Journal of CO2 Utilization 93 (2025) 103049

Contents lists available at ScienceDirect

Journal of CO2 Utilization

journal homepage: www.elsevier.com/locate/jcou

A co-feeding strategy of formate and H_2 for methanogens – Enhancing growth parameters and methane production

Björn Sabel-Becker [a,c], Nicolas Patrick Jost [c], Anne-Kristin Kaster [b], Dirk Holtmann [a,*]

[a] Institute of Process Engineering in Life Sciences 2, Electrobiotechnology, Karlsruhe Institute of Technology (KIT), Fritz-Haber-Weg 4, Karlsruhe 76131, Germany
[b] Institute for Biological Interfaces (IBG 5), Karlsruhe Institute of Technology (KIT), Hermann-von-Helmholtz-Platz 1, Karlsruhe, Germany
[c] Institute of Bioprocess Engineering and Pharmaceutical Technology, University of Applied Sciences Mittelhessen, Wiesenstrasse 14, Giessen 35390, Germany

ARTICLE INFO

Keywords:
Methanogenesis
Methanogens
Formate
Formatotrophic
Co-feeding
Mixed culture

ABSTRACT

Carbon dioxide emissions could be reduced by developing alternative production processes based on a renewable C1 building block. Formate could link the electrical and chemical sectors as its production can be realized through the electrochemical reduction of CO_2. Its function could be either a long-term energy storage medium or a starting material in a bioprocess. In this study, formate served as an energy and carbon source for methane production with a formatotrophic mixed culture. It was successfully shown that the theoretical maximum of 0.25 methane per formate can be overcome by co-feeding formate with H_2. The production yield doubled to 0.555 ± 0.021 in a CO_2-free buffer and 0.591 ± 0.032 in a bicarbonate buffer. With excess CO_2 in the bicarbonate buffered culture, it was shown that the H_2 transfer rate was the limiting factor for this process. Otherwise, the bicarbonate buffered culture outperformed other buffered cultures in terms of start-up time, formate consumption, and methane production rate. The additional CO_2 in the gas phase might have enhanced the growth of methanogens in an early stage of cultivation. 16S sequencing revealed the composition of the cultures. With nearly 25 %, the genus *Methanofollis* was one of the most dominant strains and the only detectable methanogen in the mixed culture, making it an interesting candidate for formatotrophic methane production. In summary, the co-feeding strategy might be an approach to utilizing formate as feedstock for the bioproduction of methane if hurdles like the H_2 transfer rates can be overcome.

1. Introduction

The transformation from a fossil-based to a renewable bio-economy is a protractive process with many challenges. Currently, the value-added chemicals depend on fossil carbon or monosaccharides, increasing agricultural land use, leading to a loss in biodiversity and competition between food and energy crops [1]. Hence, alternative production routes that use existing waste streams and integrate these into production processes must be developed to create a more sustainable economy. One possible carbon feedstock and waste stream could be carbon dioxide (CO_2), emitted mainly from fossil fuel combustion for energy production, the building and chemical industry, agriculture land use and forestry, and waste [2,3]. As it can be assumed that fossil-based CO_2 emissions will remain high in the following decades, converting it into building blocks for the chemical industry is one approach to reduce the accumulation of CO_2 in the atmosphere [4,5].

Numerous techniques were developed and are investigated to re-functionalize CO_2 for value-added products – electroconversion is one of them [6]. The three benefits of electrochemical CO_2 reduction (eCO_2R) compared to other techniques are mild reaction conditions, customization of products through reaction parameters, and utilizing intermittent electricity [7]. eCO_2R is a multi-step process, and various products can be formed via different pathways. With a transfer of two electrons, carbon monoxide and formic acid/formate can be produced. The latter is an organic acid that can serve as hydrogen (H_2) storage, fuel in a formate fuel cell, and feedstock to synthesize fine chemicals [8]. The demand for formic acid/formate is expected to rise in the future, but more specific applications must be developed to increase the interest in production [9]. Yishai and coworkers have developed a concept of a formate-based bio-economy [10]. Instead of focusing on an entirely physicochemical process that is often not product-specific, the group envisions a combination of physicochemical and biological methods for formate production and subsequent products like food, fuels, and fine chemicals from natural and synthetic somatotrophs. That way, formate

* Corresponding author.
E-mail address: dirk.holtmann@kit.edu (D. Holtmann).

https://doi.org/10.1016/j.jcou.2025.103049
Received 17 October 2024; Received in revised form 14 January 2025; Accepted 20 February 2025
Available online 3 March 2025

would mediate between the physicochemical and biological sectors. The concept was taken up in recent publications, demonstrating an efficient formate production from CO_2 with succeeding biopolymer production using the natural formatotroph *Cupriavidus necator* or to produce chiral building blocks with *Methylorubrum extorquens* [11,12].

The assimilation of formate is realized through three different pathways in natural formatotrophs: the serine pathway, the pentose phosphate cycle, and the reductive acetyl-CoA pathway. The energetically most efficient is the reductive acetyl-CoA pathway, which operates in acetogens and methanogens. The methanogens belong to the domain Archaea and possess a unique energy conservation called methanogenesis. Their metabolism can distinguish them into three groups: hydrogenotrophic, acetotrophic, and methylotrophic. Only the hydrogenotrophic methanogens can utilize formate for methane production, which makes them attractive candidates for formate-based bioproduction [13]. However, the number of genetic tools is limited, and the growth conditions are more difficult to maintain than for industrial strains, making it challenging to proceed to large-scale, economically feasible production [10]. Nonetheless, methanogens are already used in mixed consortia in biogas plants. Through the hydrolysis of biopolymers and fermentation of monomers, acetate, and H_2 are produced, which are substrates for methanogens. Only when remaining at relatively low levels is the degradation of other metabolites thermodynamically favorable [14]. Hence, merely the syntrophic interaction of the different microorganisms enables biogas production from complex biomass, in which methanogens play an essential role [15].

In general, using formate as a substrate for methane production does not necessarily require the application of a mixed culture. As described before, some methanogens can consume formate as an energy source to reduce CO_2. A pure culture would be sufficient for the conversion of formate to methane. However, mixed culture fermentation can offer several advantages over pure culture. It can be conducted in non-sterile conditions, complex functions can be executed, and better substrate utilization can be realized [16–18]. Further, the robustness to environmental stress factors and the resistance to contamination increases [19,20]. The latter points are advantageous when working with an open system [21,22]. However, even in a closed system, contamination might occur, for example, through culture sampling.

Utilizing formate as feedstock has many benefits regarding storage conditions and solubility compared to gaseous substrates. Nevertheless, employing solely formate as a carbon and energy source for non-fossil methane production has drawbacks. Theoretically, 4 mol formate are oxidized to H_2O and CO_2 for each mol methane, of which one carbon is fixed in methane, but 3 mol CO_2 are by-produced as shown in Eq. 1 (Eq. 1) [23].

$$4HCOOH \rightarrow CH_4 + 3CO_2 + 2H_2O\ \Delta G0 = -130\ kJ/mol\ CH_4 \quad (1)$$

Additional CO_2 released from formate oxidation would result in a biogas dilution, requiring further steps in the downstream process [24].

Therefore, this study investigated the strategy of co-feeding H_2 to a formatotrophic mixed culture. H_2 would provide additional electrons for methanogenesis, while formate would be the carbon source. So far, previous studies have focused on the kinetics of methane production and gene expression concerning the formate utilization of methanogens [23,25]. However, to our knowledge, no study has yet been carried out in which the byproduct of CO_2 from formate oxidation was used to increase the relative methane yield ($Y_{CH4/HCOO-}$). For a precise determination of whether solely formate can function as a carbon source in a co-feed process, the bicarbonate buffer was exchanged with the best-performing Good's buffer. For good comparison, the formate consumption rate (FCR), the methane production rate (MPR), and the overall yield of formate to methane ($Y_{CH4/HCOO\cdot}$) were calculated. In addition, the mixed culture composition was analyzed via Illumina sequencing for a detailed itemization of the different genera and their abundance.

2. Material and method

2.1. Medium, mixed culture and cultivation conditions

The 141 Methanogenium Medium from the DSMZ (141 medium) was used to enrich and sustain a formatotrophic mixed culture [26]. Only analytical-grade chemicals and ultrapure water were used to prepare the medium. Sodium resazurin was omitted from the medium as it interfered with the optical density measurement at 600 nm (OD_{600}), and 6.801 g sodium formate was added for a final concentration of 100 mM. After dissolving all chemicals, the solution was sparged with a nitrogen (N_2)/CO_2 mixture of 80/20 % (5.0) for 45 min with the subsequent addition of sodium hydrogen carbonate. In the case of using other buffer agents, no sodium hydrogen carbonate was added, the medium was adjusted to pH 7.0, and then sparged with 100 % N_2 (5.0) for 45 min. The tested buffers are listed in Table 1 and were chosen based on metal ion interaction and pH range. The buffer comparison study was conducted with a concentration of 50 mM each. Independent of the medium composition, 50 ml was distributed in 200 ml serum bottles in an anaerobic chamber (COY Laboratory, USA) and sealed airtight with a butyl stopper secured with aluminium seals. After sterilization and before cultivation, the corresponding gas mix was applied by exchanging three times the gas phase of the serum bottle with a final total pressure of 2.2 bar. For the bicarbonate buffered medium, a gas mix of either 80/20 % N_2/CO_2 or 80/20 % H_2/CO_2 was chosen. In contrast, medium with Good's buffers were pressurized with either 100 % N_2 or 100 % H_2. Afterward, 0.5 ml of the sterile and anaerobic L-cysteine, sodium sulfide, trace element solution (TES), and vitamin solution were added. In the fed-batch experiments, a feed solution of 5.4 M sodium formate dissolved in ultrapure water was used to supply the substrate.

The original native mixed culture was isolated from a mesophilic secondary biogas fermenter from Langendorf, Germany, using cattle manure and corn silage as feedstock. The mixed culture was subcultured on 141 media with 100 mM formate ($HCOO^-$) and N_2/CO_2 (80/20) every 2–3 weeks for over one year to ensure adaptation to the medium and stable methane production. Cultures were shaken at 37°C and 180 rpm (Infors HT, Ecotron, Switzerland). Precultures were used to adapt the enrichment culture to the different experimental conditions. After stable growth, 1 ml of 3-day-old precultures was used as inoculum. Due to sampling, the prevailing gas pressure of the serum bottle decreases over time. As soon as the total pressure was below 1.1 bar, the bottles were repressurized to 2.0 bar.

2.2. Sampling of cultures and quantification of analytes

Before and after taking gas and liquid samples, the pressure of the serum bottle was measured with an MSD 4 BAE pressor sensor and a

Table 1
The listed Good's buffers were used with a concentration of 50 mM to substitute the bicarbonate buffer. The reagents were chosen based on their metal interactions (divided into strong and weak) and their pH range [27].

Buffer name	Abbreviation	Metal interaction		pH range
		Strong	Weak	
N-(2-acetamido)−2-aminoethane sulfonic acid	ACES	Cu, Mg	Ca, Mn, Co, Ni, Zn	6.1–7.5
N,N-Bis-(2-hydroxyethyl)−2-aminoethane sulfonic acid	BES	Cu	Co	6.4–7.8
4-(2-hydroxyethyl)−1-piperazineethane sulfonic acid	HEPES	Negligible metal ion binding		6.8–8.2
1,4-Piperazinediethane sulfonic acid	PIPES	N/A	Co, Ni	6.1–7.5

digital manometer GMH 3100 Series (Greisinger, Germany).

For growth monitoring, pH determination, and formate quantification, a 1 ml sample was withdrawn from the culture medium. The absorbance at 600 nm was used as a growth indicator and measured with a cell density meter model 40 (Fisher Scientific, USA). The pH value was also determined with an InLab Micro pH electrode and the SevenMulti pH meter (Mettler Toledo, USA). Then, the sample was prepared for formate quantification by centrifugation at 16,000xg for 5 min and sterile filtration (0.22 µl, Ø 13 mm) of the supernatant. The formate quantification was realized with an Azura high-performance liquid chromatograph (Knauer, Germany) equipped with a Guard Cartridge System KJO 4282 (Phenomenex, USA) in front of a Rezex ROA-Organic Acid Aminex HPX-87H column (Phenomenex, USA). The separation was performed at 50 °C with a 5 mM sulfuric acid solution as mobile phase pumped at a volume flow of 0.5 ml/min with the quaternary 6.1 L low-pressure gradient pump. A sample volume of 30 µl was injected with an integrated 6.1 L autosampler. Formate was detected with an Azura 2.1 L High Flow RID after 17.8 min.

With a gastight sample lock syringe (Hamilton, Romania), 10 ml of gas volume was transferred from the serum bottle to a pre-evacuated Tedlar bag (Restek, USA) connected to a gas chromatograph 490 Micro GC System (Agilent Technologies, USA). A CP-Molsieve 5 Å column (10 m) was employed to separate hydrogen, oxygen, and nitrogen. Methane and CO_2 were separated via the CP-COX column (1 m). Carrier gases argon and helium were used, respectively, to realize the detection of the different gas components with a thermal connectivity detector. The temperature of the column and detector were constant at 60 °C and 100 °C, respectively. The percentages of the calibrated gas components, calculated by the OpenLab CDS software, were then further processed to compute the amount of substance. Neubert et al. [28] describe the methodology applied in this study. Firstly, the gas volume must be calculated using reference conditions. In this step, considering water vapor in the gas sample is essential as it reduces the actual volume of the gas phase. Secondly, under standard and dry conditions, the molar volume of the gaseous analytes is applied to calculate the total amount of substrate. Lastly, the amount of substrate is divided by the gas volume of the serum bottle to receive the concentration of each gas component. All production and consumption rates are the maximum values obtained in this study and were calculated at linear production and linear consumption in the same time interval within each experiment.

2.3. 16S sequencing

The dynamics of the co-fed mixed cultures were analyzed using a 16S targeted sequencing method. 100 µl of culture broth was taken and immediately suspended in 500 µl DNA/RNA Shield reagent for DNA preservation. Samples were stored at 4°C before preparing them for sequencing. The sequencing and data analysis were carried out by ZymoResearch (Freiburg, Germany) and are described in detail in the supplementary information.

3. Results

3.1. Enrichment culture growing merely on formate

The basis of this work was the enriched formatotrophic mixed culture solely fed formate as substrate in a bicarbonate buffered medium with an initial N_2/CO_2 gas atmosphere over one year. Fig. 1 visualizes the growth and pH (A), as well as formate consumption, CH_4 production, and CO_2 uptake (B) of this enriched culture in a fed-batch process over 108 h in triplicates.

The growth indicator OD_{600} increased the fastest in the first 12 h from 0.08 to 0.73, followed by a continuous but inclining rise to 1.04 (Fig. 1A). The pH value stagnated in the first 24 h around 7.30 but increased, with a few fluctuations in between, up to 8.95 at 108 h. A similar slope is visible when looking at the summed-up formate and methane concentration. The formate consumption accelerated in the first 24 h but reached a linear stage at 36 h until the end of the measurement. Methane production also had an acceleration phase between 0 and 24 h followed by a linear period. In comparison, CO_2 was formed in the first 12 h but was slowly taken up afterward, with a dip between 60 h and 65 h. The higher CO_2 uptake preceded a gas exchange at 60 h.

3.2. Investigating Good's buffers as an alternative to bicarbonate buffer

Different buffers were tested to investigate a co-feeding strategy of formate and hydrogen for methane production while omitting the additional CO_2 source from the bicarbonate buffer. This approach allowed the assumption that mainly the CO_2 produced during formate oxidation was utilized for methanogenesis with added H_2. The mixed culture was adapted to each buffer substance for two weeks before conducting the main experiment.

The OD_{600} and pH were compared between each buffer substance (Fig. 2). The abbreviations of the buffer substances are used to name the different cultures and are color-coded to distinguish one another. Each cultivation condition was carried out in duplicates. Equal to Fig. 1A, the OD_{600} increases the most in the first 12 h for all conditions. However, HEPES and BICARB had the highest OD_{600} with 0.74, closely followed by

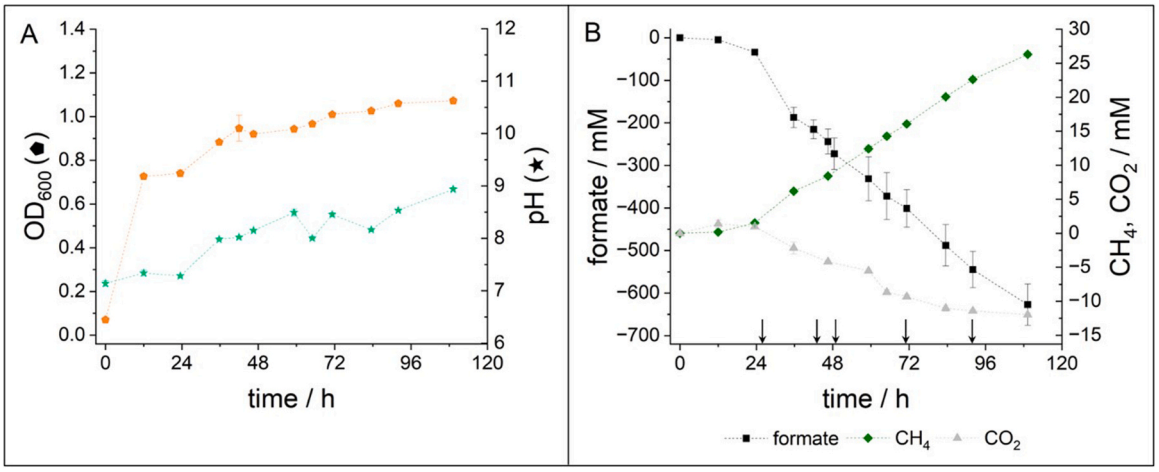

Fig. 1. Growth characteristics (A), substrate consumption, and product formation (B) of an enriched formatotrophic mixed culture. The enriched culture was grown on formate, with a 1 bar initial overpressure of N_2/CO_2, and fed Na-formate (black arrows) when the substrate was nearly depleted. Measurements were done in biological triplicates.

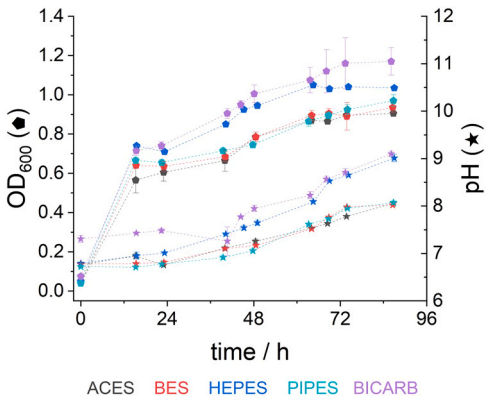

Fig. 2. OD600 and pH measurement of the buffer screening experiment. The Good's buffers were set to an initial pH of 7.0. The experiment was conducted in biological duplicates.

BES and PIPES at 0.65. ACES showed the lowest value of 0.56. Throughout the next 48 h, OD values of ACES, BES, and PIPES converged to 8.5, then separated again, leading to an end value of 0.91, 0.94, and 0.97, respectively. The OD_{600} of HEPES and BICARB increased with a similar slope until 65 h. Afterward, the HEPES OD stagnated at 1.03–1.04. The BICARB OD values continued to increase to 72 h, reaching its maximum at 1.17. Except for BICARB, all cultures started with a pH value of 7.0 and increased over time. Of the non-CO_2-containing buffers, HEPES showed the highest value of 9.05, whereas all the others reached values slightly above 8.0. The carbonate-buffered culture's pHs began at 7.30, fluctuated from 7.48 to 7.26, but then increased strongly for 8 h to 7.95. Afterward, it increased steadily, like HEPES, to a final value of 9.10. In addition to the OD_{600} and pH, the concentrations of H_2 (A), methane (B), CO_2 (C), and formate (D) were determined and are displayed over time in Fig. 3. The included arrows mark the feeding time and are colored corresponding to the culture fed. At 65 h (brown arrow), all Good's buffered cultures were fed. In the first half of the experiment, the Good's buffered cultures increased in H_2 concentration. However, the amount of H_2 detected differed, especially when comparing PIPES to the other buffers. With a maximal concentration of 5.47 mM at 48 h, it was 36 % higher than for BES, 42 % and 47 % compared to HEPES and ACES, respectively. Afterward, the H_2 concentration decreased, although ACES and BES showed a second maximum at 72 h, and for HEPES and PIPES, the slope declined between 65 h and 72 h. The BICARBs H_2 concentration had a zigzag character with a plateau from 48 h to 65 h. The CO_2 concentration (Fig. 3C) was similar to the H_2 concentration for all cultures except for BICARB, which contained an N_2/CO_2 gas atmosphere. There, the CO_2 concentration decreased after 24 h continuously up to 68 h, reaching a plateau at −3.75 mM.

The BICARB culture's formate consumption (Fig. 3D) accelerated to its maximum consumption rate in the first 40 h and continued linearly up to (−501.18 ± 4.27) mM after 86 h. The Good's buffered cultures

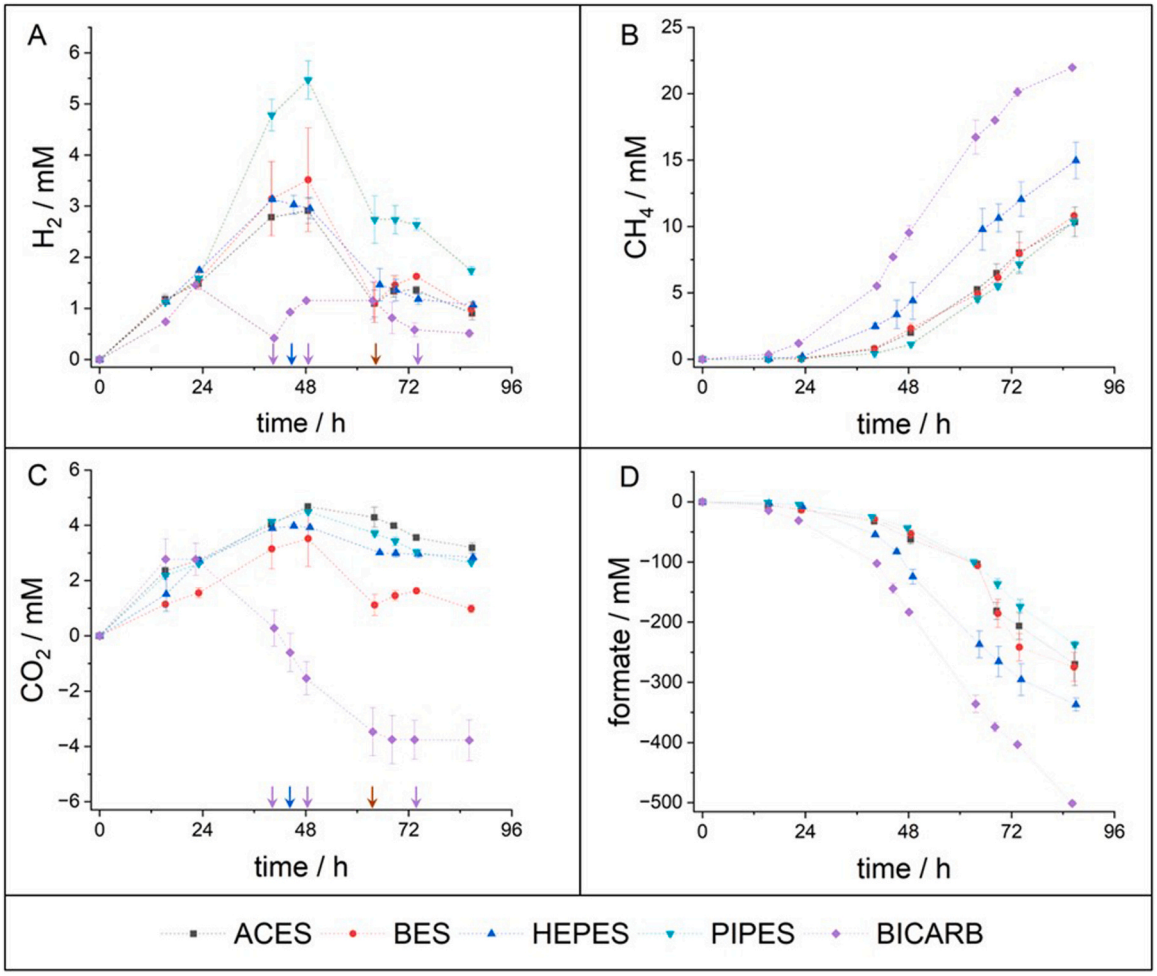

Fig. 3. Concentration of H_2 (A), CH_4 (B), CO_2 (C), and formate (D) of the buffer screening experiment. The initial headspace of the serum bottles was flushed with N_2. The cultures were fed with Na-formate when the substrate was nearly depleted. The feeding time is marked with arrows, colored according to the buffer substance. The brown arrow (65 h) indicates the feeding of ACES, BES, and PIPES.

showed a slower consumption, with HEPES decreasing the fastest after 24 h. At the end of the experiment, the HEPES culture consumed (−337.08 ± 11.00) mM formate, 43 % less than the BICARB culture. ACES, BES, and PIPES cultures took up around 100 mM of formate until 64 h. Afterward, ACES and BES slightly increased consumption, while PIPES remained the same. The total consumed formate for ACES and BES summed up to (−269.84 ± 35.75) mM and (−274.52 ± 23.90) mM, respectively, which is 46 % and 45 % less than the BICARB culture. PIPES consumed the least formate with an end value of (−237,05 ± 6.19) mM, less than half of the BICARB formate concentration. The methane concentrations (Fig. 3B) can be described similarly but with reversed signs. A slight methane increase could be detected in the first 16 h for the BICARB cultures, whereas the Good's buffered cultures needed 24 h (HEPES) to 40 h (ACES, BES, PIPES) to produce a measurable amount of methane. The BICARBs methane concentration continued to increase strongly up to 73 h, with a final concentration of 21.97 mM after 86 h. When comparing the buffers with each other, the HEPES culture showed an earlier methane production than the other buffer substances. For a better comparison of the mixed culture growth with the different buffers, the maximal formate consumption rates (FCR), maximal methane production rates (MPR), as well as the resulting methane per formate yield ($Y_{P/S}$) were calculated (Table 2).

The BICARB buffer reached the highest values in every parameter except yield. However, when focusing on the other buffers, HEPES cultures exceeded the other substances in FCR and MPR, total consumed formate, and total produced methane. Although the yield was slightly lower than for PIPES, the other parameters were favorable for continuing with HEPES as a substitution for the BICARB buffer.

3.3. Adjusting the buffer concentration

The buffer capacity is crucial for a relatively stable pH value during cultivation. It is even more relevant to buffer the medium when using formate as a substrate because of hydroxide ion formation. Therefore, 50 mM (H50), 100 mM (H100), and 200 mM (H200) of HEPES were employed to analyze the influence of the buffer concentration on the growth characteristics of the mixed culture. Fig. 4 displays the OD_{600} as the growth indicator and the resulting pH values of the cultures. The different concentrations were tested in triplicates.

The OD_{600} increased similarly during the experiment, as seen before in the buffer screening (Fig. 2). There were slight differences in OD_{600} values between the cultures after 14 h, but the slope of the OD_{600} curves continued similarly up to 48 h. Then H200 increased linearly, while H50 and H100 slightly decreased the incline. Overall, the optical densities converged after 60 h, ending with a final value of 1.05 (H100), 1.09 (H50), and 1.11 (H200) at 86 h. The pH of all cultures started at 7.00 – 7.10, which remained steady at 14 h but increased afterward. The incline of H50 was the highest and also led to the highest pH value of 8.70. H100 and H200 had the same value at 38 h but separated shortly after 6 h when H100s pH rose slightly higher than that of H200. It continued rising linearly, up to 8.40 after 86 h. H200s pH remained the lowest, with a final value of 8.03.

Furthermore, the consumption of formate and the production of methane, as well as H_2 and CO_2 evolution, were measured to ensure that

Table 2
Formate consumption rate (FCR), methane production rate (MPR), and methane per formate yield ($Y_{P/S}$) of the buffer screening experiment. The rates were calculated during the highest consumption and production phase.

Parameter	ACES	BES	HEPES	PIPES	BICARB
Max. FCR / $mM*d^{-1}$	−71.18 ± 0.68	−77.13 ± 2.58	−180.53 ± 23.68	−76.03 ± 2.05	−243.70 ± 13.62
Max. MPR / $mM*d^{-1}$	4.93 ± 0.67	5.16 ± 0.00	6.66 ± 0.18	5.69 ± 0.13	10.71 ± 0.33
$Y_{CH4/HCOO-}$ /$mol*mol^{-1}$	0.204 ± 0.035	0.210 ± 0.018	0.226 ± 0.031	0.234 ± 0.009	0.220 ± 0.003

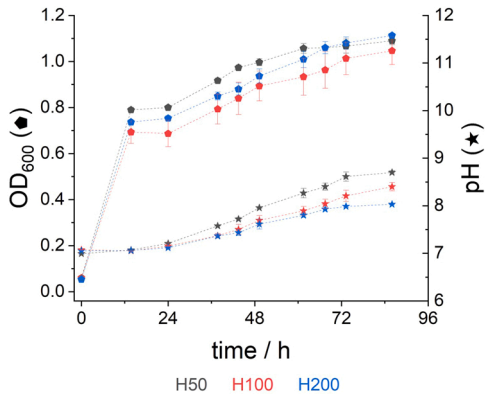

Fig. 4. OD600 and pH measurements of a mixed culture with different HEPES concentrations. 50 mM (H50), 100 mM (H100), and 200 mM (H200) HEPES were used to buffer the medium. The initial pH value was set to 7.0.

the HEPES concentration had no negative influence on the mixed culture. For comparison, the course of each analyte over time for each condition is displayed in Fig. 5 A-C. The H50 condition (Fig. 5A) showed

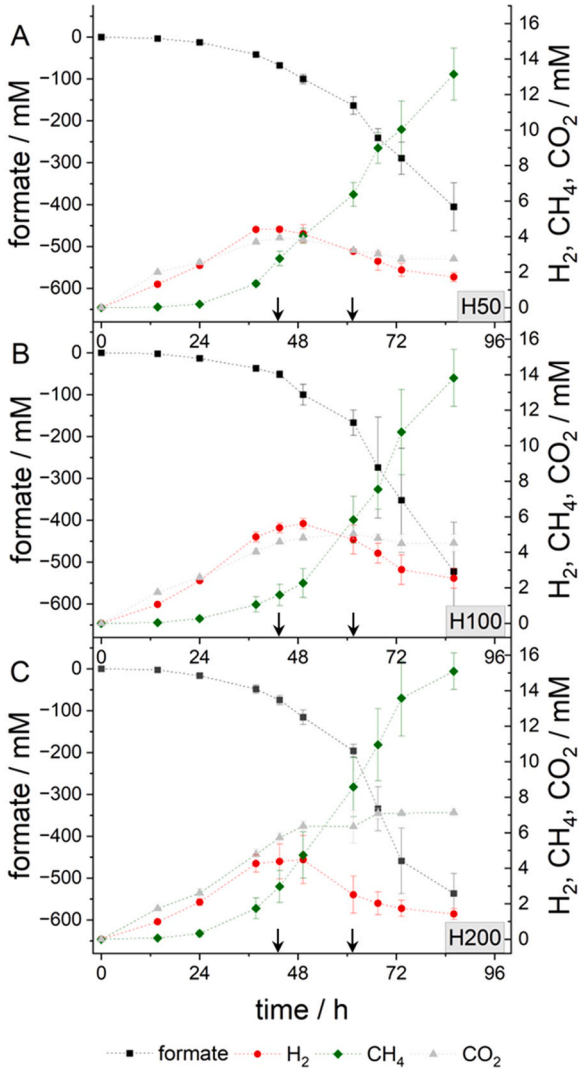

Fig. 5. Formate consumption and gas composition of mixed cultures with 50 mM (A), 100 mM (B), and 200 mM (C) HEPES concentrations. All cultures were fed with Na-formate, marked with the black arrow. The cultivation was performed in biological triplicates.

an accelerating formate consumption until 62 h with a subsequent linear uptake, reaching (−405.29 ± 57.41) mM at the end of the experiment. The methane concentration increased slowly in the first 24 h to (0.20 ± 0.01) mM but boosted afterward to a linear section with a final concentration of (13.14 ± 1.46) mM. H_2 and CO_2 concentrations rose from the start until 44 h to (4.40 ± 0.16) mM and (3.93 ± 0.13) mM, respectively. Both analytes decreased afterward. While H_2 was steadily reduced to a concentration of (1.72 ± 0.25) mM, CO_2 reached a plateau after 73 h with a value of (2.74 ± 0.10) mM. The formate consumption of H100 was nearly identical in the first 62 h. However, the formate concentration decreased strongly afterward, leading to a total of (−522.36 ± 117.45) mM consumed formate or 29 % increased consumption. The methane concentration accelerated over 49 h, longer than for H50. Yet after the acceleration period, the incline was more substantial than for H50, resulting in a minimally higher concentration of (13.82 ± 1.60) mM. The course of H_2 was similar to that of H50, but its maximum was reached 5 h later with (5.61 ± 0.28) mM. The final concentration was (2.54 ± 0.56) mM, 48 % higher than for H50. The beginning of the CO_2 course was comparable to H50. However, the maximal concentration was reached not before 62 h with (5.04 ± 0.30) mM and only sank by 0.5 mM to (4.53 ± 0.48) mM in the last 24 h.

The highest HEPES concentration exhibited the highest consumption of formate visible after 49 h with (−115.54 ± 17.67) mM. It continued to decrease even stronger with a deceleration after 73 h. It reached a total consumption of (−536.53 ± 48.04) mM. The methane concentration increased similarly to H50 but surpassed it after 49 h. The methane slope maintained high until 73 h. Afterward, the incline decreased as seen in the formate consumption. The maximal methane concentration was (15.09 ± 1.02) mM. H_2 evolution was detected, as seen in H50. The biggest difference is the non-descending CO_2 curve, which showed two plateaus from 48−62 h and 68–86 h with (6.38 ± 0.27) mM and (7.09 ± 0.30) mM, respectively.

Table 3 lists the FCR, MPR, as well as the total consumed formate and produced methane with the corresponding yield. The FCR and MPR increased with higher HEPES concentration, whereby H200 exhibited the highest values for both parameters. While H50 and H100 were similar between 37−62h, H200 already displayed 18 % higher formate consumption and 26 % higher methane production. Due to the high error bars after 62 h for formate and methane concentrations, the values were only calculated between 37 h and 62 h.

3.4. Co-feeding of formate with 100 % H_2 in the gas phase

The co-feeding of H_2 and formate was performed with the 200 mM HEPES buffered 141 medium, 100 mM initial Na-formate, and 100 % H_2 (Co-fed). As references, a set of cultures was cultivated solely with formate on 200 mM HEPES buffered medium (Ref. 1), and another set was incubated on carbonate buffered medium with a gas mixture of H_2/CO_2 (80/20) (Ref. 2). All culture conditions were examined in triplicates. Samples were taken from all three conditions for 16S sequencing to analyze the culture composition.

Table 3
Growth parameters of mixed cultures with 50 mM (H50), 100 mM (H100), and 200 mM (H200) HEPES concentration. Parameters were calculated between 37 h and 62 h of triplicates.

Parameter	H50	H100	H200
FCR / mM*d^{-1}	−123.07 ± 20.12	−131.14 ± 25.57	−149.29 ± 6.37
MPR / mM*d^{-1}	5.16 ± 0.92	5.38 ± 0.48	6.91 ± 1.17
Total consumed formate / mmol	−7.91 ± 1.00	−8.12 ± 1.51	−9.56 ± 0.81
Total produced methane / mmol	1.30 ± 0.23	1.36 ± 0.12	1.72 ± 0.29
$Y_{CH4/HCOO-}$ /mol*mol^{-1}	0.201 ± 0.047	0.181 ± 0.075	0.226 ± 0.063

Fig. 6 plots the OD_{600} and pH values of the different cultivation conditions. After the inoculation, the Co-fed and Ref. 1 OD_{600} were similar at first, 0.64 ± 0.02 and 0.68 ± 0.02, respectively. Afterward, Co-fed decreased by 0.05, while Ref. 1 remained stable. Throughout the rest of the experiment, both cultures increased almost parallel and constant incline to 0.94 ± 0.03 for Co-fed and 1.05 ± 0.02 for Ref. 1 cultures. However, Ref. 2 exhibited a stronger increase right at the beginning of the cultivation with an OD of 0.87 ± 0.01. Its OD_{600} continued to increase to a maximum of 1.26 ± 0.02. Co-fed and Ref. 1 start at the same pH value, but after 24 h, Ref. 1's pH rose higher than that of Co-fed. It continued to increase slightly stronger than Co-fed and reached a final value of 8.24 ± 0.03. Co-fed had a final pH of 7.95 ± 0.06. Ref. 2 had a different looking pH course, which started at 7.4 and increased from 17 h until 36 h. The value fluctuated in the following measurements after feeding the formate and exchanging the gas phase. The end value was 8.15 ± 0.02.

The formate consumption and gas composition of the different culture conditions and genera are illustrated in Fig. 7 A-C. The co-fed culture with 100 % H_2 (Fig. 7A) consumed formate only after 34 h linearly to (−155.84 ± 10.93) mM at 97 h. The methane concentration increased slowly at the beginning, followed by a linear increase after 49 h with an end concentration of (17.24 ± 0.10) mM. The H_2 concentration fluctuated in the first measurements but decreased after 34 h. It slightly increased at 60 h to decrease again until the end of the experiment. For the CO_2 concentration, a continuous rise was observed with a maximum of (2.23 ± 0.04) mM at 85 h. Afterward, the CO_2 decreased to (2.06 ± 0.08) mM. The solely formate-fed reference (Ref. 1) showed quicker formate consumption throughout the experimental time with (−427.74 ± 31.75) mM after 90 h. The methane concentration accelerated in the first 49 h and continued linearly subsequent, except between 62 h and 67 h when the slope slightly declined. A final concentration of (20.30 ± 1.16) mM was reached after 90 h. The H_2 concentration increased steadily to the maximum of (4.09 ± 0.09) mM after 39 h, with a final value of (1.49 ± 0.28) mM at 90 h. In comparison, the CO_2 content increased throughout the experimental time until (9.38 ± 0.13) mM. The second reference, grown on H_2/CO_2 mix and formate in a carbonate-buffered medium (Ref. 2), consumed formate from 14 h onward at a relatively constant rate with (−328.34 ± 10.67) mM as the end value. The methane concentration increased slowly at first, but after 24 h, it stabilized and rose constantly to a final concentration of (35.05 ± 1.56) mM. Both H_2 and CO_2 had a peak at 17 h but declined afterward. The uptake of CO_2 was relatively stable, while H_2 showed a plateau (36–41 h) and a local maximum (66 h) throughout the

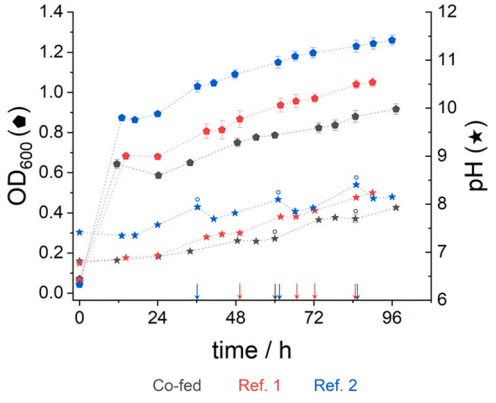

Fig. 6. OD and pH values of the different cultivation conditions in the co-feeding experiment. The Co-fed culture (formate + 100 % H_2) and Ref. 1 (formate + 100 % N_2) were grown in medium buffered with 200 mM HEPES adjusted to pH 7.0. Ref. 2 (formate + H_2/CO_2) was cultured on bicarbonate buffered medium set to pH 7.4. The arrows mark the time of formate feeding, while the circles (°) indicate a gas phase exchange. All conditions were tested in biological triplicates.

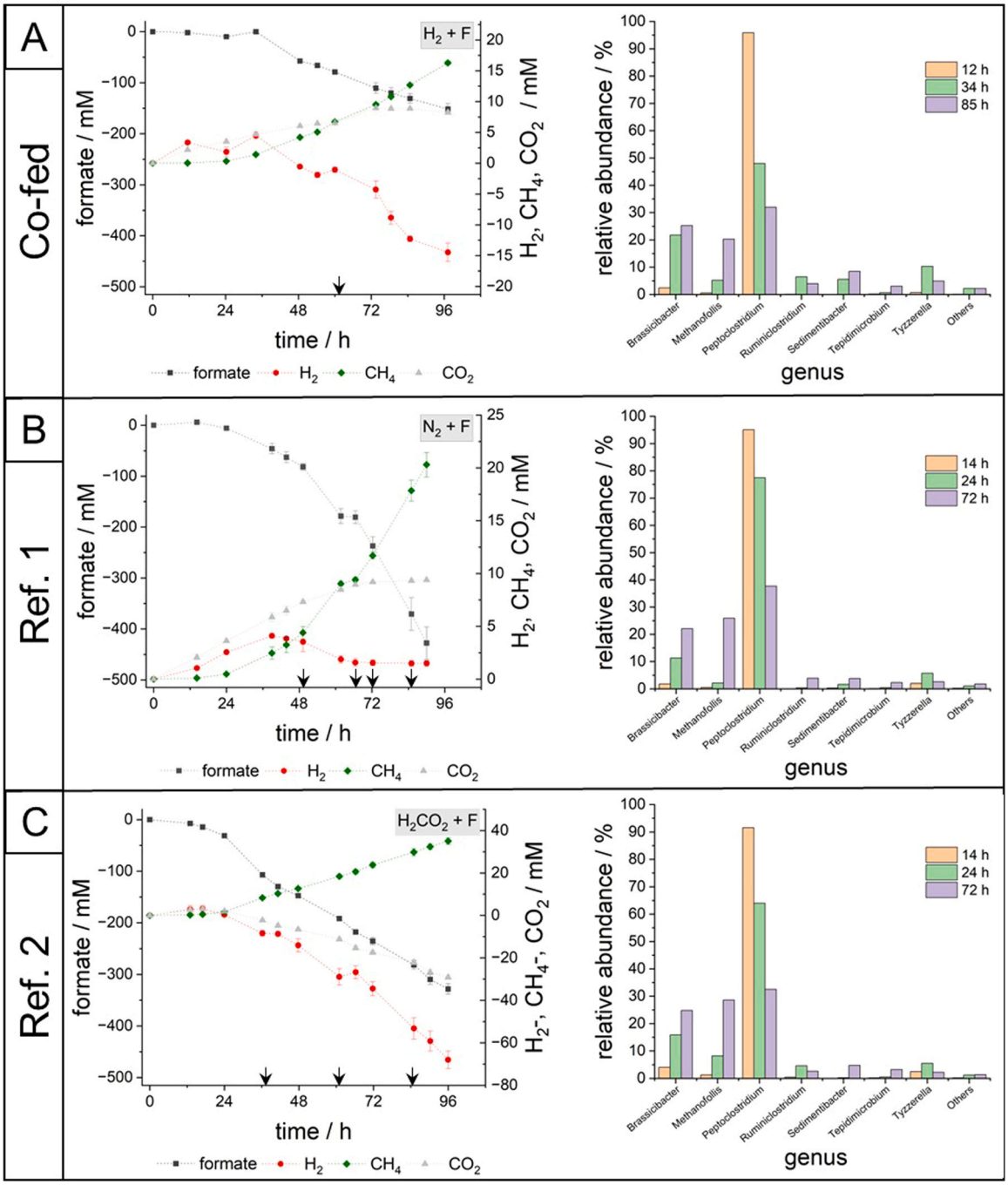

Fig. 7. Formate, H_2, CH_4, CO_2 concentrations and culture composition of different feeding conditions. (A) is the co-feeding of formate and 100 % H_2 in HEPES buffered medium, (B) is the reference 1 of purely formate-fed cultures with 100 % N_2 in HEPES buffered medium, and (C) is the reference 2, co-feeding formate and H_2/CO_2 (80/20) in a bicarbonate buffered medium. The black arrows mark the feeding time of sodium formate. Error bars represent the standard deviation of the biological triplicates.

experimental time.

The cultures' composition was analyzed using 16S sequencing, which revealed that they comprised one archaeon (*Methanofollis*) and various bacteria. The detected genera were the same regardless of the cultivation conditions. The relative abundance of *Bracissibacter*, *Methanofollis*, *Sedimentibacter*, and *Tepidimicrobium* increased for all conditions, whereas *Paraclostridium/ Peptoclostridium* decreased under each condition. The other shown genera first increased and then reduced again. When comparing the conditions, the methanogen abundance was the highest in Ref. 2 at 28.5 %. That was followed by 26.0 % and 20.5 % for Ref. 1 and Co-fed, respectively. The relative proportion of *Bracissibacter* was similar between the two co-feeding conditions at around 25 % and slightly lower for Ref. 1 at 22 %. The genera *Paraclostridium* or *Peptoclostridium*, which could not be differentiated, had the highest abundance at the beginning of cultivation but decreased afterward. The remaining genera showed the highest relative abundance in the Co-fed culture, where many exceeded 5 % during cultivation time. For the reference conditions, only *Tyzzerella* was higher than 5 % after 24 h, which decreased at 72 h again. The differences in culture composition of the used inocula can be seen in supplementary Figure S1. Co-fed and Ref. 2 received the same inoculum, while Ref. 1 was inoculated with a different pre-culture.

Besides comparing the course of formate and gas concentrations, max. consumption and production rates were calculated, as well as the methane per formate yield (Table 4). In contrast to the co-fed cultures, the pure formate-fed cultures exhibited the highest FCR by far. However, the highest MPR was achieved by Ref. 2, which was 15 % and 70 % higher than for Ref. 1 and Co-fed, respectively. Comparing the hydrogen consumption rate (HPR) of the co-fed and Ref. 2 cultures, Ref. 2 exceeded the Co-fed condition by 224 %. Regarding the production yield, Co-fed and Ref. 2 showed more than double the value achieved by the solely formate-fed culture.

4. Discussion

4.1. HEPES enabled the best methane production among Good's buffers

The typical growth of the used enriched mixed culture, using solely sodium formate as substrate in a bicarbonate buffered medium, is illustrated in Fig. 1. Although formate for methanation releases 3 mol CO_2 per mol methane, the concentration of CO_2 decreased over time. This is based on CO_2 dissolution as carbonic acid in water, driven by the pH shift to more alkaline values. The pH increase can be seen in Fig. 1A and is attributed to the consumption of formate, which requires one additional H^+ to be converted into H_2 and CO_2, the necessary substrates for methanogenesis [29]. Due to the dissociation of carbonic acid into hydrogen ions and hydrogen carbonate, the solution is buffered. For the co-feeding concept of formate and H_2, where formate represents the only carbon source for methanogenesis, it was necessary to exchange the bicarbonate buffer with a CO_2-free buffer. Other medium components, such as acetate, did not decrease in concentration (data not shown). Therefore, the medium was not modified further.

As methane detection suggests, all buffered mediums enabled the growth of methanogenic archaea (Fig. 3B). However, the production's beginning and the increase in the methane concentration varied between HEPES and the three other buffers. Methane can be used as a growth indicator for methanogens as it is a primary product coupled with generating energy equivalents [30]. Generally, it can be stated that methane formation at an earlier point indicates favorable growth conditions for methanogens. This observation could be affiliated to either a higher metabolic cell activity or higher cell number of the methanogenic organisms [31,32]. Another crucial role is the number of active methanogens in the inoculum of the pre-culture, which could influence the growth characteristics. Preliminary experiments of growing the enriched culture (grown on BICARB) on the Good's buffered medium showed similar growth as displayed in Fig. 3 (data not shown). Hence, in these experiments, the influence of the inoculum is negligible compared to the impact of the buffer substance on the growth. It can be assumed that the highest growth parameters were achieved by adapting the cultures to the different buffer substances.

Of the different tested buffers, HEPES enabled a methane concentration increase after 24 h instead of 40 h, concluding that HEPES provided the best growth condition for the mixed consortium. This is underlined by the highest FCR and MPR among buffers (Table 2), excluding BICARB. The comparably good growth and methane production of the HEPES mixed culture among the Good's buffers might be explained by the interaction of the buffers with metal ions. Ions like nickel (Ni), cobalt (Co), and iron (Fe) are necessary for the function of the Ni-Fe-hydrogenase, co-enzyme F430, and cobalamides, which are central players of methanogenesis [33]. While ACES, BES, and PIPES are described to have weak to strong interactions with different metal ions, HEPES is depicted for no metal interaction [27,34,35]. Among the complex forming buffers, cobalt (Co) is a common ion that has a weak affiliation with each of them. Bound in a cobamide-containing prosthetic arm of the subunit A of the methyl-H_4MPT:CoM-SH methyltransferase (MtrA), Co might play an essential role in binding the methyl group to transfer it to co-enzyme M [36,37]. The reduction of accessible Co and other ions through complex formation might explain the slower growth and lower methane production than HEPES. Another factor is the pH buffer range, which varied between the buffers. With a range of pH 6.80–8.20 for HEPES, its buffer capacity is fully used when starting at 7.0, and the pK_a of 7.30 (37 °C) lies close to the optimal pH of around 7.0, described for the identified *Methanofollis* genus [27,38,39]. The direct utilization of HEPES for methanogenesis can be ruled out, as two negative controls showed no difference in methane production with and without HEPES when formate was omitted (data not shown).

Further, an interesting occurrence of the buffer study is the course of hydrogen concentration when using buffers compared to bicarbonate buffer (Fig. 3A). The H_2 concentration was two times (ACES, BES, and HEPES) or 3.5 times (PIPES) higher than for BICARB cultures. Two possible factors might have accounted for this growth behavior. Firstly, the already described complex formation of metal ions and buffer substances would limit the availability of essential ions, decreasing the growth rate of methanogens. Therefore, H_2 would accumulate over an extended period as the H_2 production exceeds the consumption. Furthermore, the maximum H_2 concentration differs between the buffer agents, which might be based on the type and number of metal ion interactions. PIPES interacts weakly with Co and Ni, while ACES is described to interact with calcium (Ca), manganese (Mn), and zinc (Zn) as well [27]. As mentioned before, Co and Ni are crucial for the methanogenesis pathway, which already would impede the growth, but with a lower number of different metal ions interacting with the buffer substance, the amount of one type of metal ion bound increases relatively as there is less competition for complex formation. This might have influenced the amount of H_2 accumulating over time. No metal interaction is described in the case of HEPES, but the H_2 concentration still decreased after 40 h. Thus, another condition must be influential on the growth behavior. A primary difference between Good's buffers and BICARB is the additional CO_2 in the gas phase of BICARB cultures. This provides an immediate carbon source and might be the decisive reason for improved growth. With a threshold of pH_2 30 Pa, methanogens can use small amounts of H_2 to reduce CO_2, enabling the growth of hydrogenotrophic methanogens in an early stage of cultivation [40]. As a result, a faster decrease of hydrogen and CO_2 would be achieved compared to Good's buffered cultures.

Additionally, it can be observed that formate feeding increased the H_2 concentration in BICARB (40 h) and BES (64 h) cultures. As a mixed culture was applied, it is not possible to determine by which organism the H_2 is formed from the applied analytical methods. However, the formation of H_2 on the basis of formate by methanogens has already been described in the literature, and the culture composition from the co-feeding experiment does not include any organism known for metabolizing formate to H_2 [41].

4.2. Higher HEPES concentration showed a positive impact on methane production and has benefits for the co-feeding strategy

In Fig. 2 (pH of buffer screening), the pH of HEPES cultures increased faster compared to the other Good's buffers, possibly due to higher

Table 4
Growth parameters of mixed cultures under different feeding and cultivation conditions. Co-fed cultures were fed with formate + 100 % H_2, while reference 1 (Ref. 1) cultures were solely formate fed + 100 % N_2. Both were grown on 200 mM HEPES buffered medium. In contrast, reference 2 (Ref. 2) cultures were grown on formate + H_2/CO_2 (80/20) % in bicarbonate buffered medium.

Parameter	Co-fed	Ref. 1	Ref. 2
Max. FCR / mM/d	−59.42 ± 6.39	−266.622 ± 6.990	−89.20 ± 9.74
Max. HCR / mM/d	−10.29 ± 0.12	n.a	−33.29 ± 2.87
Max. MPR / mM/d	6.84 ± 0.30	10.08 ± 2.77	11.606 ± 0.463
$Y_{CH4/HCOO^-}$ / mol*mol^{-1}	0.555 ± 0.021	0.247 ± 0.010	0.591 ± 0.032

formate consumption. At the end of the experiment, the value of the BICARB culture was similar. However, the consumed formate of the BICARB was 43 % higher than that of HEPES. This aspect led to raising the buffer concentration to stabilize the pH and possibly increase the formate consumption in HEPES cultures. Concentrations of 50–200 mM HEPES were chosen and assessed for impact on the growth parameters. In Fig. 4, the pH values of the three different concentrations were plotted, in which H200 had the lowest pH value after 86 h. When comparing the growth parameters in Table 3, it becomes evident that higher amounts of formate and methane were consumed and produced with increased buffer capacity. Thus, it can be assumed that the growth of methanogens was optimized with comparably lower pH values. Further, a closer look at the gas composition (Fig. 5) showed a negligible change in hydrogen concentration but a shifted hydrogen peak with increasing concentration later. However, this was not accompanied by a deterioration of the methane concentration. The opposite was determined when comparing H50 with H200. After 38 h, H200 had a higher methane concentration, although the H_2 concentration was still increasing. A comparatively more stable pH between H50 and H200 (Fig. 6) could have led to a higher cell activity and cell number, which initially increases H_2 production even with increasing methane concentrations.

Another aspect is the course of CO_2, which did not decrease in the H200 condition compared to the other concentrations. This can be explained by a lower pH- value compared to H50 and H100. The more alkaline the solution, the more CO_2 reacts with H_2O to form carbonic acid as the reaction equation shifts towards carbonate ions. The carbonic acid dissociates into hydrogen ions and hydrogen carbonate, buffering the solution with additional hydrogen ions. However, this process binds the carbon chemically. Therefore, it is not directly available for methanogenesis, as the initial reduction step of methanogenesis is the reduction of CO_2 and methanofuran (MFR) to formyl-MFR [42]. Hence, stabilizing the pH is essential for high methane production and can be beneficial concerning the co-feeding strategy where accessible CO_2 and additional H_2 should increase the formate to methane yield.

4.3. Co-feeding of formate and H_2 increased the production yield

The co-feeding strategy of adding H_2 as an electron donor to a formate-fed methanogenic mixed culture increased the production yield by over two times (Table 4). This was independent of the gas phase composition, which differed between HEPES and BICARB with additional CO_2 in the BICARB cultures. Therefore, it can be concluded that the limitation in the production yield is the availability of H_2, as CO_2 was present in excess. The low solubility of H_2 compared to CO_2 underlines the necessity to increase the H_2 solubility in the medium [43]. At this point, gas exchange was only possible through the gas and liquid phase interface. Depending on the application, different reactor types could enhance the gas-liquid mass transfer into the medium [44]. Regarding biomethanation, Khesali Aghtaei *et al.* have developed a novel tubular foam-bed reactor (TFBR) that produced grid-quality methane gas (concentration $>$ 90 %). The H_2 transfer rate was improved by stabilizing a liquid foam structure with a non-ionic polymeric surfactant. [45] Besides the novel TFBR, the trickle bed reactor, a promising reactor type for gas fermentation, showed a long-term biological methanation at pilot-scale with natural gas quality ($>$ 98 %). [46]

However, the gas composition of the feeding gas must be evaluated. Based on the difference in growth between Co-fed and the references, it seems that a pure hydrogen atmosphere decelerated the growth of the mixed culture. The Co-fed culture exhibited a delayed formate and hydrogen consumption by 24 h to 36 h and the lowest consumption and production rates compared to the references. Also, the OD as a growth indicator was lower than the other conditions. It is not evident why the mixed culture performed worse in the Co-fed condition. To our knowledge, no inhibiting effect of pure hydrogen on methanogenesis has been described. In contrast, the bicarbonate buffered culture (Ref. 2) showed the highest methane production rate, indicating good growth conditions reflected in the highest relative abundance of methanogen (Fig. 7C). Combined with a higher cell density (Fig. 6), this condition enabled the highest methane concentration.

The 16S analysis revealed the culture composition and dynamic of each cultivation condition. The most dominant genera were plotted, with a relative abundance of $>$ 1 % at least once during cultivation. The culture composition analysis revealed that no additional genus was present when feeding additional hydrogen.

Methanofollis represents the only methanogen and archaea in the enriched mixed culture. All other organisms belong to the bacterial domain. Substrates of the genus *Methanofollis* can be H_2+CO_2, formate, primary, and, in some cases, secondary alcohols, depending on the strain described [47–50]. Based on the substrates utilized, *Methanofollis* can be grouped into the hydrogenotrophic methanogens [51]. By determining the genus of the methanogen, it is clarified that the organism can utilize formate directly for methanogenesis. However, discussing the other genera might give the first evidence of interaction between the organisms. The most dominant genus of this enriched culture is *Peptoclostridium*, which proliferates initially but decreases afterward. That would explain the massive OD increase in the first 12–14 h (Fig. 6), followed by a relatively low rise, portending to a change of growth parameters disadvantageous for *Peptoclostridium*. The decrease in relative abundance indicates a lower growth of *Peptoclostridium*, but it is also connected to the increase of other genera. Generally, species of *Peptoclostridium* are described as chemoorganotrophs, which can utilize peptone as a nitrogen source and yeast extract as the sole carbon and energy source [52]. Interestingly, acetate is a primary end product of their metabolism, which is listed as a necessity for methanogen growth [47,53]. Therefore, it might be possible that a higher acetate availability supports the growth of Methanofollis.

Besides *Methanofollis* and *Peptoclostridium*, *Bressicibacter* is another genus protruding from the rest of the genera. To date, two species are described in the genus *Bressicibacter*: *Brassicibacter thermophilus* and *Bressicibacter mesophilus*. As it can be deduced from the name, the species in question must be the latter one, as mesophilic conditions were applied. Besides sugars and amino acids as carbon and energy sources, peptone-yeast extract can be utilized, resulting in the products propionate, formate, acetate, ethanol, and isovalerate [54]. As described before, formate and acetate can be beneficial for the growth of *Methanofollis*, leading to the assumption that *Bressicibacter* might enhance the growth of the methanogen. A deeper understanding of the mixed culture might help reveal the reason for the difference in the growth of the co-fed cultures. One possibility would be a metaproteomic analysis approach, which would enable the analysis of microorganism interactions at the proteome level [55].

5. Conclusion and outlook

Applying formate as feedstock for methanogens has the drawback of low production yield and a dilution of the gaseous product with CO_2. The strategy of supplying additional electron donors in the form of hydrogen to reduce the surplus CO_2 has been demonstrated successfully in this study. The yield was more than doubled when hydrogen was available for methanogens. However, co-feeding formate and hydrogen had their limitations in gas exchange rates, which were connected to the reaction vessel. For further investigation, it is advisable to change the reaction vessel to a reactor type developed for gas fermentation, as hydrogen supply seemed to be the main limiting factor when co-feeding. Another aspect to consider would be the composition of the feeding gas. The gas mix of H_2 and CO_2 enabled a quick start-up time for the process. Yet, an additional CO_2 feed must be avoided as formate oxidation provides extensive CO_2. It is suggested that the off-gas of the fermentation be recirculated and externally mixed with the necessary amount of H_2. Finally, stabilizing the pH value would improve the overall amount of methane produced, as demonstrated by increasing the HEPES

concentration.

Role of the funding source

The founding source had no role in the writing of the manuscript or the decision to submit for publication.

CRediT authorship contribution statement

Sabel-Becker Björn: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Jost Nicolas Patrick:** Writing – original draft, Investigation, Data curation. **Holtmann Dirk:** Writing – review & editing, Validation, Resources, Project administration, Methodology, Funding acquisition, Conceptualization. **Kaster Anne-Kristin:** Writing – review & editing, Validation, Supervision, Conceptualization.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Dirk Holtmann reports financial support was provided by German Federal Ministry of Education and Research. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jcou.2025.103049.

Data Availability

Data will be made available on request.

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