

Sustainable Methane Production from Petroleum Produced Water via Indigenous Microbial Consortia Under Reservoir Conditions

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KEY WORDS	ABSTRACT
Petroleum Waste Microbial Methane Production Hydrogenotrophic Methanogenesis Bioenergy From Oilfield Waste Methanogenic Archaea Environmental Protection	Petroleum-produced water (PPW) is a byproduct of oil and gas production and is commonly regarded as a waste stream due to its high salinity and associated environmental risks. However, PPW contains indigenous hydrocarbon-degrading and methanogenic microorganisms providing an opportunity for microbial methane production. This study investigates the feasibility of using PPW as a microbial inoculum and nutrient-rich medium for methane production under simulated reservoir conditions. PPW samples collected from depleted reservoirs were subjected to batch incubation experiments over 10-90°C, while maintaining salinity conditions. Two experimental conditions were assessed: (i) PPW inoculated with enriched methanogenic consortia (experimental group) and (ii) PPW without enrichment (control group). After 30 days incubation, the enriched system produced substantial methane, reaching 8-10 mmol CH₄ L⁻¹ culture, STP, whereas the control group showed negligible methane production. Supplementation with H₂/CO₂ significantly enhanced methane production, resulting in approximately three-time higher methane yields and peak production rates of 0.35 mmol L⁻¹ day⁻¹ by day 15 in the experimental group. In contrast, methane production rates in the control group remained minimal throughout incubation. Microbial community analysis using 16S rRNA gene sequencing and quantitative PCR revealed enrichment of methanogenic archaea, with mcrA gene copy numbers increasing by three orders of magnitude in the inoculated system. These results demonstrate that PPW can be repurposed to valorize of residual organics and injected CO₂ into methane under reservoir conditions. The proposed approach offers a sustainable pathway for on-site methane production, enhanced gas recovery, and extension of productive life of mature reservoirs, while supporting produced-water reuse and low-carbon energy transition strategies.

1. Introduction

Microbial methane production in subsurface petroleum reservoirs has emerged as promising opportunity to enhance energy recovery. With growing global demand and concern about greenhouse gas emissions, the conversion of residual

Hydrocarbons into methane represents a sustainable strategy [1]. Unlike carbon-intensive enhanced oil recovery methods, in situ methanogenesis can increase conventional natural gas reserves using indigenous microorganisms under anaerobic conditions. Petroleum reservoirs often have diverse anaerobic

microbial communities that naturally produce biogenic gas [2], and stimulation these processes could extend the productive life of depleted fields while minimizing the need for additional drilling. Petroleum wells produce large volumes of produced water that contain salts, organics, and trace nutrients [3]. For example, In 2012 United States roughly generated 2.06×10^6 barrels of produced water [4, 5]. Instead of treating petroleum-produced water (PPW) as a waste, reinjecting it into reservoirs could supply microbes with substrate and nutrients to stimulate methanogenesis. PPW contains dissolved hydrocarbons, volatile fatty acids, and other carbon sources that methanogenic archaea and their syntrophic partner may utilize [6]. Thus, PPW reuse offers the dual benefit of waste reduction and energy generation. In petroleum reservoirs, methanogens coexist with hydrocarbon-degrading microbial communities and can generate significant biogenic methane from residual hydrocarbons [7]. It is estimated that almost 20% of the world's natural gas resources are microbial in origin [8]. Methanogens in PPW are important not only for grasping the subsurface carbon cycle but also for practical implications [9]. The use of PPW for methanogens helps reduce greenhouse gas emissions and converts unrecoverable hydrocarbons into methane [6]. While traditionally considered a waste product requiring disposal or treatment, it has potential for enhancing microbial methane production under suitable conditions. Indigenous or introduced methanogenic microorganisms can metabolize substrates derived from produced water, such as acetate, hydrogen, and other organic intermediates, to produce methane.

Depleted reservoirs, characterized by their infrastructure, trapped residual hydrocarbons, and well-defined geological settings, offer an ideal environment for microbial methane production [10]. These reservoirs are often well-suited for injecting petroleum-produced water (PPW) which can serve as a carrier for nutrients and substrate for microbial communities [11]. Moreover, integrating CO₂ injection with this approach provides the dual benefit of carbon sequestration and enhanced methane generation aligning with the global objective for carbon management and renewable energy production. Despite the promising potential of microbial methane production, several challenges hinder its application. Depleted reservoirs are often underutilized, and their significant remaining hydrocarbons are rarely utilized for biogenic methane production. Moreover, while petroleum produced water (PPW) contains organic and inorganic components that can stimulate microbial activity as shown in Figure 1. Its high salinity, presence of toxic

substances, and variability in composition pose barriers to effective methane production [12]. The relationship between produced water characteristics, reservoir conditions, and microbial communities remains poorly understood, limiting the ability to optimize this process for large-scale applications.

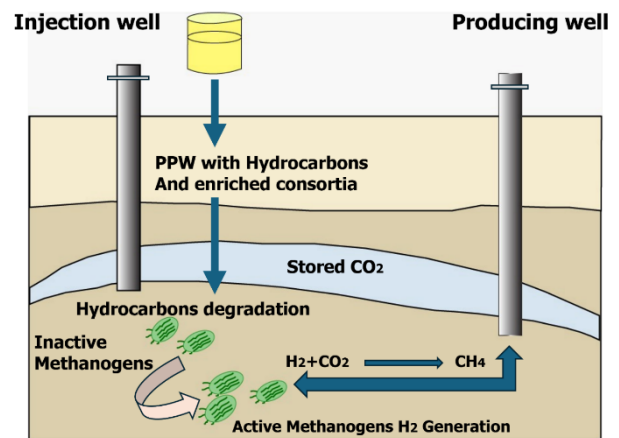
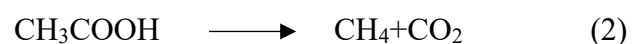


Fig. 1. Conceptual Diagram of Microbial Methane Production from Petroleum Produced Water

Methanogens utilize three biochemical pathways to produce methane, depending on the availability of substrates [13]. Among these, the hydrogenotrophic pathway is the most prominent, in which hydrogenotrophic methanogens such as *Methanobacterium* and *Methanococcus* reduce carbon dioxide using hydrogen as an electron donor. In this pathway, carbon dioxide and hydrogen react to methane and water, as shown in equation 1 [14]. Hydrogenotrophic methanogenesis dominates under anoxic conditions when hydrogen is supplied by fermentative bacterial activity [13].

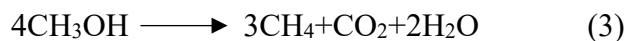


In petroleum reservoirs, this pathway becomes significant after electron acceptors, such as nitrate and sulfate, have been depleted. Under these conditions, the fermentative breakdown of residual hydrocarbons produces carbon dioxide and hydrogen, which are consumed by methanogens to produce methane [7]. The second pathway is acetoclastic methanogenesis, in which acetate-utilizing methanogens such as *Methanosaeta* and *Methanosarcina* split acetate into methane and carbon dioxide, as shown in equation 2 below [13].



These acetoclastic archaea derive two-thirds of the methane carbon from the methyl group of acetate, while the carboxyl group is released as CO₂. Acetate is a common intermediate in anaerobic degradation of organic compounds in oil reservoirs and can be produced by fermentative bacteria through β -oxidation of fatty acids. Acetoclastic methanogens

become important in methanogenic ecosystems once acetate accumulates, which often occurs after hydrogenotrophic methanogens have lowered the partial pressure of hydrogen [14]. The third pathway is methylotrophic methanogenesis, in which certain methanogens produce methane by utilizing methylated compounds such as methanol, methylamines, or methyl sulfides [13].



In this pathway one carbon from the methylated substrate is reduced to methane while the other is oxidized to CO_2 equation 3 [14]. Methanobacterium, Methanomethylovorans, and certain Methanosarcina species are examples of archaea capable of methylotrophic methanogenesis, typically through coenzyme-dependent methyl transfer systems. Methylotrophic methanogenesis becomes significant when substrates methanol or trimethylamine are available [15]. Hydrogenotrophic methanogens dominate when hydrogen and carbon dioxide are available, commonly encountered in deep, saline petroleum reservoirs, making this pathway relevant under reservoir-like conditions. In the characterized PPW samples, hydrogen and carbon dioxide were available, whereas acetate and methylated substrates were present at very low concentrations. Consequently, acetoclastic and methylotrophic methanogens were not expected to be dominant under the tested conditions and become more active when acetate or methyl compounds accumulate [16].

Previous studies have reported petroleum-produced water methanogens that did not produce methane under H_2/CO_2 alone, indicating reliance on acetate, formate, alcohols, or other methylated compounds present in the water [17]. Experimental enrichment studies from oil reservoirs further support this observation, showing that adding methylated substrates can stimulate methylotrophic methanogens and enhance methane production [18]. Similarly, the supplementation of fermentable substrates can increase hydrogen production by fermentative bacteria, thereby enhancing hydrogenotrophic methanogenesis [8]. Collectively, these findings highlight that methanogenic pathways in oilfield water operate within syntrophic microbial consortia, with each pathway becoming dominant only when its specific substrates H_2/CO_2 , acetate, or methylated compounds are available.

This research explores the potential of petroleum-produced water (PPW) in enhancing the microbial methane production in the reservoir. Various researchers have shown the viability of microbial methane production in the laboratory and field [2, 19-21]. However, understanding of the effect of

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physicochemical characteristics of PPW on microbial production in the reservoir is still limited. Additionally, the overall process of mitigating the inhibitory characteristics of the produced water and enhancing the process of methanogenesis in the reservoir has not been sufficiently explored. This research seeks to bridge this gap by examining the suitability of the PPW as a medium for the inoculum of the methanogenic microorganisms in methane production in the reservoir. The methane production in the reservoir is examined, with emphasis on the methanogenesis, use H_2 and CO_2 for methane production, and monitoring the process of methane production and the growth of the methanogenic microorganisms. The findings are likely to support development and repurposing the depleted reservoir as a bioenergy production site. Methane production in the reservoir is likely to provide an alternative solution for environmental sustainability, as using the PPW can provide a valuable alternative energy source.

2. Materials and Methods

2.1 Sample Collection and Characterization of Petroleum-Produced Water (PPW)

The samples were taken from two different depleted reservoirs of the Mari Petroleum Company Ltd, located in Pakistan. One of those reservoirs is a gas depleted reservoir located in the northern part of the Punjab district, Mianwali, as shown in Figure 2. This reservoir comprises a sedimentary sequence of rock formations, mainly composed of mudstones and sandstones. Its average depth is about 1000m. Another depleted oil reservoir comprises a sedimentary sequence of rock formations, overlying the crystalline basement rock. Its thickness exceeds 5 km, and its average depth is about 900m. These depths of the depleted oil and gas reservoirs, the salinity of the brine, the concentrations of sulfate, and the presence of residual hydrocarbons are all well within the tolerance range for subsurface methanogenic populations. These depleted oil and gas reservoirs already have established infrastructure for the production of produced water, which makes them suitable for laboratory-scale bio methanation assessment [22].

These samples were collected in sterile bottle that is completely sealed with screw cap, as per standard methods [23]. These samples were then stored in sterile gas-tight containers to maintain the anaerobic environment and then transported to the laboratory in a refrigerated condition at 4°C . The samples collected were then used within 3 to 4 days after collection. The samples were characterized to assess their suitability for microbial growth and methane production. The

samples were filtered through 0.45 μm membrane filters to remove suspended solids, as they could interfere in the measurement of dissolved ions and organic compounds. The samples were then stored at 4°C for cation and trace metal analysis. The samples were then transferred to sealed vials in an anaerobic condition for gas chromatographic analysis, particularly in the case of VFAs and dissolved methane. Chemical characterization of the sample involved the analysis of major ions such as sodium (Na^+), potassium (K^+), calcium (Ca^{2+}), magnesium (Mg^{2+}), chloride (Cl^-), bicarbonate (HCO_3^-), and sulfate (SO_4^{2-}) which were analyzed using ion chromatography. Other tests conducted were the analysis of total dissolved solids (TDS), total organic carbon (TOC), and volatile fatty acids (VFAs), which were used to determine the availability of organic matter that could be used as a source of energy for microbial growth. Oil components, sulfur compounds such as hydrogen sulfide (H_2S), and trace toxic metals were also analyzed since these may inhibit microbial growth.

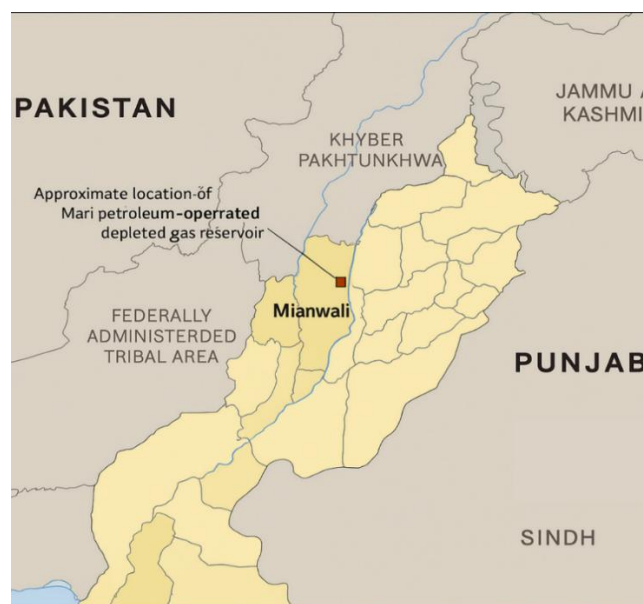


Fig. 2. Sample Collection Area Gas Depleted Reservoirs

The biological characterization involved the identification and quantification of indigenous microorganism, such as methanogenic archaea, by using molecular biology techniques such as 16S rRNA gene analysis and quantitative (qPCR) [24]. Overall, this characterization provides critical information on the suitability of petroleum-produced water for microbial methane production. Table 1 shows the instruments and techniques used to characterize a sample.

Table 1

Instruments Used in Characterization of Petroleum Produced Water

Parameter	unit	Instrument
pH	-	pH Meter
Temperature	°C	Digital Thermometer
Salinity	mg/l	Salinity Meter
Total Dissolved Solids (TDS)	mg/l	TDS Meter
Total Organic Carbon (TOC)	mg/l	TOC Analyzer
Dissolved Methane	mg/l	Gas Chromatograph
Volatile fatty acids (VFA)	mg/l	Gas Chromatograph
Major Cations (Na^+ , K^+ , Ca^{2+} , Mg^{2+})	mg/l	Ion chromatograph
Major anions (Cl^- , SO_4^{2-} , HCO_3^-)	mg/l	Ion chromatograph

2.2 Microbial Enrichment and Cultivation

To enrich the methanogenic microbial communities, experiments were conducted in 120 mL serum bottles. In total, 40 bottles were used, with 20 bottles for the experimental group and 20 bottles for the control group. Experiments were conducted in triplicate, with $n = 3$. In each bottle, 90 mL working liquid and 30 mL headspace volume were used. Working liquids were composed of 80 mL culture medium and 10 mL petroleum-produced water (PPW) used as inoculum, with the composition of the culture medium given in Table 2. The headspace volume used was 30 mL.

Anaerobic culture bottles were prepared using basal medium with sodium carbonate (1gL^{-1}), ammonium chloride (0.3gL^{-1}), potassium phosphate (0.2gL^{-1}), trace elements, and vitamins, following standard protocols for anaerobic growth media preparation [25]. After inoculum addition with PPW, the bottles were then sealed with butyl rubber stoppers, followed by flushing with a gas mixture composed of N_2 (80%) and CO_2 (20%) to obtain anaerobic conditions. The headspace was set to an initial absolute pressure of 1 bar before incubation.

All the parts, such as the bottles, stoppers, and medium, were sterilized by autoclaving at 120°C. After this, they were transferred to an anaerobic chamber under an oxygen-free atmosphere, usually a gas mixture consisting of N_2 with 2–5% H_2 as a reductant [8]. Inside the anaerobic chamber, the bottles were kept under an N_2 environment to eliminate any oxygen. Sodium sulfide (Na_2S) was added to the liquid phase with a concentration ranging from 1–2 mM as a reductant to reduce the redox potential [8]. Trace elements and vitamin solutions were added to the medium to ensure sufficiency [26].

Table 2 showed composition of Petroleum-produced water acted as both the microbial inoculum

and a part of the growth medium. To this, anaerobic mineral medium, such as sodium bicarbonate (to act as a pH buffer and a carbon source), phosphate, and ammonia, were added. Considerable Care was taken to maintain a pH close to neutral and a highly reducing environment with a redox potential < -200 mV.

Table 2

Culture Medium Composition and Their Role

Compound	Concentration	Role
Sodium bicarbonate (NaHCO_3)	1 g/l	Control pH, source of carbon
Ammonium Chloride (NH_4Cl)	0.3g/l	Provide N_2 in lab for bacteria growth
Potassium Phosphate (K_2HPO_4)	0.2g/l	As source of phosphate, act as nutrient
Trace element (Fe, - Zn, Cu, Co etc)	-	Act as cofactor
Vitamins (thiamine - B1, biotin B7)	-	-
Petroleum produced water (PPW)	10ml	Inoculum

Petroleum produced water was used as the inoculum, which is the native population of methanogens and other microbes. A volume of 10 mL of petroleum produced water was injected into the serum bottle using a gas-tight syringe under anaerobic conditions. Standard anaerobic techniques [8] were used. All the liquid handling steps were carried out with the use of degassed syringes. After inoculation, the serum bottles were sealed with the use of sterilized butyl rubber stoppers that were crimped tightly. After sealing the serum bottles, the headspace of the serum bottle was flushed with inert gas to remove the residual oxygen. An anoxic, CO_2 -buffered headspace was created using a H_2/CO_2 gas mixture at a molar ratio of 4:1. Nitrogen was used as an inert balance gas, while CO_2 maintained bicarbonate buffering in the liquid phase ($\text{pH} = 7$) and served as a carbon source for methanogens. Gas flushing was performed by sparging the headspace through sterile needles, as described in previous studies [8, 27]. In the present study, the headspace was adjusted to composition using a H_2/CO_2 (4:1) gas mixture to specifically stimulate hydrogenotrophic methanogenesis. Bottles were incubated in a temperature-controlled incubator under dark conditions to prevent photochemical effects, at temperatures representative of reservoir conditions [8].

Methanogenic enrichment in PPW typically occurs under mesophilic conditions ($25\text{--}40^\circ\text{C}$) for moderate-depth reservoirs or under thermophilic conditions ($60\text{--}80^\circ\text{C}$) for deeper, hotter reservoirs [8]. The gentle mixing was done periodically to achieve homogeneity. The process of methanogenesis was carried out in

serum bottles in which the methanogenic archaea utilized the available substrates and produced methane. The serum bottle method was useful in the simultaneous testing of various parameters (in the presence or absence of hydrogen or nutrients) to observe the effect of the parameters on methanogenesis. By sealing the bottle, an anoxic and highly reducing environment was maintained in the bottle, which is beneficial for methanogenesis [28].

2.3 Experimental Design

Experiments were conducted to test the enhancement of microbial methane production using petroleum-produced water (PPW) obtained from depleted reservoirs, and the experiments were conducted under simulated reservoir conditions. The experiments were carried out in 120 mL serum bottles, and the experimental setup is shown in Figure 3. The setup had 90 mL of working liquid in each serum bottle, and each bottle acted as a reactor that simulated anaerobic reservoir conditions. Each bottle contained sterile growth medium, PPW, and 10% (v/v) microbial consortium, where applicable. Two experimental groups were established: (i) an experimental group containing produced water (PPW) inoculated with enriched microbial consortia and (ii) control group containing produced water (PPW) without microbial inoculation. All bottles were incubated under strictly anaerobic conditions in a temperature-controlled incubator over a temperature range of $10\text{--}90^\circ\text{C}$ to evaluate the influence of temperature on methanogenic activity. Incubations were carried out for a total period of 30 days.

Methane gas samples were collected at 7 days of intervals. Independent bottles were prepared for each sampling time point, and each bottle was sampled only once to minimize the impact of headspace pressure and composition. Sterile or chemically inhibited controls, enrichment-only systems without PPW, PPW amended with antibiotics, and isotopic validation experiments were not included in the present study. These experiments are suggested as useful controls for future studies to aid in constraining abiotic production and validating the isotopic evidence for the source of the methane.

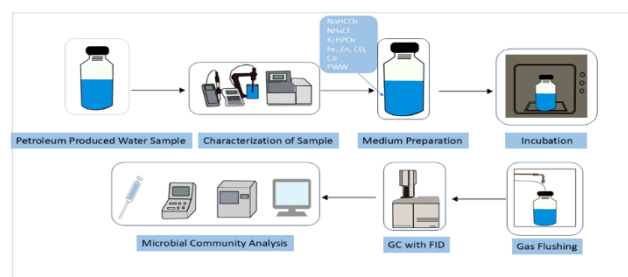


Fig. 3. Process flow diagram of Microbial Methane Production from Petroleum Produce Water

2.4 Monitoring Methane Production

Accurate monitoring of methane production in serum bottle experiments is crucial to accurately quantify methanogenic activity and to verify methane production. Gas chromatography (GC) was used as an analytical technique to accurately measure the concentrations of methane in headspace gas samples. This is a well-established technique used to accurately measure gas concentrations [29]. At scheduled weekly time points, a known volume (0.5 mL) of headspace gas was removed from each serum bottle using a gas-tight syringe. The collected gas sample was injected into GC which was equipped with thermal conductivity detector (TCD) and flame ionization detector (FID).

H₂ and CO₂ concentration were determined through TCD and CH₄ concentration was determined using FID. The concentration of gases determined using standard gas mixture which covered all ranges of concentration used in this study. The calibration curves obtained by linear regression of peak area response versus gas concentrations had an R² value > 0.99 for all gases. The detection limit obtained for this research was about 10 ppmv for methane, 20 ppmv for hydrogen, and 100 ppmv for carbon dioxide using a signal-to-noise ratio of 3. The detection limits obtained in this research were at least ten times lower than the minimum concentration used in this research. The response of detectors was within ±2% of initial calibration response throughout the analysis period.

Methane production was determined as the difference in the final and initial methane concentration at each incubation time point. All experiments were performed in triplicate unless stated otherwise. Mean ± standard deviation is used for presenting results, and the variation in results is also represented in the figures by error bars. Treatment effects were determined based on consistent and reproducible differences in the trend of methane production.

2.5 Microbial Community Analysis

To evaluate microbial community changes involved in methane production, DNA extraction from the enrichment culture samples was carried out. DNA was obtained from the enrichment culture samples by a DNA extraction kit. The obtained DNA was then amplified with universal archaeal primers for the 16S rRNA gene, which are normally used for the detection and characterization of methanogenic archaea [24]. This process helped to identify the key microbial groups involved in methane production. Correlation analysis was conducted to compare the methane production rate with methane concentration to

evaluate the relationship between microbial activity and methane production. The results obtained from microbial analysis and methane production are combined to evaluate the effectiveness of petroleum-produced water for microbial methane production.

3 Result and Discussion

3.1 Physicochemical Characteristics of Produced Water

The produced water sample was characterized to assess physicochemical as well as its microbial characteristics. The water produced from both reservoirs had similar salinity levels, pH, and microbial features that could support methanogenesis. Table 3 shows an overview of the various physicochemical parameters of the produced water. The petroleum-produced water had a highly saline environment with the total dissolved solids (TDS) ranging from 70,200 mg/l to 75,080 mg/l. The pH levels ranged from 6.5 to 7.2, indicating neutral to slightly acidic environments, which are conducive for methanogenesis. The salinity levels of the produced water ranged from 49,010 mg/l to 51,540 mg/l, which is closely related to the TDS levels. This shows that inorganic salts are the primary dissolved constituents of the produced water, a feature typical of deep subsurface petroleum reservoirs.

Table 3

Physicochemical Characteristics of Produced Water

Parameter	Unit	MX	MY
pH	-	6.5	7.2
Total Dissolved Solids	mg/l	70200	75080
Calcium (Ca ⁺²)	mg/l	26500	27000
Magnesium (Mg ⁺²)	mg/l	11503	10522
Salinity	mg/l	49010	51540
Total Fe	mg/l	84	76
Sulphate (SO ₄ ⁻²)	mg/l	285	265
Nitrate (NO ₃ ⁻²)	mg/l	1.6	1.7
NH ₃	mg/l	9.7	10.1
Cu	mg/l	0.09	0.10
Mn	mg/l	4.7	5.10
Volatile fatty acid (VFA)	mg/l	8	9.2
Dissolved Methane (CH ₄)	mg/l	0.6	0.8

The high concentration of Ca⁺² and Mg⁺², as well as levels of total iron and manganese, suggests potential risk of mineral scaling and corrosion. Sulfate levels, ranging from 265–285 mg L⁻¹, are moderate, and the low nitrates level 1.6–1.7 mg L⁻¹ suggests that the water is in an anaerobic state where sulfate-reducing bacteria (SRB) may exist. The presence of ammonia (approximately 10 mg L⁻¹) and volatile fatty acids (8–9.2 mg L⁻¹) further support ongoing organic matter degradation under reducing conditions. In addition, the detection of dissolved methane (0.6–0.8 mg L⁻¹) indicates in situ microbial gas production. The

produced waters are characterized by high salinity dominated by NaCl with moderate concentration of Ca+2, Mg+2 and bicarbonate. Sulfate concentrations were relatively low, suggesting low activity of sulphate reducing bacteria which may compete with methanogens under certain conditions. The coexistence of sulfate, iron, and manganese suggests competition between sulfate-reducing bacteria and methanogens, which may influence methane yield and pose risks of souring and corrosion during field-scale applications. Although SRB activity was not the focus of the present study, mitigation strategies including nitrate dosing, selective biocides, and scale or corrosion inhibitors can be applied suggesting that souring and corrosion risks can be managed during scale-up.

The activity of methanogens is influenced by temperature, salinity, pH, and substrate availability. Variations in these parameters can affect the metabolic efficiency of methanogenic archaea and rate of methane production. The following sections examine the effects of key parameters on methane generation under reservoir conditions.

3.2 Effect of Temperature on Methanogen Activity

Temperature significantly impacts methanogens activity. Most methanogens from reservoirs are mesophiles that grow at temperatures around 30-40°C. There are also moderate thermophile methanogens that grow at around 50-60°C [30]. Reservoir biodegradation studies indicate that as temperature rises from 80°C, the rate of microbial hydrocarbon degradation reduces [31]. This eventually affects the methane production rate. In this study, we investigate temperature effects on methanogen growth from 10-90°C.

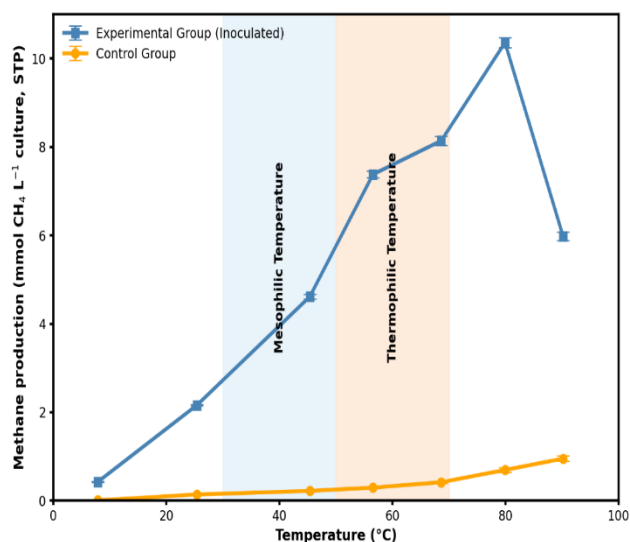


Fig. 4. The Effect of Temperature on Methanogens and Methane Production

Figure 4 shows the effect of incubation temperature on methane production in both groups. Temperature, which governs the metabolic rate, growth, and survival of methanogens, was divided into two ranges: mesophilic (30-45°C) and thermophilic (50-70°C). At temperature between 10 to 30°C, methane production is negligible, indicating that native methanogens with enriched consortia require time to activate. This is called lag phase. After 30°C Methane production in the experimental group increased with increasing temperature, reaching a maximum 10 mmol L⁻¹ culture (STP) at around 80°C. This shows increase in metabolic activity due to the temperature. This also indicates the presence of thermotolerant or thermophilic methanogens in the consortium that maintain high levels of methane production even at high temperatures. At temperatures above 80°C, methane production decreases, indicating thermal inhibition or substrate limitation at that temperature. On the other hand, the control group showed low methane production over the entire temperatures range, with values remaining below 2 mmol L⁻¹ culture (STP). This indicate that indigenous microorganisms in the PPW is either insufficient or inactive for methane production.

The shaded zone in Figure 4 show maximum methane productivity within the thermophilic temperature range, supported by previous studies [32]. Previous studies indicate that methanogens under thermophilic conditions produce faster growth rates and higher methane production compared to mesophilic conditions, provided sufficient substrates are present [33]. Therefore, the increased methane production with temperature can be attributed faster methanogens growth rates. More importantly, although the maximum methane production occurred at 80°C, considerable amount of methane production occurred within 40–60°C, which represent realistic for subsurface reservoirs temperature. This shows that the consortia are highly adaptable to temperature rather than dependent on high-temperature conditions for methane production.

3.3 Effect of Salinity

Salinity plays key role in methane production. High salinity can inhibit methanogenic archaea by exerting osmotic stress and limiting activity [34]. In general, methanogenic archaea grow better in lower

salinity environments, and activity tends to decrease as salinity rises [35]. However, there are moderately methanogens like *Methanococcus* can tolerate up to 58,440-116,880 mg/l salt [36]. In some cases, we need to adjust salinity by adding deionized water. But if methanogens are active in the sample, even with moderate or high salinity, then salinity will not affect the growth of methanogens [37]. In characterization of the sample, we found that the salinity range is 49010-51540 mg/l which falls into the moderate category. At this range, methanogens were still active. In this experiment, we did not reduce salinity because the methanogens found in the sample were already adapted to that environment.

3.4 Effect of pH

The pH of the petroleum-produced water (PPW) exhibited minor fluctuations throughout the experiment. During the early stages the pH was 7.2 ± 0.1 . Upon flushing and pressurizing the system with CO_2 the pH decreased to 6.7 ± 0.1 due to CO_2 dissolution and the addition of sodium bicarbonate. As the experiment progressed, the pH stabilized from day 30 onwards, ranging from 7.0 to 7.3. This shows the consumption of CO_2 by microbes during methane production reduces the dissolved CO_2 , and acidification. The drop in pH levels was not harmful in methane production. Despite the decrease, methane production was not negatively affected. This shows that PPW can support pH conditions conducive to methane production, particularly for hydrogenotrophic methanogens.

3.5 Microbial Methane Production in Petroleum Produced Water (PPW)

The monitoring of methane production within this experiment was conducted over a 30-day period with a batch system. Differences were noted between the control and experimental groups. The results for the control group, where petroleum-produced water (PPW) samples were not inoculated with microbes, indicated a low production rate of methane over the 30-day period. The methane concentration remained $<0.5 \text{ mmol CH}_4 \text{ L}^{-1} \text{ culture (STP)}$, indicating that either abiotic processes or the indigenous microbial community present within the PPW samples were not effective or active enough to produce methane. However, within the inoculated sample, a definite

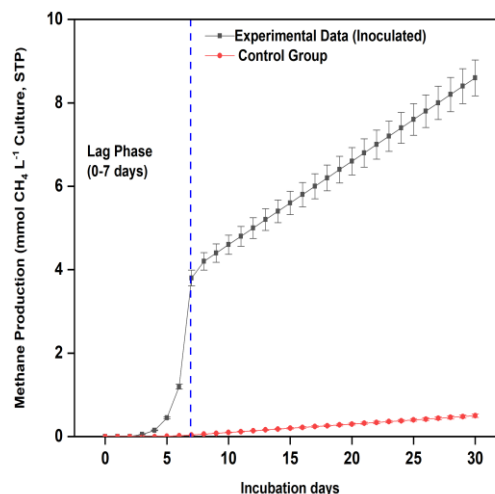


Fig. 5. Methane production for 30 days of PPW sample Incubation with and without Microbial Consortia

trend to produce methane over time was noted, as indicated within Figure 5. The initial stage of the experiment showed a lag phase during days 0-7, representing adaptation, activation, and interaction of microorganisms in the enriched methanogenic consortium. Following this period, there was an exponential methanogenic growth phase, where the rate of methane production increased between 5 and 20 days. During this period, the substrates, mainly H_2/CO_2 and intermediates, were efficiently converted to methane gas. After day 20, the rate of methane production increased gradually but at a slower rate, reaching 8-9 $\text{mmol CH}_4 \text{ L}^{-1} \text{ culture (STP)}$ by the end of day 30. The smooth methane production trend coupled with minimal fluctuations shown by the error bars indicates reproducibility across experimental replicates. Based on the differences between the inoculated and control cultures, the importance of active methanogenic consortia in the methane production from PPW is confirmed. The results showed that the presence of substrates, although favourable for methanogenesis, is not enough on their own to sustain the process in PPW under simulated reservoir conditions.

3.6 Effect of H_2 And CO_2 Supply on Methane Concentration

The addition of H_2 and CO_2 into petroleum-produced water significantly increase microbial methane. In this research, H_2 and CO_2 gas mixture was added to the experimental group and the control group. Figure 6 shows the variation of the concentration of

methane (CH_4), carbon dioxide (CO_2), and hydrogen (H_2) over the 30-day incubation period.

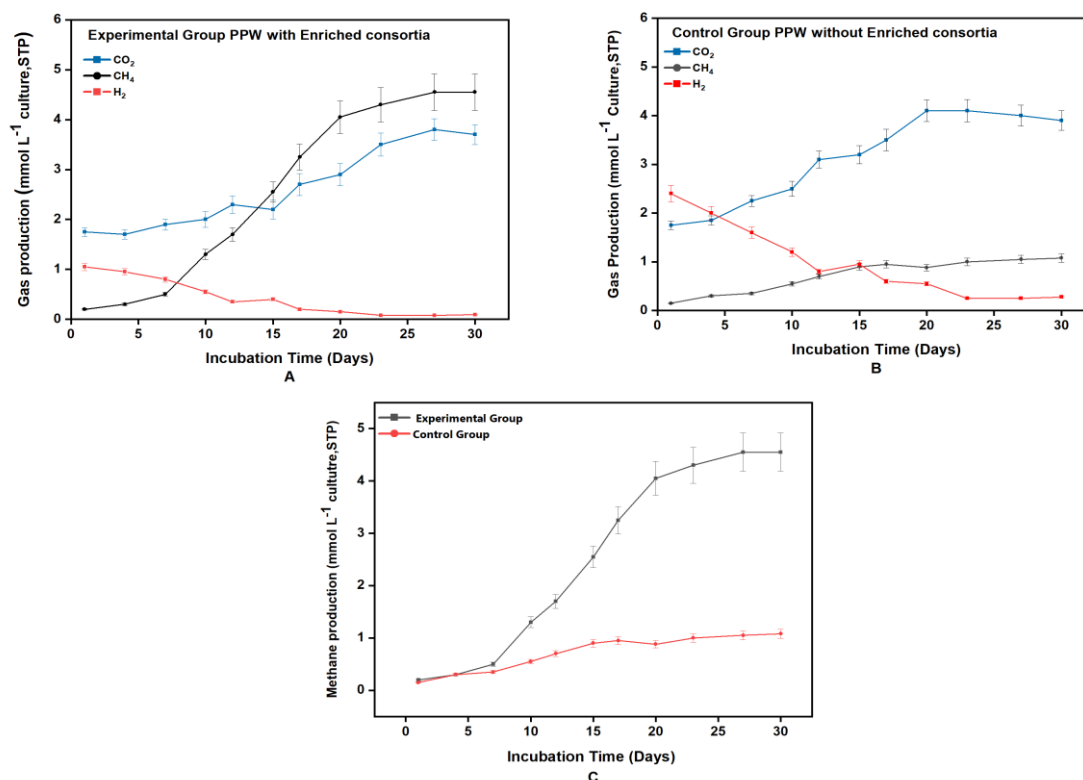


Fig. 6. Effect of H_2 and CO_2 Gas Flushing on Methane Concentration in Experimental Group (A), Effect of H_2 and CO_2 Gas Flushing on Methane Concentration in Control Group (B), Comparison of Both Groups (C)

As shown in Figure 6A in the experimental group, the rate of methane production increased over time after the initial lag period and reached 4.5 to 5 mmol L⁻¹ on days 23 to 28. Following the initial activation period, the methanogenic archaea introduced to the system became metabolically active and continued to grow, leading to an increase methane production. The rate of methane production in the system was the highest in the middle period of incubation, between days 15 and 28, showing that it is the period of maximum metabolic activity. The increased rate of methane production in the system was accompanied by a significant reduction in H_2 concentration, showing that there was consumption of hydrogen gas in the system, although there was only a moderate reduction in CO_2 concentration. The negative correlation between CH_4 accumulation and H_2 consumption confirms that hydrogenotrophic methanogenesis is the dominant metabolic process in the system. On days after 25, the rate of methane production showed slight fluctuations and stabilization, due to the progressive substrate limitation and the onset of the stationary metabolic phase.

On the other hand, the control group in Figure 6B, showed only small amounts of methane production, with only 1.1 mmol L⁻¹ culture (STP) after 30 days. The consumption of hydrogen in the control system was low, and the amount of CO_2 remained stable. These results show that the indigenous microorganisms in the PPW had low methanogenic activity. Figure 6C shows the comparison of experimental groups with the control groups. The difference in the amount of methane produced and the consumption of hydrogen is an indicator of the importance of the enriched consortia in the culture in enhancing the production of methane by microorganisms. This is in consistent with the findings of previous researchers [2] [38], which state the importance of the consortia in overcoming the limitations of the indigenous microorganisms in saline or nutrient-limited environments.

To further assess metabolic pathway consistency, molar ratios were derived from the net gas concentrations changes during the active methanogenesis period. In the experimental group, the H_2 : CH_4 molar ratios were lower than the stoichiometric 4:1 ratio for hydrogenotrophic methanogenesis. The lower ratios are due to

continuous H_2 buffering, co-occurrence of microbial metabolism, and the closed headspace environment of the serum bottle reactor. However, the significant relationship between H_2 consumption and CH_4 production clearly indicates that methane production was microbiologically related to substrate consumption, supporting hydrogenotrophic methanogenesis as the dominant pathway.

3.7 Methane Production Rate in Experimental and Control Groups

The methane production rates were further analyzed to identify the kinetic responses of the two groups. The effect of the enriched methanogenic consortia on the methane production rates was significant over the entire duration of the incubation. Figure 7 shows the methane production rate, which is given as $mmol\ CH_4\ L^{-1}\ culture\ day^{-1}$ (STP), for the two groups. Figure 7A shows the methane production rate for the experimental group. The methane production rate was high for the experimental group over the first 10 days of the incubation. The methane production rate was maximum at $0.35\ mmol\ L^{-1}\ day^{-1}$ on the 15th day. This represents the exponential growth phase of

methanogens. The high methane production rate indicates the efficient utilization of gaseous substrates by hydrogenotrophic methanogens within the enriched consortia. However, the methane production rate declined to $0.1\ mmol\ L^{-1}\ day^{-1}$ by the end of day 30. This might be due to substrate limitations, accumulation of inhibitory substances, or the stationary phase of the microbial community, as suggested by previous studies [38]. In contrast Figure 7B illustrates the methane production rate for the control groups. The methane production rate fluctuated between 0.01 and $0.08\ mmol\ L^{-1}\ day^{-1}$. However, the methane production rate did not increase over time. This indicates low methanogenic activity by the indigenous microbial population present within PPW. The methane production rate is limited by the non-enriched consortia and the non-availability of optimized substrates. This result consists of previous studies of methane production within saline produced water. The researchers suggested that the microbial community present within saline produced water might not be efficient for methane conversion due to nutrient limitations and environmental stress [37].

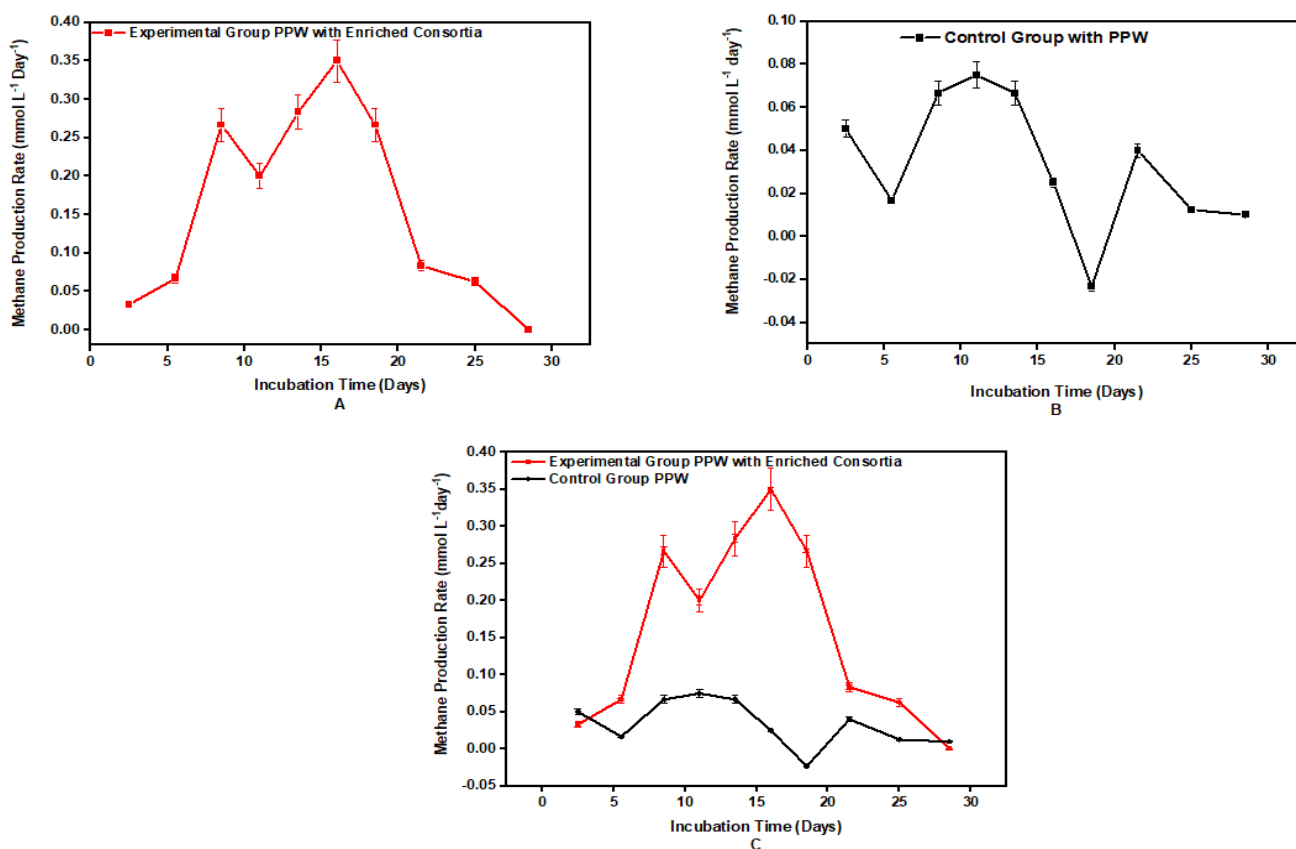


Fig. 7. Methane Production rate in Control Group (A), Methane Production rate in Experimental Group (B), Comparison of Methane production in the experimental group and the control Group (C)

A direct comparison between both groups is provided in Figure 7C. The experimental group showed considerably higher methane production rates compared to the control group. At the peak, the

methane production rate in the enriched system was 6–7 times greater than that in the control group. This figure directly shows that the process of microbial enrichment along with H_2/CO_2 supplementation

increases the rate and efficiency of methane production from PPW. These results show that the stimulation of hydrogenotrophic methanogens in PPW is vital for achieving high methane production rates.

3.8 Methanogen Population Dynamics (*mcrA* gene)

The *mcrA* gene, which encodes for the α -subunit of the enzyme methyl-coenzyme M reductase, is a functional gene used to monitor the abundance and activity of methanogens [39]. An increase in the *mcrA* gene abundance signifies an increase in the growth rate of the methanogenic community and an improvement in methanogenic potential. As seen in Figure 8, the experimental group had a sigmoid growth curve for *mcrA* gene abundance during the 30-day incubation period. After a lag phase between days 0 to 7, there was a rapid increase in *mcrA* gene abundance between days 7 to 20, which indicates an active proliferation of methanogenic archaea following a successful adaptation. After growth phase, the curve started to plateau towards the end of the 30-day incubation period, suggesting that the methanogenic community had reached a stationary phase.

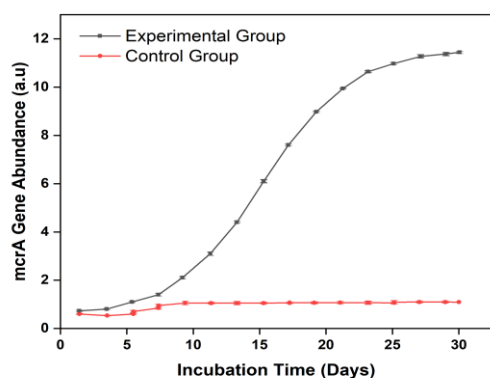


Fig. 8. Methanogen Population *mcrA* gene

On the other hand, the control group showed low and nearly constant *mcrA* gene abundance over the incubation period, indicating the lack of methanogen population. This observation is consistent with the low level of methane production measured in the control bottles of Sections 3.5 and 3.7, confirming the dependency of methanogenesis on the presence of the active methanogenic inoculum. The temporal changes of *mcrA* gene abundance closely resemble the trends of methane production as shown in Figure 5 to Figure 7. The exponential increase of *mcrA* gene abundance matches the period of the highest methane production rates. This correlation confirms the conclusion of biological-driven methane production and its direct relationship to methanogen population growth.

3.9 Microbial Community Analysis

The microbial community sequencing results showed a consortium of methanogenic archaea, along with minor populations of sulfate-reducing and fermentative bacteria. Table 4 shows the relative abundance of the important microbial populations.

Table 4

Microbial Community Composition in Petroleum Produced Water

Microbial group	Relative Abundance (%)	Functional role
Methanobacterium	35	Hydrogenotrophic Methanogens
Methanosaeta	22	Acetoclastic Methanogens
Methanosarcina	18	Versatile Methanogens
Desulfovibrio (SRB)	12	Compete with Methanogens
Other Bacteria	13	Fermentative and sulphate reducers

The dominant methanogenic populations were identified as *Methanobacterium*, *Methanosarcina*, and *Methanosaeta*, belonging to the hydrogenotrophic, versatile, and acetoclastic methanogenic populations, respectively.

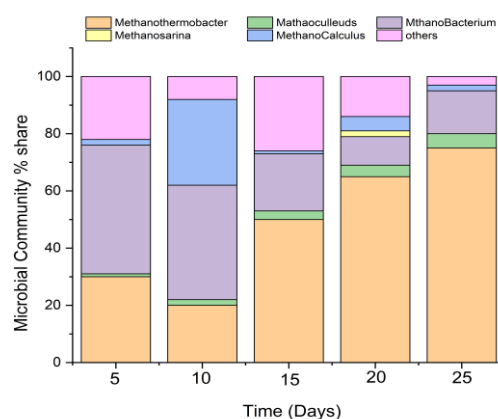


Fig. 9. Different Microbial Community % Share in PPW

Figure 9 shows the Archaeal community showed a successional pattern along the incubation time. On Day 5, the community was composed of a wide range of species. *Methanobacterium* and *Methanothermobacter* were co-dominant at 45 and 30%, respectively. Other species, including *Methanocalculus*, *Methanoculleus*, *Methanosarcina*, etc., were present at lower relative abundance. As the incubation time proceeded to Days 15-25, the proportion of hydrogenotrophic methanogens clearly increased. *Methanothermobacter* showed a steady increase along the time course and remained dominant at 65-75%, while *Methanobacterium* decreased to 10-15%. Other species, including *Methanocalculus*, *Methanoculleus*, *Methanosarcina*, etc., remained at

lower abundance (<10%). In the later stages of the incubation time, the proportion of *Methanosaeta*, which is a strictly acetoclastic methanogen, remained low (<10%), suggesting that acetate played a limited role under the H₂-rich conditions used in this study. In H₂-supplemented biogas or subsurface enrichment cultures, the same successional pattern has been observed. Overall, the observed microbial succession supports the conclusion that methane production in the enriched system was primarily driven by hydrogenotrophic methanogenesis, consistent with gas consumption trends (Section 3.6) and increasing *mcrA* gene abundance in (Section 3.8).

4. Scale-up considerations for H₂/CO₂ supply and field implementation

Field-scale implementation of H₂/CO₂-enhanced bio methanation. It requires a substrate supply strategy and risk management. Three pathways are particularly relevant: (i) H₂ delivery (tube trailers) for short pilots or early demonstration, (ii) onsite H₂ production via electrolysis using renewable electricity to reduce logistics and enable continuous supply, and (iii) use of soluble electron donors (e.g., formate, methanol, or acetate) that can be handled as liquids and may simplify metering and reduce some high-pressure hydrogen handling risks [40]. Integration with power-to-gas concepts, where renewable electricity is converted to H₂ and subsequently to CH₄ could further improve long-term feasibility, particularly where low-cost electricity is available.

A screening-level material balance can be derived from the hydrogenotrophic methanogenesis reaction Equation 1. Stoichiometrically, the process produces 1 mole of CH₄ for 4 moles of H₂ and 1 mole of CO₂. In terms of mass balance, the process requires 0.5 kg of H₂ and 2.75 kg of CO₂ to produce 1 kg of CH₄, and water vapor is produced as a by-product. The stoichiometric equations highlight the importance of the supply of hydrogen as the main limiting factor in scaling up the process and the need for critical evaluation of the injection process and the deliverability of the substrate.

Safety issues and regulatory considerations also play a role in the implementation of the fields. This includes safe handling of flammable hydrogen, which involves leak detection, ventilation, and ignition. In terms of subsurface implementation, injection permitting, operational monitoring programs, and mitigation of possible issues such as reservoir souring and corrosion, which are attributed to sulfate-reducing bacteria, are also considered [41]. From the above considerations, it is evident that the staged development concept is applicable. The process can be

scaled up to larger operational levels based on performance and risk evaluation.

Considering the results achieved from the experiments, the current serum bottle experiments can be considered as an initial step in the technology development process, corresponding to (Technology Readiness Level TRL 2-3). A logical progression from this step would involve performing experiments using a pressurized bioreactor, followed by performing a series of core flood experiments using reservoir cores and produced pore water to study flow-coupled biogeochemical reactions and geochemical stability. Subsequent steps would involve performing a single-well huff and puff test, followed by a multi-well pilot test. All these steps provide a logical and practically viable pathway for scaling up H₂/CO₂-enhanced biomethanation from the current state to the field scale.

5. Conclusion

This study proves that petroleum-produced water (PPW) can be used to stimulate microbial methanogenesis in a depleted oil reservoir. The results clearly show that methane production can be achieved if a well-adapted microbial inoculum is present. In this study, hydrogenotrophic methanogens are the key microorganisms involved in methane production. In this study, it was observed that methane production dominated in the inoculated system, which passed through the characteristic lag, exponential, and stationary phases. However, the uninoculated system remained inert. In the inoculated system, near-stoichiometric conversion of H₂ and CO₂ to methane occurred, resulting in high methane concentration and production. In the inoculated system, methane production was three times higher than that produced by the uninoculated system. This proves that the hydrogenotrophic pathway can be stimulated to produce high levels of methane. This result was further confirmed by the high *mcrA* gene copy number present in the inoculated system. Negligible methanogen populations were present in the uninoculated system. In this study, it was observed that the indigenous microbial community had the ability to tolerate high salinity, which proves that this method can be used to stimulate microbial methanogenesis. Overall, the above results suggest that the biogas generation from residual hydrocarbons is technically feasible. By fully utilizing the PPW as a microbial inoculum and a nutrient-rich growth medium, the additional energy recovery from the depleted reservoirs and the utilization of the problematic PPW as a valuable resource [42]. To apply the above research results to real industry, the future research should be conducted to evaluate the

stability of the methane production over the long term. In addition, small-scale field experiments with the injection of PPW with or without microbial inoculums should be conducted to evaluate methane production in the reservoir. As discussed above, the application of the PPW reuse, microbial inoculums, and CO₂ management is a complex strategy that may mitigate the environmental problems caused by the PPW management and CO₂ emission while creating additional renewable energy resources. With the optimization and systematic scale-up of microbial methanogenesis with the PPW, this biogas generation process has the strong potential to become a cost-effective and renewable process.

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Credit Authorship Contribution Statement

Rizwan Younas': Conceptualization, methodology Formal analysis, Writing original draft. Mohd Zaidi bin Jaafar: Supervision, data validation and critical revision of the draft, Shaziera Omar: co-Supervision in Writing, review & editing, M Shahid, provide technical guidance and manuscript revision. Atif Ismail technical guidance, writing, and revision. Sajid khan: Formal analysis, graphical guidance, Visualization. Muftahu N Yahya: Conceptualization, Formal analysis, Writing. Investigation, Writing, Visualization Ahmed R. ALBajalan: Provided technical guidance and manuscript writing and revision.

7. Declaration of competing interest

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.

8. Nomenclature

CO₂: Carbon dioxide

H₂: Hydrogen

PPW: Petroleum produced water

V/V: Volume/Volume

16S rRNA: 16S ribosomal ribonucleic acid

qPCR: Quantitative Polymerase Chain Reaction

CH₄: Methane

CH₃COOH: Acetic acid

CH₃OH: Methanol

ppt: Parts per million

mg/l: milligram per liter

ml: milli liter

TOC: Total organic carbon

TDS: Total dissolved solid

VFA: Volatile fatty acid

g/l: gram per liter

mmpv: parts per million by volume

TRL: Technology Readiness Level

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