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Temperature regulates methane production through the function centralization of microbial community in anaerobic digestion

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HIGHLIGHTS

- Temperature-regulated mechanism in AD process is revealed by metatranscriptome.
- Methanogenesis and oxidative phosphorylation are enhanced at elevated temperature.
- Function centralization is crucial for the efficiency of AD system function.
- Temperature regulates AD process by the centralization of functional pathways.

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ABSTRACT

Temperature is crucial for the performance of anaerobic digestion process. In this study of anaerobic digestion of swine manure, the relationship between the microbial gene expression and methane production at different temperatures (25–55 °C) was revealed through metatranscriptomic analysis. Daily methane production and total biogas production increased with temperature up to 50 °C, but decreased at 55 °C. The functional gene expression showed great variation at different temperatures. The function centralization (opposite to alpha-diversity), assessed by the least proportions of functional pathways contributing for at least 50% of total reads positively correlated to methane production. Temperature regulated methane production probably through reducing the diversity of functional pathways, but enhancing central functional pathways, so that most of cellular activities and resource were invested in methanogenesis and related pathways, enhancing the efficiency of conversion of substrates to methane. This research demonstrated the importance of function centralization for efficient system functioning.

1. Introduction

Temperature is one of most important factors that affect stability and performance of anaerobic digestion (AD) process (De Vrieze et al., 2015; Pap et al., 2015). In AD systems, thermodynamic equilibrium of the biochemical reactions (Wilson et al., 2008) and microbial community structure, activities and diversity (Gannoun et al., 2016; Gunnigle et al., 2015; Sun et al., 2015) can be influenced by temperature. For example, methanogens show higher growth rates at thermophilic condition, which may induce efficient methane production (Sun et al., 2015; Weiland, 2010). An increase of hydrogenotrophic methanogens at 55 °C compared to 35 °C (Tian et al., 2015), may result in alternative methanogenic pathways in AD (Li et al., 2014; Pap et al., 2015). However, most of these studies focus on microbial community composition, but rarely on metabolic activities inferred from microbial community gene expression. Microbial community gene expression, in contrast to microbial community composition, associates more tightly with system functioning (de Menezes et al., 2012; Shi et al., 2014). The gene expression based Metatranscriptomic analysis, which refers to active metabolic pathways or microorganisms, more actually reflects immediate changes in metabolic profiles corresponding to *in situ* system performance (Vanwonterghem et al., 2014). In sheep rumen microbiome, for example, the expression of methanogenesis pathway based on Metatranscriptomic analysis shows a positive correlation with methane production, whereas such a pattern cannot be shown based on metagenomics analysis (Shi et al., 2014). However, the mechanism of temperature

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effects on the microbial community gene expression to regulate methane production in AD systems has been rarely reported.

The relationship between the microbial community diversity and system functioning has been investigated comprehensively (Fredriksson et al., 2012; Wittebolle et al., 2009). The initial evenness of the microbial community is considered as one of the most important factors to guarantee functional stability (Wittebolle et al., 2009), which is supported further by the fact that a higher degree of functional stability is tightly related with the evenness of a bacterial community in AD systems (De Vrieze et al., 2013). Especially, a high microbial community evenness has been shown to improve methane production (Werner et al., 2011), likely due to the fact that high diversity provides more functional redundancy (Naeem and Li, 1997). However, some specific microorganisms, such as *Clostridium* species, are involved in multiple steps of anaerobic digestion, and some specific steps are shared by various microorganisms (Vanwonterghem et al., 2014), thus the diversity of the microbial community cannot reflect the actual diversity of system functioning.

The diversity of system functioning in AD systems is crucial for oriented and efficient conversion of organic waste to methane, because methane is not the only metabolite in AD process (Weiland, 2010). Compared to the diversity of a microbial community, the diversity of functional pathways can more synchronously and authentically reflect the diversity of system functioning. However, few researches have evaluated the relationship between the diversity of functional pathways (and even the centralization of functional pathways) and the efficiency of specific system functioning, such as methane production in AD systems. Hence, whether diversity of functional pathways shares similar mechanisms with the diversity of a microbial community to regulate system functioning needs further investigation.

In this study, the variation of functional pathways at different temperatures from 25 to 55 °C, were investigated based on Metatranscriptomic analysis, to reveal the temperature-regulated microbial effects on system functioning in AD process. We evaluated (i) the variation of functional pathways under a temperature gradient, (ii) the relationship between the variation of functional pathways and methane production, and (iii) whether the diversity of functional pathways shares similar mechanisms with microbial community diversity with respect to methane production.

2. Materials and methods

2.1. Experimental set-up

The anaerobic digestion experiment was performed with 1.5 L of digestion sludge, with a total solids (TS) content of 8% in a 2 L anaerobic flask with two holes on the upper and lower flask-wall (Supplementary Table S1). Feeding and digestate removal through the upper and lower hole, respectively, was carried out by a peristaltic pump (Cat. No. BT50 s, Leadfluid, China). At the start of the experiment, 450 mL seed sludge was inoculated. Seed sludge was prepared by semi-continuous AD of swine manure (obtained from a pig farm in Chengdu, Sichuan Province, China) under the respective experimental temperature at a hydraulic retention time (HRT) of 30 days, for at least two times the HRT, until the digestion performance was maintained at a dynamic equilibrium, with biogas containing more than 60% of CH_4. The different treatments were set up at 25, 35, 50 and 55 °C, with triplicate incubations for each temperature. Swine manure was used as substrate. After daily CH_4 production reached a first peak in the reactor, a semi-continuous feeding mode was implemented in which 150 mL of digestate was replaced with the same volume of fresh swine manure slurry every three days. The organic loading rate (OLR) was set at 2.0 g volatile solids (VS) L^{-1} day^{-1} to ensure that a dynamic equilibrium (stable period) could be maintained during the fermentation process. The reactor was shaken manually twice a day to mix the digestion sludge. The detailed parameters at the start of the experiment were shown in Supplementary Table S1.

2.2. Sampling and chemical analysis

The sludge samples were collected in the initial period (24 h after digestion start, only used for chemical analysis) and the stable period (48 h after the second feed, used for both chemical and microbial community analysis) (Supplementary Table S2). The sludge in the stable period was pelleted by centrifugation at 13,400*g* for 10 min at 4 °C, and immediately used for RNA extraction. The supernatant was filtered through a 0.22 μm filter (Cat. No. SLGP033RS; Millipore, USA). Nessler's reagent colorimetric method was used to quantify NH_4^+–N concentration (Hart et al., 1994). The volatile fatty acids (VFAs) in the supernatant were detected by Agilent 1260 Infinity liquid chromatography (Agilent Technologies, USA), equipped with a column Hi-Plex H (300 × 6.5 mm) and a differential refraction detector. The mobile phase was H_2SO_4 (0.005 M) with a flow rate of 0.6 mL min^{-1}. TS, VS and chemical oxygen demand (COD) were measured as previously described (APHA, 1998). The volume of biogas production was measured by water replacement method. The water replacement equipment was set at air pressure (about 95.86 kpa) and room temperature (about 22 °C), which avoided the bias of the measured volume caused by different pressures and temperatures. Then, the volume of the gas was normalized at standard temperature (273 K) and pressure (101325 Pa), based on the ideal gas law (Bludman and Vanriper, 1977). The CH_4 and H_2 content of the biogas were measured with an Agilent 6890 gas chromatography system (Agilent Technologies, USA), equipped with a 2 m stainless steel column packed with Porapak Q (50/80 mesh) and with a thermal conductivity detector. The injection port, column oven, and detector were operated at 100, 70, and 150 °C, respectively. The carrier gas was argon with a flow rate of 30 mL min^{-1}. Standard gases (72.15% CH_4, 3.49% H_2 and 24.36% CO_2) (Hongjin, China) were used for calibration before each measurement.

2.3. RNA extraction and Metatranscriptomic sequencing

Total RNA was extracted using the RNAprep pure Cell/Bacteria Kit (Cat. No. DP430; TIANGEN, China). Ribosomal RNA was removed from the total RNA using the RiboMinusTM kit (Lot. No. 1539791; Invitrogen, USA). The Metatranscriptomic sequencing was performed using an Illumina Hiseq 2000 (Illumina Inc., USA). The obtained sequences from total 12 samples were uploaded to MG-RAST (Meyer et al., 2008) under the project "zf-temperature-underreplication" with assigned MG-RAST ID (4606650.3, 4606661.3, 4606660.3, 4606659.3, 4606658.3, 4606657.3, 4606656.3, 4606655.3, 4606654.3, 4606653.3, 4606652.3 and 4606651.3) for further analysis. Before the Metatranscriptomic analysis, poor quality sequences were removed through the MG-RAST version 3.6 online server quality control pipeline (Meyer et al., 2008). The average uploaded sequences in each sample were 21,860,657 ± 2,077,558, of which around 57% could be annotated as predicted protein. Finally, identified functional categories in each sample were on average 119,462 ± 23,938. The annotation of phylogenetically defined microorganisms was based on the KEGG database. The annotation of functional profiles was based on the KEGG Orthologs (KO) database in which the all annotation levels were considered for a comprehensive evaluation. The functional annotation consisted of four levels. The highest level in the KEGG categories was presented as level 1, followed by level 2. Level 3 reflected the KEGG pathways, and level 4 (gene expression