Ecosystems having anoxic conditions favour the growth of methanogenic archaea leading to the production of methane. In such ecosystems, diversity of methane utilizing microbes is also present in abundance which downregulates the net CH$_4$ emission (Rusmana and Akhdiya 2009, Pandit et al. 2016). Since the last decade research worldwide is focused on finding novel genera of methane-oxidizing microbes which can be used for reducing the net methane emission for wetland ecosystems and thereby mitigating the harmful effect of methane on global warming. Evaluation of known plant growth-promoting strains for their methane degrading potential has unveiled microbes with multiple utilities (Jhala et al. 2014). We isolated diverse groups of bacteria and few types of yeast from different flooded paddy ecosystems of India to develop a microbial formulation capable of promoting paddy growth and reduction in net methane flux. The ability to use methane as a substrate for growth is mainly attributed to prokaryotic organisms. Besides prokaryotes, certain yeast isolates are also capable of utilizing single carbon compounds like methane and methanol. In the presence of methanol, enzymes involved in its metabolism get strongly induced and lead to the massive proliferation of peroxisomes containing key enzymes of methanol metabolism (Yurimoto et al. 2011). Methanol metabolism has been reported in several yeast genera Candida, Pichia, Meyerozyma, Ogataea, Kuraishia, and Komagataella (Limtong et al. 2008). However, very scanty reports are available on the ability of yeast to utilize methane. Wolf and Hanson (1979) worked on isolation and characterization of CH$_4$ utilizing yeasts but no further research confirming the CH$_4$ oxidizing ability of yeast is available. However, plant growth-promoting traits in yeast belonging to different yeast genera have been reported (Park et al. 2013; Nakayan et al. 2013). The present study was carried out in 2017-18 at the ICAR-Indian Agricultural Research Institute, New Delhi with the aim to identify and characterize yeasts inhabiting the flooded paddy ecosystem having methane oxidation and plant growth promoting attributes.

MATERIALS AND METHODS

Samples were collected (2017) from five conventional rice-growing regions of India (irrigated and rainfed), viz. lowland rainfed region of Brahmaputra Valley - Assam (26°08'14 N, 90°11'48 E), deep water paddy, Kochi - Kerala (10°03'14 N, 76°15'13 E), medium land rainfed region of Aduthurai - Tamil Nadu (11°00'29 N, 79°28'42 E), upland irrigated paddy growing region of the Indo-Gangetic plain of Gaya - Bihar (24°49'54"N, 85°03'22"E) and Varanasi - Uttar Pradesh (25°10'47 N, 82°52'33 E). From each region, a water logged paddy field with known cropping history was selected and five rice plants were uprooted randomly at the tillering stage along a zigzag path (Zigzag sampling) to account for the randomness. The samples were kept in sterile poly bags and transported to the laboratory in an insulated container at 4°C.
The samples were enriched for 7 d in ammonium mineral salt (AMS) broth containing 0.5% methanol as the sole C source (Whittenbury et al. 1970). The 0.1 mL aliquot of the serially diluted enriched samples were spread plated on AMS agar and incubated at 30 °C. After incubating for 6 d, the colonies obtained on the plates were picked and purified. All the morphotypes were assessed for their ability to utilize methane as the sole C source of growth on nitrate mineral salt (NMS) agar (Whittenbury et al. 1970) in an airtight chamber under 1% methane-air atmosphere. After incubating for 7 d at 30 °C, the colonies were screened for their ability to oxidize alkanes by the qualitative screening of naphthalene oxidation and detection of oxygenase activity in presence of diazo dye, o-dianisidine, as described by Graham et al. (1992). The isolates showing positive oxidation ability were identified through sequence analysis of the 16S rRNA gene amplified from genomic DNA (Edward et al. 1989). In certain isolates, no amplification of the 16S rRNA gene was observed; hence, they were subjected to amplification of ITS region which is specific for fungal identification (White et al. 1990). The PCR product was outsourced to Agrigenome Private Limited, Kochi, India for Sanger sequencing. A similarity search of all the sequences was done using the nBLAST at the NCBI GenBank database. The % methane utilization by the yeast isolates were quantified by growing each of the isolate in a 50 mL NMS broth having 1% methane as the sole C source in triplicate. The flasks were made airtight with a septa and 1% methane was injected through a sterile 0.2 μm syringe filter by replacing the equivalent quantity of air. Gas samples were withdrawn through a syringe and analysed for residual methane concentration using GC at 0, 3, 6 and 9 d of incubation under shaking conditions at 30 °C. The growth of the yeast isolates in the NMS broth was quantified by estimating total protein by Bradford’s method. The P and K solubilization in all the yeast isolates was estimated qualitatively by formation of solubilization halo zone around spot growth of the isolates on Pikovskaya agar (Nautiyal 1999), and Alexandrov medium (Amprayn et al. 2012), respectively. In both the medium the C sources were replaced with 0.5% methanol. For qualitative estimation of Zn solubilization activity, 3 sets of AMS agar was used, each having a different Zn salt, viz. ZnO, ZnCO₃, and Zn₃(PO₄)₂ @ 1 g/L. After the detection of solubilization halo zone around the spot growth of yeast isolates the P, K and Zn solubilization index were calculated by the formula “the ratio of the total diameter (colony + halo zone) to the colony diameter”. The isolates showing positive P solubilization were further evaluated for quantitative P solubilization by growing them in the above mentioned Pikovskaya’s broth for 5d at 30°C. The culture suspension was centrifuged at 5000g for 15 min and the supernatant was used to estimate P solubilization by ascorbic acid method (Olsen 1954).

The IAA production by the yeast isolates was quantified by growing them for 3 d (log phase) at 30°C in AMS broth supplemented with and without 100 μg/L tryptophan. The broth was centrifuged at 5000 g for 10 min after incubation and IAA was quantified in the supernatant as per the method described by Gordon and Weber (1951). The cell pellet was used for the quantification of total protein by Bradford’s method. Data were subjected to analysis of variance (ANOVA) using software SPSS ver. 10 and least significant difference (LSD) at P≤0.05 among means compared using standard error.

RESULTS AND DISCUSSION

Isolation, identification and oxygenase activity of the methane-oxidizing yeast isolated from rice ecosystem: From different sampling locations, 123 isolates showed positive oxygenase activity when grown in NMS agar with methane as the sole C source. Out of 123 isolates, 10 were identified as yeasts belonging to phylum Ascomycota and genera *Meyerozyma guilliermondii* and *Pichia guilliermondii* (NCBI accession number MG846129 - MG846138). As *Candida guilliermondii* (teleomorph *Pichia guilliermondii*) is now included in the new *Meyerozyma* genus (Kurtzman and Suzuki 2010), hence all the 10 isolates belonged to the genus *Meyerozyma guilliermondii*. The presence of methane monoxygenase or alkane oxygenases in microorganisms is screened by oxidation of naphthalene to naphthol (Graham et al. 1992) and the development of purple-red colour in presence of dye o-dianisidine. All the 10 yeast isolates were found positive for oxygenase activity and the positive result signifies that the yeast isolate could cause the conversion of naphthalene to naphthol. However, it does not necessarily confirm the presence of methane monoxygenases in these cultures. Soluble and particulate methane monoxygenases (sMMO and pMMO) are present in various methanotrophs giving them the unique ability to cause conversion of CH₄ to methanol. The presences of sMMO and pMMO gene have not been reported in yeast strains till date. However, their ability to oxidize and utilize CH₄ indicates the presence of other alkane oxygenases with broad substrate range that may possess the ability to oxidize these single carbon compounds. Alkane monoxygenases like Class I P450, Class II P450, alkane hydroxylase, sMMO, pMMO, propane monoxygenase, and butane monoxygenase are widely present in both bacteria and yeast giving them the unique ability to utilize carbon chain ranging from C₁ – C₁₆ (Beilen and Funhoff 2005). CYP52 gene (Class II P450) that can oxidize n-alkane at α- and ω- positions have been cloned from yeast strain *Candida tropicalis* (Craft et al. 2003). Zilly et al (2011) used inert chemical to change the catalytic profile of monoxygenase P450 and obtained positive results with the use of perfluoro carboxylic acid. The chemically inert compound serves as a guest in the vicinity of the alkane hydroxylase gene causing them to be genetically unstable and more prone to horizontal gene transfer (Amouric et al. 2010). Such phenomena have been observed in *Acinetobacter oleivorans* (Park et al. 2017).
Similarly, transfer of genes involved in synthesis of methane monoxygenases may impart methane oxidation ability in a diverse group of microbial forms. Ammonia monoxygenase (AMO) present in various microorganisms possess structural similarity to pMMO and can act on methane as well as methanol along with ammonia (Hooper et al. 1997). The exact mechanism through which yeast utilizes CH₄ remains unclear and requires further research. But, various possibilities may allow these eukaryotic cells to grow in an environment with CH₄ as the only C-source. Identification of only single eukaryotic genus of *Meyerozyma* from all the 5 rice growing regions indicates its strong association with rice ecosystem and adaptation potential under flooded conditions in rhizosphere.

**Quantification of the CH₄ oxidation potential of yeast isolates:** The % CH₄ reduction by the isolates ranged from 5.83–63.57% at 9th d of incubation (Table 1). MAS63 recorded the lowest reduction in methane concentration (5.83±0.80%) as well as growth (2.72 protein µg/mL), whereas, KAS143 exhibited maximum %CH₄ reduction (63.57±1.12%) and growth (35.77 µg protein/mL). The significant variation in the growth by the isolates of same isolate under 5 rice growing regions indicates its strong association of only single eukaryotic genus of *Meyerozyma* from all the 5 rice growing regions indicates its strong association with rice ecosystem and adaptation potential under flooded conditions in rhizosphere.

**Table 1 Cumulative methane utilization and % reduction by different strains of *Meyerozyma guilliermondii* at 9th d of incubation**

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Methane concentration at different time interval in 50 mL flask</th>
<th>Cumulative methane utilization</th>
<th>% Methane reduction</th>
<th>Growth (Protein µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANW10</td>
<td>10030.33 ± 23.44ab 9868.03 ± 38.39a 9471.22 ± 104.93b 9381.76 ± 95.33b</td>
<td>648.57 ± 115.87d 6.46 ± 1.14d 4.92 ± 1.23e</td>
<td>42.50 ± 3.12f</td>
<td>7.53 ± 0.71f</td>
</tr>
<tr>
<td>BAS20</td>
<td>10064.67 ± 71.73ab 8895.73 ± 129.48b 7923.96 ± 64.88d 7718.90 ± 71.77d</td>
<td>2345.77 ± 110.02b 23.30 ± 0.97b 17.37 ± 1.04d</td>
<td>21.27 ± 1.04f</td>
<td>20.58 ± 0.68c</td>
</tr>
<tr>
<td>ANSe23</td>
<td>10061.69 ± 92.02ab 8868.36 ± 124.03c 7899.77 ± 46.24e 7763.97 ± 35.75d</td>
<td>2297.71 ± 87.81b 22.83 ± 0.68b 15.63 ± 0.47c</td>
<td>20.79 ± 1.63f</td>
<td>20.58 ± 0.68c</td>
</tr>
<tr>
<td>AAAr25</td>
<td>10029.76 ± 23.23ab 7789.10 ± 77.67f 7729.03 ± 230.17e 7572.13 ± 317.13d</td>
<td>2457.63 ± 334.10b 24.50 ± 3.29b 20.58 ± 0.68c</td>
<td>20.79 ± 1.63f</td>
<td>20.58 ± 0.68c</td>
</tr>
<tr>
<td>MAAr36</td>
<td>10054.92 ± 25.15ab 9558.30 ± 164.58b 9084.84 ± 63.68c 8954.73 ± 24.47c</td>
<td>1100.19 ± 8.01f 10.94 ± 0.08c 7.53 ± 0.71f</td>
<td>11.71 ± 1.22c</td>
<td>20.58 ± 0.68c</td>
</tr>
<tr>
<td>AAAr42</td>
<td>10078.33 ± 67.40a 8799.32 ± 60.85b 7786.97 ± 54.13d 7627.94 ± 99.58d</td>
<td>2450.39 ± 163.89b 24.31 ± 1.47b 21.27 ± 0.82b</td>
<td>11.71 ± 1.22c</td>
<td>7.66 ± 0.51f</td>
</tr>
<tr>
<td>MAS63</td>
<td>10071.30 ± 2.06ab 9930.30 ± 55.50a 9581.36 ± 66.55b 9484.20 ± 81.67b</td>
<td>587.10 ± 80.68d 5.83 ± 0.80d 2.72 ± 0.80b</td>
<td>20.79 ± 1.63f</td>
<td>20.58 ± 0.68c</td>
</tr>
<tr>
<td>AAL65</td>
<td>10014.00 ± 4.48ab 9447.69 ± 19.34f 8943.01 ± 61.58e 8841.34 ± 118.07c</td>
<td>1172.65 ± 122.45e 11.71 ± 1.22c</td>
<td>20.79 ± 1.63f</td>
<td>20.58 ± 0.68c</td>
</tr>
<tr>
<td>MaAL101</td>
<td>10012.36 ± 2.83ab 8788.76 ± 162.63a 7741.24 ± 88.22b 7603.67 ± 141.30d</td>
<td>2408.68 ± 138.64b 24.06 ± 1.39b 19.49 ± 0.95c</td>
<td>20.79 ± 1.63f</td>
<td>20.58 ± 0.68c</td>
</tr>
<tr>
<td>KAS143</td>
<td>10029.82 ± 8.24ab 7060.03 ± 57.47d 4249.00 ± 85.30f 3654.12 ± 114.75e</td>
<td>6375.69 ± 107.29a 63.57 ± 1.12a 35.77 ± 2.78b</td>
<td>20.79 ± 1.63f</td>
<td>20.58 ± 0.68c</td>
</tr>
<tr>
<td>Blank control</td>
<td>171.40 ± 2.79c 169.35 ± 1.93e 169.33 ± 2.76e 168.28 ± 3.14f</td>
<td>3.13 ± 0.36c 1.83 ± 0.24e</td>
<td>20.79 ± 1.63f</td>
<td>20.58 ± 0.68c</td>
</tr>
<tr>
<td>CH₄ control</td>
<td>10007.97 ± 14.13b 9991.66 ± 7.02a 9946.16 ± 42.50b 9919.08 ± 4.42a</td>
<td>88.89 ± 14.90e 0.89 ± 0.15e</td>
<td>20.79 ± 1.63f</td>
<td>20.58 ± 0.68c</td>
</tr>
<tr>
<td>LSD_(P&lt;0.05)</td>
<td>69.11 ± 156.66 155.69 ± 206.61 230.37 ± 2.23</td>
<td>1.78</td>
<td>20.79 ± 1.63f</td>
<td>20.58 ± 0.68c</td>
</tr>
</tbody>
</table>
low molecular weight organics acids like formic, acetic, propionic lactic, glycolic, fumaric, succinic acid and acidic phosphatases like phytases (Lee et al. 2012). Yeast belonging to genera \textit{Pichia} and \textit{Candida} with the ability to solubilize phosphate (697.2 μg/mL of P) from TCP were isolated by Park \textit{et al.} (2013). Phosphate solubilizing yeast has also been isolated by other workers and checked for their efficacy to promote plant growth (Kuo \textit{et al.} 2018; Gizaw \textit{et al.} 2017) and obtained positive result.

\textit{Meyerozyma guilliermondii}, ANW10, AAAR42 and KAS143, solubilized K and Zn. (Table 2). KAS143 exhibited significantly higher K (1.79±0.13) and Zn (ZnO: 4.38±0.18; ZnCO$_3$: 4.23±0.05; Zn$_3$(PO$_4$)$_2$: 3.85±0.05) solubilization index over other two isolates. The solubility of ZnO was significantly higher as compared to other Zn source. ZnO has been reported as the preferred source of Zn over ZnCO$_3$ (Karvan et al. 2003).

\textit{Meyerozyma guilliermondii} AAAR251 and KAS143 produced significant amount of IAA in presence of tryptophan as compared to media lacking it (Table 2). KAS143 produced significantly higher IAA (58.00±1.56 and 128.72 ± 0.80 μg IAA/mg protein in the absence and presence of tryptophan, respectively) as compared to AAAR251. IAA producing root symbiotic fungi, \textit{Meyerozyma guilliermondii} (8.23±1.00 μg IAA/ml) has also been isolated earlier (Aban \textit{et al.} 2017). Besides, the ability to promote plant growth through solubilizing P, K and Zn and producing IAA, the ACC deaminase activity of \textit{Meyerozyma guilliermondii} against Botrytis cinerea, Penicillium expansum, \textit{P. italicum}, \textit{P. digitatum}, \textit{B. cinerea}, \textit{Colletotrichum capsici}, \textit{Rhizopus stolonifer}, \textit{R. nigricans} and \textit{Botryodiplodia theobromae} has been reported earlier (Zajc \textit{et al.} 2019).

Methane oxidizing yeast \textit{Meyerozyma guilliermondii} with PGP attributes were isolated for the first time from different flooded paddy growing regions of India. A steady decline in the CH$_4$ concentration and increase in growth of yeast cells was observed in the airtight culture assembly having 1% methane environment. Further research on their field evaluation should be carried out to develop novel microbial inoculants for flooded paddies which can not only promote the plant growth but also reduce methane emission.

\section*{ACKNOWLEDGMENTS}
Authors acknowledge the Division of Microbiology, ICAR-Indian Agricultural Research Institute (IARI), New Delhi and ICAR-Network project on National Initiative on Climate Resilient Agriculture for providing facilities and funds.

\section*{REFERENCES}

\begin{table}[ht]
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\begin{tabular}{|c|c|c|c|c|c|c|c|}
\hline
\textbf{Isolate No.} & \textbf{P-solubilization} & \textbf{P-} & \textbf{K-} & \textbf{Zn solubilization index} & \textbf{IAA Production (µg/mg protein)} \\
& \textbf{Index} & \textbf{Solubilization} & \textbf{Solubilization} & \textbf{ZnO} & \textbf{ZnCO$_3$} & \textbf{Zn$_3$(PO$_4$)$_2$} & \textbf{Without} & \textbf{With tryptophan} \\
& & \textbf{(mg/L)} & \textbf{Index} & & & & \textbf{tryptophan} & \\
\hline
ANW-10 & 1.37 ± 0.04 & 41.69 ± 2.79 & 1.45 ± 0.17 & 3.77 ± 0.15 & 3.27 ± 0.20 & 3.60 ± 0.10 & ND & ND \\
AAAR-42 & 1.38 ± 0.02 & 48.78 ± 3.90 & 1.49 ± 0.05 & 3.5 ± 0.10 & 3.37 ± 0.15 & 3.3 ± 0.10 & ND & ND \\
KAS-143 & ND & ND & 1.79 ± 0.13 & 4.38 ± 0.18 & 4.23 ± 0.05 & 3.85 ± 0.05 & 58.00 ± 1.56 & 128.72 ± 0.80 \\
AAAR-251 & ND & ND & ND & ND & ND & ND & 34.35 ± 1.92 & 94.11 ± 1.05 \\
LSD$_{0.05}$ & NS & 0.158 & 0.296 & 0.302 & 0.177 & NS & 2.123 & \\
\hline
\end{tabular}
\caption{P, K, Zn solubilization and IAA production by \textit{Meyerozyma guilliermondii} isolates}
\end{table}