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Variations in the denitrifying microbial community and functional genes during mesophilic and thermophilic anaerobic digestion of cattle manure

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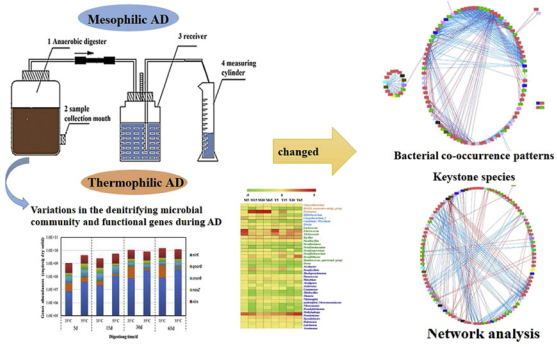
HIGHLIGHTS

- Thermophilic AD increased N_2O production.
- Thermophilic AD decreased *nosZ* abundance, which encodes nitrous oxide reductase.
- Temperature affected co-occurrence patterns of bacterial communities.

GRAPHICAL ABSTRACT



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ABSTRACT

In this study, the anaerobic digestion (AD) of cattle manure was conducted at two temperatures (mesophilic: 35 °C; thermophilic: 55 °C) to analyze the dynamics of the denitrifying functional microbial community and functional genes. The cumulative N_2O production under thermophilic conditions was 130.3% higher than that under mesophilic conditions. Thermophilic AD decreased the abundance of *nosZ*, which was more functional than other denitrifying genes. Firmicutes, Proteobacteria, and Bacteroidetes were the main phyla, and they were also related to denitrification during AD. Redundancy analysis indicated that pH, temperature, and NH_4^+-N mainly affected the functional bacterial community. Temperature altered the co-occurrence patterns of the bacterial community and the keystone genera in AD. *Desulfovibrio* in mesophilic AD and *Thiobacillus* in thermophilic AD were closely related to nitrogen transformation among the keystone genera. The variations in the abundances of members of the denitrifying microbial community and functional genes during AD suggest that thermophilic AD may have caused greater nitrogen losses.

1. Introduction

Anaerobic digestion (AD) is a widely used and economical approach for manure management. AD can produce clean energy as methane and the residue can also be used as organic fertilizer (Bacenetti et al., 2013; Möller and Müller, 2012). Microbes are the core of the AD process and they are influenced by many factors, including the reaction structure, pH, and total solids content (Sasaki et al., 2011). In particular, temperature is considered to be one of the most crucial factors for AD because the operating temperature has a considerable impact on the microbial community composition, the ability to degrade pollutants, adsorption, and the transformation of organic nutrients (Lee et al., 2012; Scaglia et

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al., 2014). Normally, the AD process is conducted under mesophilic (30–40 °C) or thermophilic (50–60 °C) conditions. The substrate degradation efficiency and methane output are better with thermophilic digestion and it significantly reduces the abundance of pathogens, but the operating cost is higher (Mladenovska and Ahring, 2000). By contrast, mesophilic AD requires less energy and it is more stable than thermophilic conditions (Shi et al., 2013).

During digestion, the characteristic distribution of the various forms of nitrogen may change greatly, which is important for effective fermentation and the utilization of the product. In anaerobic conditions, the organic nitrogen content of materials may be modified by microorganisms in various processes, such as ammonification, anaerobic ammonia oxidation, dissimilatory nitrate reduction to ammonium, and denitrification. The nitrogen cycle intermediates produced in this very complex process may also have very important roles in the AD system. Therefore, analyzing the nitrogen functional microbial community structures and how they are affected by temperature is essential for understanding nitrogen transformation during the AD of animal manure. Several studies have investigated the microbial communities and genes responsible for nitrogen metabolism in wastewater treatment systems (Gonzalez-Martinez et al., 2015; Ye and Zhang, 2013), activated sludge (Yu and Zhang, 2012), and composted livestock manure (Zeng et al., 2011) by using high throughput pyrosequencing. A previous analysis of wastewater pipe biofilms detected the presence of different microbial communities that are responsible for denitrification, ammonification, and nitrogen fixation, where taxa belonging to the class δ-Proteobacteria were the dominant members (Gomez-Alvarez et al., 2012). Denitrification may remove >76% of the total nitrogen according to a previous study that detected the genes involved with denitrification (Sul et al., 2016). The nitrogen losses in the form of N_2 and N_2O are related to the denitrification process, which is mainly catalyzed by nitrite reductase (encoded by *nirS* and *nirK*), nitric oxide reductase (*cnorB* and *qnorB*), and nitrous oxide reductase (*nosZ*) (Milenkovski, 2009). Saleh-Lakha et al. (2009) found that temperature influenced the utilization of NO_3-N and the accumulation of NO_2-N, as well as the cumulative denitrification, thereby indicating that temperature had an effect on the activities of denitrification enzymes. Functional generalists are widely distributed bacterial genera affiliated with abundant phyla and they are useful for maintaining the stability of wastewater treatment systems, where their metabolic states have significant correlations with microbial communities and ecosystem functions (Shu et al., 2016). However, little is known about the dynamics of the denitrifying functional microbial community and functional genes under different temperatures during AD.

Defining the network structure in a microbial community is challenging because of the high level of diversity present, but this problem has been alleviated by rapid advances in high-throughput methods for analyzing microbial community structures (Faust and Raes, 2012). Microbial co-occurrence patterns can also provide insights into complex microbial communities and the underlying ecological processes involved (Fuhrman, 2009). In recent years, network analysis has been used to determine microbial co-occurrence patterns, including the co-occurrence patterns of microorganisms in complex environmental samples (Postma et al., 2016; Zhang and Sun, 2014). However, co-occurrence patterns have not been employed to understand the effects of temperature on the nitrogen cycling-related bacterial communities during AD.

Therefore, in the present study: (i) the microbial community structures were compared at different digestion temperatures, (ii) the parameters that affected the distribution of the bacterial community were determined, and (iii) the keystone species were identified under different operating temperatures. The main objectives of this study were to provide a theoretical basis for understanding the effects of temperature on the functional bacterial community in AD and to obtain further insights that might facilitate the control of this process.

2. Materials and methods

2.1. Experimental setup

Cattle manure was collected from a medium-sized farm at Northwest Agricultural and Forestry University in Yangling, Shaanxi, China. The total organic carbon and total nitrogen contents of the manure were 413.0 g/kg and 16.2 g/kg, respectively. The digestion reactors comprised six identical 5-L digestion tanks, each with a working volume of 4 L. A schematic illustrating the AD system is shown in Fig. 1. The digestion system contained 2.2 kg cattle manure and deionized water was added to make the mixture equal to 8% dry manure at the beginning of AD. The digestion reactors were placed in biochemical incubators at temperatures of 35 °C (mesophilic AD) or 55 °C (thermophilic AD) and each treatment was repeated in triplicate. All of the reactors were gently mixed manually for approximately 1 min each day before measuring the biogas volume. The AD experiment was monitored for 65 days and sampled in triplicate on days 5, 15, 30, and 65. The digestion mixture samples were transferred to centrifuge tubes and centrifuged for 15 min at 5000 rpm. The supernatant was used to analyze the chemical properties. The precipitate was freeze-dried using a vacuum freeze dryer (Songyuan, China), ground to 1 mm with an ultra-centrifugal mill (Retsch Z200, Germany), and stored at −80 °C before DNA extraction.

2.2. Determination of chemical properties

The volume of the biogas produced was determined every day by measuring the volume of displaced water. The methane, nitric oxide, and nitrogen (CH_4, N_2O and N_2) contents were measured by gas chromatography using a flame ionization detector (7890A Gas Chromatograph; Agilent, USA). The concentrations of ammonium nitrogen (NH_4^+-N), nitrate nitrogen (NO_3^--N), and nitrite nitrogen (NO_2^--N) were determined using a flow injection analyzer (Westco Scientific, USA). A pH meter (Mettler Toledo, Switzerland) was used to analyze the pH values. Volatile fatty acids (VFAs) were determined by gas chromatography (Shimazu GC2010, Japan).

2.3. DNA extraction and quantitative real-time PCR (q-PCR)

DNA was extracted from 0.100 g of the freeze-dried samples using a Fast DNA Kit for Soil (MP Biomedicals, USA) according to the manufacturer's instructions. The extracted DNA concentrations were determined with a Nanodrop Spectrophotometer ND-1000 (Thermo Fisher Scientific, USA). All of the DNA extracts were stored at −20 °C until they were analyzed. The abundances of functional genes comprising *nirK*, *nirS*, *cnorB*, *qnorB*, and *nosZ* gene were determined by q-PCR (Bio-Rad CFX Connect™, USA). Details of the q-PCR methods are provided in Table S1 (supplementary materials). The extracted DNA was checked by q-PCR using serially diluted samples to minimize PCR inhibition. The squared correlation coefficient (R(Bacenetti et al., 2013)) exceeded 0.99 and amplification efficiency ranged between 90% and

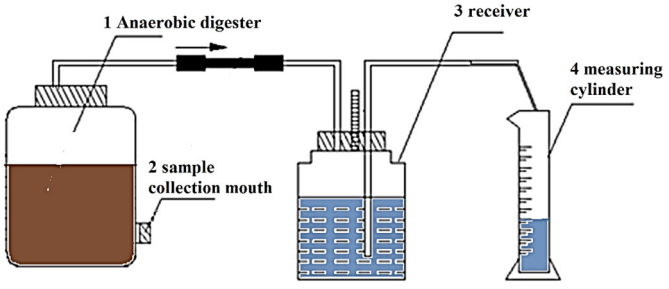

Fig. 1. Diagrammatic sketch of the digestion reactor. 1: Anaerobic digester, 2: sample collection mouth, 3: receiver, 4: measuring cylinder.