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Shaken not stirred - effect of different mixing modes during the cultivation of methanogenic pure cultures

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ABSTRACT

Numerous cultivation techniques for aerobic microorganisms have been extensively investigated in the field of microbiology. Optimisation of these techniques is important for scientific and economic reasons. Methanogenic archaea, however, are obligate anaerobic microorganisms requiring different cultivation techniques than aerobic organisms due to the fundamental differences in physiology. Mixing of aerobic cultures is generally considered as very important as it provides organisms with essential oxygen; however, for anaerobic microorganisms lacking the ability to grow with oxygen, this point in cultivation was widely neglected. This work aimed at investigating the effect of different mixing modes on cultures of the methanogenic archaea *Methanomethylovorans thermophila, Methanosarcina acetivorans, Methanosarcina thermophila* and *Methanococcus vannielii* by cultivating them anaerobically in the modes standing/lying, shaken/unshaken and large/small serum flask in order to analyse their impact on the methane and biomass production. This study showed that a shaken incubation mode had a positive impact on methane production and resulted in its accelerated production, especially in hydrogenotrophic cultures; however, higher methane production did not necessarily lead to higher biomass production.

1. Introduction

Biological systems derive their energy through biochemical reactions. Organisms either conserve this energy for their growth or release it in form of heat. During aerobic processes, complex organic substrates can be completely oxidised, using oxygen which is the strongest electron acceptor with a redox potential of + 0.82 V. Since not present in anaerobic systems, molecular oxygen (O_2) cannot be used as a terminal electron acceptor in such environments by anaerobes in general and methanogenic microorganisms (methanoarchaea) in particular. Thus, in anoxic environments electrons must be transferred to alternative electron acceptors - these can be inorganic compounds such as nitrate (NO_3), sulphate (SO_4^{2-}) and carbon dioxide (CO_2) or metals such as iron (Fe^{3+}) or manganese (Mn^{4+}). This can result in incomplete oxidation of complex substrates, which in turn leads to a lower microbial energy yield (Kim and Gadd, 2008; Madigan et al., 2022). These biochemical and therefore physiological differences between aerobic and anaerobic microorganisms during energy-yielding processes have inevitably led to different cultivation methods in the field of microbiology. Various factors such as growth media, substrate composition and availability, and various process parameters such as incubation temperature, pH, cultivation vessel or agitation/mixing (speed) can affect the growth kinetics of microorganisms (Madigan et al., 2022). The selection of appropriate culture conditions and settings is therefore essential for the optimal production of cell mass and metabolites, while minimizing cell death.

Methanogens are strictly anaerobic microorganisms that have a limited substrate spectrum but can produce a remarkable amount of methane (Enzmann et al., 2018; Liu and Whitman, 2008). These phylogenetically diverse organisms belong to the phylum *Euryarchaeota* and are classified into the following seven orders: *Methanobacteriales, Methanococcales, Methanomicrobiales, Methanosarcinales, Methanopyrales, Methanocellales* and *Methanomassiliicoccales* (Balch et al., 1979; Dridi et al., 2012; Enzmann et al., 2018; Iino et al., 2013). In 2015 and 2016, two further phyla were discovered through metagenomic analyses (MAGs), whose (as yet uncultivated) representatives would theoretically be capable of methanogenesis: *Bathyarchaeota* (Evans et al., 2015) and *Verstraetearchaeota* (Vanwonterghem et al., 2016). With regard to the metabolic pathways of the microorganisms, acetoclastic, hydrogenotrophic, methylotrophic, methoxydotrophic and alkanotrophic

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methanogenesis can be distinguished (Borrel et al., 2013; Garcia et al., 2022; Laso-Pérez et al., 2019).

Acetoclastic methanogenesis, in which acetate is metabolised to carbon dioxide and methane, is performed by archaea of the genus *Methanosarcina* and *Methanothrix* (formerly *Methanosaeta*) (Ferry, 2010, 2020; Liu and Whitman, 2008). During methylotrophic methanogenesis, methylated compounds such as methanol, methylamines or methylated sulphur compounds serve as substrates for the formation of methane and carbon dioxide (Liu and Whitman, 2008). In hydrogenotrophic methanogenesis, carbon dioxide is metabolised to methane, with hydrogen, formate or primary and secondary alcohols (e.g. ethanol, 2-propanol) serving as electron donors (Frimmer and Widdel, 1989; Liu and Whitman, 2008; Widdel, 1986). Methylated aromatic compounds, which are present in lignin or coal, are metabolised to carbon dioxide and methane during the methoxydotrophic metabolic pathway (Kurth et al., 2021; Mayumi et al., 2016). Representatives of the genus *Candidatus Methanoliparium* undergo alkanotrophic methanogenesis by using short-chain alkanes as substrate to produce methane and carbon dioxide (Borrel et al., 2019; Laso-Pérez et al., 2019; Zhou et al., 2022).

The principal characteristic of these types of methanogenesis is that acetoclastic, methylotrophic, methoxydotrophic and alkanotrophic methanogens utilise substrates contained in the liquid phase, while hydrogenotrophs use gaseous substrates when cultivated with H_2/CO_2. The microorganisms are therefore limited regarding substrate availability in the liquid or gas phase, respectively. The growth depends on the solubility of the respective substrate in the liquid phase where it becomes bioavailable (Pappenreiter et al., 2019; Seifert et al., 2014). A cost-effective method that is applied in laboratory in order to improve the gas/liquid mass transfer and solubility of the substrates, and thereby provide a homogeneous environment for the cultivated microorganisms, is using shaking incubators for mixing the whole batch cultivation system. Shaking incubators – commonly used for aerobic incubation – continuously introduce dissolved gas into the culture medium by moving the liquid onto the walls of the cultivation vessel and thereby increasing the surface area. However, excessive agitation can create shear stress and damage the microorganisms (Büchs, 2001). In addition, the gas transfer rate depends on the size, shape and material of the vessel, the filling volume and the physico-chemical parameters of the media/liquid, the shaking speed and shaking diameter of the orbital shaker (Büchs, 2001; Maier and Büchs, 2001).

Fundamental microbial processes during methanogenesis have been investigated in detail (Bryant, 1979; Ferry, 2010, 2011; Thauer et al., 2008). There is a wealth of information on the optimal cultivation conditions for methanogenic pure cultures (Jiang et al., 2005; Lackner et al., 2018; Sowers et al., 1984; Stadtman and Barker, 1951; Zinder and Mah, 1979). However, the topic of 'agitation' is still awaiting deeper investigation. Although much work has been done on cultivation methods, these efforts have primarily been confined to aerobic culture media and conditions in shaken flask studies (Büchs, 2001; Maier and Büchs, 2001). Additionally, anaerobic cultivation is largely concerned to stirred and continuous process control and optimization (Pappenreiter et al., 2019; Schill et al., 1996; Seifert et al., 2014). To the best of our knowledge, the effect of different mixing modes on the growth and methane production of methanogenic pure cultures has not been investigated in detail and the present study aimed at systematically elucidating the latter for three acetoclastic/methylotrophic and one hydrogenotrophic methanogenic species. Our findings might help scientists select an appropriate incubation setting for – often difficult cultivable – methanogenic pure cultures for their successful cultivation and biotechnological experimentation.

2. Materials and Methods

2.1. Experimental setup

The methanogenic archaea *Methanomethylovorans thermophila, Methanosarcina acetivorans, Methanosarcina thermophila* and *Methanococcus vannielii* were cultivated anaerobically in corresponding DSMZ media to investigate the effect of different cultivation modes in three replicates whereby the variants standing/lying, shaken/unshaken and large/small serum flask were investigated full factorial ending up in the following 8 variations: standing unshaken large (SUL), lying unshaken large (LUL), standing shaken large (SSL), lying shaken large (LSL), standing unshaken small (SUS), lying unshaken small (LUS), standing shaken small (SSS), lying shaken small (LSS). This resulted in 24 cultivation vessels per organism. Thus, a total of 96 experimental units were evaluated. Serum flasks (Glasgerätebau Ochs, Germany) with a nominal volume of 120 mL ("small" variant, d = 5 cm, h = 8.5 cm) or 250 mL ("large" variant, d = 6 cm, h = 10.5 cm) were used as batch reactor systems. Shaken variants were maintained under constant orbital shaking at 125 rpm (Infors HT, Switzerland) according to previous findings (Bodegom et al., 2004; Eliani-Russak et al., 2023; Sprenger et al., 2007) either standing or lying (Fig. 1). All cultures were incubated in the dark.

Depending on the growth dynamics of the respective microorganism, both gas and liquid samples were taken for further analyses at least at five distinct time points during cultivation. Firstly, the overpressure in the serum flasks was measured using a manometer. Then 1.14 mL of gas was taken from the headspace to analyse its methane, hydrogen, and carbon dioxide concentration chromatographically. Subsequently, a liquid sample of 1 mL was taken and immediately frozen at -20°C and thus preserved for further analyses. The liquid samples were used to determine the volatile fatty acids (VFA) concentration using high-performance liquid chromatography (HPLC) and to measure the protein content. The pH value was measured using pH test strips.

2.2. Cultivation

Pure cultures of *Methanomethylovorans thermophila* (DSM 17232), *Methanosarcina acetivorans* (DSM 2834), *Methanosarcina thermophila* (DSM 1825) and *Methanococcus vannielii* (DSM 1224) were originally purchased from „Deutsche Sammlung von Mikroorganismen und Zellkulturen" (DSMZ). *Methanomethylovorans thermophila* was cultivated in DSMZ medium 684 at 55°C, *Methanosarcina acetivorans* in DSMZ medium 141c at 37°C, *Methanosarcina thermophila* in DSMZ medium 120 at 55°C, *Methanococcus vannielii* in DSMZ medium 119 at 37°C. The method for the anaerobic cultivation of methanogenic archaea was based on (Wagner et al., 2019). Methanol-, trimethylammonium chloride-, sodium acetate-, and sodium formate solutions were added from sterile stock solutions according to respective requirements of the organism. A volume of 50.00 mL of sterile culture broth (using an autoclave enabling the use of closed vessels and therefore ruling out the possibility of liquid loss due to evaporation) were used and inoculated with 10 % (vol/vol) of a culture in the exponential growth phase as determined by methane production. A sterile H_2 - CO_2 (80:20 vol/vol) gas mixture was added to batch cultures of *Methanococcus vannielii* after inoculation with an overpressure of 1020 (+/−25) mbar in the large serum flasks and 1500 (+/−25) mbar in the small serum flasks representing approx. 388.65 mL and 167.27 mL, respectively. Adding the same volume of gas was not possible due to handling issues but was

Fig. 1. Experimental setup. 120 mL or 250 mL serum flasks, filling volume 55 mL, shaking frequency 125 rpm, standing or lying.

considered arithmetically when analyzing data. Growth conditions for each methanogenic culture are summarized in Table 1. In addition, the theoretical chemical oxygen demand (COD) as well as theoretical methane yield per serum flask were calculated according to Buswell and Mueller (1952) for each carbon source. Exact substrate concentrations are given in Table 1; differences from desired medium concentrations were due to carryover from inocula. It is important to emphasize that the DSMZ media utilized in this study contain yeast extract (DSMZ medium 684 contains 0.2 g L^{-1}, medium 141c contains 2 g L^{-1}, medium 120 contains 2 g L^{-1}, and medium 119 contains 1 g L^{-1}), which can serve as an additional carbon source for the microorganisms. However, due to the unavailability of precise composition data, its consumption could not be measured.

2.3. Analytical methods, sampling

Overpressure in the serum flasks was measured using a GDH 200-13 reference pressure manometer (Greisinger electronic, Germany) and the current atmospheric pressure (Central Institute for Meteorology and Geodynamics (ZAMG) (www.zamg.ac.at)). The gas composition in the headspace of the serum flasks was analysed using a GC 2010 gas chromatograph (Shimadzu, Japan) according to Wagner et al. (2011). The system was calibrated before each measurement by injecting 1 mL of reference gas (Messer, Austria) with a gas consisting of methane, carbon dioxide, and hydrogen (60:30:5 (% vol/vol), remaining rest N_2). The amount of methane produced by the respective methanogenic archaea was determined based on the results of the overpressure measurement and the gas chromatograph and was calculated according to Wunderer et al. (2022).

The composition of the volatile fatty acids was analysed using HPLC-UV/Vis at 220 and 270 nm on a Prominence system (Shimadzu, Japan) equipped with a Fast Acid column (Phenomenex, Germany) run at 70°C, a solvent consisting of 5 mM H_2SO_4 and a flow rate of 0.5 mL min^{-1} (Wagner et al., 2017). A VFA standard (CRM46975, Sigma-Aldrich, Germany) consisting of formate, acetate, propionate, i- butyric acid, butyric acid, i-valeric acid, and valeric acid, each with a concentration of 10 mM, and a methanol standard with a concentration of 1 % (w/v) were used as a reference system. The pH was determined using Dosatest® pH test strips pH 2.0 - 9.0 (VWR International, Germany).

2.4. Protein Content

Measurements of the protein content as biomass indicator were based on the method of Bradford (1976) and Read and Northcote (1981). Firstly, the protein reagent was produced by dissolving 70 mg Serva blue (100 mg Coomassie brilliant blue G-250) in 50 mL 95 % (v/v) ethanol. Subsequently, 100 mL 85 % (v/v) phosphoric acid and 50 mL aqua dest. were added to the solution. For the analyses, 1 mL sample was centrifuged at 10,000 g for 10 min. The supernatant was discarded, the pellet dissolved in 11 % (w/v) trichloroacetic acid and incubated for 10 min at room temperature. Samples were then centrifugated for 5 min at 10,000 g, the supernatant discarded and the pellet dissolved in 0.5 mL 3 % (w/v) NaOH. Bovine serum albumin served as standard and was used for the generation of a calibration curve. For the photometric measurement 100 µL sample and 1 mL diluted (1:5 in aqua dest.) protein reagent were mixed into a 1 mL cuvette. After 10 min the absorbance was measured at 595 nm.

Table 1
Growth conditions for the anaerobic cultivation of *Methanomethylovorans thermophila, Methanosarcina acetivorans, Methanosarcina thermophila* and *Methanococcus vannielii*.

Methanogenic culture	Substrate	Conc. [mM]	Gas phase	pH
Methanomethylovorans thermophila	methanol	49.44 mM	N_2:CO_2 (70:30, v/v)	7.4
Methanosarcina acetivorans	sodium acetate	12.19 mM	N_2:CO_2 (70:30, v/v)	7.6
	trimethylammonium chloride	52.32 mM		
Methanosarcina thermophila	sodium acetate methanol	30.48 mM 247.19 mM	N_2:CO_2 (70:30, v/v)	7.6
Methanococcus vannielii	sodium formate	29.41 mM	H_2:CO_2 (80:20, v/v)	7.4

2.5. Statistical analysis

The statistical and graphical data analyses were carried out using the Software package *Satistica 13* (StatSoft®) and *Microsoft Excel 2019* (Microsoft Corporation). Results are given as mean ± standard deviation from three replicate samples. The data obtained on the last day of the incubation period were selected for statistical analysis. The data were initially tested for normal distribution using *Lillefors* test. The homogeneity of variances was assessed using *Levene's* test. One-way ANOVA, *Kruskal-Wallis* ANOVA (non-parametric alternative) and multiple comparison of variance were performed as well as post-hoc analyses using the *Bonferroni* test for homogeneous groups. A significance level of 0.05 ($p < 0.05$) was set for all analyses to evaluate differences between the variants of the respective cultivated methanogens.

3. Results and Discussion

3.1. Methane yield

Cumulative amounts of methane produced by *Methanomethylovorans thermophila, Methanosarcina acetivorans, Methanosarcina thermophila* and *Methanococcus vannielii* are shown in Fig. 2. Their methane production rates during the cultivation and significant differences ($p < 0.05$) of their cumulative methane production at the last day of incubation are summarized in Table 2.

The methylotrophic methanogen *Methanomethylovorans thermophila* was incubated in the dark at 55°C for 14 days (Fig. 2A). Methane production initiated within 24 hours in the majority of variants, with the exception of the lying/shaken/large variant: all replicates of those reactors started producing methane not before the fourth day. The small variants (small cultivation vessels) reached their maximum methane content at an earlier point in time than the large variants. The maximum methane content was reached by the small variants between day three and four of the incubation period, whereas the large variants reached their maximum between day four and nine. However, statistical differences ($p < 0.05$) could only be observed between the standing/unshaken/large and the lying/shaken/large variants on the fourth day. By the end of the incubation period, the archaeon had produced a slightly higher quantity of methane in the larger serum flasks (2.1 mmol) in comparison to the smaller ones (1.8 mmol), whereby the methane content in the unshaken variants was slightly higher than in the shaken ones. However, the difference was not significant ($p > 0.05$). Obtained methane yields were slightly higher than the theoretically achievable methane content of 1.72 mmol according to the calculated COD. Increased values might be due to the yeast extract in the medium which provides important nutrients and vitamins but cannot be included in theoretical COD calculations due to its complex composition.

The archaeon *Methanosarcina acetivorans* was incubated in the dark at 37°C for 7 days (Fig. 2B). A significant difference ($p < 0.05$) in methane production between the small and the large cultivation vessels was observed already during the exponential growth phase, on the second day of incubation. The data indicated that the quantity of methane produced was significantly greater ($p < 0.05$) in the larger shaken variants than in the smaller shaken variants. Similarly, the

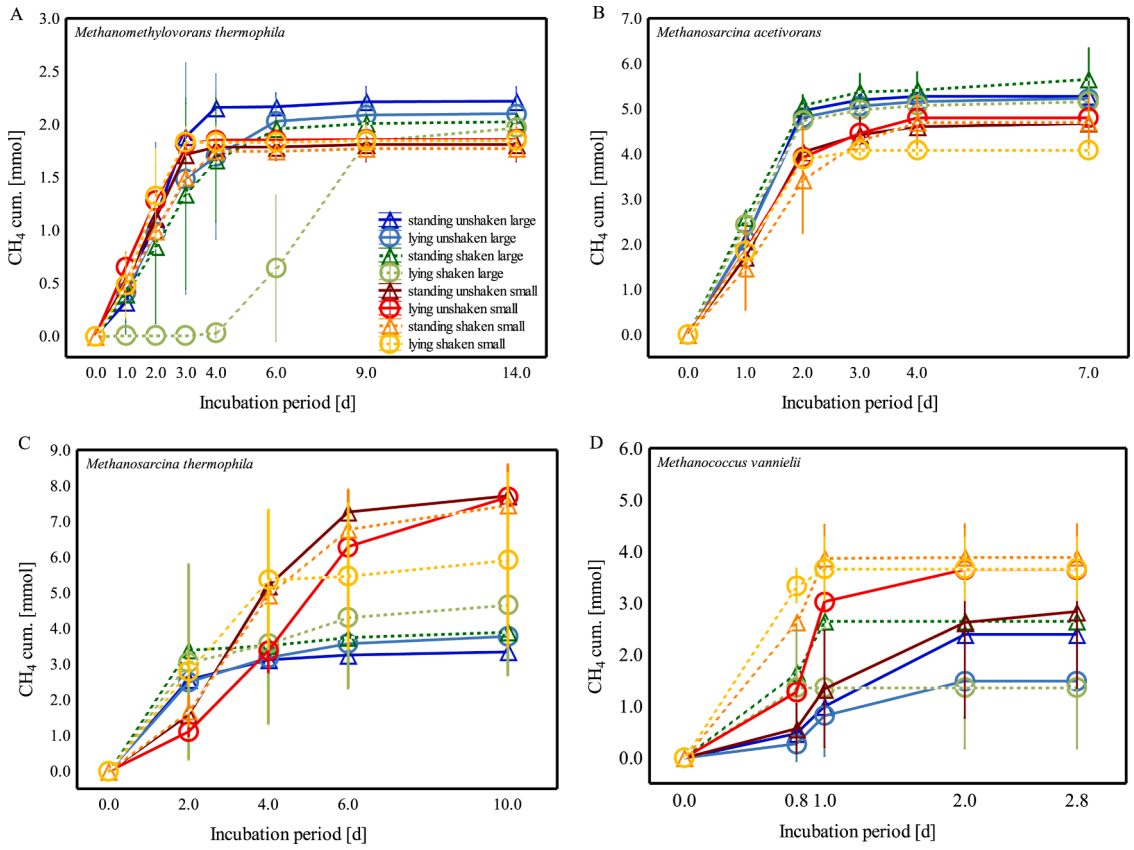

Fig. 2. Cumulative methane production over time during anaerobic cultivation of (A) *Methanomethylovorans thermophila*, (B) *Methanosarcina acetivorans*, (C) *Methanosarcina thermophila*, (D) *Methanococcus vannielii* in different incubation settings.

variants in the larger serum flasks exhibited a higher methane yield at the end of the incubation period than those in the smaller serum flasks, with an average production of 5 mmol methane and 4 mmol methane, respectively, reflecting a significant difference ($p < 0.05$). The highest amount of methane was by far produced in the standing/shaken/large variant and the least amount was produced in the lying/shaken/small one. However, the theoretically achievable methane content of 10.30 mmol according to the calculated COD could not be achieved, whereby lower values might indicate increased biomass production and reduced metabolic activity. This assumption was then confirmed by the analyses of the protein content (see chapter 3.3 Protein yield).

Methanosarcina thermophila was incubated in the dark at 55°C for 10 days. As depicted in Fig. 2C, a distinction in methane production among the various variants was noted throughout the incubation period. In contrast to the preceding two microorganisms, *Methanosarcina thermophila* yielded a significant higher ($p < 0.05$) methane concentration of 7 mmol in the smaller serum flasks on the last day of incubation compared to an average of 4 mmol in the larger ones. Additionally, the methane yield in the large/shaken flasks was marginally higher to that observed in the large/unshaken ones. However, the theoretically achievable methane content of 10.29 mmol according to the calculated COD could not be achieved, indicating increased biomass production (Fig. 4C).

The hydrogenotrophic methanogen *Methanococcus vannielii* was incubated in the dark at 37°C for 3 days (Fig. 2D). Methane production started within a few hours after the start of incubation and slight differences could already be observed after 18 hours. Organisms in shaken flasks produced more methane than those in unshaken ones. In addition, the shaken variants reached their maximum methane production within 24 hours, whereas the unshaken ones did not reach their maximum until 48 hours. From this point onwards, a difference in the amount of methane produced between the small and large variants became clearly visible. By the end of the incubation period, an average of 2 mmol methane was obtained in the large serum flasks and 4 mmol methane in the small ones, reflecting a significant difference ($p < 0.05$). However, the theoretically achievable methane content of 6.45 mmol according to the calculated COD could not be achieved. It is assumed that the higher partial pressure prevailing in the small serum flasks (overpressure of 1500 (±25) mbar on the first day of incubation) and the shaking (125 rpm) increased the solubility of the gas mixture (H_2/CO_2) and this consequently had a positive effect on methane production in these variants - as according to Henry's Law, the solubility of gases in liquids depends on the temperature and the partial pressure above the liquid phase (Henry, 1803). Additionally, the gas transfer rate depends on the geometry of the cultivation vessel, the filling volume and the physico-chemical characteristic of the media, and the operating mode of the orbital shaker (Büchs, 2001; Maier and Büchs, 2001). In another study, Seifert et al. (2014) used continuous cultures of *Methanothermobacter marburgensis*, also a hydrogenotrophic methanogen, to investigate the effects of gassing rate, reactor pressure and reactor design on its performance. Their results showed that biological methanogenesis with *M. marburgensis* is gas-limited and they concluded, that elevated mixing and reactor pressure increase the gassing rate, making gas-liquid mass transfer more effective and ultimately positively influencing volumetric methane productivity. Although a slight delay in methane production was observed in the shaken flasks in the present study, the shear forces caused by the orbital shaker seemed to be insufficient to decrease or inhibit the growth of the methanogens or cause cell lysis.

3.2. Carbon source consumption

The results of the volatile fatty acid analyses revealed that during the cultivation of *Methanomethylovorans thermophila, Methanosarcina acetivorans, Methanosarcina thermophila* and *Methanococcus vannielii*, no

Table 2
Methane production rate during anaerobic cultivation of *Methanomethylovorans thermophila* (day 2), *Methanosarcina acetivorans* (day 1), *Methanosarcina thermophila* (day 2) and *Methanococcus vannielii* (day 0.8) and significant differences ($p < 0.05$) of cumulative methane production at the last day of incubation for each methanogenic culture in different incubation settings – SUL = standing unshaken large, LUL = lying unshaken large, SSL = standing shaken large, LSL = lying shaken large, SUS = standing unshaken small, LUS = lying unshaken small, SSS = standing shaken small, LSS = lying shaken small.

Organism	Variant	CH_4 production rate [mmol/h] (mean ± SD)	CH_4 cum [mmol] (mean ± SD)	Significant difference (group)
Methanomethylovorans thermophila	SUL	0.03 ± 0.00	2.21 ± 0.14	a
	LUL	0.02 ± 0.02	2.10 ± 0.17	a
	SSL	0.02 ± 0.01	2.02 ± 0.06	a
	LSL	0.00 ± 0.00	1.96 ± 0.06	a
	SUS	0.03 ± 0.00	1.81 ± 0.16	a
	LUS	0.03 ± 0.00	1.85 ± 0.09	a
	SSS	0.02 ± 0.00	1.77 ± 0.01	a
	LSS	0.04 ± 0.01	1.85 ± 0.15	a
Methanosarcina acetivorans	SUL	0.09 ± 0.00	5.26 ± 0.15	a
	LUL	0.09 ± 0.00	5.20 ± 0.10	a
	SSL	0.11 ± 0.00	5.65 ± 0.66	a
	LSL	0.10 ± 0.01	5.15 ± 0.32	a
	SUS	0.07 ± 0.00	4.65 ± 0.49	b
	LUS	0.08 ± 0.01	4.79 ± 0.25	b
	SSS	0.06 ± 0.04	4.68 ± 0.59	b
	LSS	0.08 ± 0.01	4.07 ± 0.10	b
Methanosarcina thermophila	SUL	0.03 ± 0.03	3.35 ± 0.21	a
	LUL	0.05 ± 0.01	3.78 ± 0.33	a
	SSL	0.05 ± 0.04	3.91 ± 0.69	a
	LSL	0.06 ± 0.06	4.67 ± 1.97	a
	SUS	0.03 ± 0.00	7.72 ± 0.24	b
	LUS	0.02 ± 0.00	7.68 ± 0.34	b
	SSS	0.04 ± 0.01	7.47 ± 1.12	b
	LSS	0.04 ± 0.03	5.92 ± 2.43	b
Methanococcus vannielii	SUL	0.03 ± 0.00	2.39 ± 0.10	a
	LUL	0.02 ± 0.02	1.49 ± 1.06	a
	SSL	0.09 ± 0.00	2.66 ± 0.03	a
	LSL	0.08 ± 0.07	1.36 ± 0.97	a
	SUS	0.01 ± 0.01	2.84 ± 1.21	b
	LUS	0.03 ± 0.00	3.65 ± 0.21	b
	SSS	0.06 ± 0.00	3.88 ± 0.52	b
	LSS	0.08 ± 0.01	3.67 ± 0.50	b

concentrations of propionate, i-butyric acid, butyric acid, i-valeric acid, and valeric acid could be detected in any of the studied microorganisms. The results of the analysis of volatile fatty acids (VFAs) and thus carbon source utilisation during the cultivation of the microorganisms are shown in Fig. 3. The pure cultures were provided with acetate, methanol, trimethylammonium chloride, or formate and/or H_2/CO_2 as a carbon source.

Methanol served as the carbon source for *Methanomethylovorans thermophila*. According to DSMZ, each variant should have contained an initial concentration of 2.00 mL/L methanol. However, a slightly higher concentration of 2.07 ± 0.18 mL/L was measured at the beginning of the incubation period, possibly due to carryover from the inoculum. As illustrated in Fig. 3A, the methanol concentration decreased as expected in all variants during the cultivation of *Methanomethylovorans thermophila*. On the last day of incubation, no residual methanol could be detected in either variant, indicating complete metabolisation. The methanol concentration in all variants decreased rapidly until day 3, with the exception of the lying/shaken/large variant, in which methanol consumption was more slowly. In the small serum flasks, the methanol was completely metabolised within three days, in the large serum flasks between four and nine days. The results for methanol consumption (Fig. 3A) are consistent with those for cumulative methane production (Fig. 2A). Both, methanol consumption and methane production were delayed in the lying/shaken/large variant compared to all other variants. Furthermore, *Methanomethylovorans thermophila* used the total energy from the carbon source for methane production as methanol was completely consumed and the theoretically methane yield was reached.

The microorganism *Methanosarcina acetivorans* was provided with acetate and trimethylammonium chloride (TMA) as carbon source. As shown in Fig. 3B, the already low concentrations of acetate were hardly metabolised by the microorganism, explaining the lower obtained methane yield (Fig. 2B) than theoretically possible. It is assumed that TMA was used mainly for methane production and acetate (mainly) for anabolic purposes thus biomass production. Sowers et al. (1984) investigated the growth of *Methanosarcina acetivorans* with acetate, methanol, methylamine, dimethylamine and trimethylamine as carbon sources, and showed that the microorganism was fastest-growing on methanol (doubling time 5.2 hours), followed by the methylamines (doubling times between 7 and 8 hours) and sodium acetate (doubling time around 24 hours).

Methanosarcina thermophila used acetate and methanol as carbon source. According to DSMZ specifications, initial concentration of 30 mM acetate and 248 mM (corresponding to 10 mL/L) methanol were strived during medium preparation. However, a slightly higher concentration was measured due to carryover from the inocula at the beginning of the incubation period. As can be seen from Fig. 3C and D, both, methanol and acetate concentrations decreased in all variants during the cultivation of *Methanosarcina thermophila*.

In the prevailing literature, *Methanosarcina acetivorans* and *Methanosarcina thermophila* are predominantly categorised as acetoclastic methanogens (Ferry, 2010; Kouzuma et al., 2017), despite the fact that it has long been known that *Methanosarcina* spp. have a broad substrate spectrum (Liu and Whitman, 2008; Sowers et al., 1984; Zinder et al., 1985). The genus *Methanosarcina* favours methanol and methylamine over acetate, although many species are also able to use H_2 (Ferry, 2010, 2020; Lackner et al., 2018; Liu and Whitman, 2008). The results of the present study confirm the findings that *Methanosarcina acetivorans* and *Methanosarcina thermophila* are capable of acetoclastic as well as methylotrophic methanogenesis. Wunderer et al. (2022) observed in their study an immediate decrease in the methanol concentration when cultivating *Methanosarcina thermophila* with acetate and methanol. The acetate was only metabolised after the methanol had been completely used up. This suggests that the archaeon switched from methylotrophic to acetoclastic methanogenesis as soon as the methanol was depleted.

Methanococcus vannielii used a gas mixture of H_2/CO_2 and formate as carbon source. As shown in Fig. 3E, the hydrogen concentration

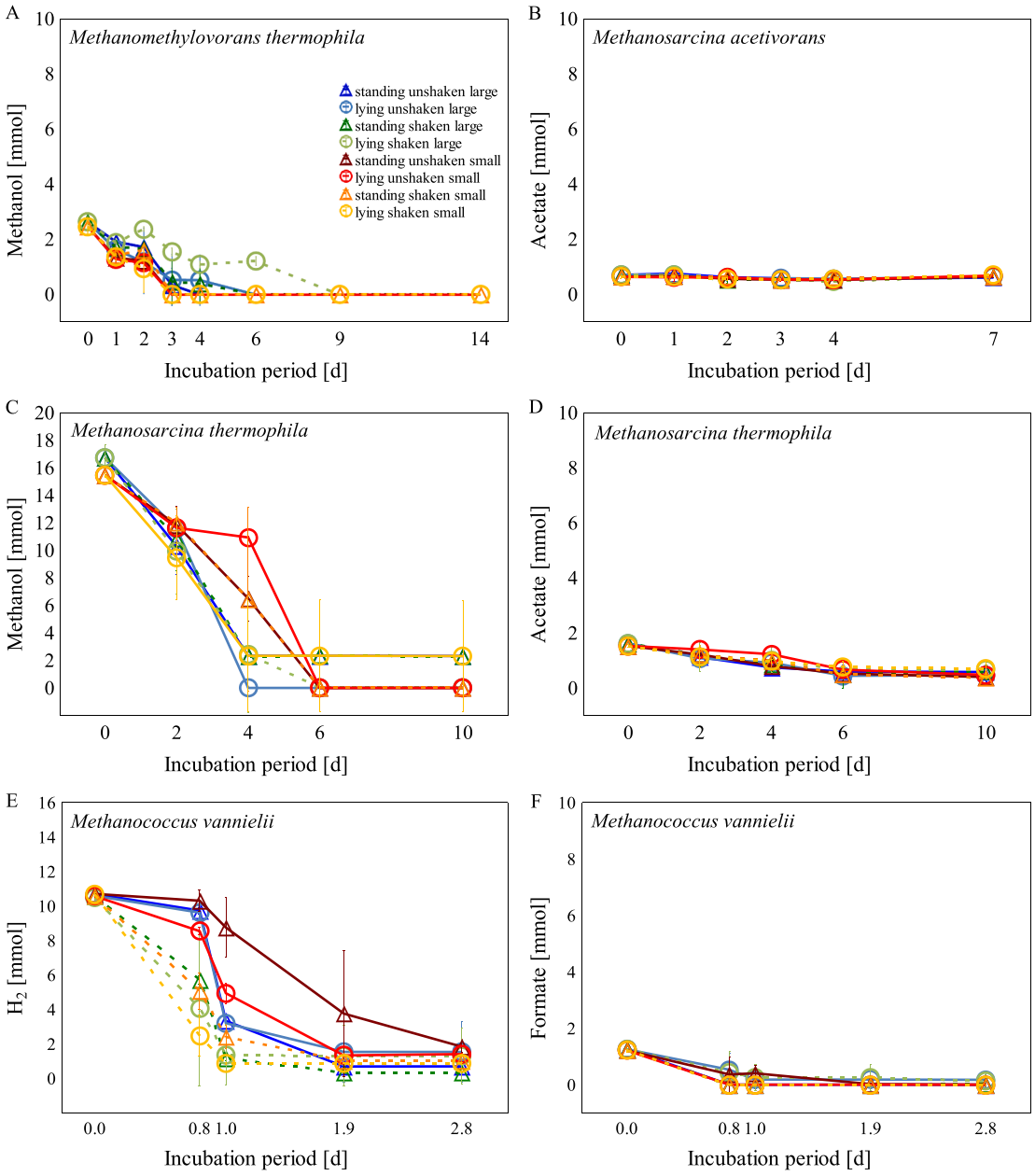

Fig. 3. Carbon source utilisation over time during anaerobic cultivation of (A) *Methanomethylovorans thermophila*, (B) *Methanosarcina acetivorans*, (C) and (D) *Methanosarcina thermophila*, (E) and (F) *Methanococcus vannielii* in different incubation settings.

decreased continuously over time, from an average of 10.61 mmol H_2 on the first day to an average of 0.96 mmol H_2 in the large serum flasks and 1.28 mmol H_2 in the small flasks on the last day. Within the first 18 hours, a notable reduction in the hydrogen concentration was evident in shaken variants, whereby the decrease was greater in the lying/shaken than in standing/shaken variants. In contrast, in the unshaken variants, the hydrogen consumption was notably delayed, particularly during the initial 18 hours. According to DSMZ specifications, each variant should have contained an initial concentration of 29 mM (corresponding to 1.45 mmol) formate. However, a slightly lower concentration (24 mM, corresponding to 1.23 mmol) was measured (Fig. 3F). During the cultivation of *Methanococcus vanniellii* both, formate and hydrogen concentrations decreased in all investigated variants. Within the initial 18 h of the incubation period, formate was completely consumed in the following variants: standing/unshaken/large, standing/shaken/large, lying/unshaken/small, standing/shaken/small, lying/shaken/small. However, on the last day of incubation, a residual amount of 0.17 mmol formate could still be detected in the variants lying/unshaken/large and 0.13 mmol formate in the lying/shaken/large.

3.3. Protein yield

The protein content of the methanogens was assessed on the last day of incubation and used as a biomass indicator (Fig. 4).

The results demonstrated a higher protein concentration in the smaller serum flasks than in the larger serum flasks during the cultivation of *Methanomethylovorans thermophila*. The lowest protein concentration was identified in the standing/shaken/large variant which differs significantly ($p < 0.05$) from the lying/unshaken/small and the standing/shaken/small variant. These data are inversely proportional with those of the produced cumulative methane production (see chapter 3.1, Fig. 2A), as a lower amount of methane was produced in the small

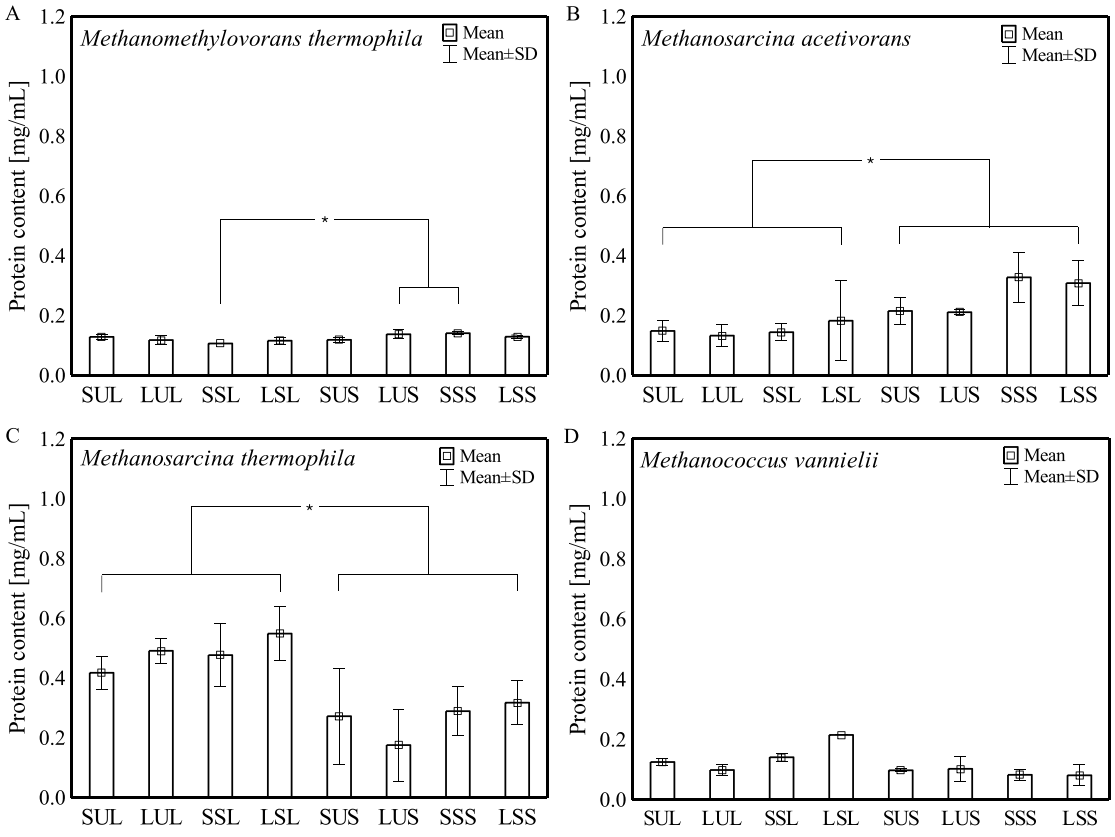

Fig. 4. Protein content determine on the last day of incubation during the anaerobic cultivation of (A) *Methanomethylovorans thermophila*, (B) *Methanosarcina acetivorans*, (C) *Methanosarcina thermophila*, (D) *Methanococcus vannielii* in different incubation settings – SUL = standing unshaken large, LUL = lying unshaken large, SSL = standing shaken large, LSL = lying shaken large, SUS = standing unshaken small, LUS = lying unshaken small, SSS = standing shaken small, LSS = lying shaken small. Significant differences ($p < 0.05$) are indicated by the symbol '*'.

cultivation vessels, yet a higher protein concentration was measured. In the case of *Methanosarcina acetivorans*, the results of protein concentration also do not align with those of methane production. Although methane production was significantly higher ($p < 0.05$) in the larger serum flasks than in the smaller ones, the protein concentration in the small serum flasks was significantly higher ($p < 0.05$) than in the large ones. The highest amount of protein concentration was detected in the standing/shaken/small variant followed by the lying/shaken/small variant. *Methanosarcina acetivorans* was not growing in flocs or forming aggregates and no biofilm formation could be observed under the applied growth conditions. Also, in the case of *Methanosarcina thermophila*, the results of protein concentration differ from those of methane production measurement. Although methane production was significantly higher ($p < 0.05$) in the smaller serum flasks than in the larger ones, the protein concentration in the smaller serum flasks was significantly lower ($p < 0.05$) than in the larger ones. It appears that *Methanosarcina thermophila* in the large serum flasks directed the substrate flow towards biomass production (Fig. 4C), whereas in small flask more methane was produced (Fig. 2C). Similarly, during the cultivation of *Methanococcus vanniellii* slightly higher but not significant differences ($p > 0.05$) in protein concentrations were measured in the larger serum flasks. In addition, the highest amount was measured by far in the lying/shaken/large variant, which had the lowest methane production and carbon source consumption, too.

Summing up, these findings suggest that an elevated methane production for some of the investigated archaea was associated with reduced biomass production. Thus, under optimal methane production conditions methanogens tended to end up in a lower biomass production. However, studies involving additional methanogens are necessary to finally resolve this.

4. Conclusion

Distinct incubation modes had a considerable influence on methane production for *Methanomethylovorans thermophila, Methanosarcina acetivorans, Methanosarcina thermophila* and *Methanococcus vannielii*. A shaken cultivation mode had a positive effect on methane production speed- and quantity-wise in hydrogenotrophic archaea. Conversely, in acetoclastic and methylotrophic archaea shear forces generated by the speed of the orbital shaker partly resulted in a delay of methane production especially in smaller cultivation vessels; however, not in the inhibition of their growth or in lysis of the cells. The mixing mode standing or lying had no significant effect neither on methane production nor on biomass production. A higher metabolic activity, as indicated by accelerated metabolisation of the carbon source and elevated methane yields, resulted in a lower protein concentration at the end of the incubation period of the investigated microorganisms, meaning that in terms of biomass production, it is not advisable to pursue an optimal methane production. The present study can give a first overview on optimizing mixing conditions; however, further analyses are necessary to investigate the physical parameters in shaken serum flasks in detail and for other methanogens in particular and anaerobic microorganisms in general. Furthermore, it would be beneficial to ascertain at what shaking frequency delayed kinetics may occur and whether there is an inhibitory effect on microbial growth.

Declaration of competing interest

None.

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Data availability

Data will be made available on request.

References

Balch, W.E., Fox, G.E., Magrum, L.J., Woese, C.R., Wolfe, RS., 1979. Methanogens: reevaluation of a unique biological group. Microbiol. Rev. 43 (2), 260–296. https://doi.org/10.1128/mr.43.2.260-296.1979.

Bodegom, P.M., Scholten, J.C.M., Stams, AJM., 2004. Direct inhibition of methanogenesis by ferric iron. FEMS. Microbiol. Ecol. 49 (2), 261–268. https://doi.org/10.1016/j.femsec.2004.03.017.

Borrel, G., Adam, P.S., McKay, L.J., Chen, L-X, Sierra-García, I.N., Sieber, C.M.K., et al., 2019. Wide diversity of methane and short-chain alkane metabolisms in uncultured archaea. Nat. Microbiol. 4 (4), 603–613. https://doi.org/10.1038/s41564-019-0363-3.

Borrel, G., O'Toole, P.W., Harris, H.M.B., Peyret, P., Brugère, J-F, Gribaldo, S., 2013. Phylogenomic data support a seventh order of methylotrophic methanogens and provide insights into the evolution of methanogenesis. Genome Biol. Evol. 5 (10), 1769–1780. https://doi.org/10.1093/gbe/evt128.

Bradford, MM., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72 (1-2), 248–254. https://doi.org/10.1016/0003-2697(76)90527-3.

Bryant, MP., 1979. Microbial methane production—Theoretical Aspects2. J. Anim. Sci. 48 (1), 193–201. https://doi.org/10.2527/jas1979.481193x.

Büchs, J., 2001. Introduction to advantages and problems of shaken cultures. Biochem. Eng. J. 7 (2), 91–98. https://doi.org/10.1016/S1369-703X(00)00106-6.

Buswell, A.M., Mueller, HF., 1952. Mechanism of methane fermentation. Ind. Eng. Chem. 44 (3), 550–552. https://doi.org/10.1021/ie50507a033.

Dridi, B., Fardeau, M-L, Ollivier, B., Raoult, D., Drancourt, M., 2012. Methanomassiliicoccus luminyensis gen. nov., sp. nov., a methanogenic archaeon isolated from human faeces. Int. J. Syst. Evol. Microbiol. 62 (Pt 8), 1902–1907. https://doi.org/10.1099/ijs.0.033712-0.

Eliani-Russak, E., Tik, Z., Uzi-Gavrilov, S., Meijler, M.M., Sivan, O., 2023. The reduction of environmentally abundant iron oxides by the methanogen methanosarcina barkeri. Front. Microbiol. 14, 1197299. https://doi.org/10.3389/fmicb.2023.1197299.

Enzmann, F., Mayer, F., Rother, M., Holtmann, D., 2018. Methanogens: biochemical background and biotechnological applications. AMB Express. 8 (1), 1. https://doi.org/10.1186/s13568-017-0531-x.

Evans, P.N., Parks, D.H., Chadwick, G.L., Robbins, S.J., Orphan, V.J., Golding, S.D., et al., 2015. Methane metabolism in the archaeal phylum bathyarchaeota revealed by genome-centric metagenomics. Science 350 (6259), 434–438. https://doi.org/10.1126/science.aac7745.

Ferry, JG., 2010. How to make a living by exhaling methane. Annu. Rev. Microbiol. 64, 453–473. https://doi.org/10.1146/annurev.micro.112408.134051.

Ferry, JG., 2011. Fundamentals of methanogenic pathways that are key to the biomethanation of complex biomass. Curr. Opin. Biotechnol. 22 (3), 351–357. https://doi.org/10.1016/j.copbio.2011.04.011.

Ferry, JG., 2020. Methanosarcina acetivorans: A model for mechanistic understanding of aceticlastic and reverse methanogenesis. Front. Microbiol. 11, 1806. https://doi.org/10.3389/fmicb.2020.01806.

Frimmer, U., Widdel, F., 1989. Oxidation of ethanol by methanogenic bacteria. Arch. Microbiol. 152 (5), 479–483. https://doi.org/10.1007/BF00446933.

Garcia, P.S., Gribaldo, S., Borrel, G., 2022. Diversity and evolution of methane-related pathways in Archaea. Annu. Rev. Microbiol. 76, 727–755. https://doi.org/10.1146/annurev-micro-041020-024935.

Henry, W., 1803. Experiments on the quantity of gases absorbed by water, at different temperatures, and under different pressures. Phil. Trans. R. Soc. 93, 29–274. https://doi.org/10.1098/rstl.1803.0004.

Iino, T., Tamaki, H., Tamazawa, S., Ueno, Y., Ohkuma, M., Suzuki, K-I, et al., 2013. Candidatus Methanogranum caenicola: a novel methanogen from the anaerobic digested sludge, and proposal of Methanomassiliicoccaceae fam. nov. And Methanomassiliicoccales ord. nov., for a methanogenic lineage of the class Thermoplasmata. Microbes. Environ. 28 (2), 244–250. https://doi.org/10.1264/jsme2.me12189.

Jiang, B., Parshina, S.N., van Doesburg, W., Lomans, B.P., Stams, AJM., 2005. Methanomethylovorans thermophila sp. nov., a thermophilic, methylotrophic methanogen from an anaerobic reactor fed with methanol. Int. J. Syst. Evol. Microbiol. 55 (Pt 6), 2465–2470. https://doi.org/10.1099/ijs.0.63818-0.

Kim, B.H., Gadd, G.M., 2008. Bacterial physiology and metabolism. editors. Cambridge University Press, Cambridge.

Kouzuma, A., Tsutsumi, M., Ishii, S., Ueno, Y., Abe, T., Watanabe, K., 2017. Non-autotrophic methanogens dominate in anaerobic digesters. Sci. Rep. 7 (1), 1510. https://doi.org/10.1038/s41598-017-01752-x.

Kurth, J.M., Nobu, M.K., Tamaki, H., de, Jonge N, Berger, S., Jetten, M.S.M., et al., 2021. Methanogenic archaea use a bacteria-like methyltransferase system to demethoxylate aromatic compounds. ISMe J. 15 (12), 3549–3565. https://doi.org/10.1038/s41396-021-01025-6.

Lackner, N., Hintersonnleitner, A., Wagner, A.O., Illmer, P., 2018. Hydrogenotrophic methanogenesis and autotrophic growth of methanosarcina thermophila. Archaea 2018 (5), 1–7. https://doi.org/10.1155/2018/4712608.

Laso-Pérez, R., Hahn, C., van Vliet, D.M., Tegetmeyer, H.E., Schubotz, F., Smit, N.T., et al., 2019. Anaerobic degradation of non-methane alkanes by "candidatus methanoliparia" in hydrocarbon seeps of the Gulf of Mexico. mBio 10 (4). https://doi.org/10.1128/mBio.01814-19.

Liu, Y., Whitman, WB., 2008. Metabolic, phylogenetic, and ecological diversity of the methanogenic archaea. Ann. N. Y. Acad. Sci. 1125, 171–189. https://doi.org/10.1196/annals.1419.019.

Madigan MT, Bender KS, Buckley DH, Sattley WM, Stahl DA. Brock biology of microorganisms. Harlow: Pearson Education Limited; 2022.

Maier, U., Büchs, J., 2001. Characterisation of the gas-liquid mass transfer in shaking bioreactors. Biochem. Eng. J. 7 (2), 99–106. https://doi.org/10.1016/S1369-703X(00)00107-8.

Mayumi, D., Mochimaru, H., Tamaki, H., Yamamoto, K., Yoshioka, H., Suzuki, Y., et al., 2016. Methane production from coal by a single methanogen. Science 354 (6309), 222. https://doi.org/10.1126/science.aaf8821.

Pappenreiter, P.A., Zwirtmayr, S., Mauerhofer, L-M, Rittmann, SK-MR, Paulik, C., 2019. Development of a simultaneous bioreactor system for characterization of gas production kinetics of methanogenic archaea at high pressure. Eng. Life Sci. 19 (7), 537–544. https://doi.org/10.1002/elsc.201900035.

Read, S.M., Northcote, DH., 1981. Minimization of variation in the response to different proteins of the Coomassie blue G dye-binding assay for protein. Anal. Biochem. 116 (1), 53–64. https://doi.org/10.1016/0003-2697(81)90321-3.

Schill, N., van Gulik, W.M., Voisard, D., von, Stockar U, 1996. Continuous cultures limited by a gaseous substrate: development of a simple, unstructured mathematical model and experimental verification with methanobacterium thermoautotrophicum. Biotechnol. Bioeng. 51 (6), 645–658. https://doi.org/10.1002/(SICI)1097-0290(19960920)51, 6<645:AID-BIT4>3.0.CO;2-H.

Seifert, A.H., Rittmann, S., Herwig, C., 2014. Analysis of process related factors to increase volumetric productivity and quality of biomethane with Methanothermobacter marburgensis. Appl. Energy 132, 155–162. https://doi.org/10.1016/j.apenergy.2014.07.002.

Sowers, K.R., Baron, S.F., Ferry, JG., 1984. Methanosarcina acetivorans sp. nov., an acetotrophic methane-producing bacterium isolated from marine sediments. Appl. Environ. Microbiol. 47 (5), 971–978. https://doi.org/10.1128/aem.47.5.971-978.1984.

Sprenger, W.W., Hackstein, J.H.P., Keltjens, JT., 2007. The competitive success of methanomicrococcus blatticola, a dominant methylotrophic methanogen in the cockroach hindgut, is supported by high substrate affinities and favorable thermodynamics. FEMS. Microbiol. Ecol. 60 (2), 266–275. https://doi.org/10.1111/j.1574-6941.2007.00287.x.

Stadtman, T.C., Barker, H.A., 1951. Studies on the methane fermentation. X. A new formate-decomposing bacterium, methanococcus vannielii. J. Bacteriol. 62 (3), 269–280. https://doi.org/10.1128/jb.62.3.269-280.1951.

Thauer, R.K., Kaster, A-K, Seedorf, H., Buckel, W., Hedderich, R., 2008. Methanogenic archaea: ecologically relevant differences in energy conservation. Nat. Rev. Microbiol. 6 (8), 579–591. https://doi.org/10.1038/nrmicro1931.

Vanwonterghem, I., Evans, P.N., Parks, D.H., Jensen, P.D., Woodcroft, B.J., Hugenholtz, P., et al., 2016. Methylotrophic methanogenesis discovered in the archaeal phylum Verstraetearchaeota. Nat. Microbiol. 1, 1–9. https://doi.org/10.1038/nmicrobiol.2016.170.

Wagner, A.O., Malin, C., Lins, P., Illmer, P., 2011. Effects of various fatty acid amendments on a microbial digester community in batch culture. Waste Manage. 31 (3), 431–437. https://doi.org/10.1016/j.wasman.2010.10.020.

Wagner, A.O., Markt, R., Mutschlechner, M., Lackner, N., Prem, E.M., Praeg, N., et al., 2019. Medium preparation for the cultivation of microorganisms under strictly Anaerobic/anoxic conditions. J. Visualiz. Experim. JoVE 150, e60155. https://doi.org/10.3791/60155.

Wagner, A.O., Markt, R., Puempel, T., Illmer, P., Insam, H., Ebner, C., 2017. Sample preparation, preservation, and storage for volatile fatty acid quantification in biogas plants. Eng. Life Sci. 17 (2), 132–139. https://doi.org/10.1002/elsc.201600095.

Widdel, F., 1986. Growth of methanogenic bacteria in pure culture with 2-propanol and other alcohols as hydrogen donors. Appl. Environ. Microbiol. 51 (5), 1056–1062. https://doi.org/10.1128/aem.51.5.1056-1062.1986.

Wunderer, M., Markt, R., Lackner, N., Wagner, AO., 2022. The glutamyl tail length of the cofactor F420 in the methanogenic Archaea Methanosarcina thermophila and Methanoculleus thermophilus. Sci. Total. Environ. 809, 151112. https://doi.org/10.1016/j.scitotenv.2021.151112.

Zhou, Z., Zhang, C-J, Liu, P-F, Fu, L., Laso-Pérez, R., Yang, L., et al., 2022. Non-syntrophic methanogenic hydrocarbon degradation by an archaeal species. Nature 601 (7892), 257–262. https://doi.org/10.1038/s41586-021-04235-2.

Zinder, S.H., Mah, R.A., 1979. Isolation and characterization of a thermophilic strain of Methanosarcina unable to use H(2)-CO(2) for methanogenesis. Appl. Environ. Microbiol. 38 (5), 996–1008. https://doi.org/10.1128/aem.38.5.996-1008.1979.

Zinder, S.H., Sowers, K.R., Ferry, J.G., 1985. NOTES: methanosarcina thermophila sp. nov., a thermophilic, acetotrophic, methane-producing bacterium. Int. J. Syst. Bacteriol. 35 (4), 522–523. https://doi.org/10.1099/00207713-35-4-522.