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Selection of methanotrophic platform for methanol production using methane and biogas

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To develop biotechnological process for methane to methanol conversion, selection of a suitable methanotrophic platform is an important aspect. Systematic approach based on literature and public databases was developed to select representative methanotrophs *Methylotuvimicrobium alcaliphilum*, *Methylomonas methanica*, *Methylosinus trichosporium* and *Methylocella silvestris*. Selected methanotrophs were further investigated for methanol tolerance and methanol production on pure methane as well as biogas along with key enzyme activities involved in methane utilization. Among selected methanotrophs *M. alcaliphilum* showed maximum methanol tolerance of 6% v/v along with maximum methanol production of 307.90 mg/L and 247.37 mg/L on pure methane and biogas respectively. Activity of methane monooxygenase and formate dehydrogenase enzymes in *M. alcaliphilum* was significantly higher up to 98.40 nmol/min/mg cells and 0.87 U/mg protein, respectively. Biotransformation trials in 14 L fermentor resulted in increased methanol production up to 418 and 331.20 mg/L, with yield coefficient 0.83 and 0.71 mg methanol/mg of pure methane and biogas respectively. The systematic selection resulted in haloalkaliphilic strain *M. alcaliphilum* as one of the potential methanotroph for bio-methanol production.

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[**Key words:** Methanol; Biogas; Methanotrophs; Methane; Biotransformation]

Greenhouse gas emission (GHG) due to anthropogenic or natural processes is harmful for the environment. In various natural and anthropogenic processes 900 Tg methane emission significantly affects climate change (1). Valorisation of these gases into value added products can be an ideal scenario for decarbonization and bio-economy. Biogas is one of the potentially accessible GHG with methane (50–70%) and carbon dioxide (25–50%) as its major constituents (2). Industrially biogas is produced from major feedstocks like distillery spent wash, food waste, municipal solid waste, sugars, and bio-oil industries. In addition to these bio-based waste residues, second generation feedstocks from agricultural residues like sugarcane bagasse, rice straw, corn cobs, sorghum straw, cotton stalk and others also have the potential to produce significant quantities of biogas. Anaerobic digestion of such agriculture residues and industrial waste into biogas provides a sustainable source of energy.

Conversion of gaseous feedstock of methane and carbon dioxide into different products will be an attractive approach to add value to overall bio-economy (3). Apart from being major component of biogas, methane is also available from natural gas reserves (113 trillion m^3) (4). Methane is also harmful to environment with ~20 folds higher global warming effect than carbon dioxide (1). Due to availability in large quantity and its effect on environment methane was identified as potential substrate in this study. Methanotrophic organisms which are known to utilize methane as sole source of carbon can be used to develop technology for biological valorization of methane (1,4,5). Methanotrophs have been demonstrated previously for their ability to produce wide variety of products including methanol, ectoin, polyhydroxy butyrate, 2,3-butanediol, single cell protein, carotenoids, vitamin B12, lactic acid and electricity generation (3,6–8). Biobased methanol is a potential biofuel because of its properties like high octane number, lesser pollutant emission and compatibility in present automobile engines (9). Methane is also known as a fuel but the advantages of methanol like high energy density (400-fold) and its ease of transportation justifies methanol production from methane (10). Along with CH_4, methanol production has also been reported using CO_2 as well as mixture of GHGs (5,11,12). Furthermore, the use of methanol derivatives as fuel additives and feedstock for fuel production is an established concept. Due to potential of methanol as a biofuel and its conversion to several intermediate chemicals, methanol is an ideal product from methane (9,13).

Methanotrophs metabolize methane to formaldehyde via methanol and gets assimilated by either ribulose monophosphate (type I) or serine (type II) as primary pathway (14). As conversion of methane to methanol is the first step of methanotroph metabolism, methanol production is essentially a biotransformation reaction. Several efforts to produce methanol using different methanotrophs belonging to type I and type II categories have been reported previously (15,16). Major limitations for bioconversion of methane are

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slow growth rate of methanotrophs, low methane solubility and metal ion dependency (16,17). Different process designs like batch mode fermentation, membrane reactor, use of co-culture and various immobilization strategies have also been investigated previously for methanol production (18–21). Table 1 (12,15,16,18,22–25) shows recent literature summary of yield, titer and specific productivity of methanol production by different methanotrophs. Studies on inhibition of methanol dehydrogenase (MDH), co-culture biotransformation, use of methane vector and regeneration of co-factor from formate have been reported to improve methanol production (1,16,20,26). Although several studies have been explored towards production of bio-methanol, the comparison of methanol production between different strains is difficult due to variation in culturing protocols, varying cell concentration in biotransformation reaction and supply of reducing agent. Important factors like selection of methanotrophic strain, from a wide diversity of methanotroph and characteristics like methanol tolerance have not been systematically investigated so far. Methanol being a bulk chemical and its production process must be designed to incur the lowest possible cost. Selection of suitable methanotrophic platform can play a key critical role in minimizing process requirements and operational costs. Key characteristics of methanotroph like optimum temperature for growth, methanol tolerance, methane conversion rate, titer, yield and productivity can have an impact on the overall cost of the bioprocess.

In the present study, an industrially suitable organism was systematically selected from wide diversity of genus and species of methanotrophs. Selected strains were investigated for methanol tolerance, requirements of reducing energy and optimization of MDH inhibitors. Methanol production using biogas generated on second generation feedstock was also evaluated.

MATERIALS AND METHODS

Data collection, segregation and processing List of 60 aerobic methanotrophs was updated from the previously reported data on culturable aerobic methanotrophic bacteria (27,28). Information like culture bank designations, physiological characteristics, presence of particulate methane monooxygenase (pMMO) or soluble methane monooxygenase (sMMO) along with the availability of subunit sequences of pMMO (*pmoA*, *pmoB* and *pmoC*) were updated as required. Strains sharing similar characteristics were identified to define a systematic strategy for screening (Fig. 1). Phylogenetic tree of *pmoA* amino acid sequences was constructed by neighbor joining method with 100 bootstrap replicates using CLC main workbench 8.1 software.

Microbial cultures, media compositions and cultivation *Methylotuvimicrobium alcaliphilum* DSMZ 19304, *Methylocella silvestris* DSMZ 15510, *Methylomonas methanica* DSMZ 25384 and *Methylosinus trichosporium* NCIMB 11131 were received from DSMZ and NCIMB culture banks. Neutrophilic organism *M. trichosporium*, *M. silvestris* and *M. methanica* were cultivated in nitrate mineral salt medium described previously (16,18). To grow *M. trichosporium* and *M. methanica*, 1 μM of $CuSO_4$ was added in nitrate mineral salt media before inoculation whereas haloalkaliphilic methanotroph *M. alcaliphilum* was cultivated in methylmicrobium medium (DSMZ medium 1180). Carbon source in the form of methane and/or methanol were provided as per experimental design. All methanotrophs were incubated at 30° C on 150 rpm shaking condition except for *M. silvestris*, which was grown at 25° C and 150 rpm. Media was dispensed to maintain 1:5 medium to headspace ratio in screw cap bottles with silicon septum. After inoculation of organisms in sealed bottles, headspace air was displaced by first applying vacuum till −10 psi gauge reading through 0.2 μm filter and sparging methane-oxygen gas mixture (1:1 ratio) till reading changed to 10 psi. This cycle was repeated for three times before culture incubation. The same gassing protocol was used for all growth and biotransformation trials at bottle level.

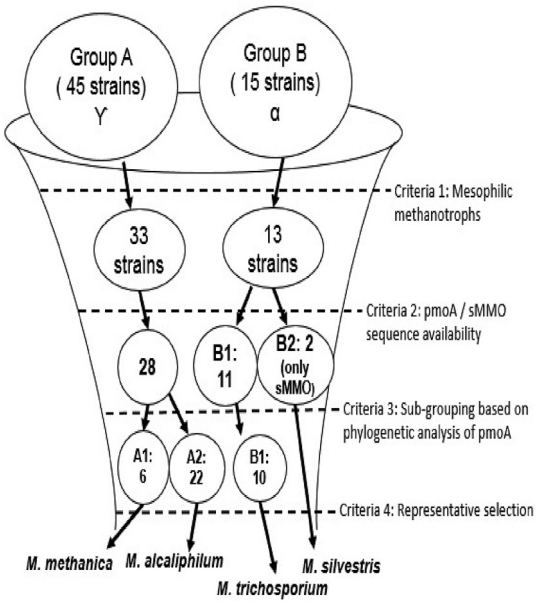

FIG. 1. Primary segregation and selection criteria for selection of representative methanotrophs for evaluation of methanol production ability.

Methanol tolerance studies To estimate methanol tolerance, exponentially grown culture was inoculated into 50 mL respective media containing 0–7% v/v methanol, and cell growth was analyzed. Methanol tolerance in the presence of methane was done in 250 mL screw cap bottles using 1:1 methane to oxygen gas mixture. Methanol tolerance in the absence of methane was performed in 250 mL Erlenmeyer flask.

Fermentor cultivation Methanotroph was inoculated into seed bottle containing 50 mL of respective media in 250 mL bottle with methane-oxygen mixture (1:1) and incubated till exponential phase (48–72 h). Grown culture was used for inoculation of 1.25 L screw cap flasks containing 250 mL media using 10% v/v inoculum. This culture was termed as pre-fermentor culture and was incubated for 48–72 h till exponential phase. New Brunswick scientific fermentor Bioflow 415 with 14 L vessel volume was used to cultivate methanotrophs in 6 L of working volume. Fermentor was operated in fed-batch mode with 150 rpm at 30° C except for *M. silvestris*, which was grown at 25° C. All methanotrophs were grown at pH 6.8 except for *M. alcaliphilum* which was grown at pH 9.0. Impellers of fermentor were positioned as one in submerged condition and another in headspace. To replace headspace gas and to saturate media, methane-oxygen mixture (1:1 ratio) was supplied at 2 L per minute flowrate for 10 min through mass flow controllers. All ports of fermentor were then closed, and fermentor was held at 10 psi gauge pressure for 10 min. After achieving gas saturation, pressure was released and fermentor was inoculated with 10% v/v pre-fermentor culture. Fresh methane-

TABLE 1. Summary of methanol production reported using methanotrophs.

Organism	C source	Max. methanol (g/L)	Methane to methanol conversion (%)	Specific methanol productivity (mg/g DCW/h)	Reaction volume (mL)	Reference
M. trichosporium OB3b	Pure methane	1.13	60	1.60[a]	300	20
Methylocaldum genus	Biogas	0.43	25	19.90[a]	3	21
Methylomonas DH-1	Pure methane	1.34	–	75.20	1	14
M. bryophila	Pure methane	0.15	–	0.69[a]	20	15
M. sporium	Synthetic gas mixture	0.23	–	0.60[a]	20	11
M. tundrae	Pure methane	0.17	–	0.38[a]	2	17
Methanotrophic consortia	Biogas	0.33	47	–	30	22
M. trichosporium OB3b	Pure methane	0.12	82	–	4	23

[a] Calculated from reported data.

oxygen mixture was sparged till 10 psi gauge reading to initiate methane fermentation. After every 6 h of incubation, headspace gas containing unconverted CH_4:O_2 mixture and CO_2 was released and fresh methane-oxygen mixture (1:1) was fed through sparger till 10 psi gauge pressure. Between every 6 h of the gassing cycle, dissolved oxygen (DO) reduced gradually and did not fall below 30%. Fermentor grown methanotrophic cells were harvested at exponential phase (48–72 h) for every culture and cell biomass was used for biotransformation reaction. For safety precautions, operated in 14 L fermentor vessel (design pressure 40 psi gauge) and methane alarms were fixed in laboratory to identify any leakage.

Suspended cell biotransformation Each methanotrophic culture grown on methane was harvested at exponential phase and centrifuged at 5000 rpm for 30 min at 4° C and washed twice with ice cold water. Cells were resuspended in buffered media containing 20 mmol L^{-1} sodium phosphate (pH 6.8) with desired concentration of sodium formate and MDH inhibitor. Cell concentration in all reactions was adjusted to 2 g of dry cells per liter of buffered medium. Reaction was performed at 30° C and 150 rpm for 48 h at both bottle and fermentor level. Biotransformation reaction at bottle level was performed in 125 mL screw cap bottle with 20 mL of reaction volume. Headspace in bottle was filled with 1:1 methane to oxygen gas mixture using vacuum and gassing protocol as explained earlier. For biotransformation trials at fermentor scale, 14 L reactor was operated with 3 L reaction volume and saturated with 1:1 methane to oxygen mixture till 10 psi gauge pressure. Biotransformation was conducted in batch mode without control of pH and dissolved oxygen and mixed gases were filled only at initiation of biotransformation reaction. During 48 h of retention time no significant drop in pH was observed. With progress of biotransformation drop in dissolved oxygen up to 40% was observed due to oxidation of methane to methanol using methane monooxygenase (MMO) enzyme. In case of trials with biogas, ratio of methane to oxygen was adjusted to achieve 1:1 based on composition of biogas. Broth samples were analyzed for methanol and formate at different time intervals. Gas samples were collected using a gas tight syringe (Hamilton Company, Reno, NV, USA) and analyzed for methane concentration on gas chromatograph.

Biogas collection, composition and use Biogas was collected in natural latex rubber bladders from anaerobic digester (70 L) operated on rice straw and sugarcane bagasse. Biogas from rice straw composed of 54.2% CH_4 and 35.4% CO_2, 85 ppm H_2S, 2.2% O_2 along with nitrogen and moisture (not analyzed), whereas the composition of biogas generated from sugarcane bagasse was found to be 49.8% CH_4 and 38.6% CO_2, 140 ppm H_2S, 1.7% O_2 along with nitrogen and moisture (not analyzed). Biogas was passed through 0.2 μm filter and stored in sterile 250 mL screw cap bottle with silicon septa. Air was replaced by applying vacuum till −10 psi gauge pressure prior to filling biogas in three repeated cycles. For biotransformation trials in screw cap bottles, headspace was first replaced with oxygen using three cycles of vacuum and fresh oxygen supply. Desired combination of biogas and oxygen mixture was achieved by replacing measured quantity of oxygen with biogas using gas tight syringe (Hamilton). For trials in 14 L fermentor, measured quantity of biogas and oxygen (1:1 ratio of CH_4:O_2) was filled till 10 psi gauge pressure.

Analysis Cell mass analysis was performed using optical density measurement at 600 nm on spectrophotometer. Dry cell weight (DCW) was measured by drying cells overnight at 80° C in a vacuum oven. For analysis of fermentation broth cells were centrifuged at 10,000 rpm for 10 min and supernatant was filtered through 0.2 μm prior to analysis. Methanol was analyzed on gas chromatograph (Agilent GC7890, Agilent, Santa Clara, CA, USA) using Heliflex AT-WAX column connected to flame ionized detector using previously reported method (20), whereas formic acid analysis was done on high performance liquid chromatograph (Agilent 1200 series H) using 0.005 M H_2SO_4 as mobile phase. The high performance liquid chromatography has Aminex Hex H+ column (300 mm × 7.8 mm, BioRad, Hercules, CA, USA) and connected to refractive index detector. For methane analysis, gas samples were collected from fermentor headspace in a gas tight syringe. The gas analysis was performed on GC (Agilent GC7890) with Agilent 19095P-MOLSEIVE column and thermal conductivity detector (2). Biogas analysis was performed on Geotech biogas 5000 portable biogas analyzer from Geotechnical Instruments Limited, Coventry, UK.

Enzyme activity assays Fermentor grown cells harvested for biotransformation were used for estimation of MMO activity. Total MMO activity was determined using propylene oxidation method as described previously by Zhang et al. (29). Formate dehydrogenase (FDH) enzyme activity determination was performed using previously reported method by Yoch et al. (30). Activity assay of FDH was performed with crude cell free extract of selected methanotrophs at room temperature. Protein analysis of crude cell free extract was performed by Folin-Lowery method. Specific enzyme activity of FDH was calculated and represented as units per mg of protein.

Redox regeneration efficiency calculation Redox regeneration efficiency was calculated based on NADH generated during formate to CO_2 conversion. Assuming 1 mole of formate produce NADH/electron required to convert 1 mole of methane into methanol, efficiency of redox regeneration was calculated based on moles of methanol formed per mole of formate consumed.

RESULTS

Systematic selection of methanotrophs In order to select industrially important methanotroph, data on 60 diverse methanotrophs including their genetic and physiological information was updated from previous published reports (27,28). These reports provided data on phylogenetic and physiological characteristics of diverse and pure isolates of methanotrophs from wide variety of habitats. Methanotrophic strains were grouped in different categories (Fig. 1) and assessed for their ability to produce methanol based on industrially relevant characteristics. Methanotrophs were segregated into major groups as gamma-proteobacteria (group A, 45 strains) and alpha-proteobacteria (group B, 15 strains). Mesophilic strains from each group were further selected to design fermentation process under ambient conditions for industrial product like methanol. Selection of mesophilic methanotrophs resulted in reduction of relevant strains to 33 and 13 from group A and B, respectively (Fig. 1). As MMO enzyme activity is the most important factor affecting methane to methanol conversion, it was selected to understand variation in types of MMO within different groups. Methanotrophs were further segregated into subgroups based on the presence and absence of particulate form of methane monooxygenase enzyme. All members of group A possess pMMO, whereas 11 strains of group B were found to have pMMO (group B1) and the rest two strains possess only soluble form of MMO (group B2). Out of three subunits of pMMO, pmoA is considered as a conserved subunit and has been used for studying diversity of methanotrophs by construction of phylogenetic tree (31). Amino acid sequence of *pmoA* was analyzed using neighbor joining analysis (Fig. 2) to find taxonomic difference within groups. Analysis of pmoA sequence separated group A into A1 (6 species) and A2 (22 species), while analyzing group B, *Methylocystis bryophila* was found to be diverse from rest of group B1. Based on reports of slower growth rate, methanol sensitivity around 0.15% v/v (32) and lower methanol production (16), *M. bryophilla* was not considered for further evaluation. Representative methanotroph from each sub-group was selected on the basis of availability in culture collection centers, availability of genome sequence and genetic engineering toolkit, genome scale metabolic model and overall research data in published literature. Selected representative methanotrophs for group A1, A2, B1 and B2 are *M. methanica*, *M. alcaliphilum*, *M. trichosporium* OB3b and *M. silvestris,* respectively (Fig. 1). Selected strains were further evaluated for methanol tolerance and methanol production ability.

Methanol tolerance Methanol tolerance of selected strains in the presence and absence of methane was analyzed and different degree of tolerance was observed in each methanotrophic strain (Fig. 3). In both presence and absence of methane, selected strains showed methanol tolerance at or above 4% v/v. Amongst selected strains *M. alcaliphilum* was found to have the highest methanol tolerance of 5% v/v in the presence of methane and 6% v/v in the absence of methane.

Methanol production using suspended cell biotransformation MDH enzyme converts methanol to formaldehyde and its inhibition is important to increase methanol accumulation. MDH was inhibited using inhibitors like potassium phosphate, $MgCl_2$, EDTA, NaCl and NH_4Cl (3,16). Considering reducing energy requirement of MMO enzyme, addition of external reducing agent like sodium formate is essential for

higher methane to methanol conversion (1,23). In the present study, MDH inhibitors were screened for selected methanotrophs and reducing agent requirement was identified to enhance methanol production. All control experiments were performed in the absence of MDH inhibitors.

Biotransformation reaction with different concentration of MDH inhibitors was performed using 40 mmol L^{-1} sodium formate as a reducing agent (Fig. 4A–E). Ammonium chloride at 20 mmol L^{-1} concentration was found to be optimum inhibitor for *M. alcaliphilum* and produced 224.06 mg/L methanol, whereas potassium phosphate at 200 mmol L^{-1} and 100 mmol L^{-1} was found to be optimum for *M. methanica* and *M. silvestris* producing 188.03 mg/L and 161.23 mg/L methanol, respectively. Interestingly, in the presence of potassium phosphate, *M. alcaliphilum* also produced high methanol (218.90 mg/L) compared to *M. methanica* and *M. silvestris*. Methanol production was found to be decreasing beyond optimal inhibitor concentration (Fig. 4).

Effect of sodium formate For achieving maximum conversion of methane to methanol, varying concentration of reducing agent in the form of sodium formate was evaluated in respective biotransformation reactions (Fig. 4F). Effect of sodium formate was evaluated in the presence of respective optimum inhibitors for selected methanotrophs. Methanol production by *M. alcaliphilum* increased maximum up to 307.90 mg/L at 80 mmol L^{-1} sodium formate. No change in methanol production was observed with change in sodium formate concentration for *M. methanica*, whereas 20 mmol L^{-1} of sodium formate was found to be optimum with *M. silvestris* producing 177.10 mg/L methanol (Fig. 4F). Methanol production by optimum MDH inhibitors (400 mmol L^{-1} potassium phosphate and 10 mmol L^{-1} $MgCl_2$) and sodium formate (20 mmol L^{-1}) for *M. trichosporium* has been previously reported by Duan et al. (22). In the present study 2 g/L dry cells of *M. trichosporium* produced up to 298.67 mg/L methanol under similar concentrations of inhibitors and sodium formate.

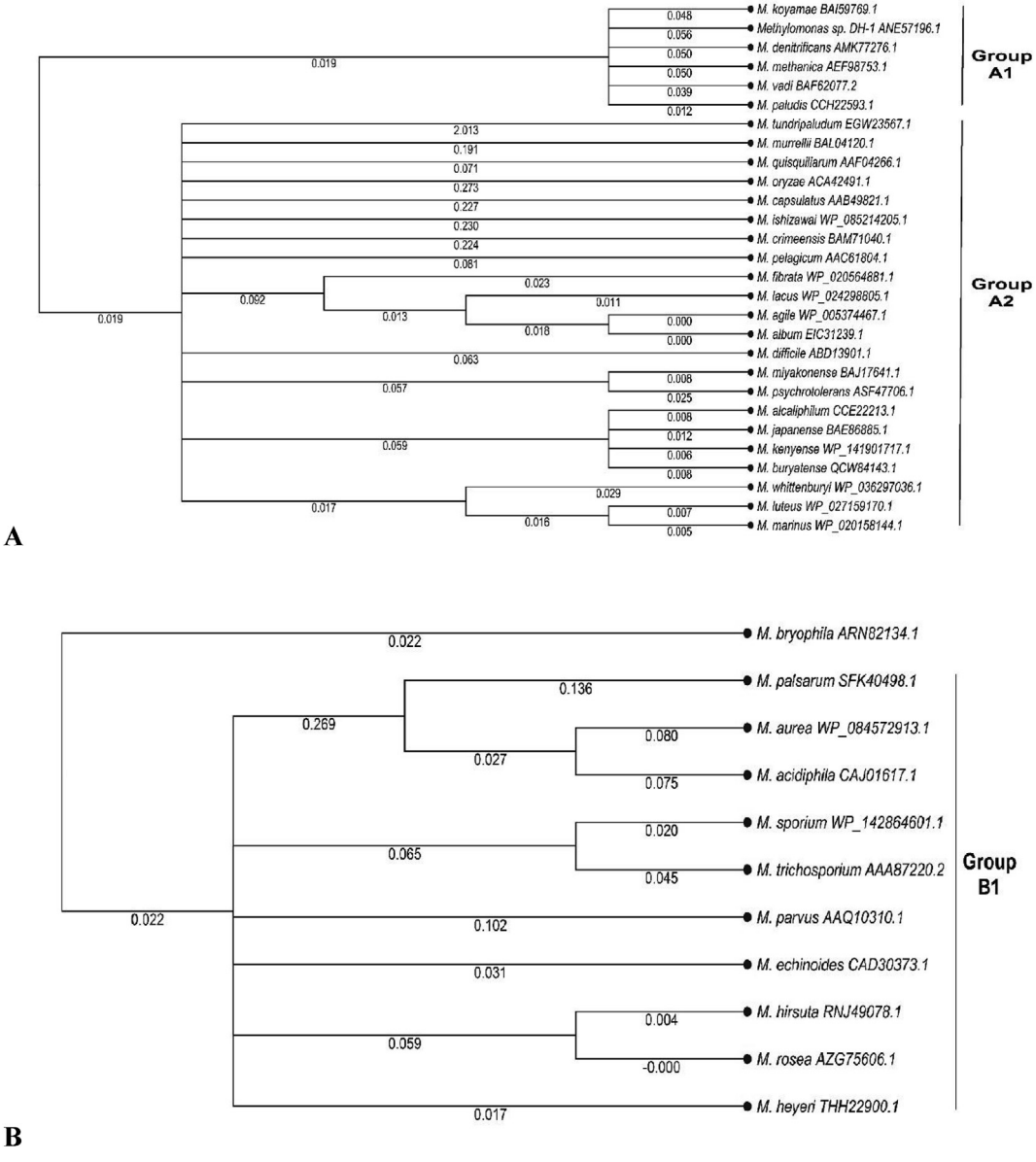

FIG. 2. Phylogenetic analysis using *pmoA* sequences performed with neighbor joining as construction method with bootstrap threshold set to 85%. (A) Mesophilic gamma-proteobacterial methanotrophs; (B) mesophilic alpha-proteobacterial methanotrophs. Branch length values displayed near lines are mentioning changes per amino acid position.

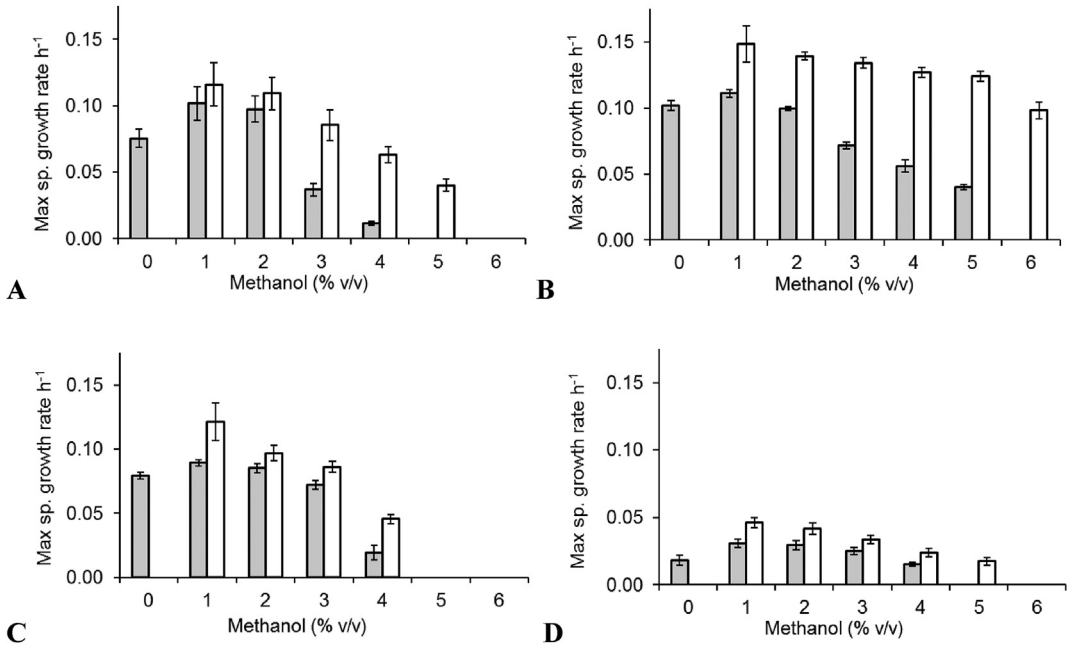

FIG. 3. Methanol tolerance data of (A) *M. methanica*, (B) *M. alcaliphilum*, (C) *M. trichosporium* and (D) *M. silvestris*. Maximum specific growth rate (h^{-1}) in the presence of methane (filled bars) and absence of methane (open bars). Data is mean representation of three replicates with standard deviation as an error bar.

MMO and FDH enzyme activity Activity assay of MMO enzyme was performed to compare methane conversion rate between selected methanotrophs. Amongst selected four methanotrophs total specific MMO activity of *M. alcaliphilum* was observed to be highest at 98.40 nmol epoxypropane per minute per mg dry cells (Fig. 5A), whereas *M. methanica*, *M. trichosporium* and *M. silvestris* showed 32.10, 68.70 and 48 nmol epoxypropane per minute per mg dry cells, respectively. All samples were analyzed in three replicates on GC and did not observe more than 10% variation.

Formate dehydrogenase activity of methanotrophs should be ensured to regenerate redox during biotransformation reaction. Activity assay of FDH enzyme from each selected methanotroph showed the ability of redox regeneration from NAD^+ to NADH. Specific FDH activity of *M. trichosporium* was found to be highest with 1.10 U/mg of protein, whereas other methanotrophs *M. methanica*, *M. alcaliphilum* and *M. silvestris* were observed to have specific FDH activity of 0.14, 0.87 and 0.31 U/mg protein, respectively (Fig. 5A). Formate utilization during biotransformation was also validated by analyzing formate consumption by all four methanotrophs. Methanotrophs *M. methanica*, *M.alcaliphilum*, *M. trichosporium* and *M. silvestris* were observed to utilize 21.75%, 39.25%, 92.86% and 81.60% of formate during biotransformation with respective optimum reaction conditions, respectively.

Biogas to methanol conversion Methanol production was further evaluated on biogas produced on rice straw and sugarcane bagasse. In biotransformation using biogas, methanol production decreased in all methanotrophs (Fig. 5B). Amongst all methanotrophs, *M. alcaliphilum* produced maximum methanol up to 204.98 mg/L and 247.37 mg/L with biogas produced from rice straw and sugarcane bagasse, respectively. In comparison of pure methane, methanol production by different methanotrophs was found to be decreased by more than 34% and 20% on biogas generated from rice straw and sugarcane bagasse, respectively.

Redox regeneration for methanol production The electrons required for methane to methanol conversion could be supplied through NADH generated by sodium formate to CO_2 conversion (26,33). Increase in sodium formate concentration affected methanol production positively but use of higher concentration of formate could not increase methanol accumulation in the presence of MDH inhibitor (16,23). To investigate this further, we estimated formate to methanol efficiency as mentioned earlier in the Materials and methods section. To evaluate redox regeneration efficiency, *M. alcaliphilum* was selected based on its higher methanol tolerance and methanol production ability. *M. alcaliphilum* was evaluated for methanol production against different sodium formate concentration in the presence and absence of ammonium chloride (Fig. 6A). Efficiency was estimated in moles of methanol produced against moles of utilized formate. In the presence of ammonium chloride, 80 mmol L^{-1} sodium formate showed 30.59% redox regeneration efficiency, whereas in the absence of inhibitor, although increase in methanol accumulation was observed with increase in sodium formate, the redox regeneration efficiency was found to be decreasing (Fig. 6A). DCW after biotransformation reaction was analyzed and no significant change in biomass concentration was observed, indicating formate was used only as source of reducing power and was not utilized for cell growth.

Methanol production at 14 L fermentor Based on the maximum methanol tolerance and methane to methanol conversion, *M. alcaliphilum* was evaluated for methanol production in fermentor culture. *M. alcaliphilum* cells were grown on methane in 14 L fermentors and used for biotransformation reaction. Biotransformation reaction was conducted in optimized reaction mixture containing 20 mmol L^{-1} sodium phosphate buffer (pH 6.8), 80 mmol L^{-1} sodium formate, 20 mmol L^{-1} of ammonium chloride and *M. alcaliphilum* at 2 g dry cells/L. In biotransformation trials with pure methane, maximum methanol up to 418 mg/L was observed while biogas from sugarcane bagasse produced up to 331.20 mg/L methanol (Fig. 6B). Based on residual gas analysis, methane consumption up to 504.74 and 469.11 mg/L of broth was observed during biotransformation reaction with pure methane and biogas, respectively.

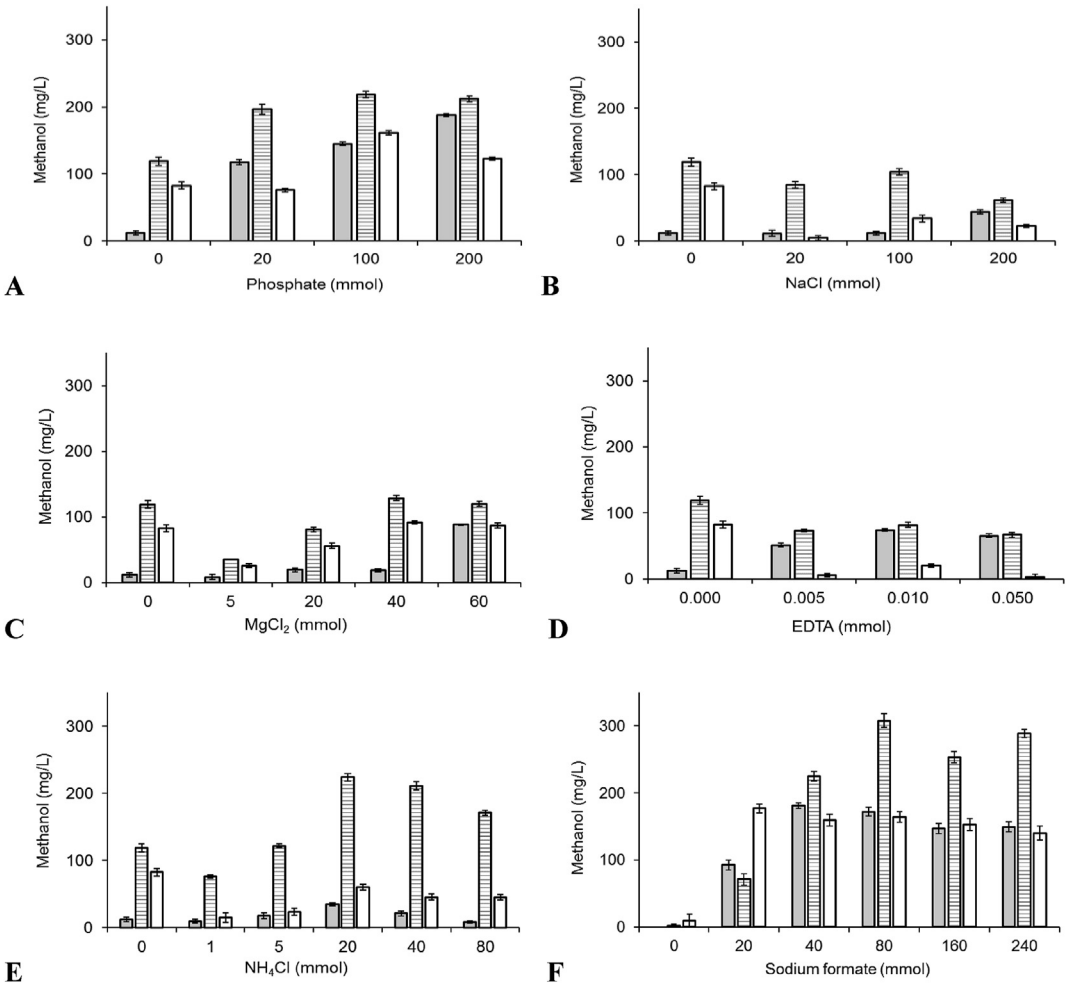

FIG. 4. Optimization of biotransformation reaction for methanol production using 20 mmol L^{-1} sodium phosphate buffer (pH 6.8), 2 g dry cells/L, 40 mmol L^{-1} sodium formate with inhibitors. (A) Potassium phosphate; (B) NaCl; (C) $MgCl_2$; (D) EDTA and (E) NH_4Cl. (F) Methanol production at varying concentrations of sodium formate at optimum inhibitor level for each strain. *M. methanica* (filled bars), *M. alcaliphilum* (horizontal-striped bars) and *M. silvestris* (open bars). Data is mean representation of three replicates with standard deviation as an error bar.

DISCUSSION

Methanotrophs are known to be present in abundance across wide geographical habitats and physiological conditions (28,34). Except for the availability of methane, no other condition rationalizes the widespread abundance of methanotrophs. Cultivation at different temperature, pH, osmolarity and combination of the key factors determines the diversity of methanotroph. It is difficult to experimentally evaluate each methanotroph from wide genera and habitats to identify few efficient and industrially suitable methanotrophs for methanol production. In the present study we applied multiple segregation and selection criteria to identify representative methanotrophs based on important characteristics. Factors like physiological conditions for growth and diversity in methane utilization was used as the basis to segregate and select representative methanotrophs.

To select suitable methanotrophic strain, data of 60 aerobic methanotrophs spread over 23 genera was analyzed. Out of 60 methanotrophic strains, particulate form of methane monooxygenase was found in 56 strains and the remaining 4 strains from *Methylocella* and *Methyloferula* genus possess only soluble form of MMO for methane utilization (27,28). Methanotrophs are known to have higher methane affinity and utilization when pMMO enzyme is selectively expressed (14). Considering the presence of pMMO activity and its importance for methane utilization, pMMO based comparison of methanotroph is highly justified. Further the sequence availability of each pMMO subunit from different methanotrophs could be useful for detailed enzyme characterisation and improvement of methane utilization ability is possible using genetic or enzyme engineering approaches. It was observed that the A1 group comprised strains from *Methylomonas* genus along with *Methylomarinum vadi*, whereas all other mesophilic gammaproteobacterium methanotrophs separated in the A2 group (Fig. 2). Although total MMO activity of methanotrophs in each sub-group can have wide variation, the diversity of pmoA sequence was used to segregate strains in different subgroups and representative from each subgroup was selected for detailed experimental investigation.

To assess methanol production ability of methanotrophs, methanol tolerance should be considered as an important aspect due to challenges associated with product inhibition. Most of the earlier studies on methanol tolerance have been done using methanol as a substrate (35,36). However, for the production of methanol, better understanding of methanol tolerance in the presence of methane is desirable. To the best of our knowledge, methanol tolerance in the presence of methane has not been investigated previously. The present study on the methanol tolerance of methanotrophs showed high tolerance both in the presence and absence of methane (Fig. 3). Interestingly, in all four selected

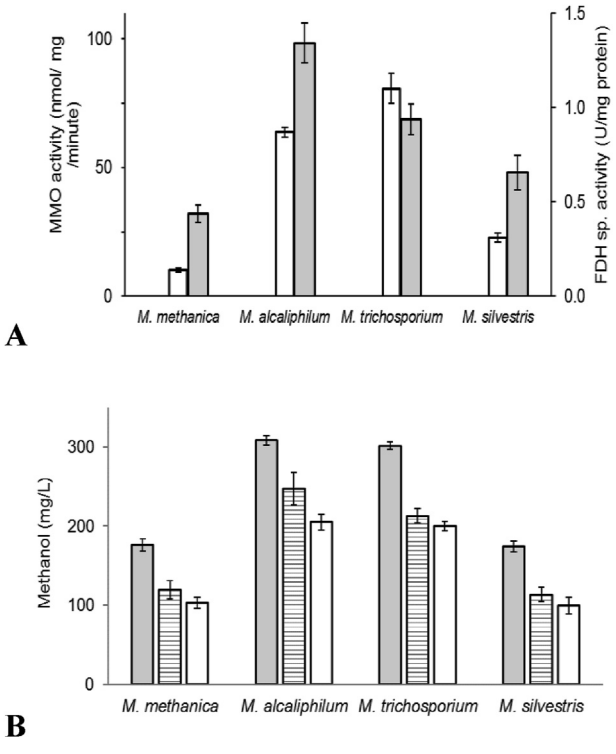

FIG. 5. Comparison of enzyme activities and methanol production using biogas. (A) Specific FDH enzyme activity (open bars) and total MMO activity of methanotrophs (filled bars). (B) Methane to methanol biotransformation reaction with 20 mmol L^{-1} sodium phosphate buffer (pH 6.8), 2 g dry cells/L, inhibitors (200 and 100 mmol L^{-1} potassium phosphate for *M. methanica* and *M. silvestris*; 20 mmol L^{-1} ammonium chloride for *M. alcaliphilum*; 400 mmol L^{-1} potassium phosphate and 10 mmol L^{-1} $MgCl_2$ for *M. trichosporium*) and sodium formate (40 mmol L^{-1} for *M. methanica*; 80 mmol L^{-1} for *M. alcaliphilum*; 20 mmol L^{-1} for *M. trichosporium* and 20 mmol L^{-1} for *M. silvestris*). Carbon sources used were methane (filled bars), biogas from sugarcane bagasse (bars with horizontal lines) and biogas from rice straw (open bars). Data is mean representation of three replicates and standard deviation is displayed as error bars.

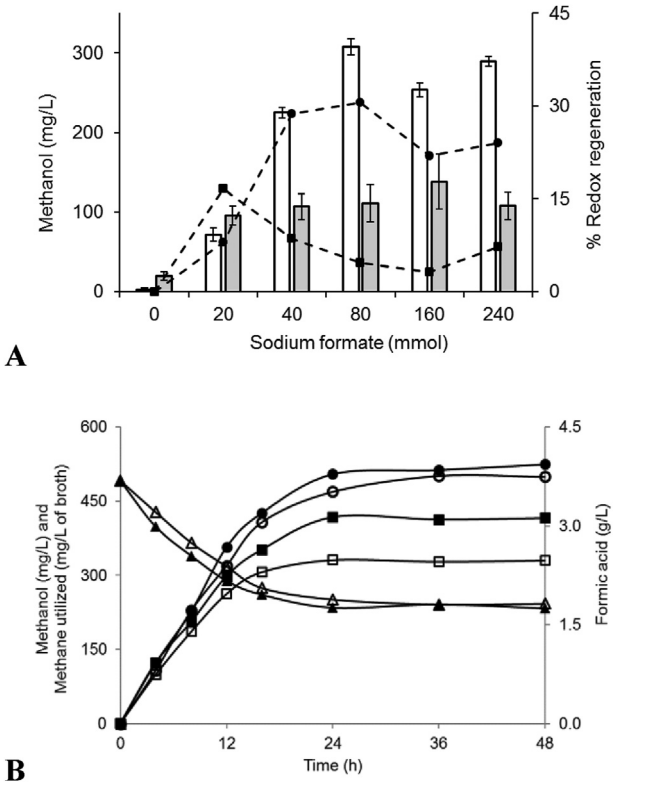

FIG. 6. (A) Effect of sodium formate on *M. alcaliphilum* methanol production in the presence of inhibitors (open bars) and in the absence of inhibitors (filled bars). Redox regeneration efficiency, in the presence of inhibitors (circles) and in the absence of inhibitors (squares). Mean of triplicate data is represented with standard deviation for bar chart. Line chart is mean representation of triplicate data for which standard deviation is within ±5% range. (B) Methanol production at 14 L fermentor using methane and biogas from sugarcane bagasse by *M. alcaliphilum*. Methanol titer using pure methane (filled squares) and biogas (open squares); methane utilized using pure methane (filled circles) and biogas (open circles); residual formic acid in pure methane (filled triangles) and biogas (open triangles). Data is mean representation of three replicates and standard deviation ≤10%.

methanotrophs, methanol tolerance was found to be decreasing in the presence of methane. In the absence of methane, methanol tolerance of *M. alcaliphilum* (6% v/v) was found to be in the similar range as reported for *Methylotuvimicrobium buryatense* (7% v/v) (36). Interestingly, both strains are halotolerant and belong to *Methylotuvimicrobium* genus. Another similar observation between *M. buryatense* and methanotrophs investigated in this study was achievement of maximum growth rate at 1% (v/v) methanol (Fig. 3).

For increasing methanol accumulation, the optimal inhibitor concentration is important for complete inhibition of MDH enzyme. Methanol production was found to be decreasing beyond the optimal inhibitor concentration (Fig. 4). This effect could be due to inhibition of MMO or formate dehydrogenase (FDH) enzyme which is responsible for supplying reducing energy for MMO activity (26). On comparison of methanol production ability using pure methane, methanol production per gram of biomass by *M. alcaliphilum* was up to 153.95 mg methanol/g DCW, whereas *M. methanica*, *M. trichosporium* and *M. silvestris* produced up to 90.49, 149.33 and 88.55 mg methanol/g DCW, respectively (Fig. 4F). As all enzymes required for biotransformation are intracellular, it is important to compare methanol production ability based on specific methanol productivity and methane to methanol yield. In earlier published reports, methanotrophs have been reported irrespective of important industrial parameters like tolerance, conversion yield, specific productivity and MMO activity (Table 1). In a study with *M. trichosporium*, maximum methanol titer up to 1.10 g/L was reported with relatively lower specific methanol productivity (22), whereas in the present study, use of 2 g/L dry cells of *M. trichosporium* resulted into 1.94 fold higher specific methanol productivity up to 3.11 mg/g DCW/h. In another study with isolate from *Methylocaldum* genus, higher specific methanol productivity and titer was reported, but the lower yield of methane to methanol conversion was achieved (Table 1). All previous studies of methanol production were performed in less than 300 mL reaction volume in serum bottles or smaller vessels (Table 1). In our studies with 3 L reaction volume *M. alcaliphilum* showed 41.36% and 35.35% methane to methanol molar conversion on pure methane and biogas with specific methanol productivity of 8.71 and 6.90 mg/g DCW/h and yield coefficient of 0.83 and 0.71 mg methanol/mg methane, respectively. Although overall carbon requirement is higher for methanol production, it could be reduced with approaches like strain engineering, improvement in MMO activity and expression of redox regeneration pathways. After the selection of industrially suitable methanotrophic strain, further enhancement in methanol production can be possible using different process modifications like use of paraffin oil (37), immobilization, co-culture, methane vector and cells recycling (1,18,20,25).

It was necessary to maintain favorable conditions for MMO activity during growth as well as biotransformation phase for achieving the maximum methanol production. In the present study, MMO activity of *M. alcaliphilum* observed to be 3.07, 1.43 and 2.05 fold higher than that of *M. methanica*, *M. trichosporium* and

M. silvestris, respectively (Fig. 5A). Specific MMO activity of *M. trichosporium* and *M. alcaliphilum* was found to be in the same range as reported in the previous studies of methane utilization using these methanotrophs (29,38). One of the major factors affecting activity of enzyme is the substrate used during growth phase. Methanotrophs grown on different substrates have shown significant variation in gene and energy regulation (35,38,39). Decrease in MMO activity of *M. trichosporium* on shifting substrate from methane to methanol (35) prompted maintenance of unlimiting concentration of methane and oxygen in both gas and liquid phase. Supply of fresh methane-oxygen mixture during growth phase at frequent intervals ensured continuous substrate availability in liquid phase. Biotransformation in stirred tank bioreactor improved mixing as well as gas liquid mass transfer. Continuous availability of methane and oxygen resulted in an increase up to 35% in methanol production by *M. alcaliphilum* as compared to that observed in serum bottles (Figs. 5B and 6B).

Methanol production ability of methanotrophs was also found to be affected by factors like stage of cell harvesting, culture motility and time lag between harvesting and biotransformation. We observed that cell harvested during the exponential phase of cell growth produced higher methanol as compared to stationary phase. In trials with *M. alcaliphilum* negative effect on methanol production in biotransformation was observed if the cell motility was not observed at the time of harvest. Similarly, in case of *M. trichosporium*, the presence of spores affected methanol production in biotransformation. Loss of motility and release of spores were observed typically during stationary phase cultures of *M. alcaliphilum* and *M. trichosporium*, respectively. For a biotransformation reaction, use of cells stored under cold conditions is expected to preserve enzyme activity. However, in our studies on multiple occasions, methane grown cells stored under cold conditions could not produce methanol and had to be utilized immediately for efficient biotransformation (data not provided). Detailed investigation to understand effect of these factors on methanol production need to be conducted.

To address the limitation of required electrons for MMO based redox reaction, supplementation of sodium formate has been an established way of increasing methanol accumulation (26,33). To investigate this further, we estimated redox regeneration efficiency and observed loss of ability to utilize formate after a threshold concentration resulting in no increase in methanol production (Fig. 6A). It was also observed that redox regeneration efficiency was up to 24–31%. This suggests that all the reducing equivalents generated by external addition of formate could not be utilized by methanotrophs for conversion of methane to methanol. This could be possible due to imbalance between $NADH/H^+$ ions produced by FDH and associated reducing equivalent requirement for MMO activity. Excess $NADH/H^+$ provided by formate supplementation could be most likely used for other cellular activities like non-growth related energy maintenance, nutrient transportation, mobility and other metabolism related energy requirements.

With increase in formate concentration, methanol accumulation was observed even in the absence of MDH inhibitors. This could be due to increase in MMO activity or due to inhibition of MDH activity by formate. In our studies, we found supplementation of formate was more critical for methanol accumulation as opposed to inhibition of MDH activity highlighting continuous supply of reducing equivalent is the most important aspect of methane to methanol biotransformation (Fig. 6A). As continuous supply of formate will increase CO_2 emission, effect of CO_2 on MMO and FDH activity need to be investigated.

Globally, methanol is produced from fossil based feedstocks like coal and natural gas. Both these feedstocks require a two-step process of methanol production involving syngas generation and conversion of syngas to methanol. Multi-step processes based on syngas to methanol require metal catalyst, high temperature and pressure conditions along with cleaning of syngas leading to large capital and operating expenditure (40). A biotechnological route based on methanotrophic organisms can provide a milder and safer fermentation-based technology for methanol production from sustainable feedstocks like biogas. Considering biogas as a potential methane rich feedstock, it was evaluated for methanol production. Use of biogas reduced methanol production significantly which could be due to the presence of CO_2, H_2S or any other inhibitors (Fig. 5B). Previously, conversion of biogas to methanol has been reported to be adversely affected due to the presence of impurities like H_2S in biogas, as well as inconsistent composition of biogas due to variation in feed like municipal solid waste (2,41). Inhibitory effect of H_2S and CO_2 on methanol production was reported with other methanotrophs at H_2S concentration less than 0.13% (2,12). Studies of *Methylosinus sporium* on pure methane produced 131.04 mg/L (4.09 mmol L^{-1}) methanol and remained unchanged on use of raw biogas with 20% methane concentration (12), while use of raw biogas with 30% and above methane concentration reduced the methanol production by more than 55% (1.82 mmol L^{-1}). In our studies with *M. alcaliphilum*, use of biogas (33% methane) resulted in only 19.77% reduction in methanol production (Fig. 5B). This is the first study reporting methanol production on biogas generated from second-generation feedstock like sugarcane bagasse and rice straw. Production of methanol using biogas produced from such agricultural waste can add value along with reduction in GHG emissions. Apart from valorization of methane, CO_2 present in biogas is the other major GHG component present in large quantities. Conversion of CO_2 to methanol can be achieved using immobilized cultures of methanotrophs as reported in previous studies (5). Other approaches like co-culture of methanotrophs with cyanobacteria can also be a promising way to utilize maximum carbon present in the biogas.

Our study presents a systematic approach for selection of industrially important methanotroph for methanol production. Overall representative selection was based on information related to microbial characterization and availability of genetic data in literature. Selected representative strains from wide habitats covering multiple genera were investigated for key characteristics like product inhibition, product formation potential and variation in source of substrate. Haloalkaliphilic organism *M. alcaliphilum* showed high methanol tolerance and methanol production ability on pure methane as well as biogas. Further improvement in methanol production is possible with *M. alcaliiphilum* due to availability of the genome sequence, genome-scale model with metabolic flux prediction and genetic engineering toolkit (7,38). We propose *M. alcaliphilum* as a potential methanotrophic platform for methanol as well as value added chemicals.

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