
P		< 0.01
CV		7.92
CD at 1%		0.95

Note: 1) SMB = Sahyadri Methylotrophic Bacteria; 2) KSI = Potassium Solubilization Index; 3) Means followed by the same letters do not differ significantly

Table.6 Zinc solubilization potentiality of methylotrophic bacterial isolates

Sl. No.	Isolate	ZnSI
1.	SMB - 5	4.56[b]
2.	SMB - 6	2.69[c]
3.	SMB - 10	6.05[a]
S. Em. ±		0.25
P		< 0.01
CV		9.20
CD at 1%		1.24

Note: 1) SMB = Sahyadri Methylotrophic Bacteria; 2) ZnSI = Zinc Solubilization Index; 3) Means followed by the same letters do not differ significantly

Table.7 Morphological and biochemical characterization of efficient methylotrophic bacterial isolates

Isolate	Morphological Test		Biochemical Test														Probable genus
	Colony characterization	Gram's staining and Cell shape	CT	H_2S	IP	NR	MR	VP	CH	CU	UA	SH	GL	AP	GP	OT	
SMB - 1	Circular convex and Smooth margin	G^{-ve} Coccus	+	+	-	+	-	-	+	+	-	+	+	+	-	+	*Methylococcus* sp.
SMB - 5	Circular raised and Smooth margin	G^{-ve} Rods	+	-	-	+	-	+	-	+	+	-	+	+	-	+	*Methylobacterium* sp.
SMB - 6	Circular and Smooth margin	G^{-ve} Sarcina	+	-	-	+	+	+	-	+	+	-	-	+	-	+	*Methylosarcina* sp.
SMB - 10	Convex and Smooth margin	G^{-ve} Rods	+	+	-	+	-	+	-	+	+	+	-	+	-	+	*Methylorubrum* sp.

Note: Positive result indicated by" +", Negative result indicated by" –", SMB = Sahyadri Methylotrophic Bacteria, CT = Catalase test, H_2S = H_2S production, IP= Indole production, NR= Nitrate reduction, MR= Methyl red test, VP=Voges Proskauer's test, CH= Casein Hydrolysis, CU= Citrate utilization, UA=Urease activity, SH= Starch Hydrolysis, GL= Gelatin Liquefaction, AP =Acid production, GP= Gas Production, OT= Oxidase test

All the efficient bacterial isolates were subjected to biochemical tests, the results of were interpreted and were presented in Table 7. The perusal of Table 7 clear reveals that, All the four isolates were positive for catalase, nitrate reduction, citrate utilization, acid production and oxidase test. All four isolates were negative for indole production and for gas production. However, the SMB-6 was positive for methyl red test, for Voges Proskauer's test is positive for SMB-5, SMB-6 and SMB-10. For casein hydrolysis SMB-1 was positive. for urease activity observed positive for SMB-5, SMB-6 and SMB-10. Further for starch hydrolysis SMB-1 and SMB-10 were positive and for gelatin liquefaction SMB-1 and SMB- 5 gave positive results and for H_2S production SMB-1 and SMB-10 were positive. Further, based on morphological and biochemical characterization the efficient methylotrophic bacterial isolates were tentatively identified as SMB - 1 as *Methylococcus* sp., SMB – 5 as *Methylobacterium* sp., SMB – 6 as *Methylosarcina* sp. and SMB – 10 as *Methylorubrum* sp. The findings are in line with the results of Senthilkumar and Krishnamoorthy (2017) who characterized methylotrophic bacterial isolates based on morphological and biochemical characterization.

In conclusion, the comprehensive study is done for the successful isolation, screening and characterization of methylotrophic bacteria from rice ecosystem, providing valuable insights into their potential as plant growth promoting activity and the efficient methylotrophic microbial consortia developed during the course of investigation will be very much useful for the sustainable production of rice and also have the wide adoptability and efficiency in the native region and also the effective native methylotrophic microbial consortia will pave a way to apply low-cost technology to increase the yield of rice.

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Author Contributions

C. K. Kavya: Investigation, formal analysis, writing—original draft. M. S. Nandish: Validation, methodology, writing—reviewing. C. J. Sridhara:—Formal analysis, writing—review and editing. G. N. Thippeshappa: Resources, investigation writing—reviewing. M. E. Shilpa: Validation, formal analysis, writing—reviewing. J. N.Ashish : Conceptualization, methodology, data curation, supervision, writing—reviewing the final version of the manuscript.

Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethical Approval Not applicable.

Consent to Participate Not applicable.

Consent to Publish Not applicable.

Conflict of Interest The authors declare no competing interests.

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