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Methane oxidizing-plant growth-promoting yeast isolated from Indian rice fields

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ABSTRACT

Potential methane-oxidizing-plant growth-promoting yeast was isolated and characterized during 2017–18 at the ICAR-Indian Agricultural Research Institute, New Delhi. Yeast isolates belonging to the genus *Meyerozyma guilliermondii* were isolated from five major flooded paddy growing regions of India. Among all the isolates, *Meyerozyma guilliermondii* KAS-143 efficiently oxidized methane up to 57.64% ± 0.83 in 6 d under *in vitro* conditions. It also produced a significant quantity of phytohormone IAA and solubilized P, K, and Zn. *Meyerozyma guilliermondii* KAS-143 can be used for devolving potential inoculants for flooded paddy which can not only promote plant growth but also simultaneously reduce methane emission by its oxidation.

Keywords: Flooded paddies, IAA production, Methane oxidizing yeast, Methane emission, *Meyerozyma guilliermondii*, P, K and Zn solubilization.

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.

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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, $ZnCO_3$, and $Zn_3(PO_4)_2$ @ 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 $P \leq 0.05$ 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 monooxygenase 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 monooxygenases in these cultures. Soluble and particulate methane monooxygenases (sMMO and pMMO) are present in various methanotrophs giving them the unique ability to cause conversion of CH_4 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 CH_4 indicates the presence of other alkane oxygenases with broad substrate range that may possess the ability to oxidize these single carbon compounds. Alkane monooxygenases like Class I P450, Class II P450, alkane hydroxylase, sMMO, pMMO, propane monooxygenase, and butane monooxygenase are widely present in both bacteria and yeast giving them the unique ability to utilize carbon chain ranging from $C_1 - C_{16}$ (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 monooxygenase P450 and obtained positive results with the use of perfluoro carboxylic acid. The chemically inert compound serves as a guest in the binding pocket of P450 BM3, thereby filling the space and reducing the translational freedom of small alkanes like propane and methane allowing its chemical conversion. Gene encoding transposases are commonly found 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 monooxygenases may impart methane oxidation ability in a diverse group of microbial forms. Ammonia monooxygenase (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 CH_4 remains unclear and requires further research. But, various possibilities may allow these eukaryotic cells to grow in an environment with CH_4 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 CH_4 oxidation potential of yeast isolate*s: The % CH_4 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 %CH_4 reduction (63.57±1.12%) and growth (35.77 µg protein/mL). The significant variation in the growth by the isolates of same genus showed their nutritional versatility and affinity towards methane. Methane utilizing yeast were initially reported in 1979 (Wolf and Hanson 1979). They used CH_4 enriched air (70%) for the initial isolation and 25% CH_4 for further experiments. Minimal growth medium with vitamins and amino acids supplement were used to obtain methane-oxidizing yeasts. They confirmed the incorporation of labelled ^{14}C carbon in cell material on incubation with $^{14}CH_4$ methane and reported the CH_4 oxidation rate to be in the range of 0.02- 0.18 µmol CH_4 per hour. Since then no further research confirming the CH_4 oxidizing ability of yeast is reported.

PGPR traits in selected yeast cultures: Out of the 10 isolates, only two *Meyerozyma guilliermondii* isolates ANW10 and AAAr42, solubilized tri-calcium phosphate (TCP) and exhibited solubilization index of 1.37±0.04 and 1.39±0.02, respectively (Table 2). The isolate AAAr42 produced 48.78±3.90 mg of P_2O_5 /L broth and was statistically at par with the isolate ANW10 (41.69±2.79 mg of P_2O_5 /L broth). Nakayan *et al.* (2013) also isolated P solubilizing *Meyerozyma guilliermondii* in the range of 97.7–190.8 mg/L and were able to improve maize productivity. Microorganisms can convert the insoluble form of phosphorus into a soluble form through producing

Table 1 Cumulative methane utilization and % reduction by different strains of *Meyerozyma guilliermondii* at 9th d of incubation NMS broth having methane as sole C source

Isolate	Methane concentration at different time interval in 50 mL flask				Cumulative methane utilization	% Methane reduction	Growth (Protein µg/mL)
	0 d	3rd d	6th d	9th d			
ANW10	10030.33 ± 23.44^{ab}	9868.03 ± 38.39^{a}	9471.22 ± 104.93^{b}	9381.76 ± 95.33^{b}	648.57 ± 115.87^{d}	6.46 ± 1.14^{d}	4.92 ± 1.23^{g}
BAS20	10064.67 ± 71.73^{ab}	8895.73 ± 129.48^{c}	7923.96 ± 64.88^{d}	7718.90 ± 71.77^{d}	2345.77 ± 110.02^{b}	23.30 ± 0.97^{b}	17.37 ± 1.04^{d}
ANSe23	10061.69 ± 92.02^{ab}	8868.36 ± 124.03^{c}	7899.77 ± 46.24^{d}	7763.97 ± 35.75^{d}	2297.71 ± 87.81^{b}	22.83 ± 0.68^{b}	15.63 ± 0.47^{e}
AAAr251	10029.76 ± 23.23^{ab}	8779.10 ± 77.67^{c}	7729.03 ± 230.17^{e}	7572.13 ± 317.13^{d}	2457.63 ± 334.10^{b}	24.50 ± 3.29^{b}	20.58 ± 0.68^{c}
MAAr36	10054.92 ± 25.15^{ab}	9558.30 ± 164.58^{b}	9084.44 ± 63.68^{c}	8954.73 ± 24.47^{c}	1100.19 ± 8.01^{c}	10.94 ± 0.08^{c}	7.53 ± 0.71^{f}
AAAr42	10078.33 ± 67.40^{a}	8799.32 ± 60.85^{c}	7786.97 ± 54.13^{de}	7627.94 ± 99.58^{d}	2450.39 ± 163.89^{b}	24.31 ± 1.47^{b}	21.27 ± 0.82^{b}
MAS63	10071.30 ± 2.06^{ab}	9930.30 ± 55.50^{a}	9581.36 ± 66.55^{b}	9484.20 ± 81.67^{b}	587.10 ± 80.68^{d}	5.83 ± 0.80^{d}	2.72 ± 0.80^{h}
AAL65	10014.00 ± 4.48^{ab}	9447.69 ± 19.34^{b}	8943.01 ± 61.58^{c}	8841.34 ± 118.07^{c}	1172.65 ± 122.45^{c}	11.71 ± 1.22^{c}	7.66 ± 0.51^{f}
MaAL101	10012.36 ± 2.83^{ab}	8786.01 ± 162.63^{c}	7741.24 ± 88.22^{e}	7603.67 ± 141.30^{d}	2408.68 ± 138.64^{b}	24.06 ± 1.39^{b}	19.49 ± 0.95^{c}
KAS143	10029.82 ± 8.24^{ab}	7060.03 ± 57.47^{d}	4249.00 ± 85.30^{f}	3654.12 ± 114.75^{e}	6375.69 ± 107.29^{a}	63.57 ± 1.12^{a}	35.77 ± 2.78^{a}
Blank control	171.40 ± 2.79^{c}	169.35 ± 1.93^{e}	169.33 ± 2.76^{g}	168.28 ± 3.14^{f}	3.13 ± 0.36^{e}	1.83 ± 0.24^{e}	-
CH_4 control	10007.97 ± 14.13^{b}	9991.69 ± 7.02^{a}	9946.16 ± 42.50^{a}	9919.08 ± 4.42^{a}	88.89 ± 14.90^{e}	0.89 ± 0.15^{e}	-
$LSD_{(P\leq0.05)}$	69.11	156.66	155.69	206.61	230.37	2.23	1.78

Table 2 P, K, Zn solubilization and IAA production by *Meyerozyma guilliermondii* isolates

Isolate No.	P-solubilization Index	P-Solubilization (mg/L)	K-Solubilization Index	Zn solubilization index			IAA Production (µg/mg protein)	
				ZnO	$ZnCO_3$	$Zn_3(PO_4)_2$	Without tryptophan	With tryptophan
ANW-10	1.37 ± 0.04	41.69 ± 2.79	1.45 ± 0.17[b]	3.77 ± 0.15[b]	3.27 ± 0.20[b]	3.60 ± 0.10[b]	ND	ND
AAAr-42	1.38 ± 0.02	48.78 ± 3.90	1.49 ± 0.05[b]	3.5 ± 0.10[b]	3.37 ± 0.15[b]	3.3 ± 0.10[c]	ND	ND
KAS-143	ND[1]	ND	1.79 ± 0.13[a]	4.38 ± 0.18[a]	4.23 ± 0.05[a]	3.85 ± 0.05[a]	58.00 ± 1.56	128.72 ± 0.80[b]
AAAr-251	ND	ND	ND	ND	ND	ND	34.35 ± 1.92	94.11 ± 1.05[a]
$LSD_{(p\leq0.05)}$	NS[2]	NS	0.158	0.296	0.302	0.177	NS	2.123

ND[1], Not detected; NS[2], Non- significant.

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 *Pichia* and *Candida* with the ability to solubilize phosphate (697.2 µg/mL of P) from TCP were isolated by Park *et al* (2013). Phosphate solubilizing yeast has also been isolated by other workers and checked for their efficacy to promote plant growth (Kuo *et al.* 2018; Gizaw *et al.* 2017) and obtained positive result.

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 than 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 $ZnCO_3$ (Saravanan *et al.* 2003).

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, *Meyerozyma guilliermondii* (8.23±1.00 µg IAA/ml) has also been isolated earlier (Aban *et al.* 2017). Besides, the ability to promote plant growth through solubilizing P, K, Zn and producing IAA, the ACC deaminase (Aban *et al.* 2017) and biocontrol ability of *Meyerozyma guilliermondii* against *Botrytis cinerea, Penicillium expansum, P. italicum, P. digitatum, B. cinerea, Colletotrichum capsici, Rhizopus stolonifer, R. nigricans* and *Botryodiplodia theobromae* has been reported earlier (Zajc *et al.* 2019).

Methane oxidizing yeast *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.

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