Biotechnol Lett (2017) 39:247–252
DOI 10.1007/s10529-016-2241-x

ORIGINAL RESEARCH PAPER

Ammonium inhibition through the decoupling of acidification process and methanogenesis in anaerobic digester revealed by high throughput sequencing

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Received: 21 August 2016 / Accepted: 13 October 2016 / Published online: 26 October 2016

Abstract
Objective To reveal the shifts of microbial communities along ammonium gradients, and the relationship between microbial community composition and the anaerobic digestion performance using a high throughput sequencing technique.
Results Methane production declined with increasing ammonium concentration, and was inhibited above 4 g l^{-1}. The volatile fatty acids, especially acetate, accumulated with elevated ammonium. Prokaryotic populations showed different responses to the ammonium concentration: *Clostridium*, *Tepidimicrobium*, *Sporanaerobacter*, *Peptostreptococcus*, *Sarcina* and *Peptoniphilus* showed good tolerance to ammonium ions. However, *Syntrophomonas* with poor tolerance to ammonium may be inhibited during anaerobic digestion. During methanogenesis, *Methanosarcina* was the dominant methanogen.
Conclusion Excessive ammonium inhibited methane production probably by decoupling the linkage between acidification process and methanogenesis, and finally resulted in different performance in anaerobic digestion.

Keywords Ammonium · Anaerobic digestion · Methanogens · Microbial communities · Swine manure · Volatile fatty acids

Introduction

Anaerobic digestion is widely applied to treat organic wastes (Bouallagui et al. 2004). It involves three main steps: substrate hydrolysis, acidification and methanogenesis. The conversion of organic matter to methane is dependent on the syntrophic interactions of functionally distinct microorganisms (Franke-Whittle et al. 2014). Decoupling of acidification and methanogenesis might result in the failure of anaerobic digestion.

Ammonium is an end-product of anaerobic digestion of proteins, urea and nucleic acids. High ammonium concentrations may severely inhibit anaerobic digestion (Yenigün and Demirel 2013). Free ammonia is the cause of the inhibition, which can passively diffuse into cells, causing proton imbalance and potassium deficiency (Chen et al. 2008; Niu et al.

Electronic supplementary material The online version of this article (doi:10.1007/s10529-016-2241-x) contains supplementary material, which is available to authorized users.

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2013). Ammonia also affects microbial communities in anaerobic digesters. Increased ammonium would select Firmicutes but inhibit syntrophic metabolism performed by specific species (Li et al. 2015).

Although the effect of ammonium on microbial communities has been studied (Abouelenien et al. 2010), many studies used low-resolution microbial profiling methods, such as terminal-restriction fragment length polymorphism (T-RFLP) and denaturing gradient gel electrophoresis (DGGE). These methods are unlikely to discern the response of sensitive particular taxonomic groups to ammonium inhibition (Kim et al. 2014). Moreover, the interaction of chemical properties are usually complex in samples collected from environment, e.g., full-scale or household anaerobic digesters, which makes it difficult to evaluate the direct influence of ammonium over microbial communities. Here we set up a series of anaerobic digesters with different concentration of ammonium, and analyzed microbial communities by high throughput sequencing techniques. The objectives were (i) to examine the direct effect of different concentration of ammonium on methane and volatile fatty acids production; (ii) to reveal the relationship between microbial communities and the anaerobic digestion performance.

Methods

Setup of fermentation system

The anaerobic digesters used the following conditions: total solids (from swine manure) 6 % (w/v), initial pH 7 ± 0.1, 35 ± 2 °C and a hydraulic retention time (HRT) of 8 days. After incubation for 6 days, NH_4Cl was added to reactors at 2.5, 4, 5.5 and 7 g l^{-1} (labeled as R1, R2, R3 and R4, respectively); the control received only 1 g l^{-1}. They were incubated for a further 15 days. All the experiments were conducted in triplicate. The reactors were manually mixed daily. The biogas production and methane content were daily monitored. The volatile fatty acids (VFAs) and microbial community composition were analyzed using samples collected at day 21.

PCR amplification, high throughput sequencing, sequencing data processing and statistical analysis

Genomic DNA was extracted using a kit (Sangon Biotech, China). PCR amplification was conducted as previously described (Li et al. 2014). PCR products were prepared for sequencing on the Illumina Miseq platform using MiSeq Reagent Kit v2.

Amplicon sequences were analyzed using the QIIME Pipeline (Caporaso et al. 2010). All sequence reads were sorted by their unique barcodes. Uchime algorithm was used to remove chimera sequences (Edgar et al. 2011). A 97 % identity of cut-off was used to cluster sequences into operational taxonomic units. Each sample was randomly resampled at 9190 reads. The phylogenetic affiliation of each sequence was assigned by the Ribosomal Database Project classifier (Wang et al. 2007). The original sequence data are available at the European Nucleotide Archive by Accession No. PRJEB14682.

Microbial community structure were assessed by principal coordinates analysis (PCoA) in Fast UniFrac (http://bmf.colorado.edu/fastunifrac/). Differences in the relative abundances of taxonomic units between samples were tested by one-way-analysis of variance (ANOVA). The linear or non-linear correlations between microbial diversity, species abundance and environmental factors were analyzed using SPSS 18.0 software.

Results and discussion

Effect of ammonium on methane and volatile fatty acids (VFAs) production

Daily methane production decreased once NH_4Cl was added at day 6. Reactors R3 (with 5.5 g NH_4Cl l^{-1}) and R4 (with 7 g NH_4Cl l^{-1}) showed faster declines than R1 (2.5 g NH_4Cl l^{-1}) and R2 (Fig. 1a). From day 12 to 21, methane production in R1 and R2 started to recover to the condition of the control, while R3 and R4 recovered to <60 % of the original level at day 6. Eventually, the accumulative methane production of R1, R2, and control were higher than that of R3 and R4 (Fig. 1b). Both the daily and accumulative methane production suggested that anaerobic digestion system can tolerate a certain level of ammonium. However, digestion process was inhibited at >4 g NH_4Cl l^{-1}, since excessive ammonia would impose an inhibition effect on microbial activity.

At the end of the process (day 21), acetic acid was the most abundant VFA in all the reactors (Fig. 2a) at 40, 69 and 72 mM in R1, R2 and the control,

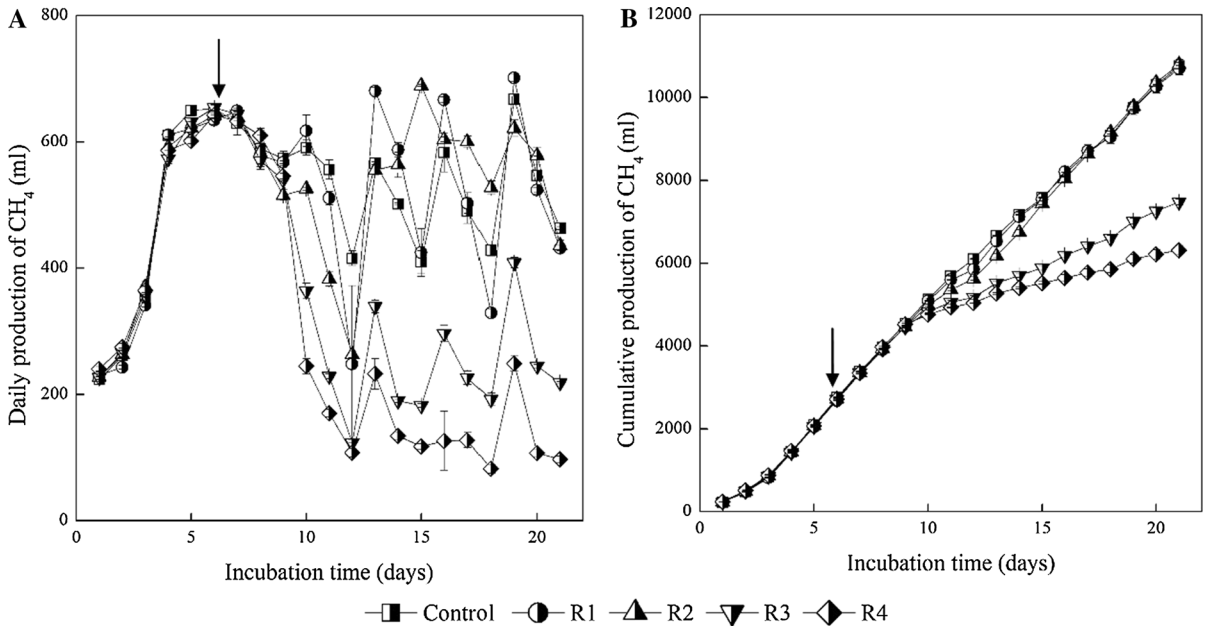

Fig. 1 Changes of daily and cumulative methane production. **a** Daily production rate of methane; **b** cumulative production of methane. The data was shown as means ± standard deviation (n = 3). The *arrow* indicates the time for NH_4Cl addition. The concentration of NH_4Cl for R1, R2, R3 and R4 was 2.5, 4, 5.5 and 7 g l^{-1}, respectively

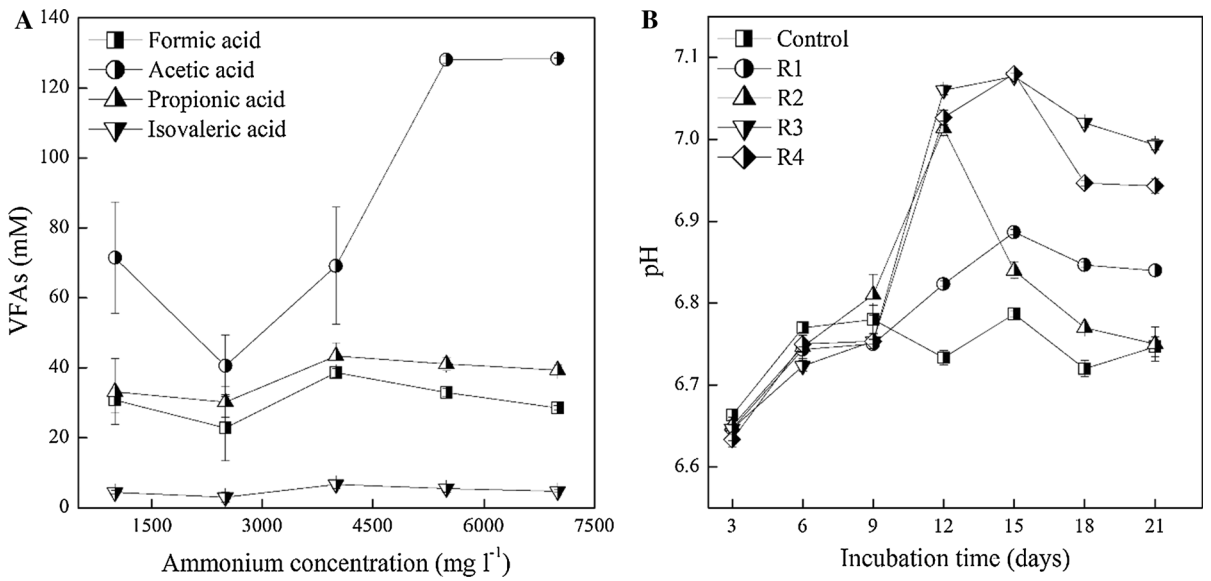

Fig. 2 **a** The concentrations of volatile fatty acids (VFAs) in different reactors at day 21; **b** dynamic changes of pH during the whole incubation time

respectively. In contrast, 128 mM acetate was detected in both R3 and R4. Formic acid and propionic acid were both <40 mM. The variation of VFAs concentration may reflect a kinetic uncoupling between acid producers and consumers (Franke-Whittle et al. 2014). The much higher level of acetate in R3 and R4 suggested that NH_4Cl >4 g l^{-1} decreased the transformation efficiency of the organic acids into methane. Usually, a high VFA concentration causes the pH value to decrease. However, due to

the buffering capacity of high ammonium and HCO_3^- contents (Sterling et al. 2001; Walter et al. 2015), the pH values of all digesters during the digestion process were relatively constant, from 6.7 to 7.1 (Fig. 2b). The optimum range is 6.8–7.4 for methane production (Khan et al. 2016). So, ammonium should be the main causative inhibitor.

Inhibition of the anaerobic digestion process is usually indicated by biogas production, accumulation of volatile fatty acids (VFAs), and the variation of pH values. This study suggested that anaerobic digestion system can tolerate a certain level of ammonium but inhibition occurs when beyond this threshold. Based on methane production and environmental variable dynamics of the system, we collected slurry samples at the end of fermentation for further microbial community analysis.

Effect of ammonium on microbial communities

The Miseq sequencing technique provides increased resolution to reveal microbial communities in anaerobic digesters (Vanwonterghem et al. 2014) and is used to study the shift of microbial community composition and structure in response to environmental variables (Li et al. 2014, 2015). Using the Miseq sequencing, we found that the microbial community diversity varied along the ammonium gradients. In general, observed operational taxonomic units, Shannon's diversity and Simpson's diversity indices increased with ammonium from 2.5 to 7 g l^{-1} (Supplementary Table 1). R1 treatment showed significant ($p < 0.05$) difference with the other treatments. Principal coordinate analysis showed that samples from different reactors were divided into three distinct groups (Fig. 3), suggesting that ammonium was the key driver to structure the microbial communities.

Among all the reactors, the relative abundances of representative phyla changed with the ammonium concentration implying that ammonium concentration changes by 1 g l^{-1} could dramatically shift microbial communities at phylum level (Table 1). The members of Firmicutes, such as *Clostridium*, *Tepidimicrobium*, *Sporanaerobacter*, *Peptostreptococcus*, *Sarcina* and *Peptoniphilus*, significantly and positively correlated with the concentrations of ammonium and acetic acid (Table 2), suggesting a high tolerance of these species to ammonium. Since Firmicutes mainly utilize

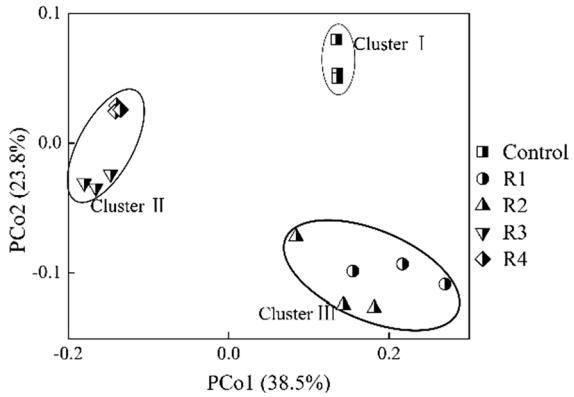

Fig. 3 Principal coordinates analysis (PCoA) of the whole microbial communities based on weighted UniFrac metrics in all the reactors

cellulose, xylan, mono- and di-saccharides, and play important roles in the hydrolysis and acid formation during anaerobic digestion (Lin et al. 2016), the inhibition exerted by excessive ammonium may not influence these stages in anaerobic digestion. This is supported by the VFAs accumulation in our experiments. However, *Syntrophomonas* was negatively correlated with ammonium, pH and acetic acid, but positively correlated with methane, which suggested that *Syntrophomonas* is vulnerable to ammonium. *Syntrophomonas* performs syntrophic metabolism in association with hydrogenotrophic methanogens during anaerobic digestion (Shen et al. 2014). Thus it is vital for the transformation of butyrate to acetate and H_2. In this study, excessive ammonium in R4 resulted in the decline of *Syntrophomonas* and, hence, the syntrophic metabolism may be inhibited.

Methanogens were mainly *Methanosarcina*, *Methanobrevibacter*, *Methanoculleus*, *Methanosphaera* and *Methanomassillcoccus* (Table 1). These methanogens produce methane through mixotrophic and hydrogenotrophic pathways. Methanogens related to different methanogenesis pathways have different tolerances to ammonium, with mixotrophic methanogens (*Methanosarcina*) > aceticlastic methanogens (*Methanosaeta*) (Lu et al. 2013). In this study, *Methanosarcina* dominated in all reactors, which is in line with the high level of acetate in the reactors. However, the accumulated acetate in R3 and R4 failed to be transformed by *Methanosarcina*, suggesting that excessive ammonium did inhibit methanogenic activity. Additionally, there was a discrepancy between the relative abundance of methanogens and methane production

Table 1 The relative abundances (%) of dominant taxa at phylum and genus level

Phylum	Genus	Control	R1	R2	R3	R4
Firmicutes		37.15 ± 0b	23.73 ± 2.02c	33.73 ± 4.26bc	44.89 ± 3.56b	58.42 ± 6.48a
	Clostridium	1.92 ± 0.23c	2.24 ± 0.25c	3.39 ± 0.56b	5.8 ± 0.12a	5.49 ± 0.22a
	Tepidimicrobium	0.09 ± 0b	0.09 ± 0.03b	0.28 ± 0.07b	2.99 ± 0.51a	2.9 ± 0.37a
	Sporanaerobacter	0.02 ± 0c	0.15 ± 0.02c	0.4 ± 0.04c	1.73 ± 0.15b	2.57 ± 0.55a
	Peptostreptococcus	0.21 ± 0.01c	0.26 ± 0.08c	0.37 ± 0.15c	1.29 ± 0.21b	2.18 ± 0.4a
	Syntrophomonas	0.34 ± 0.02bc	0.52 ± 0.09a	0.41 ± 0.04ab	0.39 ± 0.05ab	0.19 ± 0.03c
	Sarcina	0.28 ± 0.01b	0.18 ± 0.04b	0.32 ± 0.13b	0.32 ± 0.07b	0.73 ± 0.13a
	Peptoniphilus	0.07 ± 0.01c	0.1 ± 0.02c	0.21 ± 0.04c	0.61 ± 0.06b	0.82 ± 0.06a
	Sedimentibacter	0.85 ± 0.05b	0.8 ± 0.09b	1.93 ± 0.08a	0.42 ± 0.04c	0.29 ± 0.05c
Synergistetes		1.2 ± 0.14a	2.56 ± 1.39a	1.19 ± 0a	2.48 ± 0.24a	2.01 ± 0.57a
	Aminobacterium	0.38 ± 0.03b	1.36 ± 0.79ab	0.74 ± 0.04ab	2.02 ± 0.23a	1.67 ± 0.53ab
Bacteriodetes		48.93 ± 0.12ab	60.32 ± 4.67a	53.28 ± 5.19a	38.68 ± 2.51bc	28.96 ± 5.16c
	Ruminofilibacter	0.68 ± 0.06a	2.5 ± 1.56a	1.7 ± 0.35a	1.98 ± 0.64a	0.5 ± 0.02a
	Bacteroides	3.04 ± 1.01a	0.09 ± 0.03b	0.08 ± 0.03b	0.75 ± 0.21b	0.94 ± 0.21b
Euryarchaeota		0.2 ± 0.14a	0.67 ± 0.48a	0.43 ± 0.03a	1.03 ± 0.15a	0.54 ± 0.18a
	Methanosarcina	0.17 ± 0.14a	0.63 ± 0.47a	0.41 ± 0.02a	0.88 ± 0.23a	0.42 ± 0.12a
	Methanoculleus	ND	ND	ND	0.1 ± 0.01a	0.06 ± 0.04ab
	Methanobrevibacter	0.02 ± 0a	0.02 ± 0a	0.01 ± 0.01a	0.01 ± 0a	0.01 ± 0a
	Methanosphaera	0.01 ± 0a	ND	ND	0.02 ± 0.01a	0.01 ± 0.01a

Values with different letters in a row mean significant difference at $p = 0.05$. Values are means of three replicates ±SD

ND non detected

Table 2 Pearson's correlation of genera with environmental variables in all reactors

Genus	NH_4^+	pH	Acetic acid	Propionic acid	Methane
Clostridium	0.906**	0.84**	0.864**	−0.347	−0.621*
Tepidimicrobium	0.844**	0.867**	0.932**	−0.658**	−0.608*
Sporanaerobacter	0.887**	0.77**	0.905**	−0.572*	−0.75**
Peptostreptococcus	0.849**	0.723**	0.891**	−0.403	−0.811**
Syntrophomonas	−0.452	−0.236	−0.555*	0.087	0.671**
Sarcina	0.648**	0.335	0.637*	−0.116	−0.801**
Peptoniphilus	0.929**	0.811**	0.926**	−0.497	−0.815**
Sedimentibacter	−0.362	−0.744**	−0.765**	0.679**	0.407
Aminobacterium	0.531**	0.672**	0.497	−0.492	−0.295
Ruminofilibacter	−0.093	0.058	−0.225	0.062	0.295
Bacteroides	−0.392	−0.25	0.01	−0.258	0.125
Methanosarcina	0.26	0.43	0.179	−0.116	0.026
Methanoculleus	0.599*	0.77**	0.674**	−0.472	−0.317
Methanobrevibacter	−0.536*	−0.472	−0.429	−0.109	0.334
Methanosphaera	0.318	0.441	0.523*	−0.123	−0.173

** Correlation is significant at $p < 0.01$ level.

* Significant at $p < 0.05$ level

(Table 2). It is possible that the remnant DNA could still be detectable after a long time of cell death (Lu et al. 2013). Thus, further metatranscriptome analysis is needed.

Overall, anaerobic reactors fed with swine manure can tolerate a certain level of ammonium but inhibition occurs when beyond this threshold. Ammonium

exerted a strong effect on the microbial communities. Excessive ammonium decouples the linkage between acidification and methanogenesis in anaerobic digestion. The shift of specific taxa under ammonium gradients may reflect their adaptations to different niches in anaerobic digestion process, which finally results in different efficiency in anaerobic digestion.

Acknowledgements The authors thank the support by National Key Technology Support Program (2014Banaerobic digestion02B04) and National Basic Research Program of China (2013CB733502).

Supplementary information Supplementary Table 1—Microbial diversity indices at 97 % sequence similarity.

Supplementary Table 2—Variation partitioning analysis of environmental variables for the prokaryotic community structure.

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