Environmental Science & Technology

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Competitive Reactions during Ethanol Chain Elongation Were Temporarily Suppressed by Increasing Hydrogen Partial Pressure through Methanogenesis Inhibition

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Cite This: https://doi.org/10.1021/acs.est.2c09014

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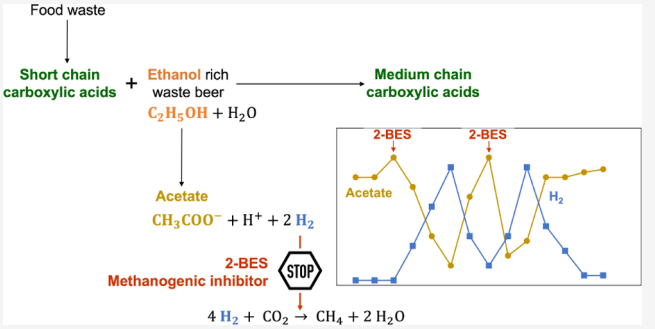
ABSTRACT: Organic waste streams can be converted into high-value platform chemicals such as medium-chain carboxylic acids (MCCAs) using mixed microbial communities via chain elongation. However, the heterogeneity of waste streams and the use of complex microbial communities can lead to undesirable reactions, thus decreasing process efficiency. We explored suppressing excessive ethanol oxidation to acetate (EEO) by increasing the hydrogen partial pressure (P_{H2}) through hydrogenotrophic methanogenesis inhibition by periodically adding 2-bromoethanesulfonate (2-BES) to an MCCA-producing bioreactor to reach 10 mM of 2-BES upon addition. The bioreactor was fed with pretreated food waste and brewery waste containing high concentrations of short-chain carboxylic acids and ethanol, respectively. While 2-BES addition initially reduced EEO, some methanogens (*Methanobrevibacter* spp.) persisted and resistant populations were selected over time. Besides changing the methanogenic community structure, adding 2-BES also changed the bacterial community structure due to its impact on P_{H2}. While we demonstrated that P_{H2} could be manipulated using 2-BES to control EEO, methods that do not require the addition of a chemical inhibitor should be explored to maintain optimum P_{H2} for long-term suppression of EEO.



KEYWORDS: *chain elongation, brewery waste, excessive ethanol oxidation to acetate, methanogenic inhibitor, 2-BES, Methanobrevibacter*

INTRODUCTION

Chain elongation of short-chain carboxylic acids (SCCAs, C1-C5) is an emerging anaerobic biotechnology to produce medium-chain carboxylic acids (MCCAs, C6-C12). It involves the stepwise elongation of the carbon chain of SCCAs to MCCAs by two carbons via the reverse β oxidation pathway.[1] The two-carbon acetyl group added to SCCAs is derived from ethanol, lactate, or other reduced compounds. Microbial-based chain elongation processes have produced MCCAs such as caproate (C6), enanthate (C7), and caprylate (C8).[2,3] MCCAs have many industrial and agricultural applications. They can be converted into longer-chain liquid fuels, or used directly as livestock feed additives, antimicrobial agents, corrosion inhibitors, and plant growth promoters, or as building blocks for producing lubricants, fragrances, and dyes.[1]

The field of waste management has been transitioning from landfill disposal and incineration to utilizing sustainable biotechnologies to recover biofuels and biochemicals from organic waste streams. The production of MCCAs from waste streams using mixed microbial communities via chain elongation[2−8] is one example consistent with this trend. One of the challenges of mixed-culture fermentation of waste streams is to control competing biochemical pathways that have the potential to take place due to the high diversity of the microbial communities, their broad metabolic capacity, and the heterogeneity of most waste streams. Methanogenesis, sulfate reduction to sulfide, excessive ethanol oxidation to acetate (EEO), carboxylic acid oxidation, and the acrylate pathway (i.e., propionate formation from lactate during lactate-mediated chain elongation) are some of the competing pathways that can affect chain elongation efficiency.[9−11] In the ethanol-driven chain elongation process, for every six molecules of ethanol, one molecule of ethanol is anaerobically oxidized into acetate to harvest one ATP via substrate-level phosphorylation.[1] Oxidation of ethanol to acetate at a proportion higher than one out of six molecules can occur along with the chain elongation process and has been termed EEO.[10] It is important to suppress EEO to ensure efficient use of ethanol, especially if costly synthetic ethanol is used. Diversion of ethanol toward EEO can reduce the amount of

Received: November 29, 2022
Revised: January 16, 2023
Accepted: January 27, 2023

acetyl-CoA available for chain elongation of SCCAs. Furthermore, acetate produced through EEO acidifies the medium leading to higher alkalinity consumption. EEO can be beneficial when the acetate produced via EEO is subsequently used in chain elongation and is referred to as ethanol upgrading.[10] However, ethanol upgrading leads to inefficient use of ethanol.[10] For example, ethanol upgrading to MCCAs via EEO consumes three moles of ethanol for every mole of caproate produced, whereas the reverse β oxidation pathway requires 2.4 moles of ethanol to elongate acetate to one mole of caproate.

EEO has been identified in several studies as an undesirable reaction,[4,6,10] and thus an adequate control strategy needs to be developed. Anaerobic ethanol oxidation to acetate has a positive standard Gibbs free energy of 49.6 kJ mole^{-1} reaction (Table S1, eq S1). This reaction is energetically feasible only when the partial pressure of hydrogen (P_{H2}) is low. The need for H_2 removal results in a syntrophic association between the H_2-producing ethanol oxidizers and hydrogenotrophic methanogens (Table S1, eqs S1−S3) or other H_2 consumers.[12] Some studies have limited EEO by controlling the CO_2 loading rate, which indirectly controls P_{H2} via hydrogenotrophic methanogenesis by limiting CO_2 availability.[4,10] Another way to control EEO is to inhibit hydrogenotrophic methanogenesis, one of the major H_2-consuming pathways in anaerobic systems, by adding methanogenic inhibitors. The most widely used methanogenic inhibitor in various applications is 2-bromoethanesulfonate (2-BES),[13] a structural analog of coenzyme M (CoM), the methyl carrier in the final step of methanogenesis. 2-BES and other methanogenic inhibitors have been used in previous chain elongation studies.[7,14−18] However, these studies focused on the effect of such inhibitors on the suppression of methane production from acetate, thus preventing the consumption of acetate, an MCCA precursor, and giving a competitive advantage to chain elongating microorganisms. As 2-BES also inhibits hydrogenotrophic methanogenesis, it can influence metabolic pathways affected by the P_{H2}. A study conducted to investigate the effects of 2-BES and chloroform on anaerobic bacterial communities showed that the use of 2-BES affected the growth of syntrophic bacteria (e.g., *Syntrophomonas* and *Syntrophobacter*) and homoacetogenic bacteria (e.g., *Moorella*) due to the accumulation of H_2.[19] Similarly, it can be expected that EEO can be altered by 2-BES addition due to its inhibition of hydrogenotrophic methanogens and the subsequent thermodynamic inhibition caused by high P_{H2}.

The objective of this study was to evaluate the effect of P_{H2} on EEO using 2-BES to inhibit H_2 consumption by hydrogenotrophic methanogens during MCCA production from pretreated food waste and brewery waste. The inhibition was evaluated by monitoring methane production, P_{H2}, ethanol consumption, and acetate production as well as by monitoring long-term changes in bacterial and archaeal population dynamics due to 2-BES addition. The knowledge obtained from this study can help assess the impact of P_{H2} in controlling undesirable reactions such as EEO during chain elongation and enhance substrate utilization toward MCCA production.

MATERIALS AND METHODS

Experimental Setup and Operating Conditions. A 7 L lab-scale bioreactor with a working volume of 5 L was operated semi-continuously as an anaerobic sequencing batch reactor (ASBR) for 339 days. The ASBR was run on a 24 h cycle through four cycles: (1) feeding (8−10 min), (2) react phase with continuous mixing and pH adjustment (22 h 40 min), (3) settling (1 h), and (4) decanting for withdrawal of effluent equal to the volume of the influent fed (8−10 min). The bioreactor was temperature controlled at 40 ± 0.5 °C until Day 73 and at 37 ± 0.5 °C from Days 74 to 339. The bioreactor pH was maintained at 5.5 ± 0.1 by the automatic addition of 3 M NaOH with the help of LabVIEW (National Instruments, Austin, TX). The biogas was collected in a 5 L Tedlar gas bag. Rumen content (17.1 ± 1.0 g volatile solids (VS) L^{-1}), obtained from a fistulated cow from a dairy farm at Michigan State University (East Lansing, MI), was used as an inoculum. The bioreactor was operated at a hydraulic retention time (HRT) of 2−4 days and an organic loading rate (OLR) of (10.5 ± 7.0 g soluble chemical oxygen demand (sCOD) L^{-1} d^{-1}). The solids retention time (SRT) was controlled around 9.7 ± 5.8 days from Days 20 to 81 by wasting suspended biomass from the bioreactor (during the react phase) and effluent (after the decant phase). The volatile suspended solids concentrations in both suspended biomass and effluent were considered for SRT calculation. Additional operational details are described by Shrestha et al.[3]

A mixture of waste beer containing ethanol and permeate extracted from an acidogenic bioreactor[20] treating food waste was fed to the ASBR once a day. The influent was prepared once a week. Waste beer was obtained from Jolly Pumpkin Brewery (Dexter, MI), where it represents 2−19% of the total volumetric beer production (Doug Knox, Sustainability Manager, personal communication). The sodium salt of 2-BES (Sigma-Aldrich, St. Louis, MO) was added to the ASBR at the beginning of the react phase roughly every 2 weeks, the first two times on Days 230 and 246, and every 10 days (equivalent to approximately three HRTs) after that on Days 259, 269, 278, 287, 296, 305, and 314 to reach a bioreactor concentration of 10 mM (~10.8 g in 5 L working volume of the bioreactor) immediately after each addition. The 2-BES dose was selected based on literature values obtained from anaerobic mixed-culture studies.[21,22] The change in 2-BES concentration over time was estimated using the initial concentration added, the volume of effluent wasted per day, and the bioreactor working volume. Thermodynamic calculations were performed to evaluate the feasibility of different reactions during the period 2-BES was added (details are given in the Supporting Information [SI]).

Chemical Analyses. Samples for various chemical analyses were collected once a week from the influent and the bioreactor content during the ASBR mixing phase and two to three times a week from the effluent during the ASBR settling phase. sCOD analyses were conducted using Lovibond medium-range (0−1500 mg L^{-1}) COD digestion vials (Tintometer, Germany). Carboxylic acids (C2−C8, including iso-forms of C4 and C5) and ethanol concentrations were determined by an Agilent Technologies 7890B gas chromatograph (Santa Clara, CA) equipped with a stabilwax-DA column (Restex) and a flame ionization detector (GC-FID). The samples for GC-FID and sCOD were acidified with phosphoric acid and sulfuric acid, respectively, centrifuged, and filtered through 0.45 μm nylon membrane filters (TISCH Scientific, North Bend, OH) before analyses. The concentrations of SCCAs and MCCAs are reported as the sum of undissociated carboxylic acids and dissociated carboxylates, even though we refer to them by their dissociated carboxylate names for simplicity. The concentrations of SCCAs (including acetate,

https://doi.org/10.1021/acs.est.2c09014
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propionate, n-butyrate, and n-valerate) and MCCAs (including caproate, enanthate, and caprylate) are expressed on a molar basis. To determine net acetate concentrations, the influent concentrations were subtracted from the corresponding effluent concentrations. Gas collected in the Tedlar gas bag was measured daily with a 0.1 L gas-tight glass syringe. Gas composition (H_2, CO_2, and CH_4) was determined two to three times a week using a Gow-Mac Series gas chromatography (Bethlehem, PA) equipped with a thermal conductivity detector. P_{H2} was calculated by considering the H_2 percentages in the gas and assuming that the headspace in the bioreactor was at atmospheric pressure. Detailed information on both of the gas chromatographs is given in the SI.

Microbial Community Analyses. Samples for biomass analyses were collected from the bioreactor effluent on Days 7, 14, 31, 66, 73, 81, 87, 115, 129, 157, 193, 206, 213, 228, 234, 241, 249, 255, 262, 270, 284, 298, 312, 319, 333, and 339. All samples were analyzed for DNA and RNA except on Day 193, when only DNA samples were collected. The biomass samples were pelletized, flash-frozen on dry ice, and stored at −80 °C until DNA and RNA extractions. DNA extractions were carried out using a cetyltrimethylammonium bromide (CTAB) method following the procedure outlined in Porebski et al.[23] with an additional 1.5 min bead beating step (Mini-Beadbeater-96, BioSpec Products, Bartlesville, OK) using 0.1 mm diameter zirconium beads. RNA extractions were performed using TRIzol reagent (Invitrogen, CA) following the manufacturer's instructions with some modifications described in Shrestha et al.[3] Co-extracted DNA was removed using ezDNase (Thermo Scientific, MA). RNA was converted to single-stranded complementary DNA (cDNA) using the SuperScript IV VILO cDNA synthesis kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. DNA and RNA were quantified using a Qubit 2.0 Fluorometer (Invitrogen, Life Technologies, CA). The samples were submitted to the Microbial Systems Molecular Biology Laboratory (University of Michigan, Ann Arbor, MI) for amplicon sequencing of the V4 hypervariable region of the 16S rRNA gene. The PCR amplification was done using primers F515 and R806[24] with the dual-index sequencing strategy.[25] Multiplexed amplicons were sequenced using the MiSeq Reagent Kit V2 (500 cycles) on the Illumina MiSeq platform (San Diego, CA).

Sequencing Data Processing. The 16S rRNA and 16S rRNA gene sequences were processed with DADA2 v1.16[26] in R following the online tutorial (amplicon sequence variant (ASV) approach) and with mothur (version 1.42.0)[27] following the MiSeq SOP (operational taxonomic unit (OTU) approach). For the ASV approach, quality filtering was done by trimming the forward and reverse reads to 240 and 200 bp, respectively, based on the read quality profiles. A maximum expected error of 2 was used to remove low-quality reads by setting truncQ to 2 followed by generating an error model for the data. A core sample inference algorithm was applied to infer true biological sequences with the pool = TRUE option to increase sensitivity to distinguish between sequencing error and real biological variation in the amplicon sequences that may be present at very low read counts across multiple samples, thus allowing detection of rare taxa. The paired-end reads were merged, and nontarget length sequences were removed from the sequence table, followed by the removal of chimeras. Finally, the sequences were taxonomically classified with the naive Bayesian Classifier method against the DADA2-formatted Ribosomal Database Project (RDP, Version 16) database. A total of 1,323,208 reads and 3480 ASVs were generated. The OTU approach is described in the SI. The raw sequences are available in the NCBI short read archive under BioProject IDs PRJNA886421 and PRJNA738485.

For phylogenetic analyses, representative 16S rRNA gene sequences obtained from DADA2 for the major methanogenic ASVs observed in the bioreactor, such as ASVs 5, 12, 20, and 29 belonging to *Methanobrevibacter*, were used. The closest relatives of the *Methanobrevibacter* ASVs given by the BLAST query search (e-value cutoff of 1×10^{-50}, 100% query cover, and 100% nucleotide identity) were chosen as reference sequences. This also included 16S rRNA genes of methanogens previously identified to be involved in ethanol metabolism, such as *Methanobrevibacter* sp. AbM4,[28] *Methanofollis ethanolicus*,[29] *Methanogenium organophilum*,[30] and *Methanosphaera* sp. WGK6.[31] The 16S rRNA gene sequences of the reference sequences were downloaded from NCBI GenBank Database. The sequences were aligned and trimmed to equal length by removing the overhangs on either side using MEGA7.[32] The evolutionary distances were computed in MEGA7 using a maximum likelihood analysis of the aligned sequences. Bootstrap testing with 1000 replicates was used to generate the final tree. Unless stated otherwise, the microbial data presented below are based on the ASV-based approach. The OTU-based results are discussed only to allow for comparison with the ASV method.

Statistical Analyses. Statistical analyses of microbial community data were performed using packages vegan (v.2.5-6),[33] phyloseq (v.1.30.0),[34] dplyr (0.8.5),[35] and ggplot2 (v.3.3.0)[36] in R (v.3.6.1). Statistical significance between groups was identified using the Kruskal−Wallis test with Benjamini-Hochberg correction for multiple testing. The Pearson correlation coefficient was calculated to determine correlations between microbial populations and various carboxylates. DESeq2 (v.1.26.0)[37] using the wald significance test was used to test the differential abundance of ASVs between the bioreactor microbial community before and after 2-BES addition. Observed ASVs for richness, Shannon diversity index, and Pielou's evenness were calculated as α-diversity estimates using the vegan package in R. Nonmetric multidimensional scaling (NMDS) plots were made using the Bray−Curtis dissimilarity matrix as implemented in the vegan package. The statistical difference in microbial community structure before and after 2-BES addition was tested with an analysis of similarities (ANOSIM) with 999 permutations. The contribution of individual ASVs to overall community dissimilarity due to 2-BES addition was determined using SIMPER as implemented in vegan.

RESULTS AND DISCUSSION

2-BES Temporarily Suppressed Excessive Ethanol Oxidation to Acetate. During the 339 days of operation, MCCAs were produced at an average rate of 4.4 ± 1.6 mmole L^{-1} d^{-1} with a maximum volumetric production rate of 9.1 mmole L^{-1} d^{-1} (Figure S1). Caproate was the dominant MCCA produced, comprising 62.7 ± 8.7% (on a carbon basis) of the total MCCAs, while enanthate and caprylate constituted on average 30.5 ± 8.5% and 6.8 ± 3.7% of the total MCCAs produced, respectively. Neither ethanol nor SCCAs were completely consumed, suggesting that their concentrations were not limiting. Ethanol, constituting on average 75% of the total influent COD, was oxidized to acetate. The acetate

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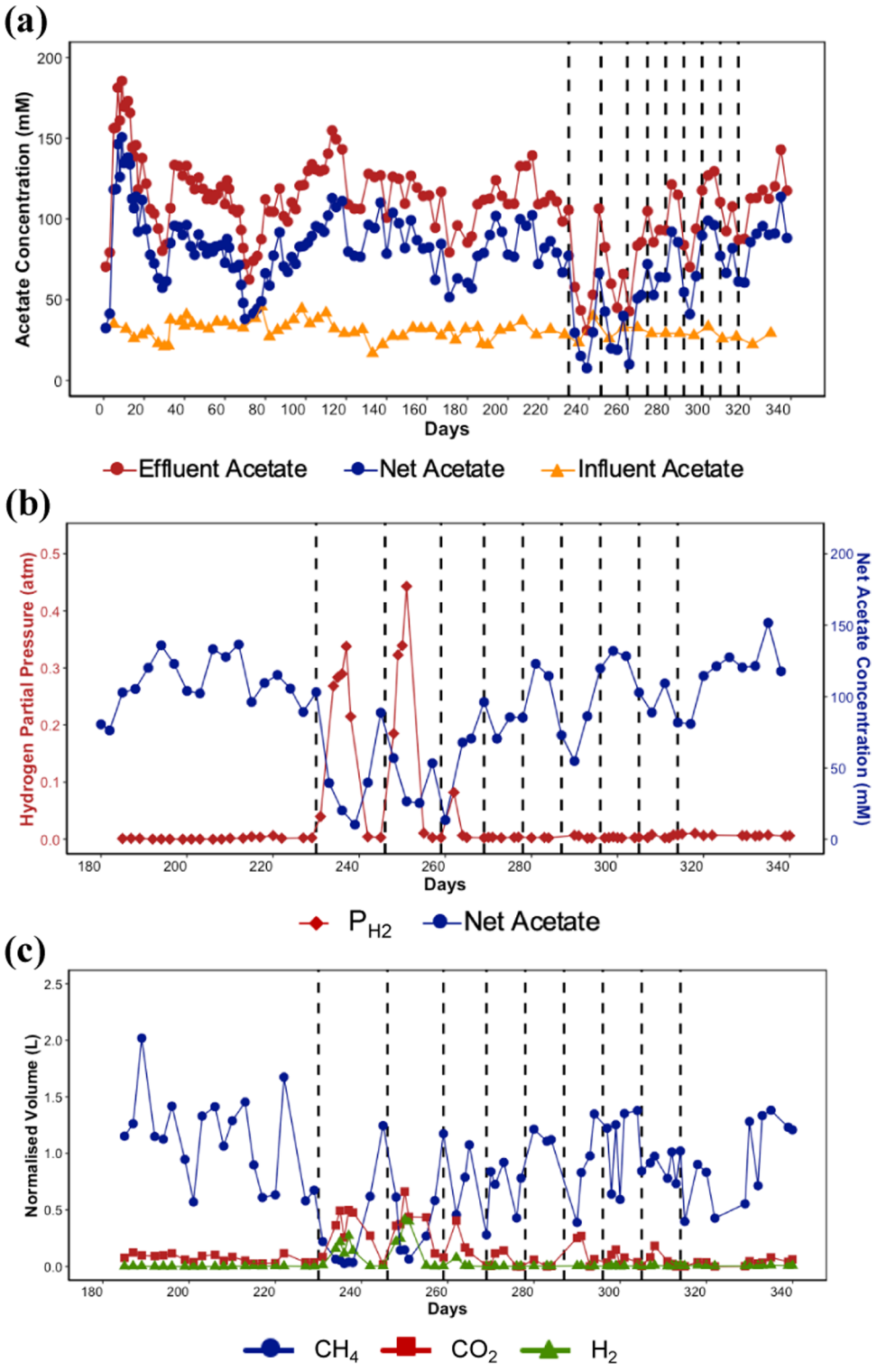

Figure 1. Effluent acetate, net acetate, and influent acetate concentrations (a), hydrogen partial pressure (P_{H2}) and net acetate concentration (secondary *y*-axis) (b), and daily gas composition after 2-bromoethanesulfonate (2-BES) addition (c) over time in the bioreactor. The dashed lines represent 2-BES additions.

accumulated in the system reaching a maximum concentration of 156.6 mM on Day 9 (Figures 1a and S2), and it was not further elongated into MCCAs despite sufficient ethanol availability. Roghair et al.[10] demonstrated that acetate derived from EEO could be involved in chain elongation, so it is unclear why acetate continued to accumulate in our study. However, it should be noted that there were several differences in operational conditions (pH 6.8 vs pH 5.5, temperature 30 vs 37 °C, HRT of 17 h vs HRT 2−4 days) and inoculum source (granular and suspended chain elongation sludge vs rumen content) between the Roghair et al.[10] paper and our study that could have affected the microbial community and thus acetate chain elongation.

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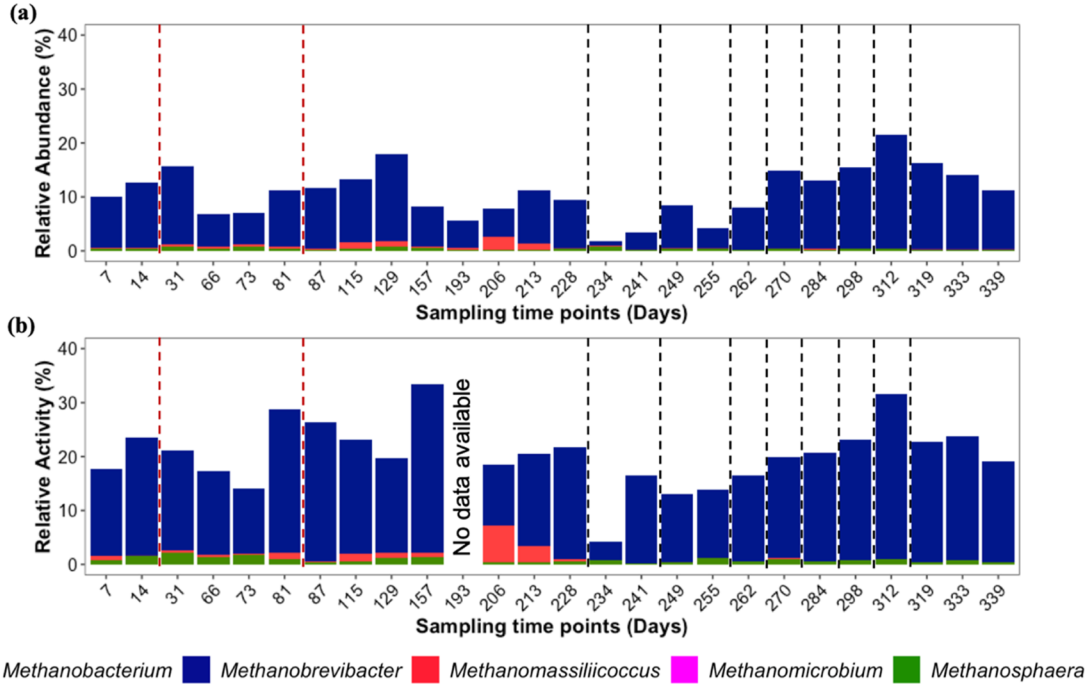

Figure 2. Relative abundance (a) and activity (b) of methanogens identified to the genus level in the bioreactor samples over time using the amplicon sequence variant (ASV)-based approach. Red dashed lines represent the start and end of wasting bioreactor content on Days 20 and 82, respectively, for controlling solids retention time, and black dashed lines represent 2-bromoethanesulfonate (2-BES) additions.

Since EEO becomes thermodynamically unfavorable at high P_{H2},[4] 2-BES was added to suppress H_2-consuming methanogens and thus inhibit EEO. P_{H2} in the bioreactor headspace averaged $2.7 \times 10^{-3} \pm 2.9 \times 10^{-3}$ atm from Days 0 to 229 before 2-BES was added. This P_{H2} is still higher than the P_{H2} required for SCCAs (1.45×10^{-4} atm for acetate, 6.65×10^{-6} atm for butyrate) and MCCAs (2.52×10^{-6} atm for caproate) oxidation via β oxidation.[5] The observed increase in P_{H2} to levels as high as 0.44 atm on Day 251 (Figure 1b) after 2-BES addition suggested that H_2 consumption by hydrogenotrophic methanogens was inhibited. This increase in P_{H2} made EEO thermodynamically unfavorable leading to a decrease in acetate concentration (Figure 1b) and a reduction in ethanol consumption. MCCAs were consistently produced, and their production did not appear to be affected negatively by this high P_{H2} (Figure S1). In fact, the total MCCA volumetric production rate significantly increased from 4.6 ± 1.8 to 5.5 ± 1.2 mmole L^{-1} d^{-1} after 2-BES addition ($p = 5.8 \times 10^{-3}$, Figure S1). As ethanol was diverted from EEO, the higher availability of ethanol might have favored higher MCCA production. Therefore, our results demonstrated that chain elongation still happened at a P_{H2} sufficiently high to suppress EEO and that maintaining a certain P_{H2} in a chain elongation system may be an effective EEO control strategy.

As 2-BES gradually washed out of the system (Figure S3), P_{H2} decreased and the net acetate concentration increased, indicating reduced inhibition. The P_{H2} again increased after additional 2-BES was introduced, with a corresponding reduction in the net acetate concentration. This trend continued until Day 268, after which P_{H2} decreased despite six more 2-BES additions indicating that the 2-BES-induced inhibition was short-lived. P_{H2} significantly decreased ($p = 6.78 \times 10^{-5}$) from average values of 0.16 ± 0.15 atm from Days 230 to 268 to $4.6 \times 10^{-3} \pm 2.3 \times 10^{-3}$ atm from Days 269 to 339. The corresponding net acetate concentrations also increased from an average of 35.5 ± 21.9 mM (Days 230−268) to an average of 78.3 ± 17.8 mM (Days 269−339). While the average P_{H2} from Days 269 to 339 was slightly higher than the average P_{H2} before the 2-BES addition had started, P_{H2} was not high enough to suppress EEO. Although Grootscholten et al.[4] reported that a P_{H2} above 0.03 atm was needed to control EEO, we observed EEO suppression at a P_{H2} higher than 0.02 atm. Besides P_{H2}, the thermodynamic feasibility of the EEO reaction is also affected by in situ conditions such as pH and temperature. Theoretical thermodynamic calculations show that the higher pH of 6.5−7.0 used by Grootscholten et al.[4] may explain the higher P_{H2} required for EEO inhibition in their study, compared to our study, which used a pH of 5.5; the slight difference in temperature between the two studies did not have an impact (Figure S4). There is little information available on the microorganisms responsible for EEO during chain elongation, so further work is needed to study how metabolic triggers for EEO play out for different populations involved.

***Methanobrevibacter* Dominated Despite the Addition of a Methanogenic Inhibitor.** We monitored 16S rRNA to study short-term changes in microbial activity induced by 2-BES. Even though using this approach to estimate activity has biases,[38] our use of both 16S rRNA and 16S rRNA gene sequencing data and comparing trends over time provides helpful insights into the microbial community responses to 2-BES additions. Using 16S rRNA sequencing data, we determined that more than one-fifth ($22.0 \pm 5.2\%$) of the active microbial community was composed of the phylum *Euryarchaeota* and was dominated by hydrogenotrophic methanogens. Aceticlastic methanogens were not detected, indicating that they were inhibited by the low bioreactor pH of 5.5. Another study has also shown that aceticlastic methanogens are more sensitive to lower pH than hydrogenotrophic methanogens and may also be inhibited to a greater extent by

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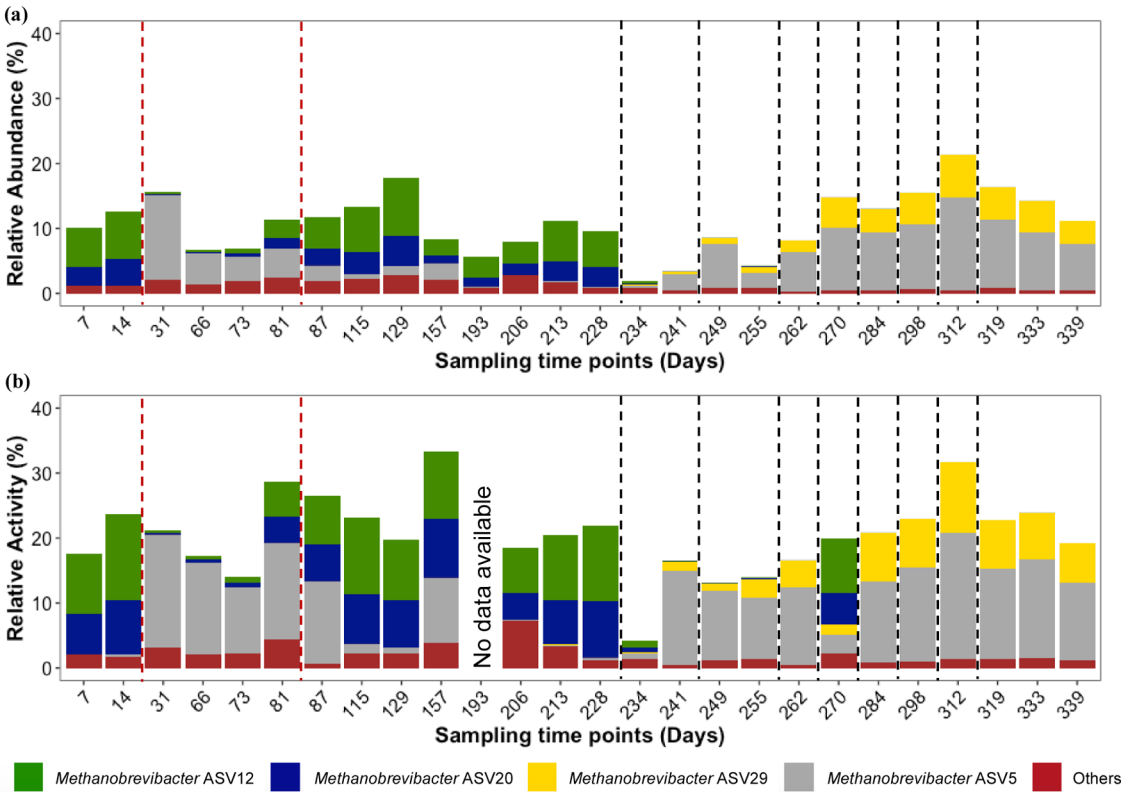

Figure 3. Relative abundance (a) and activity (b) of methanogen amplicon sequence variants (ASVs) in the bioreactor samples over time. Only *Methanobrevibacter* ASVs discussed in the text are shown, while the remaining methanogen ASVs are grouped in the "Others" category. The four *Methanobrevibacter* ASVs shown comprised 51.0−97.5% and 60.8−97.6% of the total 16S rRNA gene and 16S rRNA sequences, respectively. The red dashed lines represent the start and end of bioreactor content wasting on Days 20 and 82, respectively, for controlling solids retention time (9.7 ± 5.8 days), and the black dashed lines represent 2-bromoethanesulfonate (2-BES) additions. Note that the data up to Day 229 in this figure were previously reported[3] and that any observed reactor performance data could not explain the disparity in the Day 270 data.

undissociated SCCAs and MCCAs.[5] 16S rRNA and 16S rRNA gene sequencing data indicated that *Methanobrevibacter* was the dominant methanogenic genus at each sampling time point (Figure 2). The relative activity (as determined by 16S rRNA sequencing) of methanogens was higher than their relative abundance (as determined by 16S rRNA gene sequencing) for each time point. Over the period from Day 0 to Day 229, i.e., before 2-BES addition, the relative abundance of *Methanobrevibacter* spp. averaged 9.6 ± 3.4%, while their relative activity averaged 19.7 ± 5.7%.

After the first 2-BES addition on Day 230, the relative abundance of methanogens decreased from 9.5% on Day 228 to 1.9% on Day 234 (Figure 2a). The decrease in relative activity was even more pronounced (from 21.8% on Day 228 to 4.1% on Day 234, Figure 2b). Consistent with these observations, methane production decreased after 2-BES addition (Figure 1c) and the average methane yield decreased significantly from 7.4 ± 2.4 to 5.3 ± 3.3% of the total sCOD fed ($p = 1.8 \times 10^{-3}$) (Figure S5). The relative abundance of methanogens remained low for several weeks, but their relative activity increased substantially soon after the first 2-BES addition (Figure 2). These microbial data confirm that the periodic addition of 2-BES was ineffective in inhibiting methanogens over time, as suggested by the decrease in P_{H2} levels (Figure 1b). These long-term trends are further supported by the finding that there was no significant change in the average relative abundance ($p = 0.744$) and relative activity ($p = 0.23$) of methanogens before and after the start of 2-BES addition on Day 230.

The archaeal diversity (both Shannon index and Pielou's evenness) and richness (observed ASVs) were compared before and after the start of 2-BES addition (Figure S6). The mean number of archaeal ASVs decreased significantly ($p = 1.6 \times 10^{-6}$) from 23 ± 5 to 12 ± 3 after 2-BES addition, and a similar decrease was observed for the active archaeal ASVs ($p = 1.9 \times 10^{-2}$). The mean Shannon index and Pielou's evenness of the total and active archaeal community also consistently decreased after 2-BES addition, but the decrease was not always statistically significant. The archaeal community structures based on both 16S rRNA gene and 16S rRNA sequencing distinctly differed before and after 2-BES addition, as shown by the Bray−Curtis dissimilarity analysis (Figure S7; 78 and 71% dissimilarity, respectively). High ANOSIM R values of 0.69 ($p = 0.001$) and 0.47 ($p = 0.001$) also indicated significant changes in archaeal community composition and activity, respectively, due to 2-BES addition.

Hydrogenotrophic Methanogenesis Was the Major H_2-Consuming Pathway. Regardless of the effectiveness of 2-BES addition, inhibiting methanogens may not be sufficient to control the P_{H2} as there are other H_2 sinks besides hydrogenotrophic methanogenesis in anaerobic processes. For example, H_2 can be used by sulfate-reducing microorganisms or by homoacetogens for acetate production. While sulfate was not detected in the influent (data not reported), the sulfonate moiety of 2-BES can also serve as an electron acceptor for sulfate-reducing bacteria and thus support their growth.[39] However, sulfate-reducing bacteria (e.g., *Desulfovibrio* spp.) were present at a relative abundance and activity of less than

https://doi.org/10.1021/acs.est.2c09014
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0.1% both before and after 2-BES addition. Similarly, homoacetogenesis was not observed in the bioreactor (discussed in detail below). Since the P_{H2} increased with a simultaneous decrease in methane production (Figure 1c) and relative abundance and activity of methanogens (Figure 2) after the first few 2-BES additions, methanogenesis appeared to be the major pathway for H_2 consumption and directly affected EEO. As 2-BES inhibition of methanogens was short-lived, the low P_{H2} in the bioreactor toward the end of the experiment again favored EEO.

Periodic Addition of 2-BES Selected for Resistant Methanogens. The effective inhibitory concentration of 2-BES differs (10−50 mM) for different methanogens and environmental conditions.[13] For example, aceticlastic methanogens are more susceptible to 2-BES inhibition than hydrogenotrophic methanogens.[22,40] Several studies have reported the presence of methanogens after the addition of 2-BES,[14,15] which may be due to differences in cell envelopes resulting in the varying ability to uptake inhibitors and differences in CoM transport rates.[13] Some methanogens can adapt to 2-BES through a loss of cell permeability to 2-BES and the selection of 2-BES resistant strains.[41]

2-BES is a structural analog of CoM, the methyl carrier in the final step of methanogenesis, which catalyzes the reduction of the methyl group to methane by methyl CoM reductase.[13] Methanogens that can synthesize CoM do not depend on external CoM and likely are more resistant to 2-BES.[42] For example, *Methanobrevibacter smithii* can synthesize CoM, whereas *Methanobrevibacter ruminantium* M1 requires an external source of CoM for growth.[43,44] In our study, *Methanobrevibacter* spp. represented the highest fraction of the total and active archaeal community throughout the period with 2-BES addition (Figure 2), possibly indicating that they could synthesize CoM, making them resistant to 2-BES. However, there is no direct evidence to support this hypothesis.

ASVs 5 and 29 were the two dominant *Methanobrevibacter* ASVs observed after 2-BES addition started. As discussed in our previous publication[3] and shown in Figure 3, different *Methanobrevibacter* populations were prevalent during periods with different SRTs. Specifically, *Methanobrevibacter* ASV 5 became more prevalent after the SRT was reduced on Day 20 (Figure 3). These results suggest that ASV 5 has a faster growth rate than other *Methanobrevibacter* populations (such as ASVs 20 and 12), allowing its growth and retention when operated at a short SRT. ASV 5 reappeared after 2-BES was added (Figure 3), suggesting that the higher growth rate of this population combined with the likely ability to synthesize CoM conferred resistance toward 2-BES and allowed its growth. In addition to ASV 5, *Methanobrevibacter* ASV 29 appeared to be resistant to 2-BES (Figure 3). ASV 29, which was not detected in most samples before 2-BES addition, started appearing after Day 230 when 2-BES was added. The relative abundance and activity of ASV 29 increased from 0.8 ± 0.6 and 1.9 ± 1.5% during Days 234−262 to 6.9 ± 2.8% and 4.7 ± 1.0% during Days 270−339, respectively. This increase in relative abundance and activity of ASV 29 aligns with the observation that P_{H2} remained low despite frequent 2-BES additions (Figure 1b), showing decreased inhibition of some methanogens. The SIMPER analysis also indicated that ASVs 5, 12, 20, and 29 contributed to most of the differences (>63%) observed between the active archaeal community before and after 2-BES addition (Figure S7b). Therefore, the periodic addition of 2-BES likely provided a selective pressure to allow 2-BES-resistant populations of *Methanobrevibacter* (i.e., ASVs 5 and 29) to become abundant over time.

The sequence data were also analyzed with an OTU-based approach using mothur (Figures S8 and S9). Both OTU- and ASV-based approaches produced similar trends in changes of relative abundance and relative activity due to 2-BES addition (Figures 2 vs S8 and Figures 3 vs S9). However, the OTU method indicated that a single *Methanobrevibacter* OTU, i.e., OTU 6, was the primary population able to grow in the presence of 2-BES (Figure S9), in contrast to the ASV method, which indicated that two major *Methanobrevibacter* populations (ASVs 5 and 29) became dominant after 2-BES addition. Comparing the two methods shows that the ASV method could differentiate sequence variants down to a single nucleotide difference, thus providing improved taxonomic resolution.[45] The OTU approach clusters 16S rRNA gene sequences with 97% similarity into the same OTU, and representative OTU sequences are compared with sequences in a reference database for taxonomic identification. The increased resolution provided by the ASV-based approach captured changes in methanogen community structure in response to the 2-BES inhibition not picked up by the OTU-based approach. At the same time, the ASV method has some disadvantages, including the inability to effectively discriminate between PCR bias or sequencing error and real biological variation. Furthermore, many microorganisms harbor multiple *rrn* operons, and the 16S rRNA gene sequence diversity increases with increasing *rrn* operon copy numbers.[46] In the case of intragenomic heterogeneity, multiple ASVs can arise from a single population harboring multiple rRNA gene copies leading to more ASVs than populations present in a community.[45,47] *Methanobrevibacter* spp. have two or three 16S rRNA gene copies and intragenomic sequence variation could lead to multiple ASVs from the same *Methanobrevibacter* population.[48] So, it is possible that DADA2 assigned divergent copies of the 16S rRNA gene that belonged to one *Methanobrevibacter* population into ASV 5 and ASV 29. Comparing metagenome-assembled genomes of *Methanobrevibacter* spp. before and after 2-BES addition would resolve this uncertainty, but this analysis is beyond the scope of this study.

Syntrophic Ethanol Oxidation to Acetate Was Primarily Responsible for Acetate Production. The high relative abundance and activity of *Methanobrevibacter* populations indicated that a favorable ecological niche was created that supported their growth and activity even at low pH and during exposure to 2-BES. Some studies have pointed toward the versatility of methanogens in substrate utilization for methanogenesis.[49] For example, alcohols such as ethanol can be utilized for growth and methane production.[50−52] Bryant et al.[53] found a syntrophic association between an H_2-producing ethanol oxidizer and an H_2-utilizing microorganism such that ethanol oxidation was coupled with interspecies H_2 transfer for methane production. Some studies have reported that species of methanogens, such as *M. ethanolicus* and *M. organophilum*, can directly convert ethanol to methane and acetate.[29,30] Two moles of ethanol were oxidized to two moles of acetate for every mole of methane formed (Table S1, eq S4).

Phylogenetic analysis showed that the dominant *Methanobrevibacter* populations, ASVs 5 and 29, clustered with *Methanobrevibacter wolinii* strain SH, ASV 20 with *Methanobrevibacter boviskoreani* JH1, and ASV 12 with *Methanobrevibacter* sp. AbM4 (Figure 4). Closely related *Methanobrevibacter*

https://doi.org/10.1021/acs.est.2c09014
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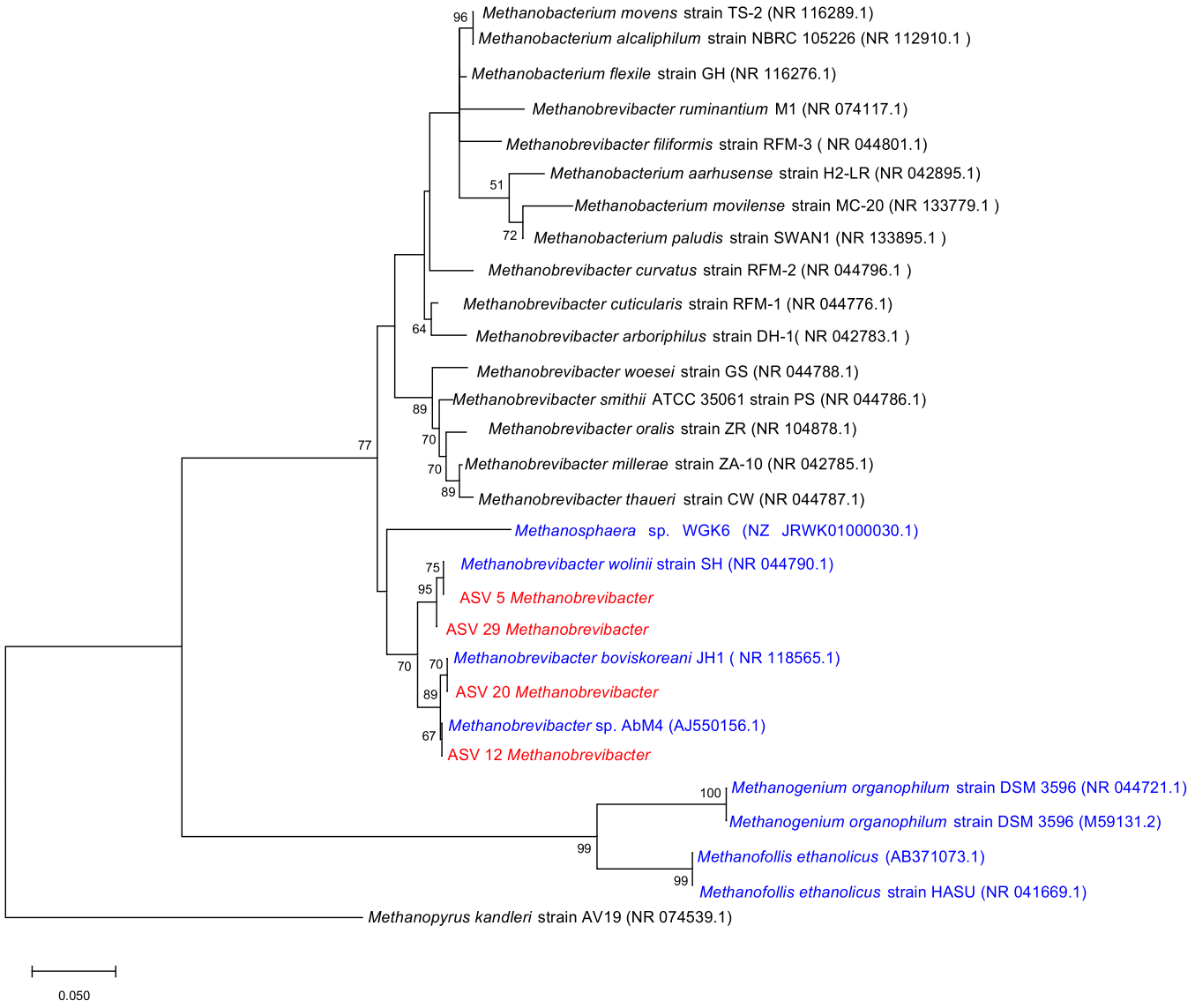

Figure 4. Phylogenetic tree of 16S rRNA gene sequences of most abundant methanogenic amplicon sequence variant (ASVs, in red). *Methanopyrus kandleri* was used as the outgroup. GenBank accession numbers are given in parentheses. Reference sequences are shown in black. Methanogens previously identified as ethanol oxidizers or capable of growth in the presence of ethanol are shown in blue. The numbers at the nodes of the branch indicate bootstrap values. The scale bar of 0.05 represents 5% substitutions per nucleotide base pair.

strains (e.g., *M. wolinii* strain DSM 11976^T and *M. boviskoreani* strain DSM 25824^T) have the genes to utilize ethanol for methanogenesis[50] and *Methanobrevibacter* sp. AbM4 is capable of growth without H_2 but in the presence of methanol/ethanol.[28] Figure 4 also shows that *Methanobrevibacter* ASVs were phylogenetically relatively closely related to *Methanosphaera* sp. WGK6, which are methanogenic ethanol oxidizers found in the foregut of macropodids (e.g., kangaroos).[31] While these results suggest the possible involvement of methanogens in ethanol metabolism, obtaining additional evidence to verify the role of *Methanobrevibacter* in EEO observed in our bioreactor was beyond the scope of this study. Future research should focus on using quantitative PCR or multi-omics tools to retrieve genome-level information to confirm if *Methanobrevibacter* populations harbor any genes required for EEO.

Acetogens can also carry out ethanol oxidation using CO_2 as an electron acceptor with no thermodynamic restriction (Table S1, eq S5). ASVs belonging to the genus *Acetobacter* were consistently present throughout bioreactor operation but at low relative abundance (1.4 ± 1.2%) and relative activity (1.4 ± 1.4%). *Acetobacter* is a typical acetic acid bacterium characterized by its ability to convert ethanol to acetate in the presence of oxygen.[54] While acetic acid bacteria such as *Acetobacter* are thought to be strict aerobes, their ability to use electron acceptors other than oxygen suggests that they may be metabolically active under anaerobic conditions.[55] However, the relative abundance and activity of *Acetobacter* were not significantly correlated to acetate production in the bioreactor, which further indicates the involvement of other microbial populations in EEO.

Homoacetogens can also produce acetate from CO_2 and H_2 (Table S1, eq S6); however, methanogens generally have a higher affinity for H_2 than homoacetogens making H_2 consumption by methanogenesis more competitive than reductive homoacetogeneis.[56] An ASV that shared 98% similarity with *Eubacterium aggregans*, a homoacetogenic

https://doi.org/10.1021/acs.est.2c09014
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bacterium,[57] was observed at a very low relative abundance and relative activity of less than 0.01%. Other homoacetogens, such as *Acetobacterium* spp. (*e.g., Acetobacterium carbinolicum*[58]), which can combine ethanol oxidation to acetate with concomitant acetate formation from carbon dioxide, were not observed in the bioreactor. Furthermore, the Gibbs free energy of hydrogenotrophic methanogenesis was exergonic over the bioreactor operating period, whereas homoacetogenesis was endergonic for part of the operating time (Figure S10), consistent with the low relative abundance and activity of homoacetogens. If 2-BES had favored homoacetogens, the acetate yield should have improved. However, net acetate production decreased after 2-BES addition showing that acetate production via homoacetogenesis was not feasible in the bioreactor. These observations confirm that syntrophic ethanol oxidation to acetate (Table S1, eq S3), which is suppressed when methanogens do not consume H_2, was the most favorable pathway for acetate production under the bioreactor conditions. However, identifying the microbial groups responsible for EEO was not possible within the scope of this study.

Implications for Ethanol Chain Elongation with Mixed Microbial Communities. While controlling competing metabolic processes such as EEO is challenging when heterogeneous waste streams are fed to a mixed community bioreactor, it is important to limit inefficient substrate usage and optimize MCCA yield and selectivity. Hydrogenotrophic methanogenesis was the critical process leading to H_2 consumption as other H_2 sinks, such as homoacetogenesis and sulfate reduction, were limited under the bioreactor conditions. The addition of the methanogenic inhibitor 2-BES limited the activity of H_2-consuming methanogens and minimized EEO due to thermodynamic inhibition caused by high P_{H2}. We observed that EEO was limited under P_{H2} higher than 0.02 atm. However, the addition of 2-BES did not provide long-term EEO suppression. The periodic addition of 2-BES created a selective environment for the microbial community making 2-BES inhibition ineffective. It would be valuable to evaluate the effect of the continuous addition of 2-BES on EEO and the microbial community dynamics. The high cost of chemical additives could most likely increase the operating cost of MCCA production, making the addition of methanogenic inhibitors challenging to scale up. Besides using chemical inhibitors, other strategies such as decreasing the SRT to wash out slow-growing methanogens, heat-shock pretreatment of the inoculum, and maintaining low pH could be evaluated to test its effectiveness for long-term methanogenesis inhibition. While this study did not address the cost and environmental impact of using a chemical inhibitor, our approach can be used to evaluate whether controlling P_{H2} is a reliable operational strategy to ensure long-term inhibition of EEO. *Methanobrevibacter* was dominant throughout the operational period, even under 2-BES-inhibited conditions. Future studies should evaluate whether methanogens such as *Methanobrevibacter* have other roles in ethanol chain elongation besides methanogenesis. Finally, future research needs to identify the microbial populations involved in EEO and study their growth characteristics to devise alternate operational strategies to control EEO.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.2c09014.

Detailed protocols of chemical analyses, thermodynamic calculations, and sequence data processing; results and discussion on 2-BES inhibition changed the bacterial community; standard Gibbs free energy for competitive reactions for acetate production; ASVs with significant differences in relative activity; volumetric production rate of total medium chain carboxylic acids, caproate, enanthate, caprylate, acetate, and butyrate; Gibbs free energy of ethanol oxidation to acetate under different pH and temperature; daily production of gas; percentage of influent soluble chemical oxygen demand used for methane production and solids retention time; α diversity indices of the archaeal community; nonmetric multidimensional scaling ordination plot; relative abundance and activity of methanogens identified to the genus or family level using operational taxonomic unit-based clustering approach; relative abundance and activity of methanogens operational taxonomic unit; and Gibbs free energy changes for hydrogenotrophic methanogenesis and homoacetogenesis (PDF)

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors acknowledge Steve Donajkowski and Ethan Kennedy for their help with building the bioreactor and Doug Knox for providing waste beer. This work was supported by the U.S. National Science Foundation (Sustainability Research Networks 1444745). S.S. was supported by an Integrated Training in Microbial Systems Fellowship funded by the Boroughs Wellcome Fund, the University of Michigan Rackham Predoctoral Fellowship, and a Water Environment Federation Canham Graduate Studies Scholarship.

https://doi.org/10.1021/acs.est.2c09014
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https://doi.org/10.1021/acs.est.2c09014
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