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# Biological conversion of methane to methanol at high $H_2S$ concentrations with an $H_2S$-tolerant methanotrophic consortium

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ABSTRACT

To develop biological biogas to methanol conversion technology without costly hydrogen sulfide ($H_2S$) removal, a high $H_2S$ tolerant methanotrophic consortium (HTMC), was enriched from an anaerobic digester effluent in presence of 5.64 g/m$^3$ $H_2S$ in the gas phase. The HTMC can grow stably and produce methanol under conditions with $CH_4$/air mixtures containing 5.64 g/m$^3$ of $H_2S$. There is no significant ($p > 0.05$) difference in cell yield or $CH_4$ to methanol conversion efficiency between trials with different $H_2S$ concentrations from 0 g/m$^3$ to 5.64 g/m$^3$. Under optimal conditions, a cell yield of 0.333 g cells/g $CH_4$, a methanol concentration of 0.28 mg/mL, and a $CH_4$ to methanol conversion efficiency of 0.22 mol/mol were obtained, respectively. Besides methanotrophs (14.85%) and other bacteria, *Cyanobacteria* were also identified in the HTMC with a high abundancy (32.16%), which could broaden the application of HTMC for simultaneous utilization of $CH_4$ and $CO_2$ from raw biogas.

## 1. Introduction

Biogas produced from organic waste has been recognized as an abundant and renewable source for bioenergy and bioproducts [1–3]. Annually, biogas captured from landfills and anaerobic digestion (AD) systems is proximally $1.84 \times 10^9$ m$^3$ in the United States [4,5]. However, the storage, transportation, and distribution of biogas are costly since the energy density of biogas is low [6,7]. Additionally, biogas is a potent greenhouse gas, which could cause environmental problems if not properly treated or utilized [8,9]. One approach to address this issue is to upgrade biogas to methanol which is a promising energy carrier for long-term storage and a valuable precursor for the production of fuels and chemicals [10].

Bio-conversion of $CH_4$ to methanol is attractive for the cleaner process, milder operating conditions, and less biogas purification requirements than thermochemical conversion methods [11]. In nature, methanotrophs can grow by using $CH_4$ as the sole carbon and energy source, acting as a $CH_4$ sink to suppress its emission to the atmosphere [12–14]. In cells of aerobic methanotrophs [14], the conversion of $CH_4$ to methanol is catalyzed by methane monooxygenase (MMO) enzyme (equation (1)), and methanol is further oxidized to $CO_2$ via a series of reactions catalyzed by methanol dehydrogenase (MDH), formaldehyde dehydrogenase (FalDH), and formate dehydrogenase (FDH) (equation (2)) [14–16].

$$CH_{4(g)} + O_{2(g)} + 2e^- + 2H^+_{(aq)} \rightarrow CH_3OH_{(aq)} + H_2O_{(aq)} \quad \Delta G(298) = -282 \text{ kJ} \Big/ \text{mol} \tag{1}$$

$$CH_3OH_{(aq)} + H_2O_{(aq)} \rightarrow CO_{2(g)} + 6H^+_{(aq)} + 6e^- \quad \Delta G(298) = -221 \text{ kJ} \Big/ \text{mol} \tag{2}$$

Methanol accumulation can be achieved by using MDH inhibitors such as phosphate, NaCl, and $MgCl_2$ which restrain the downstream pathway from methanol to $CO_2$ [17]. At the same time, exogenous reducing sources (such as formate) are supplied to maintain the metabolic activity of methanotrophs [18]. Moreover, Kalyuzhnaya [18] suggested that other important factors, such as $O_2/CO_2$ ratio, can also be controlled for methanol accumulation.

Hydrogen sulfide ($H_2S$) is commonly observed in biogas from anaerobic digestion [19] with concentrations up to 14.1 g/m$^3$ [20], especially from digesters fed with protein-rich wastes and sulfur

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containing feed stocks [21]. $H_2S$ has inhibitory effects on cell growth of methanotrophs following an inhibition competitive mode, because the gene encoding particulate MMO (pMMO) was related to competitive inhibition [13,22,23]. Compared to costly physicochemical $H_2S$ removal methods for biogas application with extra energy input, biological method with $H_2S$ tolerated methanotrophic bacteria (like whole-cell biocatalysts [24]) attracted more attentions due to mildly and directly conversion of biogas without $H_2S$ removal [11,25]. Up to now, methanotrophs have been isolated from various sources, such as natural gas fields, soils, waste treatment facilities, anaerobic digestion systems, and environments with extreme conditions [23,27]. The phylum *Verrucomicrobia* has been found in geothermal environments with high temperature and low pH [8,27–30]. A methanotrophic bacterium (SAD2) was isolated from $H_2S$-rich anaerobic digester, which shows higher $H_2S$ tolerance than *Methylocystis* sp. And *Methylomicrobium album* [13]. To the best of authors' acknowledgement, $H_2S$ content of 1.41 g/m$^3$ is still an upper limit for biogas utilization with methanotrophs.

The major objective of this study was to develop a high $H_2S$-tolerant biological system for cell cultivation and conversion of $CH_4$ to methanol under high levels of $H_2S$ stress. A methanotrophic consortium from anaerobic digester effluent was enriched under high $H_2S$ stress. The effects of $CH_4$/air ratio, pH, temperature, and $H_2S$ concentration on cell growth were studied. In addition, the effects of phosphate concentration, formate concentration, $CH_4$/air ratio, and $H_2S$ concentration on methanol production were also evaluated. Moreover, the microbial community profile of the consortium was analyzed for possible mechanisms of $H_2S$ tolerance. Results of this study suggested a feasible strategy for addressing the $H_2S$ inhibition issue in the biological conversion of raw biogas to methanol or other products.

## 2. Materials and methods

### 2.1. Enrichment of HTMC

Digester effluent was sampled from a mesophilic anaerobic digester fed with municipal sewage sludge (KB BioEnergy, Akron, OH, USA). Enrichment of HTMC (named based on functional methanotrophic bacteria) was conducted in 250-mL Erlenmeyer flasks using a method described in a previous study [25]. Each flask was set up by adding 5 g of the digester effluent and 50 mL of nitrate mineral salts (NMS) medium which contains 1.0 g/L of $MgSO_4{\cdot}7H_2O$, 0.272 g/L of $KH_2PO_4$, 1.0 g/L of $KNO_3$, 0.134 g/L of $CaCl_2{\cdot}2H_2O$, 0.284 g/L of $Na_2HPO_4$, 0.2% (*v/v*) of chelated Fe solution (1.0 g/L of ferric (III) ammonium citrate, 0.3% (*v/v*) of concentrated HCl, and 2.0 g/L of EDTA), and a 0.05% (*v/v*) of trace element solution (10 mg/L of $ZnSO_4{\cdot}7H_2O$, 500 mg/L of EDTA, 3.0 mg/L of $MnCl_2{\cdot}4H_2O$, 200 mg/L of $FeSO_4{\cdot}7H_2O$, 30 mg/L of $H_3BO_3$, 1.0 mg/L of $CaCl_2{\cdot}2H_2O$, 20 mg/L of $CoCl_2{\cdot}6H_2O$, 2.0 mg/L of $NiCl_2{\cdot}6H_2O$, and 3.0 mg/L of $Na_2MoO_4{\cdot}2H_2O$). The flask was sealed with a rubber stopper that has a gas outport controlled with a clamp. $CH_4$ (Praxair®, Danbury, CT, USA) and $H_2S$ gases were injected into the reactor to obtain a gas mixture of 80% (*v/v*) air and 20% (*v/v*) $CH_4$ (replacing 40 mL air in the 200-mL headspace by $CH_4$ using a 60-mL syringe) with 5.64 g/m$^3$ $H_2S$ in the headspace. The flask was incubated at 37 °C and 150 rpm for 5 days. Then, 5 g of the enriched culture was transferred to the next batch of flask containing 50 mL of fresh NMS medium to start the second batch of enrichment using the same procedures as described for the first batch. The HTMC was obtained after 5 successive batches of enrichment.

### 2.2. Cell growth at different $CH_4$/air ratio, pH, and temperature

Cell growth tests were conducted in 250-mL Erlenmeyer flasks with 7 mg of HTMC biomass and 50 mL of NMS medium in each reactor, and a 500-mL Tedlar gas bag was connected to the headspace via a gas outport on the stopper. The total gas volume in the headspace and the gas bag was 700 mL (200 mL of headspace + 500 mL of gas bag). All cell growth reactors were incubated at 150 rpm for 144 h with a fixed initial $H_2S$ concentration of 5.64 g/m$^3$ and designated $CH_4$/air ratios, pH, and temperatures. Cell yields (based on ash-free cell weights at 0 h and 144 h) were measured to evaluate the effects of $CH_4$/air ratios, pH, and temperatures on cell growth. Each trial was carried out in three replicates.

Trials with different initial $CH_4$/air ratios (1:2, 1:4, and 1:6, *v/v*) were first conducted at 37 °C, and the optimal $CH_4$/air ratio was determined based on cell yields. The designated $CH_4$/air ratios were obtained by replacing different volumes (233.3 mL, 140.0 mL, and 100 mL) of air in the 500-mL Tedlar gas bag with $CH_4$ (99% purity) using a 60-mL graduated syringe. For trials with different pH, 5 M NaOH or 1 M HCl were used to prepare media with pH values of 5.5, 6.0, 6.5, 6.8, and 7.5, respectively. Reactors with different pH were incubated at 37 °C with the optimal initial $CH_4$/air ratio, and the optimal pH was determined based on cell yields. Trials with different temperatures (30 °C, 37 °C, 45 °C, and 50 °C) were further performed with the optimal initial $CH_4$/air ratio and pH.

To validate $CH_4$ consumption by HTMC, Erlenmeyer flasks (250-mL) with different conditions (50 mL of NMS medium with and without 7 mg of HTMC) were also set up with an initial $CH_4$/air ratio of 1:4 and $H_2S$ concentration of 5.64 g/m$^3$ in the headspace and incubated at 150 rpm for 144 h. About 30 mL of gas sample was taken every other day for gas composition analysis.

### 2.3. Cell growth at different $H_2S$ concentrations

Cell growth tests were conducted using optimal conditions determined in section 2.2 except that flasks were set up with different initial $H_2S$ concentrations (0, 1.41, 2.82, and 5.64 g/m$^3$). The designated $H_2S$ concentrations were obtained by replacing certain volumes of mixture gas in the 500-mL Tedlar gas bag with pure $H_2S$ gas. To evaluate change of $H_2S$ concentration during cell growth and to investigate the mechanism, additional reactors were set up with a $CH_4$/air ratio of 1:4 (*v/v*) and $H_2S$ concentration of about 5.64 g/m$^3$ in headspace but with different liquid medium conditions (50 mL of NMS medium and 50 mL of NMS medium inoculated with 7 mg of HTMC). About 30 mL of gas sample and 0.5 mL of liquid sample were taken every other day from each flask for $H_2S$ analysis. Three replicates were used for each trial.

### 2.4. Methanol production with different phosphate concentration, sodium formate concentration, and $CH_4$/air ratio

Methanol production tests were set up in 250-mL Erlenmeyer flasks each containing 4 mg of HTMC biomass and 50 mL NMS medium with 5 μM $CuCl_2$. A 500-mL Tedlar gas bag was connected to the headspace of each flask and total gas volume in the headspace and the Tedlar gas bag was 700 mL. All methanol production reactors were incubated at 37 °C with 150 rpm for 48 h, with designated phosphate concentration, sodium formate concentration, and $CH_4$/air ratio. Gas composition and methanol concentration were measured every 12 h. Each trial was carried out in triplicate.

Trials with different concentrations of phosphate buffer (20, 50, 100, and 200 mmol/L of $PO_4^{3-}$ with equal mol of $KHPO_4$ and $Na_2HPO_4$) were conducted with 100 mmol/L of sodium formate in the medium and an initial $CH_4$/air ratio of 1:4 (*v/v*) in the gas phase. The optimal phosphate concentration was determined based on methanol yield. Trials with different sodium formate concentrations (50, 100, 150, and 200 mmol/L) were then performed with the optimal phosphate concentration and an initial $CH_4$/air ratio of 1:4 (*v/v*) to determine the optimal sodium formate concentration. Trials with different initial $CH_4$/air ratios (2:1, 1:1, 1:2, 1:4, and 1:6, *v/v*) were further carried out with the optimal phosphate concentration and sodium formate concentration.

### 2.5. Methanol production at different $H_2S$ concentrations

Methanol production tests were conducted using optimal conditions

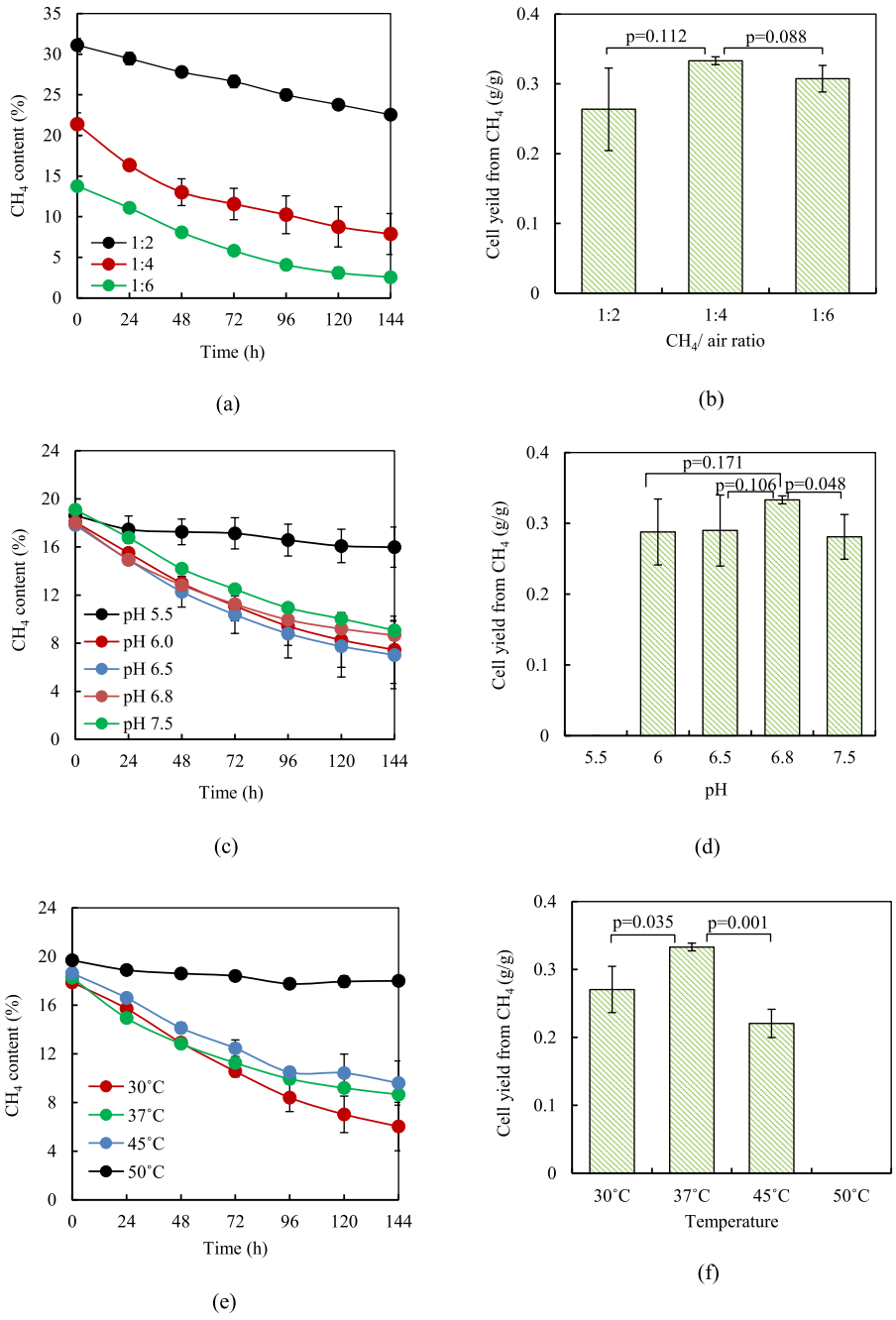


**Fig. 1.** Effect of different conditions on $CH_4$ consumption ((a) $CH_4$/air ratio, (c) pH, and (e) temperature) and cell yield ((b) $CH_4$/air ratio, (d) pH, and (f) temperature) of HTMC.

determined in section 2.4, except that flasks were set up with different initial $H_2S$ concentrations (0, 1.41, 2.82, 5.64 g/m$^3$). To evaluate the changes of $H_2S$ concentration during methanol production by HTMC and to investigate the mechanism, additional reactors were set up with a $CH_4$/air ratio of 1:4 (*v/v*) and $H_2S$ concentration of 5.64 g/m$^3$ in headspace. About 30 mL of gas and 0.5 mL of liquid samples were taken daily from each reactor for $H_2S$ analysis. Three replicates were used for each trial.

### 2.6. Analytical methods

Cell dry weight was measured according to a modified method [5]. Cell suspension (50 mL) was centrifuged at 12000 rpm for 20 min. After discarding the supernatant, the cell pellet was resuspended with 25 mL of 0.5 M $NH_4HCO_3$, and the suspension was centrifuged at 12000 rpm for 20 min. The supernatant was discarded, and the pellet was transferred to a 10-mL crucible using 3 mL 0.5 M $NH_4HCO_3$. The crucible with cell suspension was heated in an oven at 105 °C for 12 h and cooled to room temperature for measuring dry weight of total biomass. The crucible with dried sample was then heated in an Isotemp muffle furnace at 550 °C for 4 h and cooled to room temperature for measuring ash content. Ash-free dry weight was calculated as equation (3):

$$Ash\ free\ dry\ weight = Dry\ weight\ of\ total\ biomass - Resiudal\ ash\ weight \quad (3)$$

The composition of headspace gas was measured by using a GC (Agilent, HP 6890, Wilmington, DE, USA) equipped with a 30 m × 0.53 mmol/L × 10 μm Rt®-Alumina Bond/KCl deactivation column and a

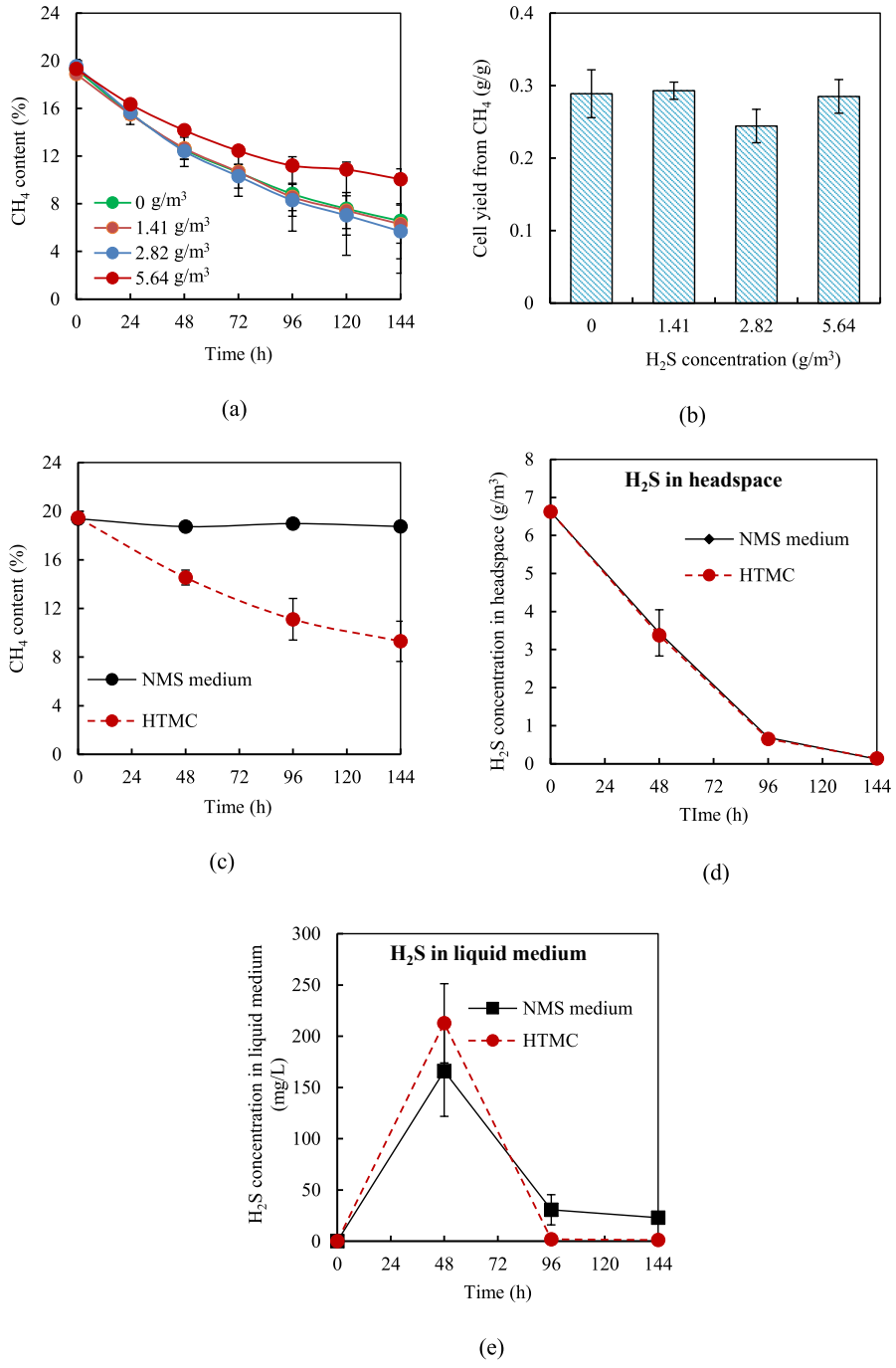


**Fig. 2.** Effect of $H_2S$ stress on (a) $CH_4$ consumption, (b) cell yield of HTM, (c) change of $CH_4$ content, and (d) $H_2S$ concentration in headspace and (e) $H_2S$ concentration in liquid medium.

thermal conductivity detector (TCD). The oven temperature was initially kept at 40 °C for 4 min, and then increased to 60 °C with an increment of 20 °C/min and maintained at 60 °C for 5 min. Temperatures of the detector and injector were set at 200 °C and 150 °C, respectively. The carrier gas was Helium.

Methanol concentration was determined by using a GC (Shimadzu, 2010PLUS, Columbia, MD, USA) equipped with a Stabilwax polar phase column (30 m × 0.32 mmol/L × 0.5 μm) and flame ionization detector (FID). The oven temperature was initially set at 50 °C and gradually increased to 80 °C in 6 min. The temperature of the injector and the detector was 250 °C, and helium was used as the carrier gas. The $CH_4$ to methanol conversion efficiency was calculated based on equation (4).

$$Methanol\ conversion\ ratio = \frac{Methanol\ production\ (mol)}{Inital\ CH_4(mol) - Finial\ CH_4(mol)} \quad (4)$$

$H_2S$ concentration was determined based on a method with slight modifications [25]. For the determination of $H_2S$ concentration in the gas phase, a sealed and vacuumed test tube (Tube A) containing 2 mL of 2% zinc acetate was used to absorb the gas sample (30 mL). After incubation at ambient temperature for 20 min, 0.5 mL of the liquid was transferred to another tube (Tube B) containing 0.5 mL of 20 mmol/L of N, N-dimethyl-*p*-phenylenediamine dihydrochloride (DPDH, in 7.2 M HCl), 0.4 mL of 30 mmol/L $FeCl_3$ (in 1.2 M HCl), and 2.6 mL of deionized water. For determination of $H_2S$ concentration in the liquid phase, 0.5

mL of liquid sample was directly transferred to the Tube B mentioned above. After incubation at room temperature for 20 min, the absorbance of the liquid mixture in Tube B was measured with a spectrophotometer (Biotek, Winooski, VT, USA) at 670 nm. The $H_2S$ concentration was then calculated based on a standard curve established before the measurement. It should be noted that this method tests the total sulfide that includes all three forms ($H_2S$, $HS^-$ and $S^{2-}$), and the result was reported as $H_2S$. The pH values were measured using a pH probe.

### 2.7. Microbial community analysis of HTMC

The HTMC was sampled and analyzed for the microbial community in triplicate. 515f-806r primers were used to amplify the V4–V5 fragment of the 16S rRNA gene of microbe following the Illumina manufacture instruction and the method as described by Caporaso et al. [31]. Then, a sequencing analysis of the amplicon library was conducted by using an Illumina MiSeq with a 2 × 300 bp paired-end protocol.

QIIME (v 1.9) [32] and the protocol reported by Ref. [33] were employed to process and analyze the sequencing data. The forward reads were used for the present analysis. Cutadapt (https://cutadapt.readthedocs.io/en/stable/index.html) was firstly used to trim the sequences corresponding to the primers from the reads. After that, a Q score no less than 20 and a length-filter were used to obtain reads of 220–280 bp. Operational taxonomic units (OTUs) were against the 2013–08 Greengenes database at 97% sequence similarity level (species equivalent). ChimeraSlayer was used to check the chimera. Further analysis was carried out after the removal of Singleton OTUs. Then the taxonomy assignments of sequences were summarized and plotted. The sequence data of this study can be found in the Sequence Read Archive (SRA) database of NCBI under the accession number SRP091046.

### 2.8. Statistical analysis

Statistical analysis in this study was conducted by analyzing the variance (ANOVA, $\alpha = 0.05$) with Minitab 17.

## 3. Results and discussion

### 3.1. Effect of $CH_4$/air ratio, pH, and temperature on cell growth of HTMC

In presence of 5.64 g/m$^3$ of $H_2S$, the HTMC showed significant $CH_4$ consumption and cell growth with different $CH_4$/air ratios, pH, and temperatures, except for some harsh conditions such as pH of 5.0 and temperature of 50 °C (Fig. 1). The decrement of $CH_4$ content in 144 h was in a range of 9%–13% for different $CH_4$/air ratios (1:2, 1:4, and 1:6, *v/v*). The maximum $CH_4$ consumption was obtained by the $CH_4$/air ratio of 1:4 (Fig. 1a). This is consistent with the $CH_4$ metabolism in methanotrophs which depend on both $CH_4$ and $O_2$ supply and require a balanced $CH_4/O_2$ ratio [18]. pH from 6.0 to 7.5 had no significant effect on $CH_4$ consumption (10% decrement in $CH_4$ content), while pH 5.5 inhibited $CH_4$ consumption considerably (3% decrement in $CH_4$ content during 144 h) (Fig. 1c). A similar phenomenon was also observed in trials of different temperatures. Only a minimal decrement (1.7%) of $CH_4$ content was obtained when the temperature was set at 50 °C, while significant $CH_4$ content decrements of 11.9%, 9.5%, and 9.0% were observed at 30 °C, 37 °C, and 45 °C, respectively (Fig. 1e). These results indicated that $CH_4$ oxidation was inhibited at low pH (5.5) and high temperature (50 °C), which is similar to those reported in literature [34]. As shown in Fig. 2c, flasks with HTMC showed a significant $CH_4$ decrease (from 20% to 10%), while flasks with only NMS medium didn't, which is also similar to results reported by other researchers [35]. As a result, the decrement of $CH_4$ content (Fig. 1) was not due to the absorption by medium but consumption by HTMC.

The maximum cell yield of 0.333 g cells/g $CH_4$ was obtained from the $CH_4$/air ratio of 1:4, although no significant difference ($p > 0.05$) was observed from that with $CH_4$/air ratio of 1:2 or 1:6 (Fig. 1b). Although the cell yield of HTMC was lower than *M. trichosporium OB3b* (0.5–0.7 g cells/g $CH_4$) and Strain MC-AD3 (0.40 g cells/g $CH_4$), it is still comparable to that of a pure methanotrophic strain, *Methylocaldum* 14B (0.2 g cells/g $CH_4$), which was isolated from the digestate of solid-state anaerobic digestion systems (Table 1). Significant cell growth was obtained when pH was in a range of 6–7.5, while no cell growth was observed with a pH of 5.5. The optimal pH was found to be 6.8 with a cell yield that is significantly higher than those obtained by pH of 5.5 and 7.5 ($p < 0.05$), but not significantly different from those by pH of 6 and 6.5 ($p > 0.05$) (Fig. 1d). In cases of temperature studies, it was found that cell yields (0.22–0.33 g cells/g $CH_4$) were achieved under different temperatures, except for 50 °C as the cell yield was 0 (Fig. 1f). Moreover, the optimal temperature was determined to be 37 °C which achieved a cell yield that is significantly ($p < 0.05$) higher than those obtained at other temperatures (30 °C, 45 °C, and 50 °C) (Fig. 1f).

**Table 1**
Comparison of cell density of methanotrophic bacteria under different cultivation conditions.

| Strains/consortia | Conditions | | | Cell density | References |
|---|---|---|---|---|---|
| | $CH_4$/air ratios | pH | Temperatures | | |
| *M. trichosporium OB3b* | 1:1 | – | 30 °C | 0.5–0.7 g cells/g $CH_4$ | [15] |
| Stain 14B | 1:4 | – | 37 °C | 0.20 g cells/g $CH_4$ | [5] |
| Strain MC-AD3 | 1:4 | 6.8 | 47 °C | 0.40 g cells/g $CH_4$ | [36] |
| HTMC | 1:4 | 6.8 | 37 °C | 0.333 g cells/g $CH_4$ | This study |

In summary, the optimal conditions for HTMC growth were $CH_4$/air ratio of 1:4, pH of 6.8, and temperature of 37 °C, which is not surprising since the HTMC was enriched under these conditions. However, the HTMC can grow stably with a wide range of $CH_4$/air ratio (1:2 to 1:6), pH (6.0–7.5), and temperature (30–45 °C), which is beneficial for commercial application. Besides, these conditions were comparable to those of two methanotrophs, 14B and SAD2, which were also isolated from anaerobic digestate [25,26].

### 3.2. Effect of $H_2S$ on cell growth of HTMC

The effect of $H_2S$ on $CH_4$ consumption and cell yield of HTMC are presented in Fig. 2. The decrement of $CH_4$ contents (12.7%, 12.7%, and 13.8%) were comparable with initial $H_2S$ concentrations of 0 g/m$^3$, 1.41 g/m$^3$ and 2.82 g/m$^3$, respectively, while the decrement of $CH_4$ content was only 9% when HTMC was exposed to 5.64 g/m$^3$ of $H_2S$, suggesting an inhibitory effect on $CH_4$ consumption (Fig. 2a). However, no significant difference in cell yield ($p > 0.05$) was observed with initial $H_2S$ concentrations from 0 to 5.64 g/m$^3$ (Fig. 2b). The maximum cell yield (0.293 g cells/g $CH_4$) was observed with 1.41 g/m$^3$ of $H_2S$, which was slightly higher than those obtained with 0 g/m$^3$ (0.289 g cells/g $CH_4$) and 5.64 g/m$^3$ (0.285 g cells/g $CH_4$) of $H_2S$ (Fig. 2b). These results indicate that HTMC can keep metabolic activities at a very high stress level of $H_2S$ (up to 5.64 g/m$^3$) (Fig. 2a and d).

$H_2S$ is known to be toxic to methanotrophic bacteria [22], and could limit the utilization of biogas as renewable and clean energy [37,38]. $H_2S$ concentration of 1.41 g/m$^3$ in headspace inhibited 35%–85% cell growth of *Methylocystis* sp., *Methylomicrobium album*, and SAD2, but showed no inhibition on cell growth of HTMC (Table 1). When $H_2S$ concentration was further increased to 5.64 g/m$^3$, cell growth of *Methylocystis* sp., *Methylomicrobium album*, and SAD2 was completely inhibited, while the cell growth of HTMC was only inhibited by 1% (Table 1). As a result, the $H_2S$ tolerance of HTMC was substantially higher than the reported methanotrophs in terms of cell growth.

Changes in $H_2S$ concentrations in the liquid medium and headspace

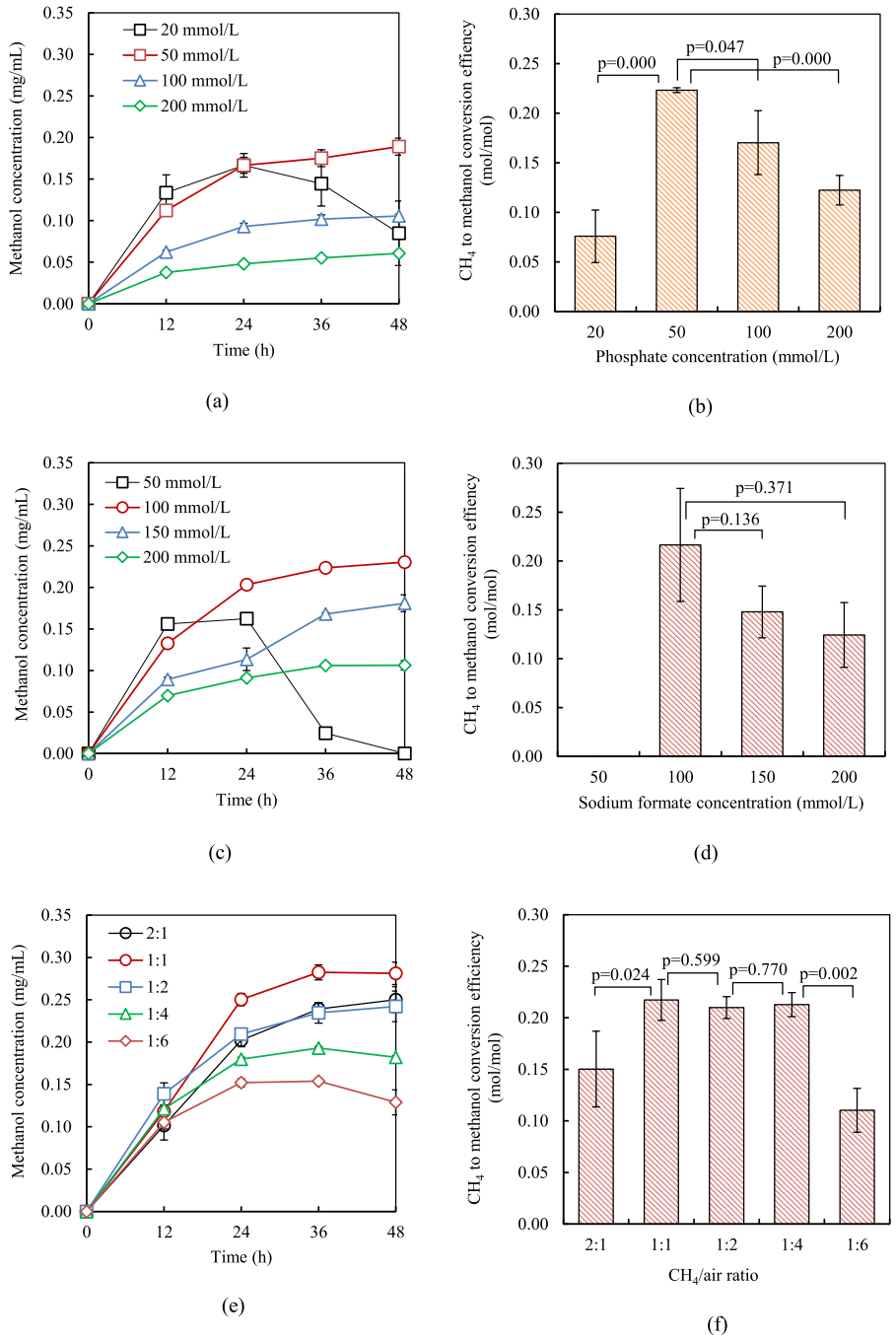


**Fig. 3.** Effect of different conditions on methanol yield ((a) phosphate addition, (c) sodium formate addition, and (e) $CH_4$/air) and $CH_4$ to methanol conversion efficiency ((b) phosphate addition, (d) sodium formate addition, and (f) $CH_4$/air ratio).

during 144 h cell cultivation process are shown in Fig. 2d and e. $H_2S$ concentration in the headspace with both conditions (with and without HTMC) dropped from 6.63 g/m$^3$ to 0.71 g/m$^3$ in 96 h, then to about 0.14 g/m$^3$ in 144 h (Fig. 2d). $H_2S$ concentrations in liquid media peaked in the first 48 h, dropped in the second 48 h, and remained relatively constant in the third 48 h, which might be due to the further oxidation of $H_2S$ to sulfate [21,35] (Fig. 2e). However, $H_2S$ concentration in liquid medium with HTMC decreased more quickly (from 213 mg/L to 2 mg/L) than that without HTMC (from 168 mg/L to 23 mg/L), indicating that presence of HTMC facilitated the consumption of $H_2S$ in the NMS medium due to the low specificities of MMO to substrate [35] (Fig. 2e).

### 3.3. *Effect of phosphate addition, formate addition, and $CH_4$/air ratio on methanol production by HTMC without $H_2S$*

Fig. 3 shows the effect of phosphate addition, formate addition, and $CH_4$/air ratio on methanol yield without the presence of $H_2S$. In general, methanol concentration increased during the 48 h production process when 20 mmol/L - 200 mmol/L of phosphate was added, except that the trail with 20 mmol/L of phosphate showed a methanol concentration decrease after 24 h (Fig. 3a). The maximum methanol concentration (0.19 mg/mL) was obtained in 48 h with 50 mmol/L phosphate addition (Fig. 3a). When increasing the phosphate concentration to 100 and 200 mmol/L, the methanol concentration decreased to 0.11 mg/mL and 0.06 mg/mL, respectively (Fig. 3a). These results indicate that MDH

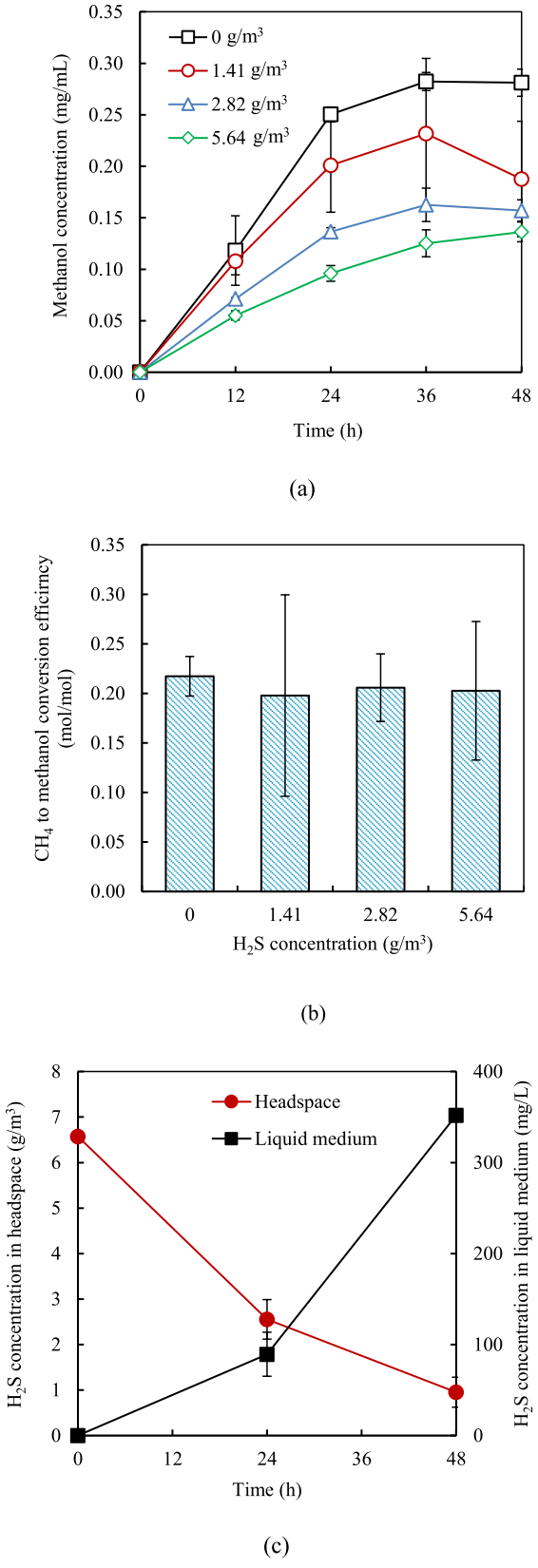


**Fig. 4.** Effect of $H_2S$ concentration on (a) methanol yield, (b) $CH_4$ to methanol conversion efficiency, and (c) $H_2S$ concentration changes in liquid medium and headspace during methanol production by HTMC.

(methanol dehydrogenase) inhibitors, such as phosphate, are essential for methanol accumulation by preventing further oxidation of methanol in HTMC [5]. However, excessive phosphate addition may also inhibit FDH (formate dehydrogenase) in HTMC, which would cause a decrease in methanol concentration under higher phosphate concentration [5].

The effect of formate addition on methanol concentration has a similar tendency to that of phosphate addition (Fig. 3c). The maximum methanol concentration of 0.23 mg/mL was obtained with a formate addition of 100 mmol/L in 48 h (Fig. 3c). When formate addition decreased to 50 mmol/L, a peak methanol concentration of 0.16 mg/mL was obtained in 24 h and then decreased to 0 in 48 h (Fig. 3c). Higher levels of formate addition (150 mmol/L and 200 mmol/L) didn't further increase the methanol concentration, obtaining peak methanol concentrations of 0.18 mg/mL and 0.11 mg/mL, respectively (Fig. 3c). These results were also consistent with other reports on conversion of $CH_4$ to methanol by *Methylosinus trichosporium* OB3b [39,40]. When MDH is inhibited for methanol accumulation, exogenous formate will function as an additional electron donor to provide electrons for cell activity [6], but too high formate could be toxic to cells as well.

When the $CH_4$/air ratio increased from 1:6 to 1:1, the methanol concentration in 48 h increased from 0.13 mg/mL to 0.28 mg/mL, but no further increment of methanol concentration was obtained when $CH_4$/air ratio increased to 2:1 (Fig. 3e). This result was also consistent with previous studies by Kalyuzhnaya et al. [18], who pointed out that the entire $CH_4$ conversion process relied on $CH_4$ and $O_2$ via the enzyme (MMO), and imbalanced $CH_4$ and $O_2$ supply would reduce the $CH_4$ consumption leading to lower methanol concentration. In addition, it was found that the methanol accumulation reached a plateau at 48 h, which could be due to the limited amount of formate in the medium.

The effect of phosphate addition, formate addition, and $CH_4$/air ratio on $CH_4$ to methanol conversion efficiency is further presented in Fig. 3. Similar to the results in Fig. 2, the highest $CH_4$ to methanol conversion efficiency was observed at 50 mmol/L of phosphate addition, 100 mmol/L of formate addition, and $CH_4$/air ratio of 1:1 (Fig. 3). Statistical analysis revealed that 50 mmol/L of phosphate addition significantly ($p < 0.05$) increased the $CH_4$ to methanol conversion efficiency (increased 1.29–13.16-fold) in comparison with other two trials with different phosphate addition (Fig. 3b). While 100 mmol/L of formate addition did not show a significant ($p > 0.05$) increase in $CH_4$ to methanol conversion efficiency compared two 150 mmol/L and 200 mmol/L of formate additions (Fig. 3d). It was noted that the $CH_4$ to methanol conversion efficiency with 50 mmol/L of formate addition was 0 since no methanol was accumulated at the termination (Fig. 3d). There were significant differences in $CH_4$ to methanol conversion efficiency between $CH_4$/air ratios of 2:1, 1:1 (or 1:2, or 1:4) and 1:6 ($p < 0.05$), but no significant difference between $CH_4$/air ratios of 1:1, 1:2, and 1:4 ($p > 0.05$) (Fig. 3f). The maximum $CH_4$ to methanol conversion efficiency of 0.22 mol/mol is comparable to that obtained by *Methylosinus trichosporium* at the same $CH_4$/air ratio [6,41].

### 3.4. Effect of $H_2S$ on methanol production by HTMC

The effects of $H_2S$ concentration on methanol concentration and $CH_4$ to methanol conversion efficiency are shown in Fig. 4. Methanol was detected at all tested $H_2S$ concentrations (0–5.64 g/m$^3$), indicating that the HTMC could produce methanol under a broad range of $H_2S$ stress (Fig. 4a). In general, methanol concentrations with different levels of $H_2S$ stress increased rapidly within 24 h, and then became stable in 36 h (Fig. 4a). It was also found that the methanol concentrations decreased with increasing $H_2S$ stress. Trials without $H_2S$ stress obtained the maximum methanol concentration of 0.28 mg/mL in 48 h, followed by those with 1.41 g/m$^3$, 2.82 g/m$^3$, and 5.64 g/m$^3$ of $H_2S$, which obtained methanol concentrations of 0.19, 0.16, and 0.14 mg/mL, respectively (Fig. 4a). The inhibition of methanol production by HTMC with 1.41 g/m$^3$ of $H_2S$ was 33%, which was lower than that of strain SAD2 (48%) (Table 2). Although methanol production of HTMC was still inhibited by

**Table 2**
Comparison of $H_2S$ tolerance between different strains/consortia.

| Strains/consortia | Inhibition of cell growth (%) | | | Inhibition of methanol production (%) | | | References |
|---|---|---|---|---|---|---|---|
| | 0.71[a] | 1.41[a] | 5.64[a] | 0.71[a] | 1.41[a] | 5.64[a] | |
| *Methylocystis* sp. | 47 | 85 | 100 | n/a | n/a | n/a | [13] |
| *Methylomicrobium album* | 39 | 49 | 100 | n/a | n/a | n/a | [13] |
| Strain SAD2 | 17 | 35 | 100 | 16 | 48 | n/a | [25] |
| HTMC | n/a | 0 | 1 | n/a | 33 | 52 | This study |

[a] $H_2S$ concentration ($g/m^3$) in the headspace.

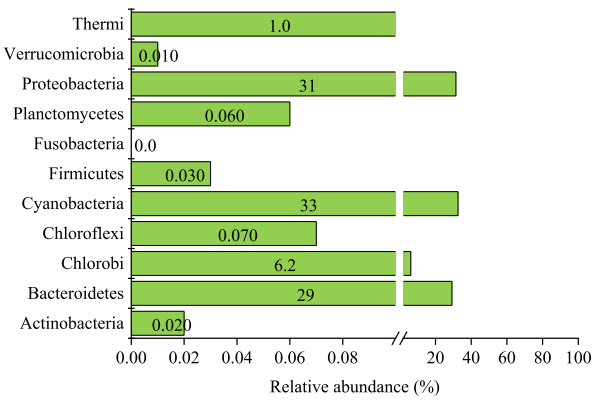


**Fig. 5.** Major bacterial phyla of HTMC.

$H_2S$ stress (1.41–5.64 $g/m^3$), its $H_2S$ tolerance was much greater than strain SAD2 [25].

$CH_4$ to methanol conversion efficiencies at all tested $H_2S$ concentrations were about 0.2 mol/mol (Fig. 4b). The $CH_4$ to methanol conversion efficiency obtained with 5.64 $g/m^3$ of $H_2S$ (0.203 mol/mol) was only 10% lower than that obtained with 0 mg/L of $H_2S$ (0.217 mol/mol) (Fig. 4b). Statistical analysis revealed that there was no significant ($p > 0.05$) difference in $CH_4$ to methanol conversion efficiency among different $H_2S$ stress levels from 0 to 5.64 $g/m^3$ (Fig. 4b). Up to date, studies on bioconversion of $CH_4$ to methanol under conditions with high $H_2S$ stress are limited with $H_2S$ concentrations no more than 1.41 $g/m^3$. This study for the first time reported biological methanol production from $CH_4$ with $H_2S$ stress up to 5.64 $g/m^3$.

$H_2S$ concentration changes in the headspace and liquid medium during methanol production process are further illustrated in Fig. 4c. The $H_2S$ concentration in headspace had an 85.5% decrease during 48 h methanol production (Fig. 4c), which was similar to that observed during cell growth (Fig. 2). However, the $H_2S$ concentration in the liquid medium increased from 0 mg/L to 352 mg/L during the methanol production process (Fig. 4B), which was 64% higher than the peaking $H_2S$ concentration in the liquid medium during cell growth (Fig. 2). This could explain the significant $H_2S$ inhibition on methanol concentration during methanol production by HTMC (Fig. 4b) compared to the minor effect of $H_2S$ on cell growth of HTMC (Fig. 2a and b).

### 3.5. Microbial community analysis of HTMC

To have a better understanding of the mechanism for the $H_2S$ tolerance of HTMC, the microbial community profile of HTMC was analyzed, and 79,601 quality-checked sequences were obtained in total. Over 99.7% of the sequences belong to the domain bacteria, while the remaining sequences were unclassified. 703 bacterial species-equivalent OTUs were found. According to the Greengenes database, almost all the bacterial sequences could be classified to a certain phylum. *Cyanobacteria* was the most predominant phylum with a 32.61% proportion of the total bacterial sequences, followed by *Proteobacteria* (31.43%), *Bacteroidetes* (29.23%), and *Chlorobi* (6.17%), while other phyla represent less than 1% of the total bacterial sequences (Fig. 5). *Cyanobacteria* are known for the ability to generate hydrogen, but their function in HTMC is unclear [42]. Consortium with methanotrophs and *Cyanobacteria* could simultaneously utilize $CH_4$ and $CO_2$ as reported by Hill et al. [43], which is highly attractive for using raw biogas (mainly $CH_4$ and $CO_2$) as feedstock for biofuel production.

On the genus level, two well-known methanotrophic genera, *Methylosinus,* and *Methylocaldum* were identified, accounting for 11.66% and 3.19% of the total bacterial sequences, respectively. Three sulfide oxidizing bacteria, *Thiobacillus* (0.13%), *Xanthobacter* (<0.01%), and *Pseudomonas* (<0.01%) were identified but with low abundancies, which indicates their limited contribution to mitigating $H_2S$ inhibition. As a result, in the HTMC, methanotrophs themselves could be tolerant to $H_2S$, although the mechanism is still unknown.

Generally, $H_2S$ can be either cytoprotective or cytotoxic to microbes depending on its concentration (protective in the μmol/L range and toxic in the mmol/L range) [44]. $H_2S$ can inhibit growth of bacteria in different ways, including inhibition of enzymes that protect cells from oxidative stress [44], inhibition of metalloenzymes by inactivating their redox centers, denaturation of protein by disulfide disruption, peroxidation of lipid and induction of DNA damage [44]. There have been studies on cellular response to $H_2S$ toxicity in *E. coli*, *A. baumanni*, *Bacillus subtilis* and *Staphylococcus aureus*, revealing genes and molecular pathways related to $H_2S$ tolerance [44]. To date, there have been very few efforts in studying $H_2S$ tolerance of methanotrophs on the level of molecular biology [45]. Recently, Schmitz et al. reported that a thermoacidophilic methanotroph *Methylacidiphilum fumariolicum* SolV can oxidize $H_2S$ to elemental sulfur likely using a type III sulfide:quinone oxidoreductase (SQR) and a sulfide-insensitive ba3-type terminal oxidase, thus alleviate the $H_2S$ inhibition on $CH_4$ oxidation [45]. Besides, putative SQR sequences have been identified in many methanotrophs, indicating that $H_2S$ detoxification is widespread in methanotrophs [45]. As a result, further transcriptomic studies, especially on SQR, could possibly reveal the mechanism of the high $H_2S$ tolerance of HTMC.

Biogas produced from organic waste via anaerobic digestion is a renewable and abundant $CH_4$ source, but is costly to store, transport, and distribute [6,7]. Methanotrophic microorganisms can be used as cellular factories for converting $CH_4$ gas into methanol, an alternative liquid fuel, which has desirable energy density and easy-to-transport [46]. One challenge to the microbial biogas-to-methanol approach is that $H_2S$ in biogas can inhibit methanotrophic microorganisms, and $H_2S$ removal is usually energy-intensive and costly [46]. The HTMC obtained in this study addressed this hurdle for its high $H_2S$ tolerance, specifically the ability to grow and produce methanol with up to 5.64 $g/m^3$ of $H_2S$ in biogas. For future applications of this technology, raw biogas can be directly used for methanol production without $H_2S$ removal process. Besides, evaluation of factors that affect HTMC cell growth and methanol production also provides base-line data for practical application of this technology for maximum methanol yield. Furthermore, the microbial community analysis provides an in-depth understanding about types and functions of microorganisms in the HTMC, and possible mechanisms for its high $H_2S$ tolerance. Future research on development of bioaugmentation strategies is promising for further improving the

methanol production performance in practical applications.

## 4. Conclusions

A high $H_2S$-tolerant methanotrophic consortium, HTMC, was enriched from anaerobic digestion effluent with high $H_2S$ stress (5.64 g/$m^3$). There was no significant decrease in cell yield and $CH_4$ to methanol conversion efficiency of HTMC when $H_2S$ concentration in the headspace was increased from 0 to 5.64 g/$m^3$. With a $H_2S$ concentration of 5.64 g/$m^3$ in the headspace, the HTMC achieved a cell yield of 0.285 g cells/g $CH_4$ and a $CH_4$ to methanol conversion efficiency of 0.203 mol/mol, respectively, which are comparable to those obtained by existing methanotrophic strains without $H_2S$ stress. The maximum cell to $CH_4$ yield (0.333 g cells/g $CH_4$) and maximum $CH_4$ to methanol conversion efficiency (0.22 mol/mol) were obtained through evaluating the effects of environmental factors on cell growth and methanol production. Microbial community analysis revealed that sulfide oxidizing bacteria may have a limited contribution to the high $H_2S$ tolerance of HTMC due to their low abundancies (<0.01%–0.13%), and high $H_2S$ tolerant methanotroph(s) could exist in the consortium. Besides, *Cyanobacteria* is the most predominant phylum (32.61%) in the HTMC, indicating a great potential for simultaneous utilization of $CH_4$ and $CO_2$ from raw biogas.

## CRediT authorship contribution statement

**Danping Jiang:** Data curation, Writing – original draft. **Xumeng Ge:** Writing – review & editing, Conceptualization. **Long Lin:** Data curation. **Zhou Chen:** Resources. **Quanguo Zhang:** Corresponding author. **Yebo Li:** Conceptualization.

## 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.

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