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Study of the performance of a thermophilic biological methanation system

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Abstract

This study investigated the operation of ex-situ biological methanation at two thermophilic temperatures (55°C and 65°C). Methane composition of 85 to 88% was obtained and volumetric productivities of 0.45 and 0.4 L CH_4 /L reactor were observed at 55°C and 65°C after 24h respectively. It is postulated that at 55°C the process operated as a mixed culture as the residual organic substrates in the starting inoculum were still available. These were consumed prior to the assessment at 65°C; thus the methanogens were now dependent on gaseous substrates CO_2 and H_2. The experiment was repeated at 65°C with fresh inoculum (a mixed culture); methane composition and volumetric productivity of 92% and 0.46 L CH_4 /L reactor were achieved in 24 hours. *Methanothermobacter* species represent likely and resilient candidates for thermophilic biogas upgrading.

Keywords: Biogas; Power to Gas; Biological Methanation; Methanogenic Archaea; Volatile Fatty Acids.

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1. Introduction

Methanation refers to the production of methane through either a catalytic or biological process. The catalytic methanation process proceeds by reacting hydrogen (H_2) with either carbon monoxide (CO) or carbon dioxide (CO_2) to form methane and water. This may be described by Eq. 1(Sabatier Equation) or by Eq. 2.

$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O \quad \Delta H_R = -165$ kJ/mol Eq. 1

$3H_2 + CO \rightarrow CH_4 + H_2O \quad \Delta H_R = -206$ kJ/mol Eq. 2

The catalytic (Sabatier) process is well understood and has been used for many years in various applications, such as for the removal of trace amounts of carbon oxides in ammonia production. A commonly utilised ammonia synthesis technique is the Haber Bosch process which is operated at an optimal temperature of 500-600 °C (Bicer et al., 2016) . A catalyst is required to reduce the activation energy of the reaction and allow it to proceed at higher rates. Such catalysts are typically nickel-based, on an alumina carrier (Charisiou et al., 2016). Biological methanation is biologically catalysed by methanogenic archaea (Shin et al., 2015). These are strictly anaerobic microbes of the Archaea domain, which carry out the final step in the anaerobic digestion process. Methanogens utilise CO_2, H_2 and acetate as substrates (Nishimura et al., 1992). The majority of methanogens are capable of utilising H_2 and CO_2 to produce methane, however, only a small number of methanogens can convert acetate to methane. Some, such as those belonging to the order methanosaeta, may only utilise acetate, while other orders such as methanosarcina are more flexible and can utilise either acetate or H_2 and CO_2. These methanogens generally grow at 35-70°C (Rittmann, 2015; Taubner et al., 2015). The free energy associated with the biological reduction of CO_2 to CH_4 using H_2 is -131 kJ/mol (Madigan, 2012), indicating that the reaction is thermodynamically favourable. Biological methanation may be carried out at industrial scales, typically in conjunction with a conventional biogas plant. The process may be carried out “in-situ” by simply injecting

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hydrogen into an anaerobic digester containing a variety of anaerobic microorganisms (Luo and Angelidaki, 2012). Alternatively, it may be carried out "ex-situ" in a separate vessel containing only methanogens (Rittmann et al., 2015).

Large-scale biological methanation is an emerging technology with stirred tank reactors capable of achieving high volumetric productivity and high methane product gas concentration at the same time (Seifert et al., 2014). At lab-scale, various reactor configurations have been trialled with a wide range of results (Bernacchi et al., 2013; Burkhardt et al., 2015; Nishimura et al., 1992; Rachbauer et al., 2016; Rittmann et al., 2012; Seifert et al., 2014). Apart from the physical layout of the reactor, a number of other process variables are critical. These include temperature, mechanical mixing rates, gas flow rates and the specific strains of methanogens utilised. A review of the various designs available in the literature is presented in Table 1.

Process variables may also vary from one reactor design to another depending on the desired outcome. Certain reactors may be designed to simply enrich the methane content of an existing biogas plant and may aim for a high gas throughput rate rather than high methane concentrations (Bensmann et al., 2014). Other facilities may wish to directly produce a green renewable gas for use as a transport fuel or for gas grid injection, and will thus aim for very high methane concentrations (in excess of 95%) in the product gas (Benjaminsson et al., 2013).

Carbon dioxide and hydrogen can only be consumed by the methanogens at the rate at which they are made available to them in the liquid methanogenic culture. Solubility of hydrogen may be improved by providing a larger transfer surface area such as trickle bed and hollow fibre membrane reactors with packing (see Table 1) or by allowing a longer period of time for the transfer to take place through increased retention time (Burkhardt et al., 2015). Where these factors are unable to be altered too severely, such as in the biological methanation

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process, mechanical mixing may provide an alternative solution such as stirring at high speeds. Mechanical mixing via stiring in a continuosuly stirred tank reactor (CSTR) is probably the simplist method of assisting H_2 to go into solution. Stirring at speeds of up to 1500 rpm have been demonstrated in lab scale reactors (Bernacchi et al., 2013; Nishimura et al., 1992; Rittmann et al., 2012; Seifert et al., 2014), however, this is energy intensive when upscaled to commercial reactor scale, where speeds below 60 rpm would be expected. The CSTR may be designed to be tall and narrow, providing a longer path for the gas to rise through and increased contact time with the methanogen culture. Another alternative to mechanical mixing is micro-sparging. In this case, the gas is released into the liquid via micro-porous material, such as a hollow fibre membrane (HFM) (Lai et al., 2008; Lee et al., 2012). This creates very small hydrogen bubbles with high partial pressure and a high ratio of surface area to volume, allowing for more effective hydrogen dissolution. Recirculation of the gas and/or liquid will also assist in the production of a product gas with a high methane content. This concept has been used very effectively in the trickle bed design described by Burkhardt and Busch (Burkhardt and Busch , 2015).

Most of the literature on biological methanation is quiet recent. There are a few studies investigating methanation with pure cultures at thermophilic temperatures and high stirring speeds (Bernacchi et al., 2014). The innovation in this paper is the detailed study of performance and identification of methanogenic communities in a closed batch system for biological methanation at two thermophilic temperatures, using mixed culture and enriched culture, with different retention times, with H_2 and CO_2 as the influent gases. The objectives of this paper are to:

- Assess the performance of the system with respect to methane concentration and volumetric productivity with H_2 and CO_2 as the input substrate gases at two different thermophilic temperatures.

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- Study the effect of time and temperature on the rate of conversion of the substrate gases to methane.
- Compare the performance of the cultures based on volatile fatty acid profile and identification of methanogens at genus or family level

2. Material and Methods

2.1 Initial inoculum and nutrient medium

The inoculum for this experiment was sourced from a thermophilic (55°C) reactor treating maize, grass and farmyard manure. The inoculum was stored at 55°C in a water bath until needed, while being fed once a week with cellulose at an organic loading rate (OLR) of 1 kg $VS.m^{-3}.d^{-1}$. As the mixed culture will only be fed with H_2 and CO_2, it needs to be supplied with certain additional nutrients to maintain growth.

A system for the preparation and dispensing of the anoxic medium was designed, based on guidelines from Wolfe (Wolfe, 2011). The anoxic medium follows the basal medium recipe described by Angelidaki and Sanders (Angelidaki and Sanders, 2004).

2.2 Reactor configuration

The reactor consists of a 1 Litre Duran bottle (actual volume 1140 mL). The cap has a rubber seal with two steel pipes drilled in to allow for refreshing of gases and the nutrient medium. A three-way Luer lock stopcock on each pipe provides a simple system for refreshing the gas and anoxic medium, while excluding air from the reactor. Each day, 25 mL of the culture was removed using a syringe (by attaching it to one of the ends of the three-way Luer lock stopcock) and replenished with anoxic medium. This system prevented any gas from entering and leaving the bottles and also helpful in pH measurement. The total liquid volume was 380 ml. As the procedure was not carried out over the weekends, the effective HRT was 21 days. At the same time as the medium replenishment, the 760 ml headspace was flushed out with

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H_2 from a gas bag and 190 mL of carbon dioxide was then injected from a gas-tight syringe to make a 4:1 stoichiometric ratio.

The daily culture samples were analysed for pH level and adjusted to ideally lie between 7.7 and 8.2 as this is generally considered optimal for anaerobic digestion (Laaber, 2011). The ideal pH will vary for different methanogens; for example, Bernacchi and co-workers obtained high methane production rates between pH 6-7.8 (Bernacchi et al., 2014). The samples were tested for pH using a syringe attached to the three way Luer lock stopcock. The pH range was maintained using 1M hydrochloric acid (HCl) and 3M NaOH. Samples were taken and frozen for future further analysis.

Each day, before refreshing the gases, a 50 mL gas sample was taken from the reactor using a gas tight syringe. This gas sample was then injected into a gas chromatograph (GC) to analyse the product gas makeup.

2.3 Chemical analyses

Gravimetric measurements including Total Solids (TS) and Volatile Solids (VS) and Volatile Suspended Solids (VSS) were determined by weighing the sample residues that were dried for 24 hours at 105° C and later burning the dried residue at 550° C for 4 hours. Volatile Fatty Acids (VFAs) were determined using a gas chromatograph (Agilent HP 6890 Series, Agilent Technologies, Santa Clara, CA, USA) equipped with a Nukol™ fused silica capillary column (Supelco, Bellefonte, PA, USA), argon as a carrier gas and a flame ionisation detector (Herrmann et al., 2015). Gas samples were measured using a gas chromatograph (Agilent HP 6890 Series, Agilent Technologies, Santa Clara, CA, USA) equipped with a Hayesep R packed column and a thermal conductivity detector. The pH was measured using a Jenway 3510 pH meter.

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2.4 Reactor start-up and continuous operation of the process.

The VSS of the inoculum was determined before inoculation. The literature indicates that a VSS value of 5-10 g/L should be used for inoculation (Krajete, 2012; Luo and Angelidaki, 2012). For this experiment, 5 g VSS/L was chosen. Three bottles were inoculated with a mixture of 47.5 mL inoculum and 332.5 mL of anoxic nutrient medium, making up a total of 380 mL. The experiment was conducted in a Thermo Scientific Incubator shaker at an rpm of 180 and initially at a temperature of 55℃. The headspace was replaced with the substrate gases (H_2 and CO_2) batch wise (as this is a closed batch system). The start-up period lasted for about 2 months till relatively stable readings were obtained and it was relatively easy to maintain pH within the range of 7-8 and a methane concentration of at least 80%.

2.5 DNA extraction and sequencing

The stages of the process were broken into (A) acclimatisation at 55^{o}C; (B) steady state at 55^{o}C; (C) initial trial at 65^{o}C and (D) reseeded reactor trial at 65^{o}C. Approximately 30ml of suspended solids from each Reactor (1, 2, and 3) for stages B, C and D were spun at maximum speed to pellet biomass (9 samples total). Nucleic acids were extracted in triplicate from these pellets using a CTAB/SDS based lysis buffer (Wilson, 2001) and two rounds of phenol-chloroform-isoamyl-alcohol extraction. Primers S-D-Arch-0349-a-S-17 (GYGCASCAGKCGMGAAW) and S-D-Arch-1041-a-A-18 (GGCCATGCACCWCCTCTC) (Klindworth et al., 2012) spanning 16S V3-V6 were selected and appraised using the SILVA testprime database (Klindworth et al., 2012) with parameters of 0 basepair-mismatches, and of 1 basepair-mismatch outside the last 3 3'-basepairs. Under these constraints, coverage was 70% and 85% for Archaea, 77% and 89% for *Euryarchaeota*, and at least 82%, 75%, 86%, and 100% of the major methanogenic clades (*Methanobacteria*, *Methanomicrobia*, *Methanococci* and *Methanopyri*) respectively.

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Coverage provided by this primer pair is likely to capture a majority of archaeal sequences. A 692bp product was generated via generic *Taq* polymerase (DreamTaq, ThermoFisher) using a PCR program of initial denaturing for 4min @ 94°C; x30 cycles of 1min @ 94°C, 54°C, and 72°C each; and a final extension of 4 min @ 72°C. Amplicons were purified via gel extraction (QIAGEN) and ligated in EZ-Competent cells (QIAGEN) before being plated on ampicillin; twelve successfully transformed colonies per Reactor per Stage (108 clones total) were used for M13 PCR before commercial sequencing by GATC (Konstanz, Germany).

2.6 Sequence Analysis

Chromatograms were manually curated in FinchTV 1.3.1 (Geospiza Inc.) for read length and accurate base-pair calling (>200bp, PHRED scores ≥20). Chimera-checking and OUT(operational taxonomic unit) clustering (<97% identity) were carried out using USEARCH v9.0(Edgar, 2010). All sequences were submitted to NCBI BLASTn (Altschul et al., 1990) to retrieve 16S reference sequences with closest identities. 16S reference sequences were also retrieved for major methanogenic groups and a bacterial outgroup (*Psychrobacter spcs.*, NR_118027.1). Gapless alignments and Neighbour-Joining phylogenetic trees were generated using MUSCLE v3.8.31 (Edgar, 2004) and formatted in MEGA7(Kumar et al., 2016). Sequences were uploaded to Genbank under accessions KY077158 - KY077249.

3. Results and discussion

3.1 Reactor performance at 55°C and 65°C with respect to methane composition, volumetric productivity, retention time and temperature.

The performance of the three reactors were monitored and process variables such as values of methane produced, pH and VFA analysis were actively recorded. Figure 1 shows mean and the mean deviation of the weekly values obtained for the triplicate reactors for 24-hour gas

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sampling. The reactors were operated for 17 weeks at 55°C for the first 12 weeks and at 65°C till week 17. It can be observed that the maximum value for methane composition and methane volumetric productivity were ca. 88% and 0.45(L CH_4 /L reactor) and later dropped to 85% and 0.4(L CH_4 /L reactor) at 65°C for the rest of the time period. The first few weeks show the acclimatisation period as the methane composition and volumetric productivities were low. Table 2 indicates the performance of the reactors at 12-hour sampling to signify the effect of gas retention time and temperature on methane composition and productivity. The 12-hour gas data at 55°C showed a methane composition and volumetric productivity of 22 % and 0.1(L CH_4 /L reactor) whereas higher values obtained when the reactor was switched to 65 °C with close to 55 % methane composition in the product gas as well as a higher productivity of 0.28(L CH_4 /L reactor). Conducting the experiment at 65°C doubled the methane composition and volumetric productivity for the 12 hour retention period. Luo and Angelidaki showed that the thermophilic (55°C) process is quicker than the mesophilic (37°C) process (Luo and Angelidaki, 2012), but did not investigate any different thermophilic and mesophilic temperatures.

3.2 Reactor performance with respect to volatile fatty acid and fresh inoculum

3.2.1 Volatile fatty acid profile of the reactors

In an anaerobic digester as the complex compounds are systematically broken down to fatty acids, there is a significant production of predominantly acetic acid followed by other acids. The profile of the VFAs also depends on the particular substrate being broken down. However in biological methanation processes as there are little breakdown of organic solid or liquid substrates since gaseous compounds are being consumed, very small quantities of VFAs are observed. Figure 2 shows the VFAs present in the three reactors.

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At 55°C the reactors contained the highest amounts of VFAs and acetic acid; this could be attributed to the initial quantities present in the stock inoculum that were slowly consumed. Although it is hoped that all the CO_2 and H_2 will be consumed directly, an alternative pathway is also possible in which acetate is produced via homoacetogenic microbial activity, in which some of CO_2 and H_2 is converted to acetate (Bensmann et al., 2014; Burak Demirel, 2008; Burkhardt and Busch, 2013; Dahiya and Joseph, 2015; Siriwongrungson et al., 2007). The acetate may then be subsequently converted to CH_4 and CO_2 by acetoclastic methanogens. The quantities of acetate reduced gradually and was probably due to the fact that there was little acetic acid production after the residual acetic acid in the inoculum was consumed and the only methane production was achieved from gaseous substrates. Residual acetic acid was consumed to form methane and the major contributor to methane production in the later stages of the reaction was the direct reduction of CO_2 by H_2 (Alitalo et al., 2015; Yu and Pinder, 1993).

3.2.2 Effect of fresh inoculum on reactor performance

As the performance of the reactors was faster at 65°C, the reactors were re-seeded with fresh stock inoculum and operated at 65°C for 24 hours and 18-hour gas sampling to determine if better and faster methane productivities and composition can be achieved. In the previous experiment, it was observed that 12 hours of biological methanation at 65°C gave nearly 55% methane composition, hence it was decided to observe the methane production at 18 hours along with the 24-hour reading. Figure 3 and Table 3 highlight the methane production at 65°C with fresh starting stock inoculum. Starting with a fresh inoculum added a few advantages. There was some residual substrate present in the stock inoculum (as the stock inoculum was fed with cellulose) along with the methanogens and bacteria that are already present in the inoculum. These together along with the gaseous substrates (H_2 and CO_2) seem

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to give slightly higher methane composition and volumetric productivity of ca. 92% and 0.46(L CH_4 /L reactor) for 24 hour sampling. Higher methane composition and productivity were obtained at 18 hours (77.5% and 0.38 L CH_4 /L reactor) when compared to the 12 hour values obtained in the previous experiment (54.6% and 0.27 L CH_4 /L reactor). It is postulated that this is due to a combination of surplus substrate in the reseeded reactor and the mixed culture of microbes, as well obviously as the longer retention time. Prolonged use of the stock inoculum leads to a more enriched culture with only the gaseous substrates to feed on. It is suggested by the authors that in a commercial industrial process that reseeding is required to maintain process efficiency.

3.3 Reactor performance with respect to Microbial community analysis

3.3.1 Community Analysis

Of the 108 clones picked and sequenced, 92 passed quality filters (average length = 626bp), and were clustered @ 97% similarity identifying 5 closely-related archaeal OTUs. An OTU table is presented in Table 4. Four OTUs aligned at sequences identities >99% with *Methanothermobacter wolfeii* (OTUs 13B, F01, B12; reference accession KT368944.1) and *Methanothermobacter thermautotrophicus* (OTU D04; reference accession HJQ346751.1). *M. wolfeii* grows optimally at 55-65°C, pH 7.0-7.7, requiring relatively high concentrations of tungsten (8uM) as a growth factor (Winter et al., 1984). *M. thermautotrophicum* grows optimally between 55-70°C over a pH range of 7.2-7.6 . Both species are capable of growing autotrophically on CO_2 and H_2 and were originally isolated from digester sludges. Additionally, *M. wolfeii* can reduce formate as a carbon source (Winter et al., 1984). A fifth OTU (E04) associated with *Methanobacterium formicicum* Mb9 (accession JN205060.1) at identities >99%. *M. formicicum* can reduce a slightly wider range of carbon sources (CO_2 and

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formate; 2-propanol and 2-butanol without methanogenesis) but is associated with a much lower thermal range of 37-45°C (Jarvis et al., n.d.). A phylogram of sequences from this study, as well as related reference sequences, is provided in (Figure 4).

Methanothermobacter-associated OTUs dominate the archaeal community in this thermophilic ex-situ reactor. OTU 13B comprises 85% of all sequences and is evenly distributed across the study, despite a slightly lower abundance in reactors at Stage D. The significance of OTUs D04 and E04 is unclear given their occurrence only once in this study. (Figure 4) shows clone sequences clearly cluster with *Methanothermobacter* references, indicating a highly homogeneous archaeal community throughout the trial. Association of OTU E04 with *M. formicicum* suggests closely related taxa at lower abundances. Notably, no sequences align with other methanogenic clades or non-methanogenic Archaea, despite expected coverage of these groups. In particular, a lack of acetoclastic methanogens (Order *Methanosarcinales*) suggests carbon-limited thermophilic conditions may be unsuitable for acetoclasts. The significance of OTUs D04 and E04 is less clear in that they occur only once in this study

3.3.2 Microbial community development

Sampling covered triplicate reactors at 55°C, 65°C, and 65°C with re-inocculation, revealing a homogeneous methanogenic population. Given the changes in reactor conditions (10° increase in temperature, re-inocculation), the consistency of these populations indicates a rapid acclimatisation from the original inocculum community and the stability of those populations once established. *Methanothermobacter* species therefore represent likely and resilient candidates for thermophilic biogas upgrading.

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Re-inocculation of the reactors at Stage D was associated with some recovery of function (from 80-90% to 90-92% CH_4 composition after 24hr) but no significant change in Archaea was observed. It is therefore unlikely that restructuring of methanogen populations had a role in the increased or decreased levels of CH_4. Instead, inocculum may have allowed rescue through the introduction of depleted organic or inorganic materials. Previous studies have identified the importance of trace elements in biogas oriented in-situ anaerobic digesters (Demirel and Scherer, 2011; Wall et al., 2014) and informed the inclusion of supplements in the reactor media for this ex-situ reactor. Response to further supplementation seen in Stage D may indicate the need for additional growth factors in thermophilic setups - in particular, a requirement for tungsten by *M. wolfeii* (Winter et al., 1984), which associated with over 90% of sequences in this study, may be relevant. Alternatively, a recovery in reactor performance without changes in archaeal taxa may reflect changes in bacterial taxa associated with methanogenic processes in this setup - bacterial taxa excluded at reactor initiation (Stage B, 55°C) may have aided stabilisation when re-inocculated (Stage D, 65°C). Although this study's microbial resolution may be constrained by primer coverage and depth of sequencing, it nevertheless outlines the major methanogenic components of this system through a consistent clustering of sequences. Although some necessary components remain uncharacterised, thermophilic (55°C-65°C) ex-situ biogas upgrading is likely to rely upon select, stable hydrogenotrophic populations of *Methanothermobacter* and *Methanobacterium*

4. Conclusion

The operation of an ex-situ biological methanation system is more efficient at 65°C than 55^0C. Methane content in excess of 90% can be achieved at volumetric productivity of 0.45 L CH4/Lreactor/day. As the inoculum ages, it changes from a mixed culture to a more enriched

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culture; in commercial operations re-seeding of the process would be required. *Methanothermobacter* species dominate the microbial communities in thermophilic ex-situ methanation systems.

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The reviewers of the paper have greatly enhanced the quality of this paper through rigorous interrogation of the original manuscript and expanded the remit to include description of the microbial community.

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Table1: Existing reactor designs and performance data

Reactor	Temp (°C)	Inoculum	Influent gas	Operation mode	Working volume (L)	Maximum methane concentration (%)	Reference
CSTR	55	Anaerobic digestate	Biogas + H_2	Continuous	0.6	95.4	(Luo and Angelidaki, 2012)
Trickle bed with packing	37	Anaerobic digestate	H_2 + CO_2	Continuous	88	96	(Burkhardt et al., 2015)
Up-flow bed	35	Anaerobic digestate	H_2 + CO_2	Continuous	7.8	-	(Lee et al., 2012)
HFM	37	Anaerobic digestate	H_2 + CO_2	Continuous	0.195	85	(Lai et al., 2008)
CSTR	37	Anaerobic digestate	H_2 + CO_2	Continuous	100	92	(Kim et al., 2013)
CSTR	60	Pure culture	Biogas+ H_2 H_2 + CO_2	Continuous	3	-	(Martin et al., 2013)
Trickle bed reactor	37	Pure culture	H_2 + CO_2		58	96	(Rachbauer et al., 2016)
CSTR	65	Pure culture	H_2 + CO_2	Continuous	10	85	(Seifert et al., 2014)
Bioreactor with packing	50	Methanogenic culture	H_2 + CO_2	Continuous	4	90	(Alitalo et al., 2015)
Closed batch system	55 and 65	Anaerobic digestate	H_2 + CO_2	Batch	1.140	90	This study

CSTR: Continuous Stirred Tank Reactor; HFM: Hollow Fibre Membrane Reactor

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Table 2: Methane composition and volumetric productivities for 12 hour gas sampling at 55°C and 65°C.

	55°C				65°C			
	% Methane	S.D	VP	S.D	% Methane	S.D	VP	S.D
Week 16	21.9	2.63	0.10	0.01	50.29	1.82	0.25	0.8
Week 17	19.8	4.65	0.099	0.02	54.6	5.75	0.27	0.24

S.D: standard deviation

VP: Volumetric productivity (L methane/L reactor)

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Table 3: Methane composition and volumetric productivities for 18 hour gas sampling at 65°C.

	65°C			
	% Methane	S.D	VP	S.D
Week 5	77.56	2.52	0.38	0.5
Week 6	75.33	1.66	0.37	0.23

S.D: standard deviation

VP: Volumetric productivity (L methane/L reactor)

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Table 4: Reference OTUs for sequences clustered at 97% as well as the closest

	Stage B			Stage C			Stage D			
Reactor	R. 1	R. 2	R. 3	R. 1	R. 2	R. 3	R. 1	R. 2	R. 3	Closest Identity
OTU 13B	8	11	10	10	8	6	5	10	10	*Methanothermobacter wolfeii*
OTU F01	2	1	1	1	0	1	3	1	1	*Methanothermobacter wolfeii*
OTU B12	0	0	0	0	0	1	1	0	0	*Methanothermobacter wolfeii*
OTU D04	0	0	0	0	0	0	1	0	0	*Methanothermobacter thermautotrophicum*
OTU E04	0	0	0	0	0	0	1	0	0	*Methanobacterium formicicum*

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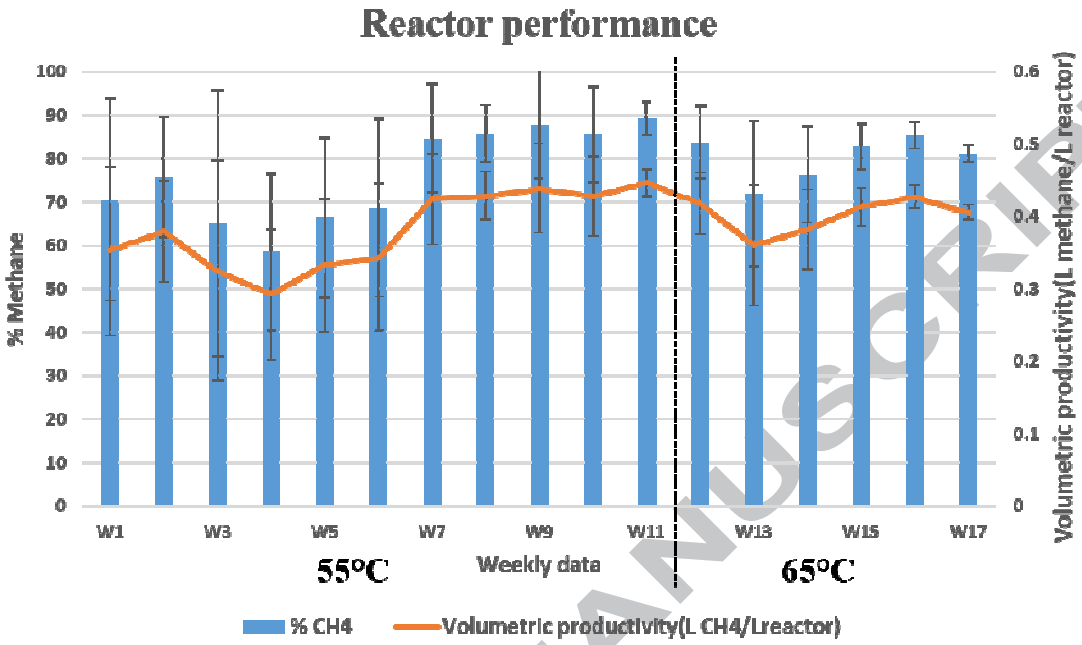


Figure 1: Methane composition and volumetric productivity at 55°C and 65°C for 24 hour retention period

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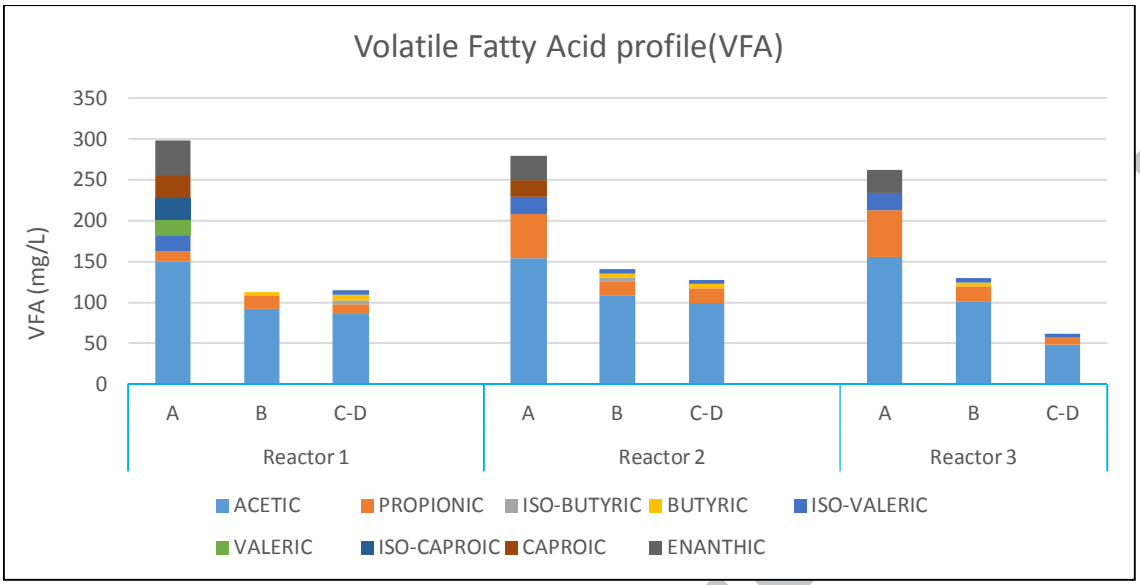


Figure 2: Volatile Fatty Acid profile of the reactors

Note: A- acclimatisation phase at 55°C; B- steady state operation phase at 55°C; C-D is the operation at 65°C

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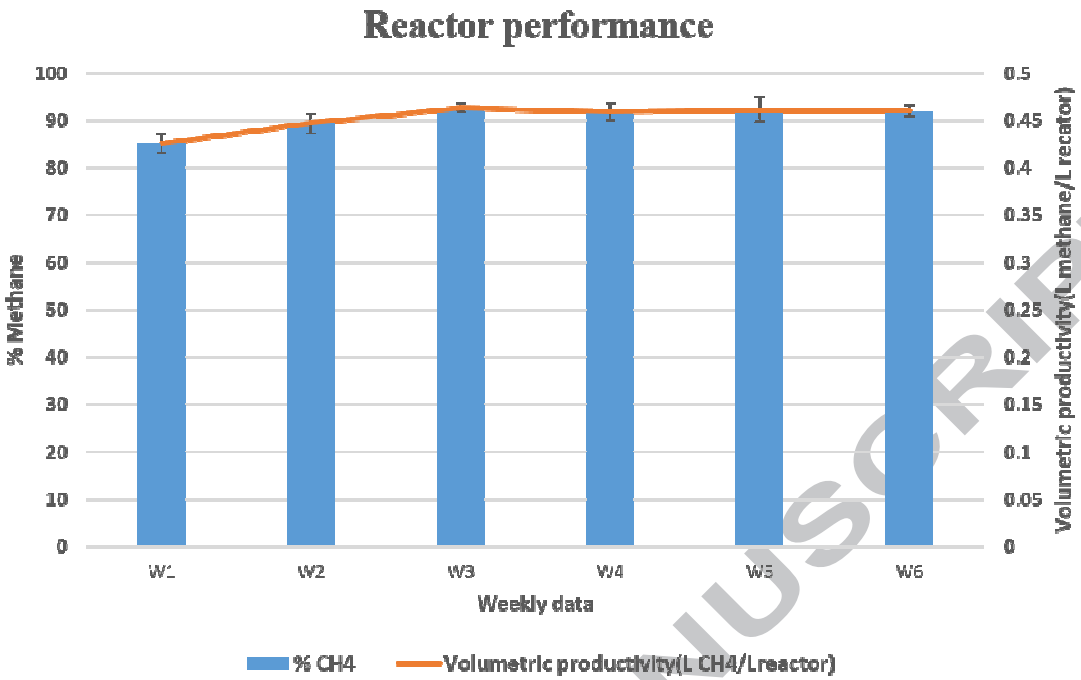


Figure 3: Methane composition and volumetric productivity at 65°C (fresh inoculum) for 24 hours

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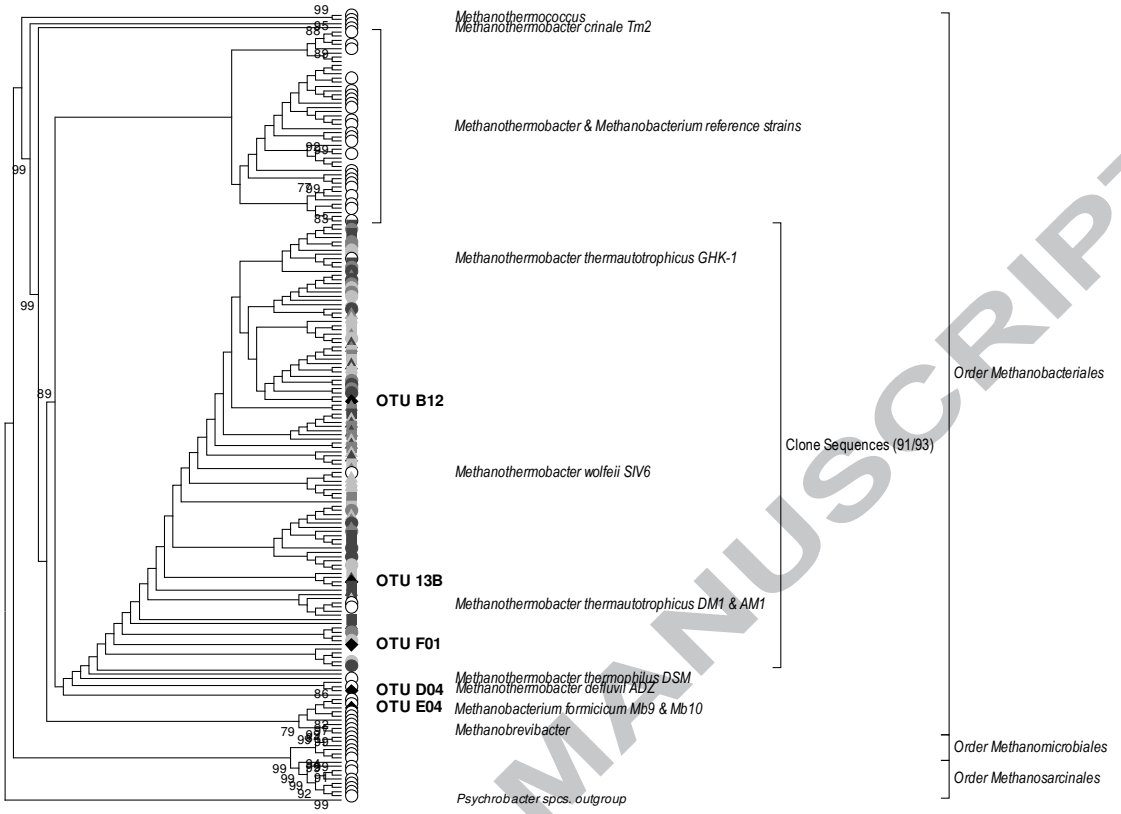


Figure 4: Consensus tree (Neighbour-Joining method with Tamura-Nei distances through 1000 iterations; MEGA) showing evolutionary distances between cloned and reference sequences in this study.

Note the segregation of Orders *Methanosarcinales* and *Methanomicrobiales* with respect to O. *Methanobacteriales* and clone sequences. The majority of cloned sequences are located among *Methanothermobacter* sequences. Tight clustering with short branch-length reflects the high sequence-similarity of the dataset. No clustering of clones by Reactor or Stage is readily apparent.
Legend: ○: reference sequences; ◆: clustered reference OTUs; ●: Reactor 1; ▲: Reactor 2; ■: Reactor 3. Stage B: ▉ ; Stage C: ▉ ; Stage D: ▉.

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- Biological methanation was assessed at 55 and 65°C with mixed culture.
- The efficiency were better at 65^0C than 55^oC
- Methane content of ca. 90% was achieved at productivity of 0.45L CH4/Lreactor/day
- Reseeding of the process is required as when innoculum ages, efficiencies decrease
- *Methanothermobacter* species dominate in the ex-situ methanation community.

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