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Methanotroph detection and bioconversion of methane to methanol by enriched microbial consortium from rice field soil

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ABSTRACT

Fast depletion of fossil fuels has triggered research in gas to liquid (GTL) technologies for synthesis of green transportation fuels. Utilization of natural gas pockets located in remote areas for methanol synthesis is a viable GTL process. This study has examined potential of microbial consortium enriched from rice field soil for bioconversion of methane to methanol. The presence of Type I methanotrophs was confirmed through PCR amplification of genomic DNA from soil using *mmoY1-mmoY2* primer set which is specific to *Methylococcus capsulatus*. Further, the methanol production profiling (without MDH inhibitors) revealed ~130 mM (4.16 g/L) methanol production from enriched consortium, which was close to 132.5 mM (4.24 g/L) methanol production by pure strain of *Methylococcus capsulatus*. The yield of methanol for enriched consortium was 78.31 % and *M. capsulatus* was 79.81 %. These results show that enriched consortium holds high potential for BioGTL process for methanol synthesis.

1. Introduction

Methane is the smallest of the hydrocarbon family in its most reduced form, which means it is packed with potential energy. It is also a major greenhouse gas (GHG) which is also called a short-term enforcer, i.e. it persists in the atmosphere for shorter duration, as compared to carbon dioxide (CO_2) that can stay in atmosphere for thousand years, but its heating effect is much more potent than that of CO_2. Thus, even with a shorter lifespan, methane has 28–34 times higher global warming potential over a period of 100 years than that of CO_2 due to its higher solar radiation capturing efficiency (Priyadarsini et al., 2020). Every year millions of tonnes of natural gas are flared at the mining sites as preventive measure for global warming due to its global warming potential as discussed above and also because it is considered as waste. As per the International Energy Agency (IEA) and World Bank's Global Gas Flaring Reduction Partnership (GGFR) reports of 2021 on flaring emissions, around 143–144 billion cubic meters of natural gas was wasted by flaring across the globe (GGFR, 2022; IEA, 2022). Also, capturing the natural gas evolved from mines is difficult and unprofitable due to lack of proper infrastructure and technologies required to make it consumable for the end-use (Wood et al., 2012). Thus, flaring causes wastage of enormous amount of potential energy feedstock, carbon monoxide emission and hazardous black carbon production by incomplete burning (Dong et al., 2017; Gvakharia et al., 2017), not to mention the fire hazards and accidents associated and reported at the oil mining and flaring sites due to human error (Brkić and Praks, 2021; Chen et al., 2022; Mignan et al., 2022).

On industrial scale, methane (natural gas) is used as feedstock for production of various hydrocarbon derivatives using Fischer-Tropsch process (Dry, 2002; Holditch, 2003; Jones et al., 2022). One of the derivatives is methanol (15.6 MJ/L) which has higher energy density than methane (36.6×10^{-3} MJ/L). As a liquid fuel, it is much easier to handle, easier to blend with gasoline, fits into current infrastructure and can be used as a feedstock for other chemical processes (Bromberg and Cheng, 2010; Ge et al., 2014a). Methanol is also produced using methane by direct methane to methanol (DMTM) technique which is also a chemical process (Wang et al., 2022b). Both Fischer-Tropsch process and DMTM process are chemical routes with very poor selectivity and low yields (Conrado and Gonzalez, 2014) for gas to liquid (GTL) conversion which are also energy intensive, uneconomic and

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environmentally unfriendly, given the source of feedstock is underrated (Adebajo and Frost, 2012; Park and Lee, 2013). To address these concerns significant amount of effort has been put into biological gas to liquid (BioGTL) conversion technology that are not energy intensive as the fermentations take place at ambient operating conditions of temperature and pressure, relatively cost effective and cleaner for the environment than chemical routes (Conrado and Gonzalez, 2014; Haynes and Gonzalez, 2014). Biological methane to methanol conversion (bioconversion) is carried out by a set of organisms known as methanotrophs. These methane utilizers capture and metabolize methane as their sole source of carbon and energy for growth and metabolism using metabolic enzymes (Dong et al., 2017; Fei et al., 2014; Ge et al., 2014).

More than 100 g-negative and strictly aerobic methane-utilizing bacteria were isolated and classified by Whittenbury et al. (1970). They extensively described the enrichment, isolation and culture methods. These methods have been modified over years and still form the foundation for media design for the growth of methanotrophs. Methanotrophs are a subset of methylotrophic group of bacteria that can utilize C1 hydrocarbons and their methylated derivatives. The methanotrophs are very specific in substrate requirements and can only grow on C1 hydrocarbons, i.e., methane and methanol (Hanson and Hanson, 1996). Methanotrophs are classified among three phyla such as Proteobacteria, Verrucomicrobia and recently added phylum NC10 (Hakobyan and Liesack, 2020). The phylum Proteobacteria is the largest among the phyla and is further subdivided into type I, type II and type X subgroups based on their metabolic pathway for carbon assimilation. Type I methanotrophs, also known as gammaproteobacteria, utilize ribulose monophosphate pathway to assimilate carbon. Similarly, type II methanotrophs, or alphaproteobacteria, use serine cycle for carbon fixation. Type X has a combination of both type I and II and can grow in higher temperatures (Colin Murrell and Radajewski, 2000; Hakobyan and Liesack, 2020; Hanson and Hanson, 1996; Whittenbury et al., 1970).

Major sources of methanotrophs include agricultural rice fields, natural gas fields, dairy and cattle industries, etc. They are ubiquitous and are almost present wherever methane exists. India is an agriculture-based country with abundant paddy cultivation. Paddy cultivation is one of the largest sources in the global budget of methane emissions (Wang et al., 2022a). It is interesting to note that majority of methane (90 %) emitted in the wetland rice fields comes from the vascular transport system within the rice plants instead of ebullition or diffusion through the rhizosphere. The rate at which methane is emitted is much less (<20 %) than the actual rate of production in the rice fields. The difference in the rate of production and emission can be explained by methane oxidation by the methanotrophs present in the oxic regions of the rice roots and rhizospheres (Conrad and Rothfuss, 1991). Thus, they are present in abundance in rice fields and they act as the main methane sink where they capture around 20 % of methane evolved due to decaying of water-logged organic matter (Rahalkar et al., 2021). The conversion efficiencies of transported methane by methanotrophs remain in the range of 70–95 % in the soil-water interface (Epp and Chanton, 1993; Gilbert and Frenzel, 1995). The rice field rhizosphere community comprises of type I and type II methanotrophs. However, very few have been successfully cultivated *in situ* due to various reasons like resistance to growth in laboratory formulated medium, lack of specific nutrient for growth, extremely strict association with heterotrophic satellite community, etc. (Pandit et al., 2016). In fact, no Type I methanotrophs had been isolated from rice rhizosphere, rice roots, bulk soil or floodwater and only their presence was confirmed using molecular techniques (Dianou et al., 2012; Pandit et al., 2016; Takeda et al., 2008). Several methanotrophs with *pmoA* lineages from rice paddy cluster (e.g., RPC1–3) have been detected but remain uncultured till date (Knief, 2015; Lüke et al., 2014). Only recently, Type Ia (*Methylomonas, Methylomicrobium, and Methylocucumis*) have been cultivated and isolated (Rahalkar et al., 2021). Methanol production capability of these methanotrophs is yet to be studied. Thus, rice field was chosen for this study since they are a breaming source of methanotrophs and they are commonly found across India.

Methane is a very stable molecule and difficult to dissociate chemically. However, methane to methanol bioconversion is easily catalysed by a family of enzymes called the methane monooxygenases, i.e. particulate methane monooxygenase (pMMO) and soluble methane monooxygenase (sMMO), depending upon the location of the enzymes. *Methylococcus capsulatus* (Bath) is a type X methanotroph that possesses both pMMO and sMMO enzymes (Khider et al., 2021). The presence of extracellular concentration of copper in the growth medium strongly influences the expression of each enzyme. The pMMO enzyme essentially requires copper (both Cu(I) and Cu(II)) for its catalytic activity whereas sMMO contains iron (Fe). Thus, pMMO is predominantly expressed when extracellular copper concentration is higher and alternatively, sMMO is expressed when extracellular copper concentration is low (Larsen and Karlsen, 2016; Lieven et al., 2018). The sMMO enzyme complex is made up of protein A, B and C. Protein A, the hydrolase constituent, is made up of two copies of three subunits-α (coded by *mmoX*), β (coded by *mmoY*) and γ (coded by *mmoZ*). Protein B, coded by *mmoB* gene, is a regulatory protein that functions as the effector of electron transfer during catalytic reaction. Protein C is a reductase coded by *mmoC* gene (McDonald et al., 1995). The pMMO enzyme has three subunits called *pmoA*, *pmoB* and *pmoC*. Here, the *pmoA* and *pmoC* are the transmembrane subunits and *pmoB* has the soluble region which exhibits methane oxidation activity (Culpepper et al., 2012). In comparison to sMMO, not much has been understood about pMMO yet. Further studies are required to understand the structure, function and mechanism of each subunits and pMMO as a holoenzyme.

India has numerous natural gas reserves located in areas of difficult terrain with little or no infrastructure for transportation of gas. The motivation for this preliminary study was to address the natural gas wastage in remote and diffused sites by developing technology for on spot utilization of natural gas pockets. Several studies on methanol production have been reported using pure strains (Duan et al., 2011; Ghaz-Jahanian et al., 2018; Patel et al., 2016a, 2017, 2019, 2020d, 2023; Sahoo et al., 2023). A summary of literature on methanol production using methanotrophic bacteria is presented in Table 1. As can be referred from the table, most of the previous studies have employed single isolated strains of methanotroph for methanol production. However, very few studies have used mixed cultures with methanol titre of 1.49 g/g (Han et al., 2013), 28.13 mM (Sheets et al., 2017) and 15.16 mM (AlSayed et al., 2018). In addition, no previous study has used methanotrophic consortium enriched specifically from rice field soil for methanol production. Although the basic concept of conversion of methane to methanol has been previously reported in literature, several practical constraints and hurdles have to be overcome to convert the basic concept into a viable solution. One major hurdle is the use of natural isolates (such as consortia enriched from natural source of rice fields) which are robust and stable for large scale operation.

The main objectives of this preliminary study include (i) detection and enrichment of methanotrophic bacteria from rice field soil community, (ii) methanol production using enriched microbial consortium. As already discussed, rice fields are a substantial source of massive methanotrophic communities. Unfortunately, not all the bacterial species are culturable. So, early and easy detection tools can help in understanding, strategizing and refining the enrichment and isolation process. Significant levels of methanol production have been observed in this preliminary study, as described in subsequent sections.

2. Materials and methods

2.1. Chemicals and regents

All the chemicals and reagents, apart from kits, including nuclease-free water, used in the experiments were molecular biology grade and purchased from Sigma Aldrich and HiMedia.

Table 1
Summary of literature on methanol production by methanotrophs.

Microbial culture	Substrate	Fermentation conditions	Methanol yield	References
Methylosinus trichosporium OB3b	CH_4: air (1:5 *v*/v)	Batch mode, 25 °C, pH 7.0, 200 mM NaCl, 20 mM sodium formate	7.7 mM	(Sang et al., 2004)
Methylosinus trichosporium OB3b	CH_4: O_2 (1:1 *v*/v)	Batch mode, 30 °C, pH 7.0, 400 mM phosphate, 20 mM sodium formate	35 mM	(Duan et al., 2011)
		Continuous Bubble free membrane reactor	29.69 mM	
Methylosinus trichosporium OB3b	CH_4: air (1:3 v/v)	Batch mode, 25 °C, pH 7.0, 100 mM NaCl, 1 mM EDTA, 20 mM sodium formate	13.2 mM	(Kim et al., 2010)
	CH_4: air (1:1 *v*/v)	Continuous mode	13.7 mM	
Mixed methanotroph consortium from landfill soil	Artificial biogas: air (4:6 *v*/v)	Batch mode, 30 °C, 100 mM NaCl	1.49 g/g (g CH3OH per g CH4)	(Han et al., 2013)
Methylosinus trichosporium OB3b	CH_4: air (1:1 v/v) V_{gas}/V_{liq} = 9	Batch mode, 25 °C, pH 7.0, 12.9 mM phosphate, 20 mM sodium formate, 100 mM NaCl, 1.0 mM EDTA	9.06 mM	(Pen et al., 2014)
Methylosinus trichosporium OB3b	CH_4: air (30:70 *v*/v)	Batch mode, 30 °C, pH 6.3, 0.5 mM EDTA,40 mM formate, 5 μM Cu^{2+}	12.28 mM	(Hwang et al., 2015)
M. sporium	Raw biogas (20 % CH_4)	Batch mode, 30 °C, pH 7.0, 20 mM $MgCl_2$, 5 μM Cu^{2+}, 10 μM Fe^{2+}	6.68 mM	(Patel et al., 2016b)
Methylocella tundrae	CH_4: air (1:1 *v*/v)	Batch mode, 30 °C, pH 7.0, 50 mM sodium formate, 50 mM $MgCl_2$, 5 μM Cu^{2+}, 10 μM Fe^{2+}	5.18 mM	(Mardina et al., 2016)
Strain *Methylocaldum* sp. SAD2	Biogas:air (1:2 *v*/v)	Batch mode, 37 °C, pH 6.7, 100 mM sodium formate, 5 μM Cu^{2+}	10.72 mM	(Zhang et al., 2016)
Strain *Methylocaldum* sp. 14B	Biogas:air (1:2.5 *v*/v)	Batch mode, 37 °C, pH 6.6–6.8, 80 mM formate, 1 μM Cu^{2+}	13.44 mM	(Sheets et al., 2016)
Methylomonas sp. DH-1	CH_4: air (40:60 *v*/v)	Batch mode, 30 °C, pH 7.0 40 mM sodium formate, 0.5 mM EDTA	41.86 mM	(Hur et al., 2017)
Mixed culture dominated by *Methylocaldum* sp. 14B	Biogas:air (1:2.5 *v*/v)	Trickle-bed reactor with Liquid circulation	28.13 mM/d	(Sheets et al., 2017)
Immobilized *Methylocystis bryophila*	CH_4:CO_2 (30:70 *v*/v)	Batch mode, 30 °C, pH 7.0, 100 mM Formate, 50 mM $MgCl_2$	25.75 mM	(Patel et al., 2018a)
Co-culture (*M. tundrae and Methylomonas methanica)*	CH_4:CO_2 (30:70 *v*/v)	Batch mode, 30 °C, pH 7.0, 100 mM Formate, 50 mM $MgCl_2$, 5 μM Cu^{2+}, 10 μM Fe^{2+}	9.65 mM	(Patel et al., 2018b)
Strain AS1	CH_4: air (1:1 v/v)	Batch mode, 28 °C, External loop airlift bioreactor,	50 mM	(Ghaz-Jahanian et al., 2018)
Mixed culture	CH_4: O_2 (4:1 *v*/v)	Batch mode, RT, pH 7.0, 120 mM Formate, 10 mM $MgCl_2$, 5 μM Cu^{2+}	15.16 mM	(AlSayed et al., 2018)
Encapsulated *Methylomicrobium album*	CH_4: CO_2 (4:1 *v*/v)	Batch mode, 30 °C, pH 7.0 100 mM Formate, 20 mM $MgCl_2$, 5 μM Cu^{2+}, 10 μM Fe^{2+}	4.96 mM	(Patel et al., 2020a)
	Raw biogas (CH_4 30 %)		6.92 mM	
Immobilized co-culture of *Methylocystis bryophila* and *Methyloferula stellata*	CH_4: air (30:70 *v*/v)	Batch mode, 30 °C, pH 7.0, 100 mM Formate, 20 mM $MgCl_2$	5.37 mM	(Patel et al., 2020b)
Methylocystis bryophila	CH_4: air (30:70 *v*/v)	Repeated batch mode, 30 °C, 100 mM Formate, 20 mM $MgCl_2$, 5 μM Cu^{2+}, 10 μM Fe^{2+}	52.9 mM	(Patel et al., 2020c)
Encapsulated *M. album*	CH_4: CO_2 (2:1 v/v)	Batch mode, 30 °C, pH 7.0, 100 mM Formate, 50 mM $MgCl_2$	7.46 mM	(Patel et al., 2020d)
Encapsulated *Methyloferula stellata*			7.14 mM	
M. tundrae	Raw biogas (CH_4 30 %)	Batch mode, 30 °C, pH 6.8–7.0, 100 mM Formate, 20 mM $MgCl_2$, 5 μM Cu^{2+}, 10 μM Fe^{2+}	4.97 mM	(Patel et al., 2021)
M. tundrae immobilized on banana leaves			6.05 mM	
Methylosinus trichosporium NCIMB 11131	CO_2 and air (1:1 *v*/v)	30 °C, pH 6.8, 20 mM phosphate buffer, 5 mM $MgCl_2$, customized air-tight batch reactors	18.13 mM	(Sahoo et al., 2022)
Methylosinus trichosporium NCIMB 11131	CO_2 and air (1:1 *v*/v)	Batch mode, 30 °C pH 6.8, 20 mM phosphate buffer medium, 5 mM $MgCl_2$, 20 mM phosphate buffer medium, high pressure stirred tank reactor	61.88 mM	(Sahoo et al., 2023)

(*continued on next page*)

Table 1 (*continued*)

Microbial culture	Substrate	Fermentation conditions	Methanol yield	References
Co-culture *M. sporium* and *M. bryophila*	CH_4 (30 %) and H_2 (15%)	Batch mode, 30 °C, pH 7, 100 mM phosphate buffer, Fe^{2+} (10 µmol/L), Cu^{2+} (5 µmol/L), formate (100 mM), and $MgCl_2$ (20 mM)	64.6 mM	(Patel et al., 2023)
Enriched microbial consortium from rice field soil	CH_4: air (1:1)	Batch mode, 30 °C, pH 6.8	130 mM	**This study**

2.2. Collection of environmental samples and procurement of pure strains

The soil samples were collected from upper 10-cm soil layer (essentially the rhizosphere) in sterile 50 mL falcon tubes from a rice field in Bongaigaon district of Assam. The samples were stored in 4 °C until further experiments. The pure strains of *Methylococcus capsulatus* (ATCC 33009) were obtained from ATCC (USA), while strains of *Methylomicrobium buryatense* were gifted by Prof. Mary Lidstrom, University of Washington, USA.

2.3. Enrichment of rice field soil cultures

The enrichment was done in 20 mL of NMS medium in 120 mL airtight serum flasks using extinction dilution technique. The soil samples were diluted in distilled water (1 g in 10 mL) and seeded in nitrate mineral salt medium (NMS) composed of $MgSO_4{\cdot}7H_2O$ (1.0 g/L), KNO_3 (1.0 g/L), KH_2PO_4 (0.272 g/L), Na_2HPO_4 (0.284 g/L), $CaCl_2{\cdot}2H_2O$ (0.134 g/L), chelated Fe solution (0.2 % *v*/v), and a trace element solution (0.05 % v/v) (Sheets et al., 2016). 1 mL of the diluted environmental sample was inoculated in fresh NMS medium (pH 6.8) and the bottles were sealed under pressure with equimolar mixture of methane and air (1:1) in the headspace. The cultures were then incubated at 30 °C and 200 rpm for 5 days to produce a mixed culture. After 5 days, 1 mL from the mixed culture was inoculated into fresh sterile NMS medium in triplicate and incubated for 3 days with methane as before. Every third day, the enriched culture was successively transferred to fresh sterile NMS medium and fed with equimolar methane and air mixture (1:1). This process was repeated for 30 days.

2.4. Genomic DNA extraction and gene detection

2.4.1. DNA extraction

300 mg of the rice field soil was weighed and used for the genomic DNA extraction without dilution in duplicate. The extraction was done using soil DNA extraction kit (MP Biomedicals) method which allows genomic DNA extraction by feeding methanotroph rich rice field soil directly into the lysis column. The extracted genomic DNA were resolved in 1 % agarose gel in TBE buffer using ethidium bromide for imaging alongside 1Kb DNA ladder from New England Biolabs (NEB).

2.4.2. Gene detection

The gene detection was done through PCR amplification in a thermal cycler (Applied Biosystems) using the primers mentioned in Table 2 (McDonald et al., 1995; Miguez et al., 1997; Narihiro and Sekiguchi, 2011). The PCR amplification was performed in 0.5 mL flat cap PCR tubes. The total volume of PCR mix was 50 µL containing 5 µL genomic DNA template, 4 µL forward and reverse primer each, 25 µL master mix (MP Biomedicals) and 12-µL nuclease-free water.

2.4.3. PCR program

The PCR program started with initial denaturation at 95 °C for 5 min. Then, the program cycled through 94 °C for 30 s, 54 °C for 45 s and 72 °C for 60 s for 35 cycles. The final amplification step was carried out at 72 °C for 10 min. The amplified products were stained with ethidium bromide and imaged in a 1 % horizontal agarose electrophoresis gel with TBE buffer at 80 mA for 30 min.

2.5. Methanol accumulation

The pure strains of *Methylococcus capsulatus* and *Methylomicrobium buryatense* and the isolated consortium from rice field sample were grown in 20 mL NMS medium (120 mL serum vials) at pH 6.8 and pH 9 in batch mode. The cultures were sealed, injected under pressure with equimolar methane and air mixture (1:1) in the headspace, and incubated at 30 °C at 200 rpm. Due to small volume of the vials (120 mL) we couldn't measure the pressure of the headspace gas. However, the methane and air mixture was injected using a sterile syringe under pressure and we expect the approximate headspace pressure to be between 1.5 and 1.8 atm. The methanol production was estimated using methanol standards through gas chromatography analysis in GC Clarus 590 (Perkin Elmer). The cultures were grown for 5 days and 1 mL of culture was centrifuged at 10000 rpm (≈11,170 g) for 10 mins. The supernatant was collected. The samples were filtered through 0.2 µm syringe filters and prepared for methanol estimation through GC analysis.

Table 2
PCR primers used for amplification of the pMMO and sMMO genes from environmental samples.

Probe	Primers	Sequence (5′-3′)	Target	Target strain	References
mmoX	*mmoX1/mmoX2*	CGGTCCGCTGTGGAAGGGCATGAAGCGCGT/ GCCTCGACCTTGAACTTGGAGCCATACTCG	α subunit, sMMO	*Methylococcus capsulatus*	(Miguez et al., 1997)
mmoY	*mmoY1/mmoY2*	CGAGACCACGGAGCTGCGCACCGTCGACTG/ CGGCCTTCGGCACCGCTGTGGACTCGTCGA	β subunit, sMMO	*Methylococcus capsulatus*	
Met	*met86f/met1340r*	GCTCAGTAACACGTGG/CGGTGTGTGCAAGGAG	16S rRNA	Most methanogens	(Narihiro and Sekiguchi, 2011)
mmoX	*mmoX f882/mmoX r1403*	GGCTCCAAGTTCAAGGTCGAGC/TGGCACTCGTAGCGCTCCGGCTCG	α subunit, sMMO	Common for all methanotrophs	(McDonald et al., 2008, 1995)
mmoY	*mmoY f198/mmoY r820*	CCGACTGGATCGCCGGCGGCCT/CGCTGGAAGAACTCGCGGCGG	β subunit, sMMO	Common for all methanotrophs	(McDonald et al., 1995)
mmoZ	*mmoZ f133/mmoZ r483*	CGCCGTTCCGCAAGAGCTACGA/TTGCGCAGCCCTTCCAGCGGCGTG	γ subunit, sMMO	Common for all methanotrophs	
mmoB	*mmoB f77/mmoB r369*	AGTTCTTCGCCGAGGAGAACCA/TGCCCAGGGTGTAGGCGCGGCCGA	Protein B, sMMO	Common for all methanotrophs	
mmoC	*mmoC f542/mmoC r986*	GGTTCTGCTGTGCCGCACC/ATCCCGTGCCGCCGGCGACG	Protein C, sMMO	Common for all methanotrophs	

2.5.1. Gas chromatography

The filtered samples (1 μL) were run in GC using Elite-wax column (30 m × 0.32 mm × 25 μm) from Perkin Elmer and detected using Flame ionization detector (FID). The temperature of injector and detector were 110 °C and 250 °C, respectively. The initial oven temperature was 40 °C with a hold time of 3 min, which increased to 100 °C with a hold time of 5 min at 5 °C/min ramp rate, and finally the temperature increased to 200 °C with a hold time of 3 min at 5 °C/min ramp rate. Nitrogen gas was used as carrier at 2 mL/min flow rate and split ratio 20:1. Air and hydrogen were fed at 450 mL/min and 45 mL/min, respectively. The final quantity of methanol produced was measured using the slope equation from the calibration curve obtained using the peak area of various methanol standards plotted against corresponding methanol concentrations.

3. Results

3.1. Preamble: enrichment of rice field soil cultures

Isolation of methanotrophic bacteria is a time-consuming and tedious process. The methanotrophs are strongly associated with heterotrophic satellites around them in their community (Dedysh and Dunfield, 2011). So, the initial growth is often attributed to the growth of satellite bacteria growing on products from methanotrophic bacteria. The bacterial growth was observed in the enrichment cultures after 3 days which could be identified by increase in the turbidity of the cultures. Since, the growth was also observed in control experiments within 3 days, which were not fed with methane, it can be inferred that initial growth attributes to the heterotrophic community and not the methanotrophs. However, methanotrophic bacteria have been reported to grow in 4–5 days from inoculation. Pure cultures are isolated bacteria that help in study of single species. In contrast, use of enriched culture has economic and practical advantage over pure cultures. Microbial consortium has more tolerance to potential contaminants in the feed gas (Jiang et al., 2023). So, the cost associated with the purification of feed gas for fermentation can be significantly reduced. This shows that methanotrophic bacteria has better adaptability and applicability towards changing environment while growing in a community (Han et al., 2013). Moreover, isolation of pure strains needs longer incubation time period and multiple transfers rendering the entire process not only unreliable and extremely challenging but also time consuming; further, it requires expertise to avoid contaminations in between transfers (Kulkarni et al., 2022). On the contrary, consortial cultures are reliable, easier to culture and have above mentioned advantages over pure cultures in fermentations. The use of enriched mixed cultures (AlSayed et al., 2018) and co-cultures (Patel et al., 2018b) have also been previously reported for methanol production with end concentrations of 15.16 and 9.65 mM, respectively, but the source was mostly activated sludge. So, in this preliminary study the approach was focused on enriched consortium from rice field soil for methanol production.

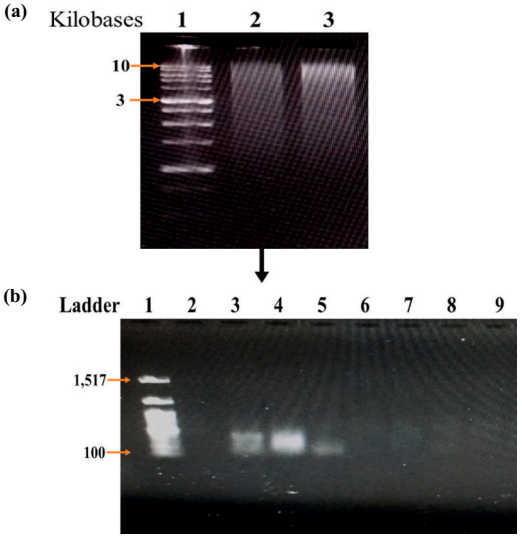
3.2. Genomic DNA extraction and gene detection

PCR amplification of target gene sequences is an easy tool for direct detection of microorganisms in environmental samples. This tool is extremely useful especially when the microorganisms in the samples are difficult to culture in laboratory for studies (as discussed in previous sections). The best genomic DNA extracted yielded 63.7 ng of DNA/μL with 260/280 ratio of 1.61 among the duplicate isolations from 300 mg rice field soil. This DNA was stained with ethidium bromide and run in 1 % agarose gel alongside of 1 KB ladder. The genomic DNA was used as a template and checked for the presence of soluble methane monooxygenase genes. The presence of methanogenic community was also examined in the rice field soil samples using the *met86f/met1340r* primers for the 16S rRNA for most methanogens.

In Fig. 1, lane 6–9 also show amplification, although low but detectable levels, indicating the presence various methanotrophic bacteria. This implies that the rice field methanotrophic community harbours a diverse group of methanotrophs. The *mmoY1-mmoY2* primer set (Lane 4) is derived from the *β* subunit of hydrolase protein A and is specific for the *mmoY* gene from *M. capsulatus* (Miguez et al., 1997). From Fig. 1 (b), it can be observed that maximum amplification has been obtained in Lane 4. From this result, the presence of either *M. capsulatus* or *M. capsulatus*-like methanotrophs in the original rice field soil community is confirmed. As expected, slight amplification can also be observed in Lane 5 representing the methane producing community in the rice field soil sample.

3.3. Methanol production

The methanol production by the enriched rice field consortium and the pure strains of *M. capsulatus* and *M. buryatense* in 5 days (or 120 h) of incubation at pH levels of 6.8 and 9 are shown in Fig. 2. The pH of the NMS media for *M. buryatense,* a haloalkaliphile, was 9 because this



Lane 1: 1 Kb ladder
Lane 2 and 3: Genomic DNA extracted from rice field soil sample

Lane 1: 100 bp ladder
Lane 3: For *mmoX*, *Methylococcus capsulatus*
Lane 4: For *mmoY*, *Methylococcus capsulatus*
Lane 5: For most methanogens
Lane 6 to 9: For sMMO, common methanotrophs

Fig. 1. (a) Genomic DNA extraction from rice field soil samples and (b) methane monooxygenase and methanogenic 16S rRNA gene detection using PCR amplification.

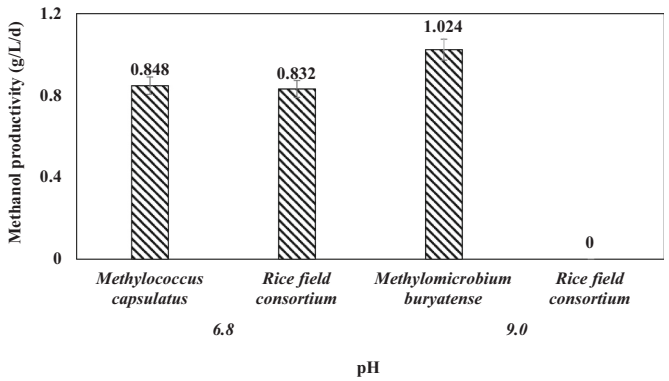


Fig. 2. Methanol production in M buryatense, *M. capsulatus* and enriched rice field soil consortium after 5 days of incubation.

culture grows well in slightly alkaline pH. This could be attributed to its origin of soda lake samples (Fei et al., 2018; Groom and Lidstrom, 2021; Kaluzhnaya et al., 2001). The *M. capsulatus* was grown in slightly acidic pH of 6.8 (Yu et al., 2003). The rice field soil consortium was grown in both the pH. There was no methanol production in rice field soil samples grown in pH 9. However, the methanol productivity in rice field sample (0.832 g/L d^{-1}) grown in pH 6.8 was similar to that in *M. capsulatus* (0.848 g/L d^{-1}). Most notably, the maximum methanol production by rice field consortium was 130 mM (4.16 g/L), which was at par with the methanol production by *M. capsulatus* (132.5 mM or 4.24 g/L) and by *M. buryatense* (160 mM or 5.12 g/L). To the best of our knowledge, these values are the highest among literature on methanol production by methanotrophs.

3.4. Determination of methanol yield

As mentioned earlier, headspace pressure was approximately in the range of 1.5 to 1.8 atm. The moles of headspace gas (equimolar mixture of air and methane) can be calculated using ideal gas equation: $PV = nRT$. Considering an average pressure of 1.65 atm, V = 100 mL, R = 0.08206 L atm K^{-1} mol^{-1}, T = 303 K, the total number of gas moles (n) in the headspace are 6.64 mmoles, half which accounts for methane. Considering the metabolic stoichiometry of 1 mol of methanol per mol of methane, the total percentage consumption of methane (or in other words methanol yield per mole of methane) is: 2.6/3.32 = 0.783 or 78.3 %.

4. Discussion

Methanotrophs efficiently convert methane to methanol in a single step using methane monooxygenase enzymes at ambient reaction conditions. The production of methanol occurs intracellularly by both pMMO and sMMO. Extracellular methanol accumulation is usually achieved by methanol dehydrogenase (MDH) inhibition (Patel et al., 2016c). In this study, methanol production was achieved without addition of MDH inhibitors. The samples from initial culture and fifth day enriched cultures were taken and examined for the presence of methanol using gas chromatography. The methanol production in the pure strains of *M. capsulatus* and *M. buryatense* were also examined after 5 days of incubation (Fig. 2). It is very interesting to note here that these results are consistent with the gene detection results. The presence of bacteria in rice field soil similar to *M. capsulatus* (confirmed by PCR gene amplification) shows similar methanol production as well. The maximum methanol production reported in previous literature was 50 mM (1.6 g/L) by Strain AS1 isolated from active anaerobic sludge grown under 50 % methane at 28 °C in an external loop airlift bioreactor where the airlift reactor was constantly supplied with methane enriched NMS medium (Ghaz-Jahanian et al., 2018). Recently, a methanol titre of 61.88 mM was achieved a customized airlift with a draft tube and microsparger using *Methylosinus trichosporium* NCIMB 11131. The design of the bioreactor and use of methane vector enhanced the volumetric mass transfer rate of methane resulting in a high methanol titre (Sahoo et al., 2023). The highest methanol titre reported so far is 64.6 mM that has been achieved by using co-culture of *Metholosinus sporium* and *Methylocystis bryophila* and a feed gas composition of methane (30 %) and hydrogen (5–20 %) that was derived from anaerobic digestion and dark fermentation of rice straw (Patel et al., 2023). Table 1 compares our results with previous literature.

5. Conclusions

Here, we have shown that the use of enriched cultures from rice field soil in place of pure cultures (*M. buryatense* and *M. capsulatus*) can also produce similar quantities (130 mM, 160 mM and 132.5 mM, respectively) of methanol. An added advantage of microbial cultures enriched from natural resources is that they are highly stable and robust against environmental variations, which makes them most suitable for large scale operations. This study, thus, opens up a spectrum of new possibilities for methanol production from an economically friendly route of BioGTL process and also provide a possible solution for effective on-spot utilization of isolated natural gas pockets.

CRediT authorship contribution statement

Aradhana Priyadarsini: Conceptualization, Methodology, Investigation, Writing – original draft. **Rekha Singh:** Methodology, Investigation. **Lepakshi Barbora:** Supervision. **Subhrangsu Sundar Maitra:** Conceptualization, Supervision. **Vijayanand Suryakant Moholkar:** Conceptualization, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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