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**Bioaugmentation strategy for overcoming ammonia inhibition
during biomethanation of a protein-rich substrate**

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Abstract

High ammonia levels inhibit anaerobic digestion (AD) process and bioaugmentation with ammonia tolerant methanogenic culture is proposed to alleviate ammonia inhibition. In the current study, hydrogenotrophic *Methanoculleus bourgensis* was bioaugmented in an ammonia-inhibited continuous reactor fed mainly with microalgae (a protein-rich biomass), at extreme ammonia levels (i.e. 11 g NH₄⁺-N L⁻¹). The results showed 28% increase in methane production immediately after bioaugmentation. Moreover, volatile fatty acids decreased rapidly from more than 5 g L⁻¹ to around 1 g L⁻¹, with a fast reduction in propionate concentration. High throughput 16s rRNA gene sequencing demonstrated that the bioaugmented *M. bourgensis* doubled its relative abundance after bioaugmentation. “Microbiological domino effect”, triggered by the bioaugmented *M. bourgensis* establishing a newly efficient community, was proposed as the working mechanism of the successful bioaugmentation. Additionally, a strong acetoclastic methanogenesis was found at the end of the experiment evidenced by the dominant presence of *Methanosarcina soligelidi* and the low abundance of syntrophic acetate oxidising bacteria at the final period. Overall, for the first time, this study proved the positive effect of bioaugmentation on ammonia inhibition alleviation of the microalgae-dominating fed reactor, paving the way of efficient utilization of other protein-rich substrates in the future.

Keywords

Methane; ammonia inhibition; microbial community; *Methanoculleus bourgensis*; hydrogen partial pressure.

1 Introduction

Biogas from anaerobic digestion (AD) is a renewable energy carrier, which plays an important role in substituting fossil fuel and contributing to the reduction of greenhouse gas emission (Chen et al., 2016). A variety of biomasses is used already as feedstock of AD process, e.g. agricultural biowaste, forestry residual and municipal sewage sludge, etc. (Chynoweth et al., 2001). Recently, protein-rich biomass, such as microalgae, which is considered as 3rd generation biomass, is becoming an attractive feedstock due to its high methane production potential (Mahdy et al., 2017). However, high ammonia levels released from the protein degradation have been reported to cause process instability indicated by volatile fatty acids (VFA) accumulation and low methane production, and even complete process collapse (Alvarez and Lidén, 2008; Chen et al., 2008).

AD is a complex biological process mediated by different microorganisms, mainly bacteria and archaea. Archaea (i.e. methanogens) are more sensitive to ammonia toxicity compared to bacteria, rendering methanogenesis as the most vulnerable step of AD process (Yenigün and Demirel, 2013). Acetate is the most important precursor for methane production and is catabolized by two major pathways: 1) aceticlastic pathway mediated by aceticlastic methanogens, and 2) hydrogenotrophic pathway mediated by hydrogenotrophic methanogens coupled with syntrophic acetate oxidising bacteria (SAOB) (Zinder and Koch, 1984). Moreover, hydrogenotrophic methanogens were reported to have a higher tolerance to ammonia toxicity compared to aceticlastic methanogens (Werner et al., 2014; Dai et al., 2017; Yang et al., 2018), together with a relatively high relative abundance of SAOB at the high ammonia levels. Ammonia concentration higher than 3 g NH₄⁺-N L⁻¹ is generally accepted to cause ammonia

inhibition to most of the methanogens (Nielsen and Angelidaki, 2008). Additionally, free ammonia (NH_3 , FAN) was reported to be the most inhibitory ammonia form, which can result in inhibition when the FAN concentration is above $0.15 \text{ g NH}_3\text{-N L}^{-1}$ (Massé et al., 2014).

Ammonia inhibition can cause more than 30% loss of the methane potential of full-scale biogas reactors digesting protein-rich substrates (Fotidis et al., 2014). In order to alleviate ammonia inhibition and utilize protein-rich substrates more efficiently, many methods have been proposed in the past decades, for example, air stripping (Yuan et al., 2016), absorbing material addition (Kougiass et al., 2013), dilution with tap water/ digested manure/ fresh manure (Nielsen and Angelidaki, 2008), etc. However, almost all the proposed solutions are cost-expensive and some of them even create new problems, such as increasing the total waste volume when dilution with tap water, which limits the application of these solutions (Fotidis et al., 2014). Additionally, some researchers also claim that ammonia inhibition can be alleviated by indigenous microorganisms themselves because of microbial acclimation (Yenigün and Demirel, 2013; Tian et al., 2018a). However, acclimation is usually time-consuming and thus causes indirect extra economic loss for biogas plants. Recently, bioaugmentation, a process that delivers specialised microorganisms into the system to improve a specific system function, was applied successfully to recover the methane production and improve reactor performance from organic overloading (Tale et al., 2015) and lignocellulosic feedstocks degradation (Tsapekos et al., 2017). Thus, bioaugmentation with ammonia-tolerant methanogenic culture was also proposed as a time-saving solution to increase methane yield in ammonia inhibited reactors (Hao et al., 2015). Successful bioaugmentation studies performed with pure hydrogenotrophic methanogen

and enriched ammonia-tolerant consortia on manure-based continuously stirred tank reactor (CSTR) were reported to improve the methane production up to 36% (Fotidis et al., 2014; Fotidis et al., 2017). However, bioaugmentation on the ammonia inhibited continuous reactor fed with natural protein-rich substrates, such as 3rd generation biomass microalgae, has never been reported. To confirm the effectiveness and develop the applicability of this technology, it is inevitable to apply bioaugmentation into reactors fed with a natural protein-rich substrate. Meanwhile, it is reported that bioaugmentation usually results in the establishment of a more efficient microbiota (Fotidis et al., 2014), thus the microbial community difference before and after bioaugmentation is of great importance to elucidate the working mechanism of this technology.

Therefore, the main aim of the current study was to perform bioaugmentation on an ammonia inhibited reactor fed with protein-rich substrate. The additional aim was to elucidate the bioaugmentation mechanism by deciphering the microbial community dynamics. To fulfil these aims, a mesophilic CSTR reactor fed mainly with microalgae *Chlorella vulgaris*, was operated under extreme ammonium levels ($>10 \text{ g NH}_4^+ \text{-N L}^{-1}$), and bioaugmentation was performed on this reactor during the ammonia-induced inhibited period. Moreover, 16S rRNA amplicon sequencing was applied to analyse the microbial community changes before and after bioaugmentation.

2 Material and methods

2.1 Inoculum and feedstock

The inoculum used to start-up the CSTR reactor was derived from a mesophilic ($37 \pm 1 \text{ }^\circ\text{C}$) lab-scale reactor (Tian et al., 2018a). A mixed substrate of cattle manure and

microalgae *C. vulgaris* (20/80, VS/VS) was used in this study. The cattle manure, taken from Hashøj Municipality (Denmark), was sieved through 1 mm diameter mesh, and then it was stored at -21 °C and thawed at 4 °C for 2-3 d before use. The protein-rich substrate (protein content 49.6% of the dry mass) microalgae *C. vulgaris*, was purchased from med24.dk in the form of capsules (detailed information is shown in Table. S1, supplementary material). The capsular *C. vulgaris* was grinded into powder using mortar and pestle before mixing it with the cattle manure. The biochemical methane potential (BMP) of the microalgal biomass and the cattle manure were determined according to Holliger et al. (2016) and the detailed results can be found in Fig. S1 (supplementary material). The basic characteristics of the inoculum and the substrates are shown in Table 1.

Table 1. Characteristics of the inoculum and substrates.

Parameter	Inoculum	Cattle manure	Microalgae
	value $\pm$ SD ^a	value $\pm$ SD ^a	value $\pm$ SD ^a
Total solids-TS (g kg ⁻¹)	47.83 $\pm$ 1.22	32.90 $\pm$ 0.02	985.07 $\pm$ 1.06
Volatile solids-VS (g kg ⁻¹)	41.14 $\pm$ 1.10	23.00 $\pm$ 0.04	916.57 $\pm$ 1.16
Total Ammonium nitrogen-TAN (g NH ₄ ⁺ -N kg ⁻¹)	9.97 $\pm$ 0.13	1.10 $\pm$ 0.12	6.57 $\pm$ 0.37
Total Kjeldahl nitrogen-TKN (g N kg ⁻¹)	10.28 $\pm$ 0.04	1.49 $\pm$ 0.01	78.33 $\pm$ 1.22
Volatile fatty acids-VFA (g HAc kg ⁻¹)	2.26 $\pm$ 0.18	8.94 $\pm$ 0.51	-
Biochemical methane potential-	-	332.49 $\pm$ 7.60	330.61 $\pm$ 9.43

BMP (mL CH₄ g⁻¹ VS)

^a Standard deviation

2.2 Bioaugmentation culture

A pure strain, hydrogenotrophic *Methanoculleus bourgensis* MS2^T (DSM No. 3045), purchased from DSMZ GmbH Company, was used as the bioaugmentation culture. It was cultivated in the specific medium suggested by DSMZ (DSMZ, 2014) using batch reactors with 238 and 80 mL total and working volume, respectively. The culture was acclimatized to high TAN (12 g NH₄⁺-N L⁻¹) and FAN (730 mg NH₃-N L⁻¹) levels by adjusting the pH to 7.7. A mixture gas of H₂ and CO₂ (80/20, v/v) was added (1 bar extra) in the headspace as substrate. At the end of the exponential phase, the optical density (OD₆₀₀) was determined to be 0.11 and the volatile suspended solids (VSS) were 20.78 mg VSS L⁻¹. The growth curve of *M. bourgensis* used as the bioaugmentation culture is presented in Fig. S2 (supplementary material).

Furthermore, in order to achieve a high biomass concentration for the bioaugmentation, the original methanogenic culture (directly from cultivation bottles) was centrifuged at 8000 rpm for 10 min, and then a specific amount of the supernatant was removed under N₂ gas headspace to up concentrate the active biomass concentration by 7.5 times. Before bioaugmentation, the precipitated methanogens of the bioaugmentation culture were resuspended using vortex.

2.3 Experimental setup

A lab-scale mesophilic (37±1 °C) CSTR reactor with 2.3 and 1.8 L total and working volume, respectively, was used in this study. The whole system consisted of a main reactor, an influent and effluent bottle, a feeding peristaltic pump, an electrical

heating jacket, a water-displacement gas meter and two magnetic stirrers. The hydraulic retention time (HRT) and the organic loading rate were set as 23 d and 2.00 g VS L⁻¹ d⁻¹, respectively. The TAN levels in the reactor were increased from 10 to 11 g NH₄⁺-N L⁻¹ using ammonium chloride (NH₄Cl, CAS: 12125-02-9, Sigma-Aldrich) as extra ammonia source (data not shown), and then were kept at 11 g NH₄⁺-N L⁻¹ throughout the experiment. The whole experimental period was divided into three phases: P1 (d 0-51); P2 (d 52-55) and P3 (d 56-139). The bioaugmentation with 20 mL concentrated *M. bourgensis* culture was performed once per day for four consecutive days during P2. Throughout the experiment, methane production, pH, VFA and TAN levels of the reactor were measured regularly, and sampling for microbial analyses was also conducted.

2.4 Analyses

2.4.1 Physicochemical analyses

TS, VS, VSS, TKN and TAN were measured based on APHA (2012). Biogas composition and VFA concentration were determined by two different gas-chromatographs as was described before (Tian et al., 2018b). The pH of samples retrieved from the reactor was determined immediately by PHM99 LAB pH meter.

2.4.2 Microbial analyses

Triplicate samples were taken from day 49 (before bioaugmentation, P1), day 60 (immediately after bioaugmentation, P3_a) and day 134 (at the end of the experiment, P3_b), to identify the microbial dynamics. Genomic DNA extraction was done using PowerSoil® DNA Isolation Kit (MO BIO laboratories Inc., Carlsbad, CA USA). Universal primers 515F/806R was used for polymerase chain reaction (PCR)

amplification on the V4 region of 16S rRNA gene, and further amplicon sequencing was performed by Illumina MiSeq platform (Ramaciotti Centre for Genomics, Kensington, Australia).

The raw sequencing reads were deposited in Sequence Read Archive (SRA) database (<http://www.ncbi.nlm.nih.gov/sra>) under the project of PRJNA483801. Data treatment of the raw reads was performed with CLC Workbench software (V.8.0.2) with microbial genomics module plug in (QIAGEN), and more details were previously described (Tian et al., 2018a). Rarefaction curve (Fig. S3, Supplementary material) was used to evaluate the sequencing depth. BLASTN search against 16S ribosomal RNA sequences (Bacteria and Archaea) database (National Center for Biotechnology Information, NCBI) was used to confirm the taxonomic assignment of the most interesting OTUs (relative abundance higher than 0.5% for bacteria and 0.01% for archaea, Table. S2, supplementary material). Principal Component Analysis (PCA) of the different samples was performed by STAMP software (Parks and Beiko, 2010), while relative abundance and fold changes of the interesting OTUs at different phases were depicted as heat maps through Multi experiment viewer software (MeV 4.9.0) (Saeed et al., 2006).

2.5 Calculations and statistics

The FAN concentration was calculated according to the following equation (Tian et al., 2017):

$$FAN = \frac{TAN}{1 + \frac{10^{-pH}}{K_a}} \quad \text{Eq. (1)}$$

Where K_a is the dissociation constant and equals to 1.29×10^{-9} at mesophilic (37 ± 1 °C) condition.

The OriginLab program (OriginLab Corporation, Northampton, Massachusetts) was used for statistical analyses and the plots of the reactor performance. STAMP software was used to determine the significantly changed ($p < 0.05$) OTUs with respect to relative abundance between different samples.

3 Results and discussion

3.1 Reactor performance

3.1.1 Methane production efficiency

During P1, the reactor reached an inhibited steady state (steady state was defined by the variation of the methane yield less than 10% for at least ten consecutive days (Hansen et al., 1998)) with methane yield of 185 ± 12 mL CH_4 g^{-1} VS at TAN levels of 11 g NH_4^+ -N L^{-1} (Fig. 1), which was used as the baseline to evaluate the reactor performance in the following phases. Compared to the BMP results of the substrates (Table 1), around 46% loss of the methane potential was observed during P1, which was mainly due to the high ammonia stress, thus the base line condition can be considered as an inhibited steady state.

After bioaugmentation (P3), methane yield increased immediately and reached 236 ± 9 mL CH_4 g^{-1} VS establishing a new steady state (Fig. 1). Compared to the baseline, 28% increase in methane yield was achieved after bioaugmentation, which demonstrated a successful strategy in recovering methane production from ammonia inhibited reactor. The result was similar with a previous study by Fotidis et al. (2014)

reporting that a 31% methane increase was observed after bioaugmentation in reactors operated under TAN levels of only 5 g $\text{NH}_4^+\text{-N L}^{-1}$. Unfortunately, due to a technical problem, (i.e. the temperature of the reactor increased up to 42 °C during days 78 and 79 due to stirrer malfunction), the methane yield of the reactor decreased rapidly, and it took almost one and half HRT to recover. This phenomenon agreed with a previous study (Luo et al., 2015) treating cattle manure, where the methane yield dropped close to zero when the digestion temperature increased from 37 to 45 °C, and recovered after one HRT. Moreover, due to the high ammonia levels in the current study, the reactor became more sensitive to the temperature increase disturbance (Angelidaki and Ahring, 1994), which also explained the relatively long and unstable period of the reactor. However, after the instability, the reactor entered a new steady state until the end of the experiment with an average production of $242 \pm 7 \text{ mL CH}_4 \text{ g}^{-1} \text{ VS}$, which was 31% higher than the baseline. Overall, after bioaugmentation, the reactor showed a significantly higher methane production efficiency ($p < 0.05$) compared to the inhibited steady state, which for the first time demonstrated the positive effect of the bioaugmentation strategy to alleviate ammonia inhibition of AD process fed with mainly protein-rich, 3rd generation AD biomass.

Fig. 1. Methane yield, TAN and FAN levels throughout the experimental period

3.1.2 VFA and pH variation

The significant decrease of total VFA concentration ($p < 0.05$) from more than 5 g L⁻¹ to around 1 g L⁻¹ after bioaugmentation (Fig. 2a) coincided with the increase of methane yield discussed above. Immediately after the bioaugmentation, the total VFA concentration started dropping, which indicated a robust bioaugmentation effect and agreed with previous studies (Fotidis et al., 2014; Fotidis et al., 2017). Moreover, synergistic inhibition of high ammonia and VFA levels was reported before (Lu et al., 2013). It seems that the decreased VFA levels due to bioaugmentation could mitigate the synergistic inhibition, thus resulted in a higher methane yield. Among the individual VFAs, acetate and propionate were the major intermediates accounting for at least 77% of the total VFA concentration during the inhibited steady state at P1, followed by isovalerate and other VFAs (Fig. 2b). Previous studies (Angelidaki and Ahring, 1994; Calli et al., 2005; Nielsen and Angelidaki, 2008) also showed similar results with mainly acetate and propionate accumulation when ammonia inhibition occurred to the reactors. Moreover, propionate concentration was even higher than acetate and kept

constantly higher than 2 g L^{-1} during the inhibitory period, which agreed with reported severe inhibition when propionate concentration was higher than 0.9 g L^{-1} (Wang et al., 2009). After bioaugmentation, propionate levels decreased rapidly from around 2.5 g L^{-1} to 0.1 g L^{-1} , indicating a positive effect on propionate degradation resulted from bioaugmentation. However, acetate levels dropped relatively slower compared to propionate from 1.6 g L^{-1} to 0.8 g L^{-1} . This is mainly because acetate is the intermediate product of propionate degradation (Van Lier et al., 1993). Thus, it seems that propionate concentration and consumption rate could be an indicator in term of ammonia inhibition alleviation. Finally, pH increased slightly after bioaugmentation, which might be due to the VFA consumption, but it remained within the optimal pH range (6.5-8.5) of AD process (Lay et al., 1998).

Fig. 2. VFA and pH variations throughout the experimental period: a) total VFA and pH, b) individual VFAs.

3.2 Microbial community composition and dynamics

In total, around 1.8 million sequences were generated from all the triplicate samples and 1.3 million high quality sequences were assigned to OTUs. The triplicate samples of each experimental period clustered together in the PCA plot, in which principal components (PC) 1 and 2 explained 77.9% and 17.0% of the microbial composition

difference, respectively. It indicated that the composition of the microbial community changed significantly after bioaugmentation (Fig. 3), which agreed with previous study showing big difference of the microbial community before and after bioaugmentation (Fotidis et al., 2017). Moreover, it seems that temperature disturbance also strongly shaped the community composition (Fig. 3) since temperature is a key factor that can influence the microbial community of AD process (Luo et al., 2015; Treu et al., 2018). However, even with big difference, the microbial community of all the samples still consisted of mainly bacteria with relative abundance varied from 97.7 to 98.5% at different phases, and archaea (methanogens) with a much smaller percentage. At phylum level, only *Euryarchaeota* was found within archaea, while *Firmicutes* (62.0-69.4%) and *Bacteroidetes* (22.7-26.6%) composed more than 90% of bacteria, followed by *Proteobacteria*, *Spirochaetes*, *Actinobacteria* and *WWE1*, etc., which had the similar overview of the microbial community compared to other studies (Gao et al., 2015; Kovacs et al., 2015).

Fig. 3. Principal components analysis (PCA) of the triplicate samples from different periods. PC1 and PC2 stand for principal component 1 and 2, respectively.

Among the archaea community, the top three abundant OTUs in all the samples were *Methanobrevibacter acididurans* 1, *Methanosarcina soligelidi* 2 and *Methanoculleus bourgensis* 3 (Fig. 4a), which were identified as *Methanobrevibacter acididurans*, *Methanosarcina soligelidi* and *Methanoculleus bourgensis*, respectively, with the similarity value higher than 98% through BLASTN search. Furthermore, these three OTUs were also identified to be the methanogens that changed significantly ($p < 0.05$) in relative abundance after bioaugmentation (Fig. 5a). The *M. bourgensis*, which was used for bioaugmentation of the reactor, was found in all the samples. However, the relative abundance with respect to the archaea community increased from 3.6% before bioaugmentation to 6.7% after bioaugmentation. Even though *M. bourgensis* was not the dominant methanogen, it seems that it was sufficient to drive the archaea community towards a more efficient methanogenesis process, which agreed with the reported “microbiological domino effect” (Fotidis et al., 2014). According to this theory the bioaugmented culture, even at a relative low abundance, is sufficient to drive the establishment of a new community. In fact, the bioaugmented *M. bourgensis* in this study triggered microbial community changes (Fig. 6). The most possible explanation was that the bioaugmentation of hydrogenotrophic *M. bourgensis* reduced the hydrogen partial pressure by consuming hydrogen, which created thermodynamically favourable conditions for VFAs degradation (Fig. 2), such as acetate and propionate due to their sensitivity to hydrogen (Massé and Droste, 2000; Hao et al., 2017). The reduced VFA levels further mitigated the synergistic inhibition of ammonia and VFA, and allowed the growth of other methanogens, such as aceticlastic

M. soligelidi, which was found to be dominant at high ammonia levels in previous studies (Calli et al., 2005; Tian et al., 2018a). Therefore, as a result, the relative abundance of *M. soligelidi* increased from 36.3% to 66.1% immediately after bioaugmentation and reached 86.9% at the end of the experiment as the most dominant species. However, *M. acididurans*, the most dominant methanogen before bioaugmentation, accounted for more than half of the relative abundance of archaea. It decreased in relative abundance to less than 20% and 5% immediately after bioaugmentation and at the end of the experiment, respectively. Although *M. acididurans* was identified as a hydrogenotrophic methanogen, its growth rate can decrease more than 100% when the pH was increased from 7.2 to 7.5 (Savant et al., 2002), which happened in the current study, where the pH increased up to 7.6. This pH increase can explain the decrease of *M. acididurans* and the poor reactor performance during the inhibited steady state.

Fig. 4. Relative abundance (%) (left part) and the corresponding folds change (right part) for the interesting a) archaea and b) bacteria at different phases.

Within bacteria community, the first interesting and abundant OTU was *Syntrophaceticus schinkii* 11 (Fig. 4b), that together with *Syntrophaceticus* sp. 33, were assigned to a well-known SAOB, *Syntrophaceticus schinkii* (Westerholm et al., 2010). Moreover, another mesophilic SAOB, *Clostridium ultunense* (Schnürer, 1996), was also

found in this study as OTU *Clostridium ultunense* 16 (Fig. 4b). Overall, relative abundance of SAOB was similar ($p>0.05$) between P1 and P3_a (17.1-20.9%) but decreased significantly ($p<0.05$) down to 6.2% at the end of this experiment P3_b, indicating a stronger hydrogenotrophic methanogenesis at P1 and the period immediately after bioaugmentation, compared to the end of the experiment. This change was also in accordance with the changes between hydrogenotrophic and acetitlastic methanogens discussed above. However, considering the similar levels of SAOB, but worse reactor performance at P1 (before bioaugmentation) compared to P3_a (immediately after bioaugmentation), it seems that the bioaugmented *M. bourgensis* was more efficient than *M. acididurans* on hydrogen consumption in the current experimental conditions. Another interesting OTU, *Bacteroidales* sp. 14, which was identified to be a significantly ($p<0.05$) changed OTU and had the biggest contribution to the whole community change (Fig. 5a) compared to other OTUs, was 82% similar to *Parafilimonas terrae*, who has the ability to utilize propionic acid (Kim et al., 2014). Furthermore, *P. terrae* was phylogenetically closest to *Filimonas*, which was reported to have a positive relationship with the propionate assimilation (Albert et al., 2016). Therefore, the significant increase in relative abundance of *Bacteroidales* sp. 14 was attributed to the sharp decrease of propionate concentration after bioaugmentation (Fig. 2b). It is also noteworthy that *Bacillaceae* sp. 12, which was either the second or the highest abundant OTU throughout the experimental period, cannot be allocated at species levels, but belongs to uncultured SHA-98 order, which was reported to ferment different organic matters at high ammonia levels (Mei et al., 2016; Müller et al., 2016). Considering other five OTUs (*Thermoanaerobacteraceae* sp. 19, *Thermoanaerobacteraceae* sp. 21, *Synergistaceae* sp. 23, *Thermoanaerobacteraceae* sp.

26, *Synergistaceae* sp. 38) that belong to SHA-98, in total their relative abundance increased gradually from 20.1% before bioaugmentation to 26.4% at the end, indicating more efficient substrate degradation after bioaugmentation, which agreed with the higher methane yield compared to the inhibited steady state (Fig. 1). Furthermore, some other groups of OTUs that were suspected to contribute to the substrate utilization also increased gradually after bioaugmentation, for example, three OTUs (*Syntrophomonadaceae* sp. 17, *Syntrophomonas sapovorans* 24 and *Lysinibacillus alkalisoli* 36) that related to lipid degradation and several OTUs (*Thermoactinomycetaceae* sp. 15, *Bacteroides coprosuis* 18, *Catabacteriaceae* sp. 20, *Dysgonamonadaceae* sp. 28, etc.) that were involved with carbohydrate/ cellulose fermentation.

Fig. 5. OTUs that changed significantly ($p < 0.05$) among the most interesting OTUs a)

P3_a compared to P1, and b) P3_b compared to P1.

3.3 A proposed working mechanism for the bioaugmentation

Overall, considering the improved reactor performance and changes of the microbial composition, the success of the bioaugmentation in this study, i.e. the most possible mechanism, lies on the “microbiological domino effect” triggered by the bioaugmented *M. bourgensis*, which means that an efficient community could be established even with the less dominant bioaugmentation culture (Fig. 6). Specifically, during the ammonia-induced inhibited steady state, both bacteria and archaea were inhibited mainly by high hydrogen partial pressure and synergistic inhibitory effect of ammonia and VFA. However, the hydrogen partial pressure was reduced due to hydrogen consumption by the bioaugmented hydrogenotrophic *M. bourgensis*, thus resulted in VFAs degradation and synergistic inhibition mitigation. Therefore, overall favourable conditions were created for the growth of ammonia-tolerant bacteria and archaea. For example, propionate concentration decreased rapidly because of the increased abundance of *Bacteroidales* sp. 14. Finally, as a result of the aforementioned “microbiological domino effect”, the whole microbial community was driven towards a more efficient community degrading protein-rich substrate, evidenced by the increased relative abundance of ammonia-tolerant methanogens, propionate degraders and fermentative bacteria, etc. discussed above.

Fig. 6. A schematic diagram depicting the proposed working mechanism of the successful bioaugmentation. *M. bourgensis* was the bioaugmentation culture. Red solid and green dashed arrows present the change of the compound concentration and the OUT's relative abundance, respectively.

4 Conclusions

Bioaugmentation with pure hydrogenotrophic methanogen was proved, for the first time, to alleviate ammonia inhibition of continuous AD reactor fed mainly with a natural protein-rich biomass, namely microalgae, which are a 3rd generation biomass, under extremely high ammonia levels. Specifically, the bioaugmentation strategy improved the methane yield by around 30% and decreased the VFA levels rapidly by more than 80%. Moreover, it seems that the removal of hydrogen and propionate played an important role in alleviating inhibition and recovering methane production. The microbial analysis demonstrated the significant community difference before and after bioaugmentation, especially the increase in relative abundance of ammonia-tolerant methanogens, propionate degraders (e.g. *Bacteroidales* sp. 14) and fermentative bacteria (e.g. SHA-98). Furthermore, aceticlastic *M. soligelidi* was found to be the dominant

methanogen at the end of the experiment. Finally, “microbiological domino effect” triggered by the bioaugmented culture shaping a more efficient community was proposed to be the main mechanism for the success of bioaugmentation.

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Appendix A. Supplementary material

Supplementary data of this article can be found in online version of the paper.

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Highlights

- Bioaugmentation alleviated ammonia inhibition of a reactor fed with microalgae
- Bioaugmented *M. bourgensis* increased methane yield by 28% and decreased VFA by 80%
- Reduction of hydrogen pressure and propionate is crucial in alleviating inhibition
- The bioaugmentation success lies on the triggered “microbiological domino effect”
- *M. soligelidi* was the dominant methanogen three HRTs after bioaugmentation