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Novel bioaugmentation strategy boosted with biochar to alleviate ammonia toxicity in continuous biomethanation

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HIGHLIGHTS

- Ammonia tolerant methanogens stored in gel for long period and used on demand.
- Bioaugmentation boosted with Biochar enhanced the methane yield by 28.6%.
- A biochar and biogel synergism was found to alleviate ammonia inhibition.
- Bioaugmented M. thermophilus sp. contributed largely to rapid methane recovery.
- Biochar addition created a long-term ammonia tolerance of Methanosarcina spp.

GRAPHICAL ABSTRACT

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ABSTRACT

This study investigated for the first time if ammonia tolerant methanogenic consortia can be stored in gel (biogel) and used in a later time on-demand as bioaugmentation inocula, to efficiently relieve ammonia inhibition in continuous biomethanation systems. Moreover, wood biochar was assessed as a potential enhancer of the novel biogel bioaugmentation process. Three thermophilic (55 °C), continuous stirred-tank reactors (R biogel, R char, and R mix), operated at 4.5 g NH₄⁺-N L⁻¹, were exposed to biogel, biochar and mixture of biogel and biochar, respectively, while a fourth reactor (R ctrl) was used as control. The results showed that the methane production yields of R biogel, R char and R mix increased by 28.6%, 20.2% and 10.7%, respectively compared to R ctrl. The highest methane yield was achieved by the synergistic interaction between biogel and biochar. Additionally, biogel stimulated a rapid recovery of Methanooccus thermophilus sp. and syntrophic acetate oxidising bacteria populations.
1. Introduction

Anaerobic digestion (AD) is a waste-to-energy alternative for organic waste treatment with low environmental footprint and efficient energy recovery (Braguglia et al., 2018). However, the efficiency of AD process and thus the methane production yield are susceptible to several factors, with ammonia being the most significant among them (Chen et al., 2008). Ammonium nitrogen produced by protein and urea decomposition is toxic to the AD process, causing process imbalance and decrease of methane production (Tian et al., 2018a). It has been reported that biogas plants inhibited by ammonia (greater than 1.5 g NH₄⁺-N L⁻¹) could constantly lose up to 1/3 of their practical biogas production, with significant economic and environmental consequences (Nielsen and Angelidaki, 2008). Furthermore, free ammonia nitrogen (FAN) levels that are dependent on the total ammonium nitrogen (TAN) levels, the temperature and the pH, are considered to be the major source of ammonia toxicity (Angelidaki and Ahring, 1994).

Methanogenesis is the most vulnerable step of the AD process because methanogens (methanogenic archaea) are more susceptible to ammonia toxicity compared to anaerobic bacteria (Rajagopal et al., 2013). The most significant mechanisms of ammonia inhibition on methanogens proposed to date are as follows: (i) FAN is diffusing through the cell membrane to the intracellular space, it is taking up one proton and is ionised to ammonium ion (NH₄⁺). Since protons (H⁺) inside the cells are consumed, antiporters are activated exporting K⁺ to maintain the cation balance inside the cell. This costs energy to the cell and is reducing its efficiency (Yan et al., 2020b); (ii) FAN has negative effect on the enzyme system that synthesizes methane (Lie et al., 2012).

In recent years, bioaugmentation was suggested as a solution to solve the ammonia inhibition problem in AD processes (Fotidis et al., 2014). Bioaugmentation is a strategy to introduce microorganisms with desirable properties into a biological system to boost specific microbial functions (Stephenson and Stephenson, 1992). Unlike other methods to alleviate inhibition of ammonia in anaerobic digestion (e.g. as adjusting the operating pH and temperature, reactor content dilution, or changing the substrate composition), bioaugmentation does not require additional infrastructure, dilution, or longer hydraulic retention time (HRT). Specifically, bioaugmentation could be applied once or during a specific short period, without stopping or alternating the reactors’ operation or changing the ammonia-rich substrates, and its results are expected to last for many HRTs (Yan et al., 2020b).

Tian et al. (2019b) has demonstrated the crucial role of hydrogenotrophic methanogens in the success of bioaugmentation while achieving an 11–13% improvement of methane yield and 45–52% decrease of volatile fatty acids (VFAs) during thermophilic continuous biomanethanation processes. Furthermore, Yang et al. (2019) reported that bioaugmentation of Methanobrevibacter and Syntrophaceticus schinkii increased methane yield by 71% at 4 g NH₄⁺-N L⁻¹. Overall, the successful bioaugmentation mechanism has been described as the development of microbial syntrophy between the bioaugmented consortia and the reactors’ existing methanogenic microbiome (Megaw and Gilmore, 2017). This newly formed syntrophy favors significantly the electron transfer during methanogenesis (Ruiz-Sánchez et al., 2018). Additionally, The removal of these intermediates (i.e., VFA and H₂), creates better growth conditions for microorganisms and stimulates rapid microbial reproduction for bacteria and archaea (Yan et al., 2019).

Many previous studies emphasized that successful bioaugmentation demands adequate amounts, in terms of microbial biomass, of ammonia tolerant bioaugmentation inocula (i.e. “crucial biomass”) (Fotidis et al., 2017). However, ammonia tolerant methanogens are fastidious, slow growing microorganisms that can be impractical to transfer and store for large-scale applications (Zeng et al., 2015). Recently it was proven in batch reactor experiments that ammonia tolerant methanogenic consortia can be immobilized in gel and stored for long periods (up to six months) while maintaining their AD process recovery abilities (Yan et al., 2020a). This development reduces the requirements for transfer and storage and provides a significantly longer shelf-life for the methanogenic consortia. However, the bioaugmentation performance of gel-immobilized bioaugmentation consortia in continuous reactors have not been assessed yet.

Biochar is the carbonaceous solid product of the thermal conversion of biomass in oxygen-depleted conditions (e.g., pyrolysis or gasification). Biochar addition in AD has been proposed as an alternative to increase methane production and it is another promising strategy to counteract ammonia inhibition, by supporting microbial cell immobilization on the surface (Cai et al., 2016). A plausible explanation could be that biochar addition alternates the microbial community structure and shifts and/or enhances the anaerobic metabolic pathways (Sossa et al., 2004). For example, Su et al. (2019) added biochar in continuous AD reactors at room temperature with TAN levels of 1500 mg L⁻¹ and observed an increase of Methanoregulaceae, Bacteroidales, Anaerolineales, and Syntrophobacterales. Meanwhile, Giwa et al. (2019) have shown that introducing biochar in mesophilic AD reactors resulted in increasing the abundance of Methanobrix spp. Moreover, biochar is an electron conductor, which stimulates direct interspecies electron transfer (DIET) between syntrophic methanogenic and acetogenic species (Rotaru et al., 2014). However, the long-term performance of bioaugmentation with ready-to-use inocula in continuous reactors is rarely reported. Furthermore, the combined effect of bioaugmenting ammonia tolerant methanogens with biochar addition in continuous reactors has not been investigated yet.

The primary aim of this study was to use, for the first time, a gel-immobilised ammonia tolerant methanogenic consortium (biogel) as bioaugmentation inoculum in a thermophilic continuously stirred tank reactor (CSTR), to alleviate ammonia toxicity effect and thus improve methane production efficiency. An additional aim was to further enhance the bioaugmentation effect by combining biogel and biochar in an ammonia-inhibited thermophilic CSTR reactor. A secondary aim was to investigate potential synergistic interactions between biogel and biochar on alleviating ammonia inhibition, using a two-way analysis of variance (ANOVA), thus another CSTR reactor with only biochar was used. Finally, microbiological analysis was performed to elucidate how the biogel and/or biochar affected the microbial composition and interactions.

2. Material and methods

2.1. Inoculum and feedstock

Fresh and highly active thermophilic inoculum was obtained from Lemvig Biogas A.m.b.A (Pillevej, Denmark) and immediately used to start-up the CSTR reactors. Biopulp, the pre-treated organic fraction of food waste by a mechanical pulper (Khoshnevisan et al., 2018), was taken from HCS A/S transport & Spediting (Denmark) and was used as feedstock. Biopulp was stored in a freezer (-21 °C) until use, where thawed and diluted. The basic characteristics of the inoculum and the feedstock used in the experiments are shown in Table 1.

2.2. Bioaugmentation cultures

Two different ammonia tolerant methanogenic consortia were used
in the current study that were enriched separately and then combined to prepare the biogel inoculum. Glucose (3.0 g L⁻¹) and a gas mixture of H₂ and CO₂ (80/20, v/v) were used as sole substrates for culture growth, respectively. Anaerobic batch reactors (1000 mL total volume and 400 mL working volume, respectively) were used to conduct the cultivation process of the enriched cultures with basal anaerobic medium (BA medium) at 55°C (Angelidaki et al., 1990). The two enriched cultures were acclimatized by stepwise-exposure (with 1 g NH₄Cl-N L⁻¹ each step) up to 5.0 g NH₄⁺-N L⁻¹ at pH 8.0. The final volatile suspended solid (VSS) of two consortia were 96 mg L⁻¹ and 112 mg L⁻¹, respectively. Then, the two different enriched consortia were combined into the final mixed bioaugmentation inoculum (1:1, biomass w/w). To ensure adequate biomass inside the biogel, the mixed inoculum was centrifuged at 4500 rpm for 10 min under N₂ gas headspace and up-concentrated 16 times. Finally, to prepare the biogel, the mixed inoculum was immobilized by 10 g L⁻¹ agar solution at 55°C, with a ratio 70%/30% (v/v) respectively. After 3 mins flashing with nitrogen, the biogel storage bottles were sealed by rubber plugs and parafilm, then stored in a dark place at 24°C.

The sequencing results showed that the relative abundance of total methanogens was 12% in biogel. The hydrogenotrophic methanogenesis was the dominant methanogenic pathway. Methanoculleus thermophilus sp. and Methanothermobacter thermautotrophicus sp. were the dominant methanogenic species with 52.4% and 27.9% relative abundance (among methanogens), respectively, followed by Defluviitoga tunisiensis sp. was the dominant bacteria species with 76.4% relative abundance of total bacteria (among methanogens), respectively, followed by Methanosarcina sp. of 19.3%. Defluviitoga tunisiensis sp. was the dominant bacteria species with relative abundance of 76.4%. The syntrophic bacteria Tepidanaerobacter syntrophicus sp., Thermoanaerobacter sp., Synergistaceae sp. and Syntrophaceae schnitki sp. were identified with more than 1% relative abundance in biogel.

### 2.3. Biochar preparation

The wood biochar used as additive in this work was obtained via two-stage gasification (Hansen et al., 2015) and sieved to particles in the size range of 1–3 mm. The choice of this biochar was based on the results obtained from a previous work (Yan et al., 2021), where it outperformed straw-biochar and digestate-derived biochar on enhancing biogas production under ammonia toxicity conditions in batch experiments.

### 2.4. Bioaugmentation strategies

Four CSTR reactors (R<sub>Ctrl</sub>, R<sub>Bgel</sub>, R<sub>Char</sub> and R<sub>Mix</sub>), with 2.3 L total and 1.8 L working volumes, were set up and operated for 90 days. Electrical heating jackets were mounted on each one of the reactors to maintain thermophile (55 ± 1°C) operation temperature. Biopulp was mixed homogeneously in influent bottles equipped with stirrers and injected into each reactor twice per day to a total of 120 mL per 24 h. All reactors were fed with 3.0 g VS L⁻¹ d⁻¹ (organic loading rate-OLR) and were operating with 15 days HRT until the final day of the experiment. The experiment was separated into four different periods: P1 (days 0–30, uninhibited period); P2 (days 31–45, ammonia shock); P3 (day 46, bioaugmentation and/or biochar addition); P4 (days 47–90, recovery). Specifically, during P1, the reactors were operating at a quasi-steady state (as described previously (Hansen et al., 1998)) under 0.84 g NH₄⁺-N L⁻¹. The ammonia shock was applied by spiking ammonium chloride (NH₄Cl, CAS: 12125–02–9, Sigma-Aldrich) into the reactors and the feedstock, at the beginning of P2, which immediately increased the TAN levels to 4.5 g NH₄⁻-N L⁻¹, where they remained until the end of the experiment (days 31–90). R<sub>Bgel</sub> was bioaugmented with 60 mL biogel, which contained 72 mg dry biomass. 2 g L⁻¹ biochar was added into R<sub>Char</sub>, both 2 g L⁻¹ biochar and 60 mL biogel were added into R<sub>Mix</sub>. In P4, biochar was continuously added into the influent bottles of R<sub>Char</sub> and R<sub>Mix</sub>, keeping a biochar concentration at 2 g L⁻¹ inside two reactors. In addition, R<sub>Ctrl</sub> was used as control group. Main characteristics of the reactors for each period shown in Table 2.

### 2.5. Analyses

TS, VS, VSS, TAN and TKN were measured using the Standard Methods (APHA, 2005). The pH fluctuation inside the reactors was measured using a PHM99 LAB pH meter. The biogas production of the reactors was determined using water-displacement gas meters. The biogas composition and VFA accumulation were determined using a gas-chromatograph as described previously (Yan et al., 2019).

### 2.6. Calculations and statistical analysis

The FAN was estimated using the following equation (1) with K<sub>a</sub> = 3.91 × 10⁻⁹ (at 55°C).

\[
FAN = \frac{TAN}{1 + \frac{M_{\text{TAN}}}{K_a}} 
\]

The methane production recovery rate R was calculated by the equation (2).

\[
R = \frac{V_4 - V_1}{V_0} \times 100\% 
\]

Where, V<sub>n</sub> (in mL CH₄ g⁻¹ VS) is the average methane yield of the steady state in P1, V<sub>i</sub> (in mL CH₄ g⁻¹ VS) is the average methane yield of inhibited steady state in P2 and V<sub>n</sub> (in mL CH₄ g⁻¹ VS) is the average methane yield of each HRT in P4 (n = 4, 5, 6).

### 2.7. Microbial analyses

At days 26 (before TAN increase, P1), 45 (after TAN increase but one day before bioaugmentation, P2), 73 (after bioaugmentation while methane yield become stable, P4) and 89 (three HRTs after bioaugmentation, P4) triplicate samples were taken from each reactor. The following samples were retrieved: R<sub>Ctrl</sub>-P1, R<sub>Bgel</sub>-P1, R<sub>Char</sub>-P1, R<sub>Mix</sub>-P1, R<sub>Ctrl</sub>-P2, R<sub>Bgel</sub>-P2, R<sub>Char</sub>-P2, R<sub>Mix</sub>-P2, R<sub>Ctrl</sub>-P3, R<sub>Bgel</sub>-P3, R<sub>Char</sub>-P3, R<sub>Mix</sub>-P3, R<sub>Ctrl</sub>-P4, R<sub>Bgel</sub>-P4, R<sub>Char</sub>-P4, R<sub>Mix</sub>-P4, R<sub>Ctrl</sub>-P4end, R<sub>Bgel</sub>-P4end, R<sub>Char</sub>-P4end, R<sub>Mix</sub>-P4end and Inoculum. Genomic DNA was obtained using the DNeasy PowerSoil Kit (QIAGEN GmbH, Hilden, Germany). Polymerase chain reaction (PCR) amplification was performed on the V4 region of 16S rRNA gene with universal primers 515F/806R, and high throughput sequencing was performed by Illumina MiSeq platform (Majorbio, Shanghai, China). The raw sequences were submitted to Sequence Read Archive database (http://www.ncbi.nlm.nih.gov/stra), named SUB 9908894. The triplicate

<table>
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<th>Period</th>
<th>Days</th>
<th>R&lt;sub&gt;Ctrl&lt;/sub&gt;</th>
<th>R&lt;sub&gt;Bgel&lt;/sub&gt;</th>
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<td>P4</td>
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#### Table 2

The experimental setup of the four CSTR reactors.
samples combined before operational taxonomic units (OTU) clustering by averaging the number of reads for each OTU. Based on 16S ribosomal RNA sequences (Bacteria and Archaea) database on the website of National Center for Biotechnology Information, OTU clustering was defined at 97% sequence similarity. The heat map was used to plot relative abundance of interesting OTUs (relative abundance higher than 0.5% and 0.01% for bacteria and archaea, respectively). The principal co-ordinates analysis was used for beta diversity calculation.

3. Results and discussion

3.1. Reactors’ performance

At the end of P1, the four reactors achieved a steady state with a fluctuation of methane yields lower than 5% for more than 10 days (Fig. 1). The average methane yields were 412.3 ± 17.1, 417.6 ± 13.6, 439.1 ± 14.1 and 425.9 ± 15.8 ml CH4 g-1 VS for RCtrl, Rbiogel, Rbiogel−Char and Rbiogel−CharMix, respectively. The CSTR reactors’ production yields were 86.9–92.5% compared to the BMP value of the substrate (i.e., 474.5 ml CH4 g-1 VS), indicating an efficient AD process. After the ammonia shock of 4.5 g NH4-N L-1 at the beginning of the P2, the methane yield decreased rapidly and achieved an inhibited steady state with 51.3%, 45.7%, 45.7% and 41.8% average methane production loss for RCtrl, Rbiogel, Rbiogel−Char and Rbiogel−CharMix, respectively. In this case, reactors fed with food waste suffered more methane production loss compared with previous work, which lost 34–39% methane production in manure-feeding thermophilic CSTR reactors when 5.0 g NH4-N L-1 ammonia shock performed (Tian et al., 2019b).

At the end of P4, Rbiogel (bioaugmentation with the biogel and biochar) established a recovery steady state with methane yield of 387.4 ± 9.7 ml CH4 g-1 VS, which correspond to 90.9% methane production of P1, and 31.7% increase compared with P2. Rbiogel, which was bioaugmented only with biogel, experienced a statistically significant (p < 0.05) methane production increase within two days after bioaugmentation. The final methane yield of Rbiogel was 293.4 ± 9.2 ml CH4 g-1 VS, 12.4% increase compared to P2, which was 66.5% of methane production compared to P1. Methane production of Rbiogel−Char, which was bioaugmented with the fastidious methanogens (Schwede et al., 2017).

Interestingly, the biochar enhanced bioaugmentation strategy achieved both rapid recovery speed and high recovery rate. Rbiogel−Char recovered 69.7% of its missing methane production yield compared to 20.9% and 46.8% of Rbiogel and Rbiogel−Char under steady state. A clear synergy interaction effect (p < 0.05) between biochar and bioaugmented AMT in alleviating ammonia inhibition was found in AD process with two-way ANOVA analysis. In addition, the methane yield in Rbiogel−CharMix reached 90.0% (P4) of the uninhibited one (P1) with biochar/biogel enhanced bioaugmentation strategy, which was higher than the 78% to 80% final methane production recovery achieved with normal bioaugmentation (Tian et al. 2019).
shows that biochar addition enhances the effectiveness of the bioaugmented methanogenic consortia and thus the overall AD process efficiency.

The total methane production recovery in \( R_{Mix} \) was 4576 mL higher compared to \( R_{Ctrl} \) during P4. According to the cost assessment method reported by previous study (Ma et al., 2021), the output profit of the biochar enhanced bioaugmentation in 1.8 L lab-scale was 3.68 dollar while the input were 14.4 g biochar and 60 mL biogel with cost require of 0.006 and 0.15 dollar, respectively. The results showed that the retained profit was 43.5 $ t^{-1}$ working volume $d^{-1}$ (unit price of methane 0.8 $ L^{-1}$, unit price of biochar 400 $ t^{-1}$, unit price of biogel 2500 $ t^{-1}$, respectively). Therefore, if this novel technology is applied in full-scale biogas plants to alleviate ammonia inhibition, could reduce economic losses by hundreds of thousands of dollars every month.

In general, VFA accumulation in all four reactors followed the methane production fluctuations. Specifically, the VFA levels of all the reactors remained inside the defined healthy levels (<1500 mg HAc L$^{-1}$) (Angelidaki et al., 2005) for CSTR reactors at P1 (Fig. 2a). The VFA levels in all four reactors increased fast after ammonia shock and exceeded 2000 mg HAc L$^{-1}$ before the bioaugmentation. Methanogenesis, which is more sensitive to ammonia toxicity compared to acetogenesis that leads to the accumulation of the intermediate VFA with the degradation of methanogens metabolic activity (Bui et al., 2019). The VFA in \( R_{Ctrl} \) increased continuously during the first 10 days of P4 and then stabilised at around 3000 mg HAc L$^{-1}$ throughout the rest of the experiment. This was a clear indication of a typical ammonia-induced inhibited steady state (Tian et al., 2019a), which appears as a stable production process, but has a great economic and environmental cost for the affected reactor.

Both \( R_{Bgel} \) and \( R_{Mix} \) experienced a rapid VFA decrease immediately after bioaugmentation. The ammonia tolerant hydrogenotrophic methanogens introduced by biogel, strengthened the metabolism balance (Boe et al., 2010). The subsequent utilisation of acetate by the ammonia tolerant acetoclastic methanogens that improved the kinetics of the overall AD process. The VFA accumulation in \( R_{Char} \) kept stable

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Fig. 3. Beta diversity of the four CSTR reactors. (\( R_{Bgel} \): CSTR reactor bioaugmented with biogel, \( R_{Char} \): CSTR reactor added with biochar and \( R_{Mix} \): CSTR reactor bioaugmented with both biogel and biochar, \( R_{Ctrl} \): CSTR reactor used as control group).

Fig. 4. Relative abundance of the interesting archaea and bacteria at different periods in the four reactors: a) archaea in \( R_{Ctrl} \), b) archaea in \( R_{Bgel} \), c) archaea in \( R_{Char} \), d) archaea in \( R_{Mix} \), e) bacteria in \( R_{Ctrl} \), f) bacteria in \( R_{Bgel} \), g) bacteria in \( R_{Char} \) and h) bacteria in \( R_{Mix} \). (\( R_{Bgel} \): CSTR reactor bioaugmented with biogel, \( R_{Char} \): CSTR reactor added with biochar and \( R_{Mix} \): CSTR reactor bioaugmented with both biogel and biochar, \( R_{Ctrl} \): CSTR reactor used as control group).
(above 1500 mg HAc L\(^{-1}\)) for 10 more days after the addition of biochar, before it returns inside healthy levels (bellow 1500 mg HAc L\(^{-1}\)), which could be explained by the slow rate that microorganisms accumulate on the surface of biochar and form biofilms (Sossa et al., 2004).

Overall the pH levels of all reactors remained within the acceptable pH range (6.5–8.5) (Nisslía et al., 2012) for AD process. Specifically, the pH fluctuated between 7.56 and 7.74 before the addition of ammonia for the four reactors (P1, Fig. 2b). After ammonia shock, pH in R\(_{\text{Ctrl}}\), R\(_{\text{Char}}\) and R\(_{\text{MIX}}\) decreased between 7.21 and 7.56 due to the VFA accumulation (P2-P4). R\(_{\text{Bgel}}\) experienced bigger decline to 7.08 than the other three reactors and showed a lag in pH rebound, which was consistent with its delayed VFA reduction. The FAN levels were analogous with the pH fluctuation in the four reactors. FAN increased from 125 up to 295 mg L\(^{-1}\) after the ammonia spike and then decreased under 350 mg L\(^{-1}\) by the end of the experiment due to the VFA accumulation, which subsequently decreased the pH. The reactors where biochar was added (R\(_{\text{Char}}\) and R\(_{\text{MIX}}\)) were able to perform more efficiently and at higher FAN levels compared to R\(_{\text{Bgel}}\) and R\(_{\text{Ctrl}}\).

3.2. Microbiological analysis

3.2.1. Beta diversity

Beta diversity, which shows the change in microbial community diversity between samples, was expressed based on PCoA (Fig. 3). The PCoA showed the initial common microbiota inside four reactors experienced an analogous shift throughout the ammonia shock. However, it was driven into forming distinct communities by different bioaugmentation strategies. Specifically, longest matrix distance was found between P1 and P4b in R\(_{\text{Ctrl}}\), indicating the largest impact on microbial community composition under long-term ammonia stress. Bioaugmentation with biogel showed a capacity to optimise microbial community structure, which was also proved in a previous study (Yan et al., 2004). It was reported by previous ammonium adaption experiments that biochar addition groups R\(_{\text{Char}}\) and R\(_{\text{MIX}}\) were able to perform more efficiently and at higher FAN levels compared to R\(_{\text{Bgel}}\) and R\(_{\text{Ctrl}}\). The microbiota consistency was credited to the biochar pore structure, where methanogens cells colonized and were protected from inhibitors (Giwa et al., 2019). Moreover, the shortest matrix distance in R\(_{\text{MIX}}\) from P1 to P4b proved that the microbial composition was re-established to be like P1. Overall, the biochar enhanced bioaugmentation strategy harboured further potential to protect the microbial community structure under ammonia-stress, which contributed to the rapid recovery of methane production yield.

3.2.2. Microbial composition

The microbial community composition changes in the four reactors, at species level, are visualized with six archaea OTUs (Fig. 4). At the P1, the most dominant methanogenic OTU was Methanoculleus thermophilus sp.1 varied between 61.0 and 75.1% (among total methanogens) inside all four reactors, indicated hydrogenotrophic methanogenesis was the most dominant methanogenic pathway. Methanosarcina spp. (Methanosarcina sp.2 and Methanosarcina thermophila sp.4) occupied the second and third most abundant OTUs, with relative abundance between 22.2% and 34.7%. The relative abundance of Methanobacterium thermotrophicus sp.3, Methanobacterium sp.5 and Methanomassiliicoccus sp.6 were between 0.9% and 2.9%. The relative abundance of the dominant species Methanoculleus thermophilus sp.1 decreased significantly (p < 0.05) in all reactors after the ammonia shock. Conversely, the relative abundance of Methanobacterium thermotrophicus sp.3 increased more than twofold at P2. In this study, Methanosarcina spp., even if it has been reported as ammonia-sensitive in some cases, showed better ammonia tolerance at P2 compared to hydrogenotrophic methanogens (Li et al., 2017; Sossa et al., 2004). It was reported by previous ammonium adaption experiments that Methanosarcina spp. became the dominant species by 5 g NH\(_4\)-N L\(^{-1}\) ammonia stress (Yan et al., 2019). Both Methanobacterium sp.5 and Methanomassiliicoccus sp.6 thrived at P2 in all reactors with doubled relative abundance. Previous studies reported that Methanoculleus thermophilus sp.1 was a crucial methanogen species in thermophilic AD process (Tian et al., 2018a). Therefore, the decline of Methanoculleus thermophilus sp.1 could be the reason for the rapid methane yield drop, after the ammonia shock.

After the bioaugmentation, the most noteworthy species was Methanoculleus thermophilus sp.1. Its relative abundance in R\(_{\text{MIX}}\) recovered from 55% to 61% at P4a compared with R\(_{\text{Ctrl}}\), R\(_{\text{Bgel}}\) and R\(_{\text{Char}}\), where the relative abundance continuously decreased between 54% and 13%, and even stopped being the dominant methanogens in R\(_{\text{Char}}\). Conversely, the relative abundance of Methanobacterium thermotrophicus sp.3 was 25.5%, 15.0% and 23.0% in R\(_{\text{Bgel}}\) and R\(_{\text{Char}}\), respectively, but only 7.7% in R\(_{\text{MIX}}\) at P4a. Additionally, another hydrogenotrophic methanogenic species, Methanobacterium sp.5, decreased to <0.4% in all four reactors, indicated its limited long-term tolerance to ammonia toxicity. The combine addition of biogel and biochar brought speedy recovery of Methanoculleus thermophilus sp.1, which seems to explain the rapid increase of methane production in R\(_{\text{MIX}}\). This can be explained by the increased abundance of Methanoculleus sp., which enhanced the electron transfer between syntrophic microbes and accelerated the VFA consumption process (Yan et al., 2020b). During P2 to P4b, the relative abundance of Methanosarcina spp. dropped from 29% to 19% in R\(_{\text{Char}}\) and 37% to 23% in R\(_{\text{Bgel}}\). On the contrary, the final relative abundance of Methanosarcina spp. were 42% in R\(_{\text{MIX}}\) and 40% in R\(_{\text{Char}}\), indicating a protective effect to Methanosarcina spp. by biochar. This can be explained by adhesive ability of Methanosarcina spp. that allow them to bind on biochar and each other, creating ammonia gradient to alleviate the toxicity and form methanogenic zones (Calli et al., 2005; Luo et al., 2015).

Throughout the experimental period, the most dominant bacteria OTU in the four reactors was always Defluviitoga tunisensis sp.8 (Fig. 4), which is widely found in AD systems, performing the conversion of carbohydrates (e.g., cellulose and glucose) to acetate, H\(_2\) and CO\(_2\). In P1, Thermoclostridium sp.14 was the second most abundant bacteria species, followed by Thermoanaerobacter syntrophus sp.12, acetocrobium mobile sp.13, Thermoaerobacteriales sp.18, Symbiobacterium schinkii sp.20, were all assigned to well-known syntrophic H\(_2\)-producing taxa commonly found in AD systems (Wagner et al., 2013). After the ammonia shock, the relative abundance of well-known syntrophic acetate oxidizing bacteria (SAOB), i.e., S. schinkii sp.20 and A. mobile sp.13 were decreased in all four reactors. After bioaugmentation, the relative abundance of S. schinkii sp.20 increased more than twofold in R\(_{\text{Bgel}}\) and R\(_{\text{MIX}}\) at P4a, contrary to R\(_{\text{Ctrl}}\) and R\(_{\text{Char}}\), where continuously decreased. The additional biomass of S. schinkii introduced by the biogel contributed to the shorter recovery phase in bioaugmented groups. On the other hand, the A. mobile sp.13 became significantly more abundant in the R\(_{\text{Char}}\) and R\(_{\text{MIX}}\) with both 7.8% relative abundance compared to 2.0% relative abundance in R\(_{\text{Ctrl}}\) at P4b. These results suggest that the novel bioaugmentation strategy provided an environment that promoted the growth of SAOB, which consecutively enhanced the methane production efficiency (Werner et al., 2014).

Methanosarcina spp. were proved to be the main methanogens involved in interspecies electron transfer (IET) mechanism and had twofold higher relative abundance in R\(_{\text{Char}}\) and R\(_{\text{MIX}}\) compared with R\(_{\text{Bgel}}\) and R\(_{\text{Ctrl}}\). In addition, Tepidanaerobacter syntrophus sp.10, Bacteroides sp.19, Syntrophomonas bryantii sp.15 and Syntrophothermus sp.24 were reported as the syntrophic partners with several methanogenic species during IET (Yamada et al., 2015). These species thrived in biochar adaption groups R\(_{\text{Char}}\) and R\(_{\text{MIX}}\) compared to R\(_{\text{Ctrl}}\). Recent studies suggested that biochar can promote DIET, which has 10\(^5\) times transfer velocity than indirect interspecies electron transfers (Cruz Viggi et al., 2014). Syntrophic acetogens and methanogens maybe enriched on the surface of biochar and utilize it as temporary electron acceptor (Zhao et al., 2016). Specifically, DIET is vital in ammonia inhibited AD processes because of the metabolic inactivity of methanogens (Wang et al., 2018). Although the DIET mechanism in biochar mediating AD processes is unclear, it is known that several species can interact with
biochar and accelerate methanogenesis (Qiu et al., 2019). Previous studies reported that Syntrophomonas sp., Tepidanaerobacter syntrophicus sp. and Methanosarcina spp. were abundant and tightly-bound to biochar, which showed potential to utilize biochar for electron transport (Liu et al., 2012; Lü et al., 2016). Therefore, in the current study, the biochar enhanced bioaugmentation achieved the highest methane recovery rate by enrichment of syntrophic partners and utilising their DIET potential to accelerate methanogenesis.

3.2.3. Microbial network

Based on the significant correlation on relative abundances were identified with network analysis (Fig. 5), Methanoculleus thermophilus sp.1 had direct correlation with the methane production yield. Its important role in ammonia-stressed AD systems was once again confirmed. The significant negative correlation between Methanoculleus thermophilus sp.1 and VFA producers (e.g., D. tunisiensis sp.8 and Thermaanaerobacteraceae sp.16) demonstrated its sensitivity to acid accumulation. However, some species of SAOB had positive correlation with these conflicting acetogens and hydrogenotrophic methanogens (e.g., Thermoanaerobacteraceae sp.16, S. schinkii sp.17 and Methanothermobacter thermotrophicus sp.3), indicting SAOB harboured a potential to reconcile the systems. Specifically, Clostridium sp.31, the only species that had positive correlation with Methanoculleus thermophilus sp.1, was reported to be a member of a potential acetate-oxidizing community (Dyksma et al., 2020). The important role of the SAOB to maximize biomethane recovery in ammonia inhibited AD process was supported by previous study (Werner et al., 2014; Yan et al., 2020c).

Consequently, bioaugmentation with biogel enabled the rapid recovery of AD due to the immediate supplement of SAOB and the Methanoculleus thermophilus sp.1, which were identified as “crucial methanogens” in this case. Biochar addition promoted the long-term methane production performance by improving the ammonia tolerance of acetoclastic methanogens Methanosarcina spp. The synergistic interaction between biogel and biochar create a co-operative microbial system against ammonia toxicity.

4. Conclusions

This study proposed an innovative bioaugmentation strategy to improve the methane yield in ammonia-stressed thermophilic continuous anaerobic reactors. A synergistic interaction between biochar and biogel was found on alleviating ammonia inhibition and recovering more than 90% of the uninhibited methane yield. The rapid recovery of SAOB and the Methanoculleus thermophilus sp.1 contributed primarily to the successful bioaugmentation. Moreover, the enrichment of Methanosarcina spp. on surface of biochar rebuilt the balance of acetoclastic methanogenesis. Overall, this cost-efficient, high-efficiency and readily available bioaugmentation strategy is recommended to recover ammonia-stressed commercial bioreactors.

The relative abundance of interesting OTUs, the detailed growth of the inoculum is presented in the E-supplementary.

CRediT authorship contribution statement

Yixin Yan: Methodology, Writing - review & editing, Conceptualization. Miao Yan: Methodology, Writing - review & editing. Giulia Ravenni: Methodology, Writing - review & editing. Irini Angelidaki: Writing - review & editing, Funding acquisition. Dafang Fu: Writing - review & editing, Funding acquisition. Ioannis A. Fotidis: Writing - review & editing, Conceptualization, Methodology, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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**Appendix A. Supplementary data**

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**References**


