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Published in:
Bioresource Technology

Link to article, DOI:
10.1016/j.biortech.2021.126146

Publication date:
2022

Document Version
Publisher's PDF, also known as Version of record

Citation (APA):
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.

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 (Rbgel, Rchar, and Rmix), 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 (Rctrl) was used as control. The results showed that the methane production yields of Rmix, Rchar and Rbgel increased by 28.6%, 20.2% and 10.7%, respectively compared to Rctrl. The highest methane yield was achieved by the synergistic interaction between biogel and biochar. Additionally, biogel stimulated a rapid recovery of Methanoculleus 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 (Bráuglia 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., 2020c); (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 biomethanation 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 microbe (Megaew and Gilmore, 2017). This newly formed syntrophy favours 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 (Zheng 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 immobilisation on the surface (Cai et al., 2016). A plausible explanation could be that biochar addition alters 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 Syntrophobacteriales. Meanwhile, Giwa et al. (2019) have shown that introducing biochar in mesophilic AD reactors resulted in increasing the abundance of Methanothrix 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 (Pillevye, Denmark) and immediately used to start-up the CSTR reactors. Biopulp, the pre-treated organic fraction of food waste by a mechanical pulper (Khoshevisan 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.

<table>
<thead>
<tr>
<th>Table 1: Characteristics of the inoculum and feedstock.</th>
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<tr>
<td><strong>Parameter</strong></td>
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<td>----------------</td>
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<tr>
<td>Total solids-TS (g L⁻¹)</td>
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<tr>
<td>Volatile solids-VS (g L⁻¹)</td>
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<tr>
<td>Total ammonium nitrogen-TAN (g NH₄⁺-N L⁻¹)</td>
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<td>Total Kjeldahl nitrogen-TKN (g N L⁻¹)</td>
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<td>Volatile fatty acids-VFA (g HAc L⁻¹)</td>
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<tr>
<td>Biochemical methane potential (ml g⁻¹)</td>
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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 H\(_2\) and CO\(_2\) (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 NH\(_4\)-N L\(^{-1}\) each step) up to 5.0 g NH\(_4\)-N L\(^{-1}\) at pH 8.0. The final volatile suspended solid (VSS) of two different 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 N\(_2\) 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 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 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 stage gasification (Hansen et al., 2015) and sieved to particles in the size range of 1–3 mm. The wood biochar was continuously added into the influent bottles of R\(_{\text{Ctrl}}\), R\(_{\text{Mix}}\), R\(_{\text{Bgel}}\) and R\(_{\text{Char}}\) biochar at 60 mL biogel g L\(^{-1}\) biochar. In P4, biochar was continuously added into the influent bottles of R\(_{\text{Char}}\) and biochar at 60 mL biogel g L\(^{-1}\) inside two reactors. In addition, R\(_{\text{Char}}\) 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\(_a\) = 3.91 × 10\(^{-9}\) (at 55 °C).

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

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

\[
R = \frac{V_o - V_i}{V_o}
\]  

The OriginLab program (OriginLab Corporation, Northampton, Massachusetts) was used for the statistical analyses of the data and the results’ illustrations. A two-way analysis of variance (ANOVA) was applied to assess the interactions between biogel and biochar.

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\(_{\text{Ctrl}}\)-P1, R\(_{\text{Bgel}}\)-P1, R\(_{\text{Mix}}\)-P1, R\(_{\text{Char}}\)-P2, R\(_{\text{Bgel}}\)-P2, R\(_{\text{Mix}}\)-P2, R\(_{\text{Char}}\)-P2, R\(_{\text{Bgel}}\)-P4, R\(_{\text{Mix}}\)-P4, R\(_{\text{Char}}\)-P4 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/sra), named SUB 9908894. The triplicate
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, Rbgel, Rchar and Rmix, 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, Rbgel, Rchar and Rmix, 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, Rmix (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. Rbgel, 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 Rbgel was 293.4 ± 9.2 ml CH4 g-1 VS, which was 12.4% increase compared to P2, which was 66.5% of methane production compared to P1. Methane production of Rchar experienced a stable increase for 20 days and finally achieved 78.5% methane yield compared to P1 (24.2% recovery compared to P2), with an average yield of 327.9 ± 13.5 ml CH4 g-1 VS. The methane yield of Rmix was 213.8 ± 9.0 ml CH4 g-1 VS at the last HRT of P4. This small increase of only 3.1% by the end of the experiment indicating, an expected slow adaptation potential to the ammonia toxicity of microorganisms mediating the AD process (Fotidis et al., 2014; Tian et al., 2018b).

In the current experiment, Rbgel and Rchar significantly (p < 0.05) improved the methane production efficiency by 9.3% and 21.1%, respectively compared to RCtrl during the recovery steady state period at the end of P4. The improvement of biogel bioaugmentation, under thermophilic conditions, were analogous to previous batch study (Yan et al., 2020a). Batch reactors boosted with biogel showed 99% higher max methane production rate than control group, which was explained by accelerating catalabism of Avicel and intermediates (e.g., acetate) conversion. This clearly indicates that ammonia tolerant methanogenic inocula can be stored in gel under ambient conditions and used on-demand as bioaugmentation inocula in a later time, to efficiently alleviate ammonia inhibition in continuous biogas reactors.

Even though the final methane yield of Rbgel was 12% lower than Rchar, the adaptation period to ammonia-stress was < 48 h in the Rbgel compared with 20 days in Rchar. However, biochar addition, even in microbial-sensitive thermophilic conditions (Hansen et al., 1998), achieved a significantly higher methane yield recovery compared to e.g. Giwa et al. (2019), where only a 5% higher methane yield was achieved by biochar addition in mesophilic continuous reactors. This indicates that addition of wood-derived biochar, the relatively cheap material that is easy to purchase, transfer, store and apply, could play a pivotal role in the fast response to unexpected ammonia toxicity events in commercial biogas reactors. As supported by previous research, DIET has been defined as a new mechanism in biochar enhanced AD systems, which promoted the methanogenic adaptation to extra ammonia (Rotaru et al., 2014). Another possible explanation is that microorganisms immobilised into the specific surface area of biochar, forming layered biofilm to protect fastidious methanogens (Schwee et al., 2017).

Interestingly, the biochar enhanced bioaugmentation strategy achieved both rapid recovery speed and high recovery rate. Rmix recovered 69.7% of its missing methane production yield compared to 20.9% and 46.8% of Rbgel and Rchar under steady state. A clear synergy interaction effect (p < 0.05) between biochar and biogel on alleviating ammonia inhibition was found in AD process with two-way ANOVA analysis. In addition, the methane yield in Rmix 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). This
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\textsubscript{Mix} was 4576 mL higher compared to R\textsubscript{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\textsuperscript{-1} working volume d\textsuperscript{-1} (unit price of methane 0.8 $ L\textsuperscript{-1}, unit price of biochar 400 $ t\textsuperscript{-1}, unit price of biogel 2500 $ t\textsuperscript{-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\textsuperscript{-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\textsuperscript{-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\textsubscript{Ctrl} increased continuously during the first 10 days of P4 and then stabilised at around 3000 mg HAc L\textsuperscript{-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\textsubscript{Bgel} and R\textsubscript{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\textsubscript{Char} kept stable
(above 1500 mg HAC L$^{-1}$) for 10 more days after the addition of biochar, before it returns inside healthy levels (below 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$_{Ctrl}$, R$_{Char}$ and R$_{Mix}$ decreased between 7.21 and 7.56 due to the VFA accumulation (P2-P4). R$_{Bgel}$ experienced a 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 subsequently decreased the pH. The reactors where biochar was added (R$_{Char}$ and R$_{Mix}$) were able to perform more efficiently and at higher FAN levels compared to R$_{Bgel}$ and R$_{Ctrl}$.

### 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$_{Ctrl}$, 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., 2020a). On the other hand, the R$_{Char}$-P4b and R$_{Char}$-P1 clustered more closely compared to R$_{Ctrl}$. 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$_{Mix}$ 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 Methanococcus thermophilus sp.1 varied between 61.0 and 75.1% (among total methanogens) inside all four reactors, indicated hydrogenotrophic methanogenesis was the 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 Methanococcus 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 ammoniumadaptation 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 Methanococcus thermophilus sp.1 was a crucial methanogen species in thermophilic AD process (Tian et al., 2018a). Therefore, the decline of Methanococcus 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 Methanococcus thermophilus sp.1. Its relative abundance in R$_{Mix}$ recovered from 55% to 61% at P4a compared with R$_{Ctrl}$, R$_{Bgel}$ and R$_{Char}$ where the relative abundance continuously decreased between 44% and 13%, and even stopped being the dominant methanogens in R$_{Char}$. Conversely, the relative abundance of Methanobacterium thermotrophicus sp.3 was 20.5%, 15.0% and 30.0% in R$_{Ctrl}$, R$_{Bgel}$ and R$_{Char}$, respectively, but only 7.7% in R$_{Mix}$ 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 combination addition of biogel and biochar brought speedy recovery of Methanococcus thermophilus sp.1, which seems to explain the rapid increase of methane production in R$_{Mix}$. This can be explained by the increased abundance of Methanococcus 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$_{Ctrl}$ and 37% to 23% in R$_{Bgel}$. On the contrary, the final relative abundance of Methanosarcina spp. were 42% in R$_{Mix}$ and 40% in R$_{Char}$, 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 tumensinsis sp.8 (Fig. 4), which is widely found in AD systems, providing 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 bacterium species, followed by Thermoanaerobacterium thermophilum sp.12, Acetomicrobiurn mobile sp.13, Thermoanaerobacteriales sp.18, Symbiobacterium schincki 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. schincki sp.20 and A. mobile sp.13 were decreased in all four reactors. After bioaugmentation, the relative abundance of S. schincki sp.20 increased more than twofold in R$_{Bgel}$ and R$_{Mix}$ at P4a, contrary to R$_{Ctrl}$ and R$_{Char}$, where continuously decreased. The additional biomass of S. schincki sp. 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$_{Char}$ and R$_{Mix}$ with both 7.8% relative abundance compared to R$_{Ctrl}$. 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$_{Char}$ and R$_{Mix}$ compared with R$_{Bgel}$ and R$_{Ctrl}$. In addition, Tepidanaerobacterium syntrophicus sp.10, Bacteroidetes 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 addition groups R$_{Char}$ and R$_{Mix}$ compared to R$_{Ctrl}$. Recent studies suggested that biochar can promote DIET, which has 10$^3$ times transfer velocity than indirect interspecies electron transfers (Cruz Viggi et al., 2014). Syntrophic acetogens and methanogens may benefit 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 *Thermoanaerobacteraceae* 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 *Methanothrophic bacter thermotrophicus* sp.3), indicating 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 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).

### 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.

**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, Conceptualization, Methodology, 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.

**Acknowledgements**

Yixin Yan appreciate the financial support from China Scholarship Council. Ioannis A. Fotidis acknowledges the financial support of National Natural Science Foundation of China, International Cooperation and Exchange Program “LyoCH4-Development of novel lyophilized...

![Fig. 5. The microbial network of relevant species with the acetic acid and methane yields.](image-url)
bioaugmentation inocula to alleviate ammonia toxicity in anaerobic reactors” (518504152).

Appendix A Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2021.126146.

References


