Asia-Pacific Journal of Science and Technology :Volume :30 Issue :06 Article ID :APST-30-06-10 Research Article

APST

Asia-Pacific Journal of Science and Technology

https//:www.tci-thaijo.org/index.php/APST/index

Published by Research and Innovation Department,
Khon Kaen University, Thailand

High methane production by *Methanobacterium ferruginis* Mic6c05^T, a hydrogenotrophic methanogen isolated from a depleted oil and gas field in a neighboring country.

Nusara Yinyom[1], Kusuma Rintachai[2] and Siriwan Wichai[1*]

[1]Department of Microbiology, Faculty of Medical Science, Naresuan University, Phitsanulok, Thailand
[2]Department of Civil Engineering, Faculty of Engineering, Naresuan University, Phitsanulok, Thailand
[*]Corresponding author: siriwanwichai@nu.ac.th

Received 7 January 2025
Revised 16 May 2025
Accepted 30 June 2025

Abstract

Some depleted oil and gas fields harbor anaerobic microbes, including methanogens. In this study, methanogens were isolated from a depleted oil and gas field in a neighboring country using enrichment techniques in methanogenic media (ATCC 1340). Five isolates were obtained. Among them, three isolates- ATCC 06.2-1, ATCC 06.2-2, and ATCC reactor 1- demonstrated a strong ability to produce high levels of methane in the ATCC 1340 medium. This culture medium is specifically designed to convert H_2 and CO_2 at a ratio of 4:1, leading to a theoretical total yield of 249.82 µmol of methane. The results indicated that ATCC 06.2-1, ATCC 06.2-2, and ATCC reactor 1 achieved methane production percentages of 94.07%, 75.65%, and 72.05% of the theoretical yield, respectively. Additionally, all three isolates could utilize formate and methanol as substrates for methane production and were classified as hydrogenotrophs. Genetic identification revealed that the three methane-producing isolates- ATCC 06.2-1, ATCC 06.2-2, and ATCC reactor 1- belong to the species *Methanobacterium ferruginis* Mic6c05^T. The other two isolates, ATCC 03 and ATCC 04.2, were identified as *Clostridium sporogenes* DSM 795^T. The three methane-producing isolates and the two Clostridium isolates show significant potential for optimizing alternative energy sources.

Keywords: methanogenesis, archaea, biogenic gas, methanogenic media, phylogenetic analysis, MPN.

1. Introduction

Methanogens are strictly anaerobic archaea belonging to the phylum Euryarchaeota, known for their ability to produce methane through well-defined methanogenic pathways. These pathways utilize C1 and C2 substrates as intermediates. In the acetoclastic pathway, acetate is converted into methane and carbon dioxide, as represented by the equation: $CH_3COOH \rightarrow CH_4 + CO_2$. In the hydrogenotrophic pathway, also referred to as CO_2-reduction methanogenesis, carbon dioxide is reduced with hydrogen to produce methane and water: $CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O$. The methylotrophic pathway involves the conversion of one-carbon compounds, such as methanol and methylamines, into methane: $4CH_3OH \rightarrow 3CH_4 + HCO_3^- + H_2O + H^+$ [1]. These pathways underscore the metabolic versatility of methanogens in utilizing various substrates for methane production.

Methanogens are obligate methane producers that derive energy exclusively through methanogenesis. They inhabit a broad range of anaerobic environments, from temperate ecosystems to extreme habitats characterized by high or low temperatures, varying salinity, and fluctuating pH. Common niches include marine and freshwater sediments, flooded soils, the gastrointestinal tracts of humans and animals, termite hindguts, anaerobic digesters, landfills, geothermal systems, and even the heartwood of trees [2].

In a study analyzing effluent from a palm oil mill, hydrogenotrophic and acetoclastic methanogens were detected, with hydrogenotrophic types being more dominant [3]. A mesophilic hydrogenotrophic methanogen, designated strain T53BJ, was isolated from an anaerobic digester continuously supplied with agro-industrial waste. Furthermore, *Methanospirillum* spp., known hydrogenotrophs, have been isolated from anaerobic digesters treating fruit waste, press mud, and neem seed cake. These organisms are proposed as candidates for

bioaugmentation in anaerobic reactors or for upgrading biogas and syngas to methane. Recent studies also highlight rice paddies as significant sources of atmospheric methane emissions [4]. In these ecosystems, both H_2/CO_2 and acetate serve as key substrates for methanogenesis. Dominant methanogenic families reported in rice fields include Methanomicrobiaceae, Methanobacteriaceae, Methanosarcinaceae, and Methanosaetaceae [5].

A 2023 study published in Microbiome investigated methanogenic communities in mangrove sediments and reported the predominance of hydrogenotrophic methanogens, such as Methanolinea and Methanoregula (order Methanomicrobiales), in deeper sediment layers. These archaea are suggested to participate in syntrophic interactions with other microbes, contributing to methane production in situ [6]. Investigations of reservoir sediments on the Qinghai Plateau revealed that acetoclastic and hydrogenotrophic methanogenesis account for approximately 70% and 30% of total methane production, respectively. This study also demonstrated that environmental factors such as pH, temperature, and sediment depth significantly influence methanogenic activity [7]. Similarly, research on shrimp pond sediments found that aquaculture practices enhance methanogenesis, with acetoclastic methanogenesis contributing up to 45% of methane generation during and after farming. Dissolved organic carbon concentrations and carbon-to-nitrogen ratios were key drivers of pathway selection [8].

A study published in the International Journal of Hydrogen Energy reported that hydrogenotrophic methanogenesis predominates in coal-bearing environments. In these settings, methanogenic archaea produce methane primarily via CO_2 reduction using hydrogen, underscoring the significance of this pathway in subsurface coal-associated ecosystems [9]. In regions bordering Myanmar, biogas is generated in coastal areas comprising the Ayeyarwady Delta and Bago Yoma Basins. These basins contain an estimated 4.735 trillion cubic feet of gas reserves, accounting for approximately 35% of the reserves in the Rakhine and Mautama Basins [10].

Although methanogenic pathways have been extensively studied, regional comparisons remain limited. Rintachai et al. reported biogenic methane production in the bioreactor containing a mixture of sediment and anaerobic microbes from a nearby depleted field, achieving methane production from H_2 and CO_2 at 77.46% of the theoretical yield [11]. However, methanogen isolation and species identification in the study area remain unexplored. Therefore, this study aimed to quantify methanogenic archaea and select high CH_4-producing isolates for phylogenetic analysis to explore their potential for sustainable energy applications.

2. Materials and methods

2.1 Methanogenic medium preparation

The anerobic medium, methanogenic media (ATCC 1340), was prepared by mixing all components, excluding the vitamin solution and reducing agent, and adjusting the pH to 7.0 [12]. The prepared medium was then transferred into serum bottles, sealed with butyl rubber stoppers, and covered with aluminum caps. The medium was sterilized by autoclaving at 121°C for 15 min. After sterilization, the medium was maintained in a water bath at 95-100°C, and the vitamin solution was added, followed by the reducing agent (Table 1).

Table 1 ATCC 1340 medium composition and preparation.

Component	Quantity	Composition details
Mineral Solution I	15 mL	6.0 g K_2HPO_4 in 1 L distilled water
Mineral Solution II	15 mL	6.0 g KH_2PO_4, 6.0 g $(NH_4)_2SO_4$, 12.0 g NaCl, 2.6 g $MgSO_4 \cdot 7H_2O$, 0.16 g $CaCl_2 \cdot 7H_2O$ in 1 L distilled water
Yeast Extract-Trypticase Solution	4 mL	20.0 g yeast extract, 20.0 g trypticase peptone in 100 mL distilled water
Other Components	6 mL	20% sodium formate, 25% sodium acetate, 0.2% $FeSO_4 \cdot 7H_2O$, 0.1% resazurin
Vitamin Solution	4 mL	2 mg biotin, 2 mg folic acid, 10 mg pyridoxine hydrochloride, 5 mg thiamine-HCl, 5 mg riboflavin, 5 mg nicotinic acid, 5 mg calcium D-pantothenate, 0.1 mg vitamin B12, 5 mg p-aminobenzoic acid, 5 mg thioctic acid in 1 L distilled water
Trace Mineral Solution	4 mL	1.5 g nitrilotriacetic acid, 3.0 g $MgSO_4 \cdot 7H_2O$, 0.5 g $MnSO_4 \cdot H_2O$, 1.0 g NaCl, 0.1 g $FeSO_4 \cdot 7H_2O$, 0.1 g $CoCl_2 \cdot 6H_2O$, 0.1 g $CaCl_2$, 0.1 g $ZnSO_4 \cdot 7H_2O$, 0.01 g $CuSO_4 \cdot 5H_2O$, 0.01 g $AlK(SO_4)_2 \cdot 12H_2O$, 0.01 g H_3BO_3 in 1 L distilled water
Sodium Bicarbonate Solution	2.4 g	Dissolve in 334 mL of distilled water
Reducing Agent	16 mL	0.5 g $Na_2S \cdot 9H_2O$ in 10 mL distilled water

A gas mixture of H_2 and CO_2 in an 80:20 ratio was injected into the serum bottles. In the absence of oxygen, the culture medium causes resazurin to lose its color. Solid media were prepared following the same procedure. (Figure 1).

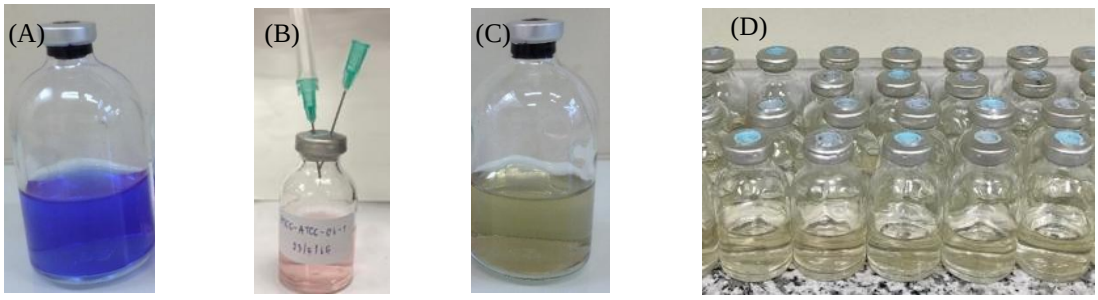

Figure 1 Photo of ATCC 1340 media preparation in an anaerobic condition; (A) media presence oxygen, (B) gas mixture H_2:CO_2 injection, (C) media absence oxygen, (D) prepared ATCC 1340 media.

2.2 Sample collection and methanogen enumeration

In an oxygen-free environment, water samples were collected from depleted oil and gas fields in a neighboring country using sample-collecting equipment. The most probable number (MPN) technique was employed to enumerate and isolate methanogenic archaea using the ATCC 1340 media, with three tubes per dilution. These samples were then transferred into methanogenic media using a syringe and incubated at 37ºC for 31 days in an anaerobic chamber for the presumptive test. For the confirmed test, the roll-tubed method was used after the incubation period; a 1 mL sample from the syringe was injected into a 50 mL serum bottle containing 10 mL of melted solid methanogenic medium. The samples and culture media were thoroughly mixed. A mixture of H_2 and CO_2 gases in a ratio of 80:20 was added to the serum bottles, which were then incubated at 37ºC until colonies were observed. A positive tube was identified when both colony growth and CH_4 content were observed in the same tube. The MPN/100 mL was calculated from three tubes at three serial dilutions using MPN tables.

Various characteristics of colonies were selected and purified to obtain single colonies on solid media using the roll-tube method. The characteristics of each colony on the culture medium were studied, including color, shape, edge, colony surface, and features observed under a microscope, such as Gram staining, cell shape, and cell arrangement.

2.3 Biogas production in methanogenic media (ATCC 1340)

Each isolate was grown in serum bottles containing ATCC 1340 medium. The bottles were closed with Mininert valves, and a 10% starter culture was added. An 80% H_2: 20% CO_2 gas mixture was also added. The bottles were then incubated in an anaerobic chamber at 37°C. CH_4, H_2, and CO_2 content were measured on days 0, 3, 6, 9, 16, 21, 26, and 31.

Biogas components, including CH_4, H_2, and CO_2, were measured using gas chromatography, specifically the Agilent 6890 model with an HP-PLOT/Q column. The sample volume used was 0.5 mL, employing the split method. Thermal conductivity detected CH_4 gas, while the flame ionization detector detected H_2 gas and CO_2. The argon gas flow rate was set at 6 mL/min, with the temperature of the inlet at 55ºC. The amount of CH_4 obtained must be ten times greater than the control set, with no samples added to the liquid medium. The amount of CH_4 was calculated from the standard curve [11].

2.4 Substrates for CH_4 production

The substrates used in the CH_4 production test included acetate, formate, methanol, and a mixture of H_2 and CO_2 in an 80:20 ratio. The culture medium for the acetate test was deutsche sammlung von mikroorganismen und zellkulturen (DSMZ) DSMZ 334, with 83 mM acetate. A basal medium was employed with 10 mM formate added for the formate test [13]. The test utilizing H_2 and CO_2 also used a basal medium with an 80:20 ratio. Lastly, the methanol test used DSMZ 317 medium containing 0.5% methanol. A 1 mL of culture was transferred to each 10 mL of media. The mixture was then incubated in an anaerobic chamber at 37°C for 15 days. The CH_4 gas produced was measured.

2.5 Phylogenetic analysis of 16S rRNA

2.5.1 DNA extraction, purification, and amplification

The selected isolates, ATCC 06.2-1, ATCC 06.2-2, and ATCC reactor1, were grown in ATCC 1340 under anaerobic conditions at 37ºC for 15 days. The resulting bacterial suspension was then centrifuged at 10,000 g for 1 min. The centrifuged sample was further processed using the TIANamp Bacteria deoxyribonucleic acid (DNA) Kit, and the extracted DNA was stored at -20ºC for further study. The DNA of the archaeal 16S rRNA partial gene sequence was amplified using the variable regions V4-V5, with Arch519f (5'-CAGC-CGCCGCGGTAA-3') as the forward primer and Arch915r (5'-GTGCTCCCCCGCC-AACCTCT -3') as the reverse primer, in a 50 µL polymerase chain reaction (PCR) master mix under specific amplification conditions. The reaction conditions included an initial denaturation at 95 °C for 2 min, followed by 30 cycles of denaturation at 95 °C for 30 seconds, annealing at 55 °C for 20 seconds, and extension at 72 °C for 30 seconds, with a final extension at 72 °C for 5 min. Negative controls (without template DNA) were included in all PCR reactions to ensure no contamination occurred during amplification. PCR products were stored at 4 ° C. Ten microliters of the PCR product were examined using gel electrophoresis at a 1.2% agarose gel concentration in 0.5X TBE buffer, and then the DNA band was measured using UV light transilluminators to detect any possible degradation or contamination.

2.5.2 DNA sequencing

The purified PCR products of isolates ATCC 06.2-1, ATCC 06.2-2, and ATCC reactor1 were sequenced by Macrogen Korea and aligned using GenBank's basic local alignment search tool (BLAST) function and the National Center for Biotechnology Information on their website. Samples were sequenced in duplicates to verify the reproducibility and accuracy of sequencing data. The evolution tree of the data set was inferred using the Maximum Likelihood method based on the Kimura-2-parameter model. Evolutionary analyses were conducted using MEGA software version 7 [14].

The other selected isolates, ATCC 03 and ATCC 04.2, were also grown in ATCC 1340 under anaerobic conditions at 37ºC for 15 days and subsequently sequenced by Macrogen Korea.

2.6 Data analysis

The kinetic behavior of CH_4 production was evaluated using a modified Gompertz model, a common approach for modeling cumulative biogas production [15]:

$$P\ (t) = PMax\ \cdot \exp\{-\exp\left[\frac{R\max.\,\mathrm{e}}{Pmax}(\lambda - t) + 1\right]\}$$

Where: $P(t)$ = cumulative CH_4 production at time t,
P max = maximum CH_4 production potential (µmol),
R max = maximum production rate (µmol/day),
λ = lag phase duration (days),
e = Euler's number (~2.718).

To assess the statistical significance differences in CH_4 production among isolates, a one-way analysis of variance (ANOVA) was performed, followed by Tukey's honesty significance difference (HSD) post hoc test at a significance level of $\alpha = 0.05$.

3. Results and Discussion

3.1 Sample collection and methanogen enumeration

The enumeration of methanogens was measured using the three-tube MPN method. MPN is a technique for enumerating microorganisms in samples with low microbial content. It is a statistical, multi-step assay that consists of presumptive and confirmed phases. In this study, the methanogens were enumerated using selective ATCC 1340 media incubated at 37°C for 31 days during the presumptive phase and confirmed through CH_4 content analysis and growth on ATCC 1340 solid media. The count of positive tubes was used to estimate the number of microorganisms present, as specified by the U.S. food and drug administration (FDA). The results indicated a concentration of 240 MPN methanogens per 100 mL, suggesting a significant presence of bacteria capable of producing high amounts of CH_4, measured at 29.87 ± 0.31%. The culture medium, ATCC 1340, measures total methanogens, specifically in the hydrogenotrophic and acetoclastic groups. It contains acetate,

formate, and H_2/CO_2, allowing the methanogen groups to grow and multiply. These results align with reports of methanogen concentrations from marine sediment samples collected from various locations in Truong Sa Dong Island (part of the Truong Sa archipelago, Vietnam), which showed higher densities (up to $1.2x10^2$ MNP/mL) and found 15% CH_4 content by enrichment in artificial seawater with 10 mM CH_3COONa substrate [16]. Moreover, reports indicate that CH_4 levels in various areas, such as anaerobic digesters continuously fed with agro-industrial waste, released CH_4 concentrations of 30% in the headspace of each serum bottle [4].

Chemical and geochemical analyses indicated that the sampling site from a depleted oil and gas field in a neighboring country was suited for biogas production. According to Rintachai's thesis [17], chloride concentrations ranged from 11,677 to 17,069 mg/L, which was below 1,800 millimolar (mM) or 63,810 mg/L, favorable for the survival of CH_4-producing archaea [18]. Moreover, nutrient levels in a depleted oil and gas field included iron (Fe) at 4.572 -220.977 ppm, manganese (Mn) ranging from 0.482 to 20.573 ppm, and a pH between 6.72 and 7.52, indicating a nearly neutral environment. Furthermore, organic matter was represented by a total petroleum hydrocarbon concentration of 12.3 to 23.1 mg/L, and total organic carbon values ranged from 130 to 413 mg/L, which also suited the activity of CH_4-producing archaea [19]. Overall, the results indicated that the chemical and geochemical characteristics of the water samples were appropriate for anaerobic processes and conducive to the survival of CH_4-producing archaea.

3.2 Morphological and physiological characteristics of the isolated methanogens

The sample from a depleted oil and gas field in a neighboring country had an oxidation-reduction potential (ORP) of -32 mV, indicating optimal conditions for methanogens (17). The ORP of oxygen-free growth media, ATCC 1340, must be controlled below -330 mV [20]. By adding a reducing agent, $Na_2S·9H_2O$, at a concentration of 0.05%, the ORP value was reduced to -571 mV, in which a change of resazurin from pink to colorless could be observed.

After purification using the roll-tube method in anaerobic conditions, five pure isolates were obtained, including ATCC 03, ATCC 04.2, ATCC 06.2-1, ATCC 06.2-2, and ATCC reactor1. Most colonies' characteristics were round, white, and opaque with smooth edges. Microscopic analysis revealed that all isolates had gram-positive and rod-shaped cells with rounded ends (Table 2).

Table 2 Cultural and morphological characteristics of isolated methanogen and related species cultured in the absence of oxygen ATCC 1340 media.

Culture media	Isolated no.	Cultural characteristics	Morphological characteristics
ATCC 1340	ATCC 03	Small white colony	Gram-positive, rod-shaped, pair
ATCC 1340	ATCC 04.2	White flat colony and smooth edge	Gram-positive, rod-shaped, dispersed into single cells
ATCC 1340	ATCC 06.2-1	Gray amorphous colony	Gram-positive, long-shaped, chain
ATCC 1340	ATCC 06.2-2	Brown amorphous colony	Gram-positive, rod-shaped, dispersed into single cells
ATCC 1340	ATCC reactor 1	White flat colony and smooth edge	Gram-positive, long-shaped, chain

ATCC 1340 medium is used to isolate hydrogenotrophs and formate degraders. Other media, such as ATCC 1045 and basal medium, can also be used [21]. To isolate methanogens, it is crucial to use a nutrient-rich medium containing carbon sources, nitrogen sources, mineral solutions, trace mineral solutions, vitamin solutions, reducing agents, and indicators, all within strictly anaerobic conditions. ATCC 1340 medium in the presence of H_2: CO_2 (80:20; v/v) at 35 °C after 20 days showed CH_4 productivity of 46%, and microscopic observation revealed the presence of spiral-shaped methanogens and non-methanogens. Fermentative bacteria that can utilize acetate and/or formate but produce little to no CH_4 can also grow in this medium [4]. So, the ability of five isolates to produce CH_4 was tested in the next step.

3.3 Biogas production in the methanogenic media: ATCC 1340

The results of CH_4 production for all five microbial isolates are shown in Figure 2, indicating that isolates ATCC 06.2-1, ATCC 06.2-2, and ATCC reactor1 could produce methane. The isolate ATCC 06.2-1 exhibited a log phase of CH_4 production up to day 9, producing the highest amount of CH_4, after which the production decreased. However, ATCC 06.2-2 and ATCC reactor 1 showed the highest CH_4 content on day 16, after which their CH_4 content also decreased. Additionally, the other strictly anaerobic isolates, ATCC 03 and ATCC 04.2, were found to grow but did not produce methane.

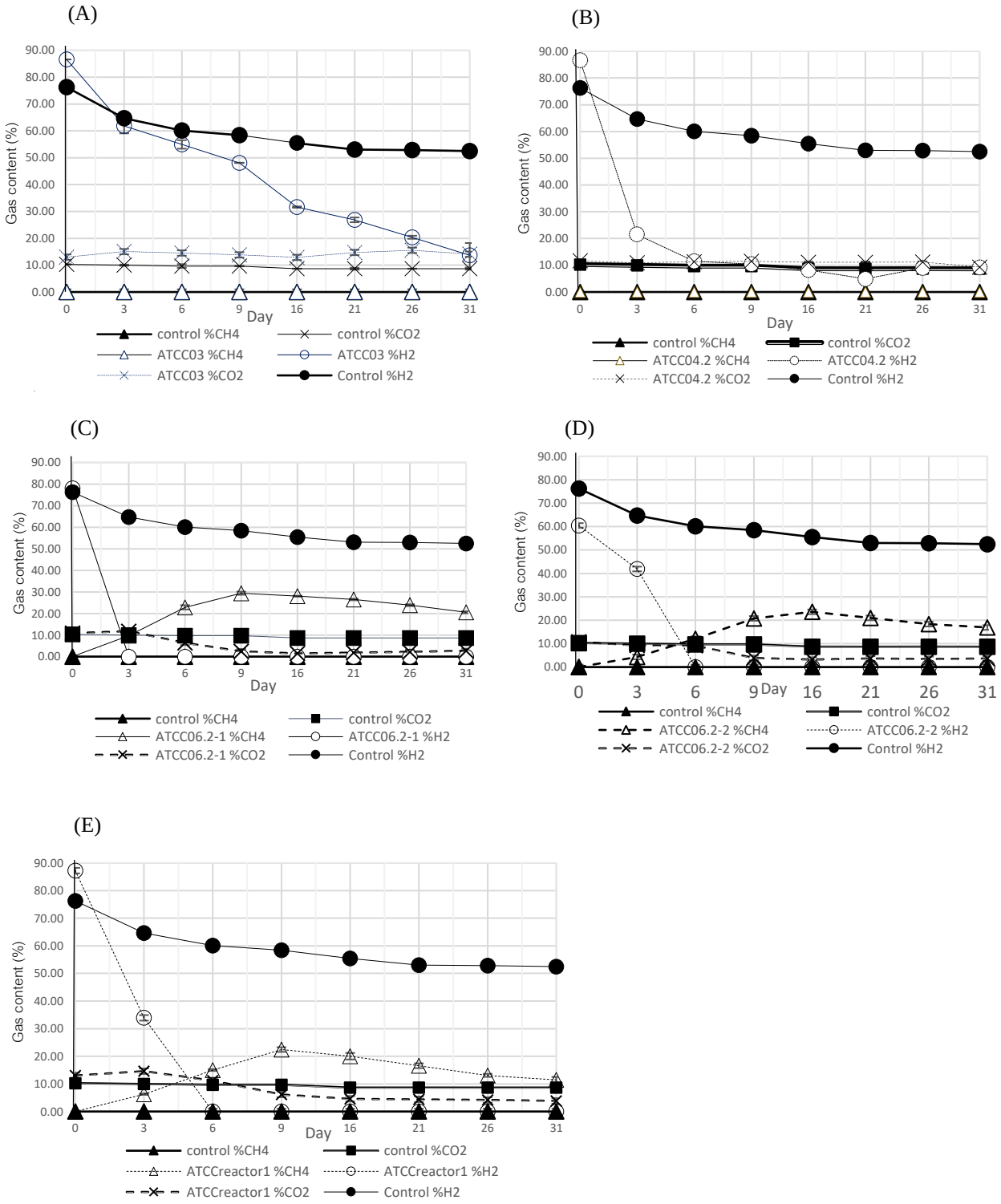

Figure 2 Biogas production of each isolate in methanogenic media (ATCC 1340) on days 0, 3, 6 9, 16, 21, 26, and 31; (A) ATCC 03, (B) ATCC 04.2, (C) ATCC 06.2-1, (D) ATCC 06.2-2, (E) ATCC reactor1.

Isolate ATCC 03 was cultured in ATCC 1340 medium with H_2/CO_2 gas at an 80:20 ratio, indicating the CO_2 levels showed little to no change, while the H_2 levels significantly decreased. On day 0, the H_2 concentration was approximately 80%, gradually decreasing. By day 31, only 20% of the initial H_2 concentration remained. A similar pattern of change was observed for isolate ATCC 04.2. The reduction of H_2 in the presence of H_2/CO_2 may occur due to homoacetogenic bacteria that can utilize H_2 and CO_2 to produce acetate. Therefore, isolates ATCC 03 and ATCC 04.2 may belong to the group of homoacetogenic bacteria. Homoacetogens are divided into different

genera, including Acetobacterium, Clostridium, Morella, Eubacterium, Sporomusa, and Ruminococcus Homoacetogens have been found to grow in diverse habitats alongside various other microbes, including methanogens, due to their ability to grow autotrophically on $H_2/CO_2/CO$ and heterotrophically on various substrates, including hexoses, pentoses, alcohols, acids-formic acid, and methyl groups. They are present in numerous anaerobic environments, including sediments, soils, and the gastrointestinal tracts of animals [22].

For the three isolates, ATCC 06.2-1, ATCC 06.2-2, and ATCC reactor 1, high CH_4 production was observed in the ATCC 1340 medium. This culture medium can theoretically convert the substrate into CH_4 gas by adding mixed gas H_2/CO_2 at a ratio of 80:20, yielding 249.82 µmol [23]. The results show that ATCC 06.2-1 generated a maximum of 235 micromoles of CH_4, accounting for 94.07% of the theoretical amount. Isolate ATCC 06.2-2 produced a maximum of 189 micromoles of CH_4, accounting for 75.65% of the theoretical yield. The ATCC reactor 1 isolate produced a maximum of 180 micromoles of CH_4, accounting for 72.05% of the theoretical yield. Table 3 presents the key kinetic values derived from the Gompertz model. The high R^2 values indicate a strong fit of the model to the experimental data, supporting the reliability of the kinetic predictions. Among the tested isolates, ATCC 06.2-1 demonstrated the highest methane production efficiency, achieving 94.07% of the theoretical CH_4 yield under the experimental conditions.

Table 3 Gompertz model kinetic parameters for CH_4 production.

Isolate	*P*max (µmol)	*R*max (µmol/day)	Lag phase (λ, days)	R^2
ATCC 06.2-1	235	28.2	1.8	0.987
ATCC 06.2-2	189	20.6	2.5	0.981
ATCC reactor1	180	18.9	2.3	0.975

To assess the statistical significance of differences in CH_4 production among the isolates, a one-way analysis of variance (ANOVA) was performed, followed by Tukey's HSD post hoc test ($\alpha = 0.05$). The results revealed a significant difference ($p < 0.05$) in CH_4 production between ATCC 06.2-1 and the other two methane-producing isolates, highlighting its superior performance. In contrast, no significant difference was observed between ATCC 06.2-2 and ATCC Reactor 1, indicating similar CH_4 production capacities (Table 4). The combination of kinetic modeling and statistical validation confirms the potential of these isolates for biotechnological applications in methane production.

Table 4 Statistical significance of CH_4 production among isolates (One-Way ANOVA with Tukey's HSD, $\alpha = 0.05$).

Isolate	*p*-value	Significance
ATCC 06.2-1 vs ATCC 06.2-2	0.017	Significance
ATCC 06.2-1 vs ATCC reactor1	0.009	Significance
ATCC 06.2-2 vs ATCC reactor1	0.271	Not Significance

These results indicate that those three isolates are high CH_4-producing methanogens. Similar reports indicate that utilizing CO_2 and H_2 at a ratio of 1:5 as feedstock yields a theoretical CH_4 yield of 0.0167 mole. During 21 days of continuous operation in a bioreactor, the maximum yield of CH_4 formation by methanogens, without any optimization or manipulation, ranged from 0.87 to 77.46% of the theoretical yield per day [11].

Methanogens can utilize various substrates to produce CH_4. Therefore, the next study analyzed the substrates for CH_4 production.

3.4 Substrates for CH_4 production

The substrates used in the testing consisted of 0.68% acetate in the DSMZ 334 medium. The H_2/CO_2 ratio of 80:20 was used in the basal medium. The 0.068% formate was added to the basal medium w/o H_2/CO_2. The last medium contained 0.5% methanol in the DSMZ 317 medium. Three isolates- ATCC 06.2-1, ATCC 06.2-2, and ATCC reactor 1- produced methane effectively using H_2/CO_2 as a substrate. These isolates did not generate CH_4 from acetate. However, when methanol was used as a substrate, all isolates produced only a small amount of methane (Figure 3).

In general, CH_4 production increases and accumulates in the system depending on the incubation time until the substrate is completely decomposed. Changes in CH_4 production rates from using different substrates in an oxygen-free environment were observed, with CH_4 production highest in H_2/CO_2 > Formate > Methanol > Acetate. Formate is an important precursor in the anaerobic digestion process. Certain species of hydrogenotrophic methanogens can utilize formate as both an electron donor and acceptor to produce CH_4 and CO_2. The decomposition of formate produces H_2 and CO_2, which can then be converted into CH_4 through the hydrogenotrophic pathway. Alternatively, formate can be converted into acetate through a process called homoacetogenesis.

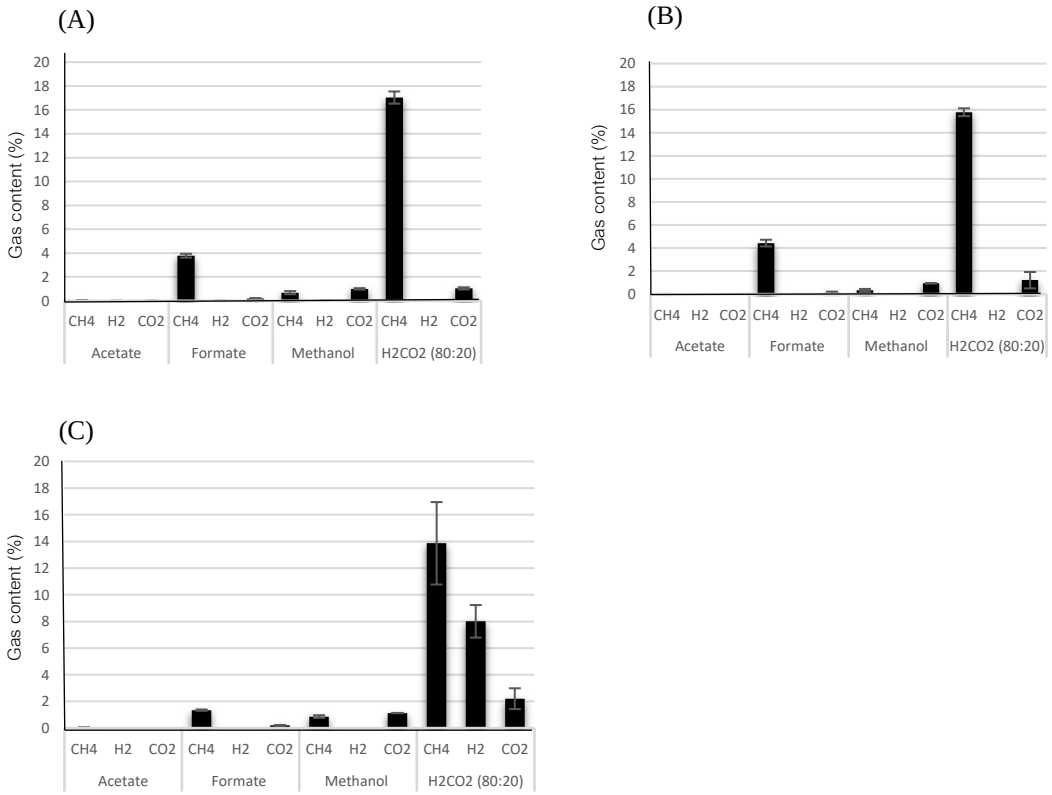

Figure 3 Biogas production of each isolate for substrate testing, which included acetate, formate, methanol, and a mixture of H_2 and CO_2 in an 80:20 ratio: (A) ATCC 06.2-1, (B) ATCC 06.2-2, and (C) ATCC reactor1.

When using H_2/CO_2 as a substrate, a lag phase was observed because the injection of hydrogen gas (in the gas phase) required some time to transition into a liquid phase. This change is necessary for methanogens to utilize H_2 effectively [24]. The results indicated that no isolate used acetate to produce methane, suggesting that these isolates may not belong to the acetoclastic methanogen group. The use of methanol to produce CH_4 was observed in all isolates, although it generates only a small amount of CH_4. This study investigated using substrates that produce CH_4, revealing that the isolated methanogens were hydrogenotrophic archaea. The results demonstrated that those isolates could produce high levels of CH_4. Therefore, the phylogenetic tree was classified in the next step.

3.5 Phylogenetic analysis of 16S rRNA

Phylogenetic analysis was conducted for ATCC 06.2-1, ATCC 06.2-2, and ATCC Reactor 1. A previous study conducted by Wei et al. in 2019 demonstrated that the primer pair Arch519f/Arch915r is effective for amplifying DNA in archaea, particularly those found in deep-sea sediments [25]. The analysis revealed that three isolates showed high similarity to *Methanobacterium ferruginis* Mic5c12^T (Figure 4). This identification is strongly supported by the phylogenetic analysis of the 16S rRNA gene, which provides sufficient resolution to differentiate species within the Methanobacterium genus. In this context, further sequencing would likely provide limited additional value for species-level identification.

The other isolates, ATCC 03 and ATCC 04.2, underwent phylogenetic analysis of the partial 16S ribosomal RNA gene. The PCR products, approximately 688 bases long, were identified as belonging to *Clostridium sporogenes* DSM 795^T (Figure 5). *Clostridium sporogenes* is an anaerobic spore-forming bacterium genetically related to *Clostridium botulinum* but lacking toxin genes. *Clostridium sporogenes* are found in various environments, including soil and sediment from both marine and freshwater sources, as well as preserved meat and dairy products [26].

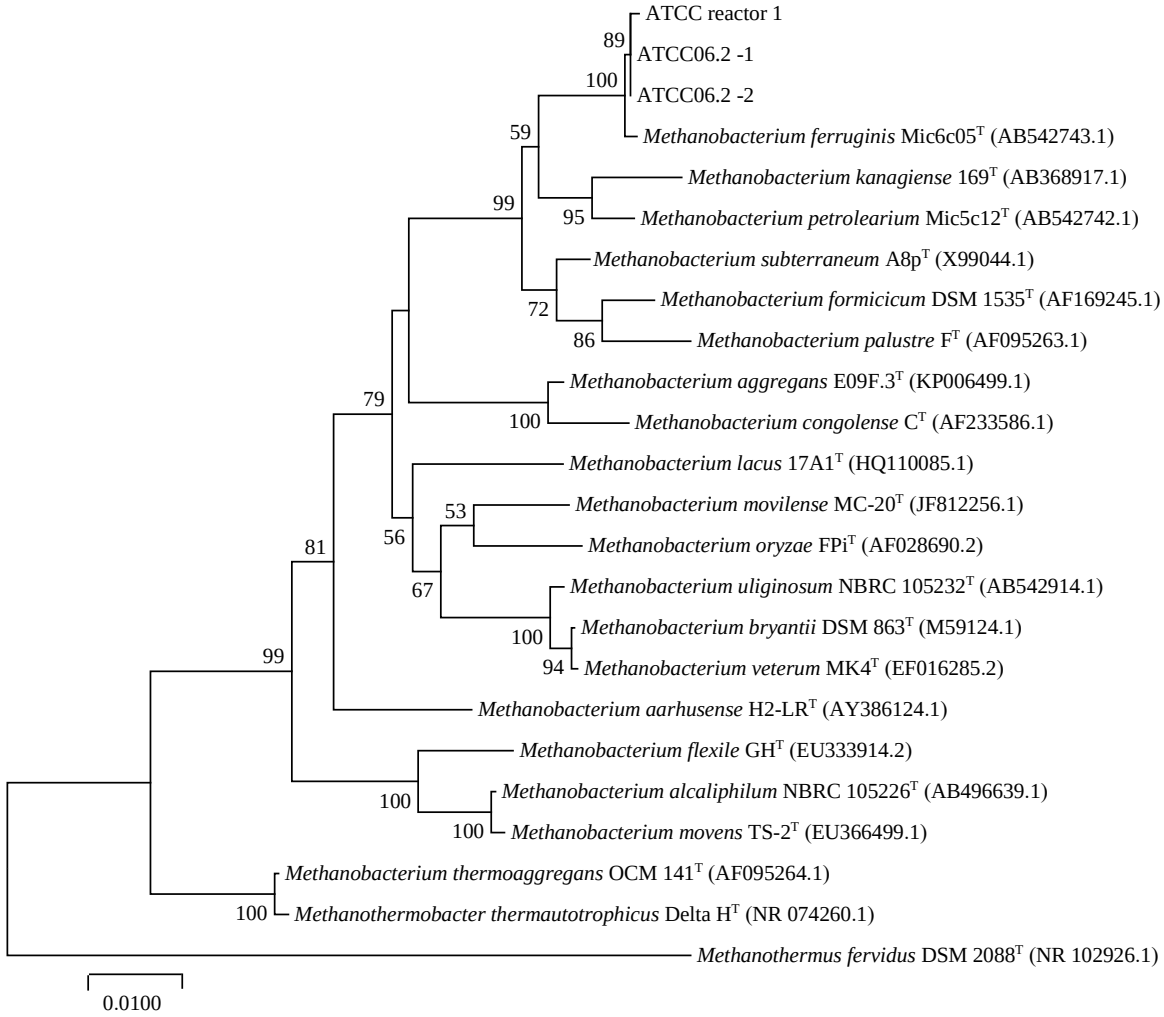

Figure 4 Phylogenetic tree of archaeal 16S rRNA partial gene sequence (Bar =0.0100).

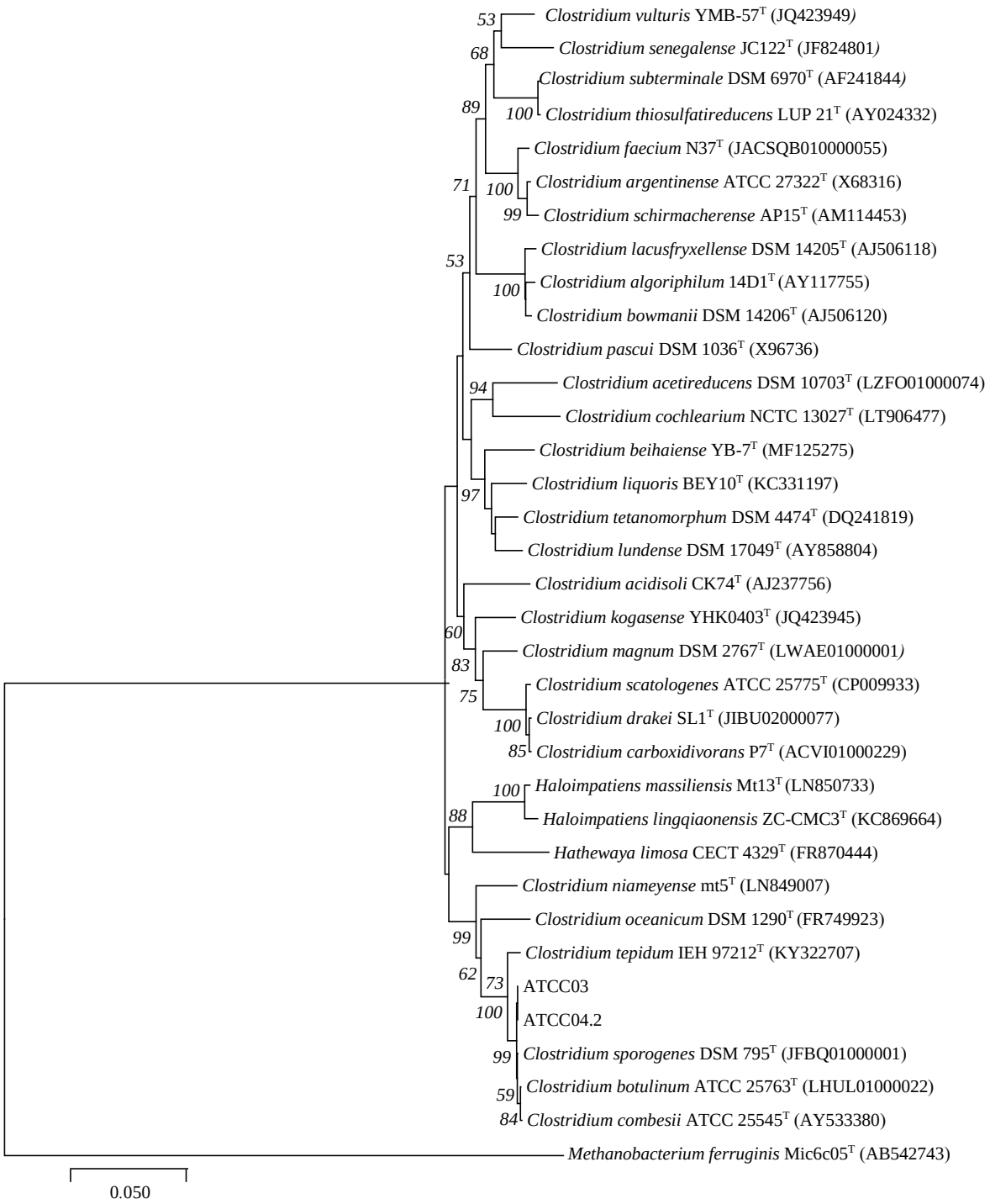

Figure 5 Phylogenetic tree of partial 16SrRNA gene sequence (Bar = 0.05).

Depleted oil and gas fields are anaerobic, carbon-rich environments where diverse microbial communities, including methanogens and Clostridium species, play essential roles in carbon cycling and methane production. Clostridium species exhibit metabolic versatility, capable of both producing and consuming hydrogen (H_2), depending on environmental conditions and species characteristics. Many Clostridium species, such as *C. butyricum*, *C. pasteurianum*, and *C. sporogenes*, are fermentative anaerobes that degrade carbohydrates and amino acids, producing H_2, CO_2, and short-chain fatty acids (e.g., acetate and butyrate) as major end products [27,28]. This hydrogen production is facilitated by ferredoxin-dependent hydrogenases during the oxidation of pyruvate [29]. In contrast, particular species—most notably *C. ljungdahlii* and *C. aceticum*—are acetogens that utilize H_2 and CO_2 through the Wood–Ljungdahl pathway to synthesize acetate, playing a critical role in syngas fermentation

and anaerobic carbon fixation [30]. Environmental factors-including substrate availability, partial pressure of hydrogen (pH_2), pH, temperature, and microbial community dynamics-strongly influence the metabolic direction of *Clostridium* species. In the present study, elevated pH_2 levels may have inhibited H_2 production in *C. sporogenes* ATCC 03 and ATCC 04.2, shifting its metabolism toward increased acidogenesis. In subsurface ecosystems such as oil reservoirs, syntrophic interactions between fermentative bacteria (e.g., *Clostridium* spp.) and hydrogenotrophic methanogens are vital for the complete degradation of organic matter. Fermentative bacteria break down complex substrates into simpler compounds, releasing H_2 as a byproduct. This hydrogen is then consumed by hydrogenotrophic methanogens, such as *Methanobacterium* spp., for the production of methane. These cooperative interactions are fundamental to the anaerobic food web and significantly contribute to biogenic methane formation in oil-bearing formations [31].

The metabolic activities of *Clostridium sporogenes* and *Methanobacterium ferruginis* present promising potential for biogas production in depleted oil and gas fields through the inoculation of microbial strains and the controlled optimization of the H_2/CO_2 balance within bioreactor systems. Harnessing these microbial processes may lead to more efficient resource utilization and support the advancement of sustainable energy solutions. Furthermore, investigating the interactions between methanogens and *Clostridium* within the methane, nitrogen, and sulfur cycles could offer valuable insights for applications in ecological systems, including the detoxification of pollutants, promotion of plant growth, and regulation of greenhouse gas emissions.

4. Conclusions

Five isolates were obtained through an enrichment technique with ATCC 1340 medium from a depleted oil and gas field in a neighboring country. The ATCC 1340 is designed to convert H_2 and CO_2 at a ratio of 4:1, resulting in a total theoretical yield of 249.82 µmol of CH_4. The isolates ATCC 06.2-1, ATCC 06.2-2, and ATCC reactor 1 achieved CH_4 production percentages of 94.07%, 75.65%, and 72.05% of the theoretical yield, respectively. These isolates were identified as *Methanobacterium ferruginis* Mic6c05^T and are classified as hydrogenotrophic methanogens. The other two isolates, ATCC 03 and ATCC 04.2, were identified as *Clostridium sporogenes* DSM 795^T. Further study evaluating their potential application in industrial biogas production and carbon capture technologies could contribute to the development of sustainable energy solutions.

5. Acknowledgements

This research was funded by Associate Professor Tanapon Phenrat at the Faculty of Engineering, Naresuan University. It is supported by the Program Management Unit for Human Resources and Institutional Development, Research and Innovation (Grant Number B40G660037).

6. References

[1] Mei R, Kaneko M, Imachi H, Nobu MK. The origin and evolution of methanogenesis and archaea are intertwined. PNAS Nexus. 2023;2(2):1-10.

[2] Wang J, Yao X, Xu H, Lou H, Hu B. Methane cycle in subsurface environment: A review of microbial processes. Environ Res. 2025;265:120404.

[3] Woraruthai T, Kunno J, Pongsopon M, Yansakon K, Phoopraintra P, Chantiwas R, et al. Identification and cultivation of hydrogenotrophic methanogens from palm oil mill effluent for high methane production. Int J Energy Res. 2020;44(13):10058–10070.

[4] Jain KA, Suryawanshi PC, Chaudhari AB. Hydrogenotrophic methanogen strain of Methanospirillum from anaerobic digester fed with agro-industrial waste. Biologia. 2021;76:255–266.

[5] Ray S, Kuppam C, Pandit S, et al. Biogas upgrading by hydrogenotrophic methanogens: An overview. Waste Biomass Valor. 2023;14:537–552.

[6] Qian L, Yu X, Gu H, et al. Vertically stratified methane, nitrogen and sulphur cycling and coupling mechanisms in mangrove sediment microbiomes. Microbiome. 2023;11:70-71.

[7] Liu Z, Mao X, Wu Y, Xia L, Yu H, Tang W, et al. Methanogenic community characteristics and its influencing factors in reservoir sediments on the northeastern qinghai plateau. Biology (Basel). 2024;13(8):61-65.

[8] Tan J, Lichtfouse E, Luo M, Liu Y, Tan F, Zhang C, et al. Aquaculture drastically increases methane production by favoring acetoclastic rather than hydrogenotrophic methanogenesis in shrimp pond sediments. Aquaculture. 2023;563(2):738999.

[9] Akimbekov, N. S., Digel, I., Tastambek, K. T., Kozhahmetova, M., Sherelkhan, D. K., & Tauanov, Z. Hydrogenotrophic methanogenesis in coal-bearing environments: Methane production, carbon sequestration, and hydrogen availability. Int J Hydrogen Energy. 2024;52(D):1264–1277.

[10] Myint L. Biogenic gas potential of Myanmar. In: 4th AAPG/EAGE/MGS Myanmar oil and gas conference: Myanmar: A global oil and gas hotspot. AAPG Asia Pacific Region; 2019.

[11] Rintachai K, Phenrat T, Wichai S, Limmongkon A, Yinyom N. Design and commissioning of continuously stirred anaerobic bioreactor for upcycling carbon dioxide (CO_2) to methane (CH_4) via methanogenesis. Naresuan Univ Eng J. 2023;18(2):41–50.

[12] ATCC. ATCC Medium 1340 (MS medium for methanogens). Manassas (VA): American Type Culture Collection; 2025

[13] Zhou J, Smith JA, Li M, Holmes DE. Methane production by Methanothrix thermoacetophila via direct interspecies electron transfer with *Geobacter metallireducens*. mBio. 2023;14:e00360.

[14] Machado RAR, Muller A, Hiltmann A, Bhat AH, Půža V, Malan AP, et al. Genome-wide analyses provide insights into genetic variation, phylo- and co-phylogenetic relationships, and biogeography of the entomopathogenic nematode genus Heterorhabditis. Mol Phylogenet Evol. 2025;204:108284.

[15] Santaweesuk A, Artnaseaw A, Benjapiyaporn C. Optimization of methane production through co-digestion of pig manure with napier grass. Clean Eng Technol. 2025;26:100931.

[16] Nguyen TH, et al. Using metagenomics tool to evaluate the enrichment efficiency of methanogens in marine sediment in Truong Sa archipelago, Khanh Hoa province, Vietnam. E3S Web Conf. 2023;407:2010.

[17] Rintachai K. Feasibility study of methane production from carbon source by deep underground microorganisms in the gulf of martaban, Myanmar [Master's thesis]. Naresuan University; 2021.

[18] Shuai Y, Zhang S, Grasby SE, Hou W, Chen Z, Huang L, et al. Microbial consortia controlling biogenic gas formation in the Qaidam Basin of western China. J Geophys Res Biogeosci. 2016;121(8):2296–2306.

[19] Wellinger A, Murphy J, Baxter D. The biogas handbook: Science, production and applications. Woodhead Publishing; 2013.

[20] Hungate R. Chapter IV A roll tube method for cultivation of strict anaerobes. In: Norris JR, ribbons DW, editors. J Microbiol Methods. 1969;3:117–132.

[21] ATCC. ATCC Medium 1045 (Methanobacteria Medium) [Internet]. Manassas (VA): American Type Culture Collection; 2025.

[22] Karekar S, Stefanini R, Ahring B. Homo-acetogens: Their metabolism and competitive relationship with hydrogenotrophic methanogens. Microorganisms. 2022;10(2):397-399.

[23] Susilawati R, Esterle JS, Golding SD, Mares TE. Microbial methane potential for the South Sumatra Basin coal: Formation water screening and coal substrate bioavailability. Energy Procedia. 2014;65:282–291.

[24] Garcia-Robledo E, Ottosen LD, Voigt NV, Kofoed M, Revsbech NP. Micro-scale H2–CO2 dynamics in a hydrogenotrophic methanogenic membrane reactor. Front Microbiol. 2016;7:1276.

[25] Wei S, Cui H, Zhang Y, Su X, Dong H, Chen F, et al. Comparative evaluation of three archaeal primer pairs for exploring archaeal communities in deep-sea sediments and permafrost soils. Extremophiles. 2019;23(6):747–757.

[26] Brown JL, Tran-Dinh N, Chapman B. *Clostridium sporogenes* PA 3679 and its uses in the derivation of thermal processing schedules for low-acid shelf-stable foods and as a research model for proteolytic *Clostridium botulinum*. J Food Prot. 2012;75(4):779–792.

[27] Cai G, Jin B, Monis P, Saint C. A genetic and metabolic approach to redirection of biochemical pathways of *Clostridium butyricum* for enhancing hydrogen production. Biotechnol Bioeng. 2013;110(1):338-342.

[28] Gupta S, Fernandes A, Lopes A, Grasa L, Salafranca J. Microbes and parameter influencing dark fermentation for hydrogen production. Appl Sci. 2024;14(23):10789.

[29] Albuquerque MM, Sartor GB, Martines-Burgos WJ, Scapini T, Edwiges T, Soccol CR, Medeiros ABP. Biohydrogen produced via dark fermentation: A review. Methane. 2024;3(3):500-532.

[30] Sun X, Atiyeh HK, Hunhnke RL, Tanner RS. Syngas fermentation process development for production of biofuels and chemicals: A review. Bioresour Technol Rep. 2019;7:100279.

[31] Christman GD, León-Zayas RI, Zhao R, et al. Novel clostridial lineages recovered from metagenomes of a hot oil reservoir. Sci Rep. 2020;10:8048.