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The chemical properties and microbial community characterization of the thermophilic microaerobic pretreatment process

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HIGHLIGHTS

- Chemical properties and microbial community of TMP were characterized.
- During TMP, cellulase activity under microaerobic condition was obvious higher.
- The abundances of phylum *Firmicutes* and class *Bacilli* obviously raised after TMP.
- The microbial community shift explained the improved AD performance after TMP.

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ABSTRACT

Thermophilic microaerobic pretreatment (TMP) was recently reported as an efficient pretreatment method of anaerobic digestion (AD). In this study, the chemical properties and microbial community were characterized to reveal how TMP working. Compared with thermophilic treatment under anaerobic condition (TMP0), cellulase activity obviously improved under microaerobic condition (TMP1), which was 10.9–49.0% higher than that of TMP0. Reducing sugar, SCOD and VFAs concentrations of TMP1 were 2.6–8.9%, 1.8–4.8% and 13.8–24% higher than those of TMP0, respectively. TMP gave obvious rise to phylum *Firmicutes*, which associated with extracellular enzymes production. The proportion of class *Bacilli* (belongs to phylum *Firmicutes* and mainly acts during hydrolysis) in TMP1 was 124.89% higher than that of TMP0, which reflected the greater hydrolytic ability under microaerobic condition. The improved abundance of phylum *Firmicutes* (especially class *Bacilli*, order *Bacillales*) under microaerobic condition could be the fundamental reason for the improved AD performance of thermophilic microaerobic pretreated corn straw.

1. Introduction

Lignocellulosic materials (particularly agricultural residues) are the most abundant renewable organic compound as well as an attractive bioenergy sources (Brethauer and Studer, 2014; Monlau et al., 2012). Anaerobic digestion (AD) has been widely studied as an efficient way for the comprehensive usage of lignocellulosic materials and biogas production (Parawira, 2012). However, the long retention time and/or the low overall degradation efficiencies, which probably associated with the hydrolysis process, limited the AD process (Carballa et al., 2011). The native recalcitrant structure of lignocellulosic materials due to their natural physicochemical barriers confers a resistance to hydrolysis of AD (Monlau et al., 2014). Pretreatment is essential to disrupt the recalcitrant structure of lignocellulosic materials, therefore, improve the efficiency of AD.

Recently, microaerobic pretreatment has been demonstrated to be a potential pretreatment method in several studies (Botheju and Bakke, 2011; Botheju et al., 2010; Jang et al., 2014; Johansen and Bakke, 2006; Lim and Wang, 2013; Mshandete et al., 2005). However, the mechanism of microaerobic pretreatment was still less known. According to Charles et al. (2009) and Zhu et al. (2009), the higher facultative bacteria grow rate, more cellulase production and higher enzyme activity under microaerobic condition which then led to the higher hydrolysis could be the mechanism behind microaerobic pretreatment. Botheju and Bakke (2011) also reported that facultative bacteria have a quick grow rate and

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consequently produce more cellulose and protein hydrolytic enzymes. According to Lim et al. (2014), the more diverse bacterial population and higher abundance of phylum *Firmicutes* under microaerobic condition led to the higher hydrolysis rates. In our previous study, a thermophilic microaerobic pretreatment step improved the cumulative methane yield of corn straw for 16.24%, and the structural change during TMP was thought to be the ground for the better AD performance of thermophilic microaerobic pretreated corn straw (Fu et al., 2015b). However, what happened during TMP process is still less known and study on the whole thermophilic microaerobic pretreatment process is also lacking.

Following our previous study, the objective of this study was to characterize the chemical properties and microbial community of the TMP process. Thus explain the reason for the improved AD performance of lignocellulosic materials like corn straw after TMP.

2. Methods

2.1. Substrate and inoculum

Corn straw collected from field of pingdu (Shandong province) was used as substrate. Before further use, the corn straw was chopped and sieved to a size of less than 0.5 cm by a 0.5 cm sieve. The TS (total solid) and VS (volatile solid) of corn straw were 92.44% and 93.44% (% of TS), respectively (TS and VS were determined according to standard methods (APHA, 2006)). The C/N and C/H ratio are 28.97 and 7.73, respectively (The element contents of corn straw were detected with an elemental analyzer). The proportions of cellulose, hemicellulose and lignin are 45.43%, 22.73% and 10.79% (% of TS), respectively (Cellulose, hemicelluloses and lignin were determined according to the method as Goering and van Soest (1970) described).

Biogas slurry collected from a 500 m^3 size of biogas plant (Qingdao, Shandong province, China) was used as inoculum. The biogas plant is operating at 37 °C with corn straw as substrate and retention time of 40 days. The TS and VS of biogas slurry were 6.64% and 70.62% (% of TS), respectively. The biogas slurry was stored in a refrigerator at 4 °C until further use. Before inoculation, biogas slurry was first incubated at 37 °C for 3 days.

2.2. Thermophilic microaerobic pretreatment (TMP)

Triplicate thermophilic microaerobic pretreatment was carried out in 250 ml bottles. During TMP, 5.77 g (wet weight) corn straw, 20 ml biogas slurry and deionized water were mixed to a total volume of 100 ml. The bottles were flushed with N_2 for 5 min to replace air and closed with rubber stopper. Then 25 ml oxygen at atmosphere pressure was injected into the bottles (marked as TMP1) with a syringe to reach an oxygen load of 5 ml/g $VS_{substrate}$, which was studied to be the optimized oxygen load in our previous study (Fu et al., 2015b). Another 3 bottles without oxygen injected were also carried out as control (marked as TMP0). During the TMP process, all the bottles were placed in a shaking water bath at 55 °C with 130 rpm.

2.3. Reducing sugar and carboxymethyl cellulase test

The samples used for reducing sugar and carboxymethyl cellulase test were collected from bottles with a syringe every 3 h. Before further use, the samples were centrifuged at 5000 rpm for 14 min at 4 °C. The supernatants were used for reducing sugar and carboxymethyl cellulase test. The reducing sugar and carboxymethyl cellulase were assayed using the dinitrosalicylic acid (DNS) method described by GHOSE (1987).

2.4. Volatile fatty acids (VFAs) and soluble chemical oxygen demand (SCOD)

The samples for VFAs and SCOD test were firstly centrifuged at 10,000 rpm for 5 min and filtered through a 0.22 μm glass microfiber filter. VFAs were tests using high performance liquid chromatography with UV detection (210 nm) and refractive index detection (Aglient HPLC 1200 series) using an Aminex HPX-87P cation exchange column (Bio-Rad Laboratories, Hercules, CA). SCOD was tested using spectrophotometric method (SEPA, 2002).

2.5. Biomethane potential (BMP) test

Batch mesophilic (37 °C) anaerobic digestion tests were performed in triplicates, the bottles after pretreatment were added with 20 ml biogas slurry and deionized water to a total volume of 0.2 L. Another 3 bottles (marked as WP) were also employed to find out the biogas yield from corn straw without pretreatment. The bottles were placed in a shaking water bath at 37 °C with 130 rpm. The biogas yield and methane content in biogas were measured according to the method in our previous study (Fu et al., 2015b).

2.6. Microbial community structure analysis

10 ml of different inocula were collected from bottles with a syringe at the end of TMP process and then stored in a refrigerator (−80 °C) until further microbial community structure analysis.

During the next generation sequencing library preparations and Illumina MiSeq sequencing, DNA extraction was conducted by FastDNA® Spin Kit for Soil (CWBIO) according to the manufacture's protocols. Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA) was used for quantifying the DNA samples and 0.8% agarose gel was used for check the DNA quality. DNA was used to generate amp icons using a MetaVx™ Library Preparation kit (GENEWIZ, Inc., South Plainfield, NJ, USA). A panel of proprietary primers was designed to anneal to the relatively conserved regions bordering V3, V4, and V5 hypervariable regions. The forward primers containing the sequence "CCTACGGRRBGCASCAGKVRVGAAT" and reverse primers containing the sequence "GGACTACNVGGGTWTC TAATCC" was used for amplifying the v3 and v4 regions. The forward primers containing the sequence "GTGYCAGCMGCCGCGGTA A" and reverse primers containing the sequence "CTTGTGCGGKC CCCCGYCAATTC" was used for amplifying the v4 and v5 regions. Besides the 16S target-specific sequence, the primers also contain adaptor sequences allowing uniform amplification of the library with high complexity ready for downstream NGS sequencing on Illumina MiSeq.

Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) was used for validating the DNA libraries, Qubit and real time PCR (Applied Biosystems, Carlsbad, CA, USA) were used for quantifying the DNA libraries. DNA libraries were multiplexed and loaded on an Illumina MiSeq instrument according to manufacturer's instructions (Illumina, San Diego, CA, USA). Sequencing was performed using a 2 × 250 or 2 × 300 paired-end (PE) configuration; image analysis and base calling were conducted by the MiSeq Control Software (MCS) on the MiSeq instrument. The sequences were processed and analyzed by GENEWIZ. Taxonomy analysis was carried out on Qiime platform.

3. Results and discussion

3.1. The oxygen consumed and biogas production

The oxygen consumed curve and biogas production during TMP were shown in Fig. 1(1–2). The oxygen injected during TMP was used up at the 12th hour of TMP. Compared with absolute anaerobic condition, the hydrogen and methane yield under microaerobic condition obviously decreased. The methane yield of TMP0 was 11.3 ml, which was 94.8% higher than that of TMP1. It could be concluded that the activity of methanogens was obviously inhibited at the oxygen load of 5 ml/g $VS_{substrate}$ during TMP. The hydrogen yield of TMP1 was also lower than that of TMP0, which reflected the substrate loss during TMP under microaerobic condition was less.

The methane yield from corn straw was shown in Fig. 1(3). Consistent with our previous study (Fu et al., 2015b), the TMP process before AD could obviously improve the methane yield of corn straw. The maximum cumulative methane yield of 335.2 ml/g $VS_{substrate}$ was obtained from TMP1, which was 10.2% and 15.7% higher than those of TMP0 and WP.

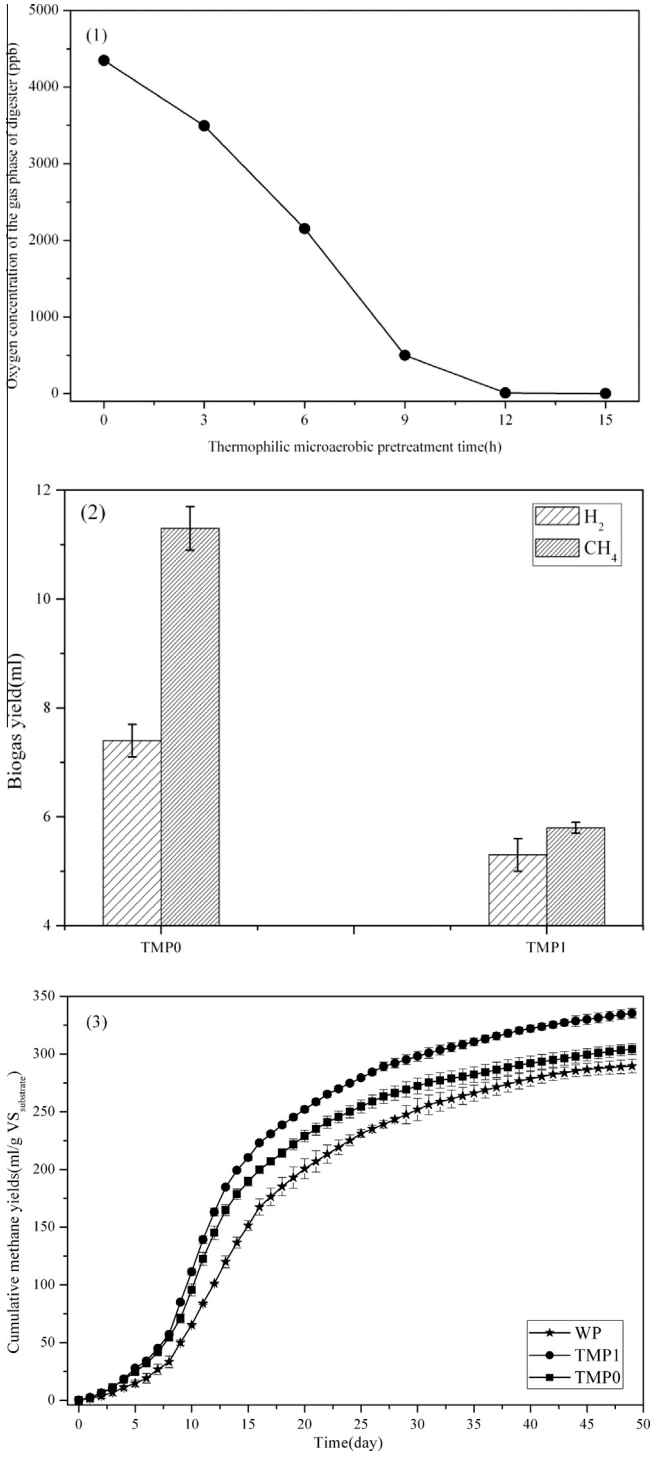

Fig. 1. The oxygen concentration and biogas yield: (1) the oxygen concentration during TMP, (2) biogas yield during TMP, (3) methane yield during AD of corn straw.

3.2. The carboxymethyl cellulase activity and reducing sugar concentration curve during TMP

The macromolecular components such as polymeric carbohydrates, lipids and proteins present in the substrate cannot be taken by the microbial cells, therefore, hydrolytic enzymes were produced during the hydrolysis process by microorganism to break down the macromolecular components into soluble matter (e.g. reducing sugar, volatile fatty acids) (Parawira, 2012). The activity of cellulase was critical during the hydrolysis in AD of cellulosic substrate like corn straw. The carboxymethyl cellulase activity and reducing sugar concentration curve were shown in Fig. 2. The activity of carboxymethyl cellulase rose obviously from the start of TMP, and reached the maximum at the 9th hour for

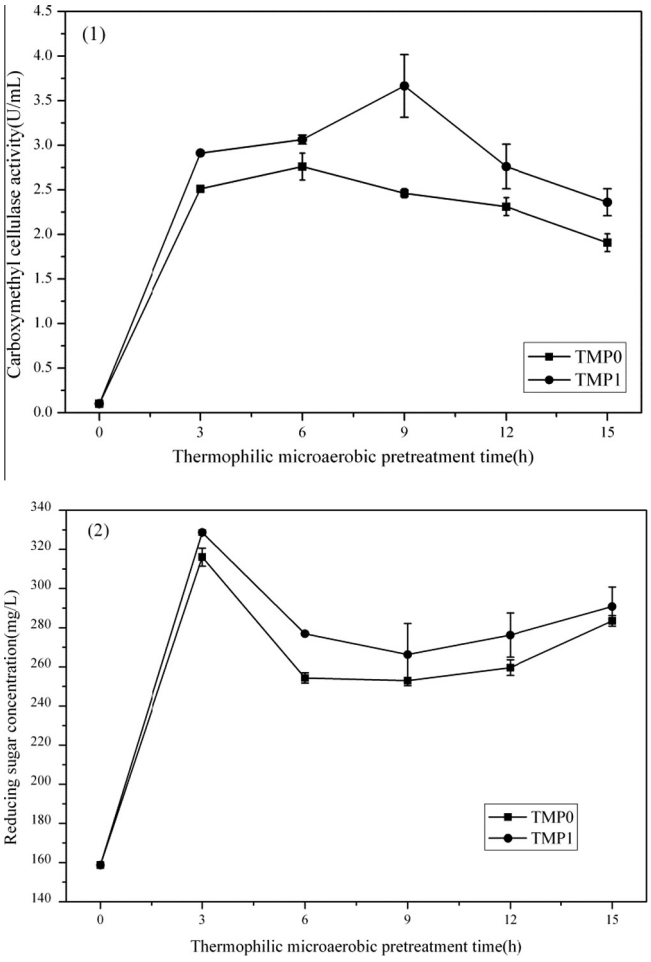

Fig. 2. The cellulase activity and the reducing sugar concentration curves during TMP, (1) the activity cellulase curve, (2) the reducing sugar concentration curve.

TMP1 and at the 6th hour for TMP0. Then, the cellulase activity decreased slightly. In addition, the cellulase concentration of TMP1 was 10.9–49.0% higher than that of TMP0, which means the oxygen introduced during TMP was beneficial for the improving of cellulase activity. The maximum cellulase concentration of 3.67 U/ml was obtained from TMP1 at the 9th hour of TMP, which was 49.0% higher than that of TMP0. The higher cellulase activity under microaerobic condition was also reported by Mshandete et al. (2005) in mesophilic aerobic pretreatment of sisal pulp for anaerobic digestion. Reducing sugar is the main hydrolytic product of cellulase. The concentration curve of reducing sugar during TMP was shown in Fig. 2(2). The reducing sugar concentration of TMP1 was also higher than that of TMP0 during the whole TMP process. The maximum reducing sugar concentration of TMP1 and TMP0 was obtained at the 3rd hour of TMP. And then, the reducing sugar concentration remained more or less constant. The reducing sugar concentration reflects the balance between the reducing sugar production by cellulase activity and uptake by bacteria metabolism. At the 15th hour of TMP, the concentration of reducing sugar increased slightly, which was due to the production of reducing sugar by cellulase exceeded the uptake by bacteria metabolism.

3.3. SCOD and VFAs variation during TMP

During the pretreatment process, the substrate was disintegrated and the macromolecular components were degraded into soluble matter (volatile fatty acids (VFAs) were among the main production) and the concentration of soluble chemical oxygen demand (SCOD) could represent the extent of solubilization (Appels et al., 2011; Fu et al., 2015a). The concentration curves of SCOD and VFAs were shown in Fig. 3. The concentrations of SCOD and VFAs were raised with the running time of thermophilic microaerobic pretreatment, which meant more hydrolysis of substrate to soluble matter. The SCOD and VFAs concentrations of TMP1 were always higher than that of TMP0, which means the higher acidification and hydrolysis process under microaerobic condition. The maximum SCOD concentration of 9802.9 mg/L and the maximum VFAs concentration of 2985.9 mg/L were simultaneously obtained from TMP1, which were 4.8% and 14.6% higher than those of TMP0. The higher concentration of SCOD and VFAs under microaerobic condition was also reported by Lim and Wang (2013) and Mshandete et al. (2005).

3.4. The microbial community structure analysis

According to the previous study on the microaerobic pretreatment, the oxygen supplied during microaerobic pretreatment was used to support the growth and metabolism of facultative bacteria, which led to more hydrolytic enzyme production, and then higher hydrolysis rate was obtained (Botheju and Bakke, 2011; Charles et al., 2009; Lim et al., 2014; Zhu et al., 2009). In this study, the microbial community structures of TMP1, TMP0 and inoculum were analyzed and the microbial community structures at different taxa levels from phylum to order were shown in Fig. 4.

At the phylum level, inoculum, TMP0 and TMP1 showed obvious differences at phylum *Firmicutes* and *Bacteroidetes*. Biogas slurry after TMP gave significant rise in the relative abundance of phylum *Firmicutes*, which is known to produce extracellular enzymes (e.g. cellulase, lipase and protease) and plays an important role in degradation of cellulose, protein, lignin and lipids (Lim et al., 2014; Wirth et al., 2012). The proportion of *Firmicutes* in TMP1 was 50.36%, which was 21.55% and 425.13% higher than those of TMP0 and inoculum, respectively. Therefore, there might be more extracellular enzymes produced in TMP1, the hydrolysis process in TMP1 was therefore quicker. Improved abundance of *Firmicutes* under microaerobic condition was also reported by

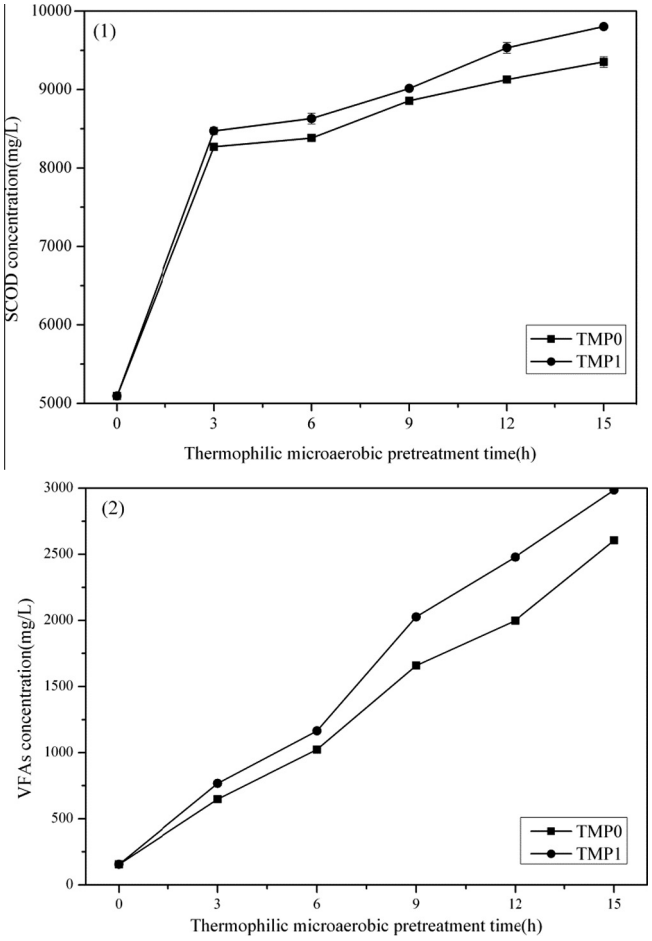

Fig. 3. The of SCOD and VFAs concentration curves during TMP: (1) SCOD concentration curve during TMP, (2) VFAs concentration curve during TMP.

Lim et al. (2014) during the co-digestion of brown water and food waste. Comparatively, the relative abundance of phylum *Bacteroidetes* which plays an important role in protein degradation (Kampmann et al., 2012) decreased sharply. *Bacteroidetes* in inoculum accounted for 51.5% of the total clones, which was 150% and 236.16% higher than those of TMP0 and TMP1. The reason for the decreased proportion of *Bacteroidetes* in TMP0 and TMP1 could be the low protein content in corn straw.

Class *Clostridia*, *Bacilli* affiliated to phylum *Firmicutes* and class *Bacteroidia* affiliated to phylum *Bacteroidetes* showed the biggest difference among TMP0, TMP1 and inoculum. After TMP, the abundance of class *Clostridia* and class *Bacilli* increased obviously. The proportion of class *Clostridia* in TMP0 and TMP1 was almost the same, which was 33.9% and 33.81%, respectively, the most of which belong to the order *Clostridiales*. *Clostridia* were known to play a key role in biogas-producing process (Briones et al., 2014; Kampmann et al., 2012), members belong to order *Clostridiales* contributed to the breakdown of polysaccharide molecules and ferment sugars to organic acids (Guo et al., 2015; Shi et al., 2013; Wirth et al., 2012). The relative abundances of class *Clostridia* and order *Clostridiales* in TMP0 and TMP1 were more than three times higher than that of inoculum, which indicated the greater ability of TMP0 and TMP1 to breakdown of polysaccharide molecules and ferment sugar to organic acids. Class *Bacilli* in TMP0 and TMP1 accounted for 6.91% and 15.54% of the total clones, respectively, the most of which belong to the order *Bacillales*. The class *Bacilli* mainly acts during the hydrolysis process of AD (Schluter et al., 2008). The relative abundance of class *Bacilli* in

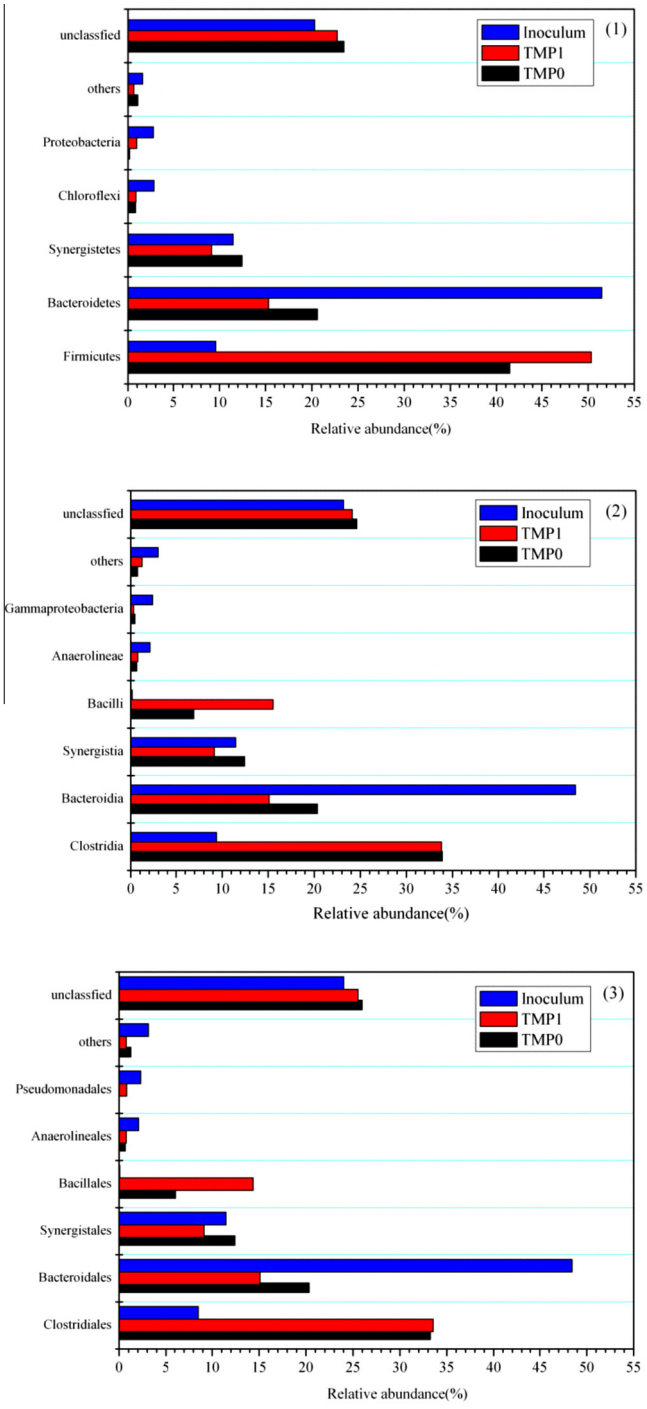

Fig. 4. Taxonomic classifications of the microbial communities from TMP1, TMP0 and inoculum at the (1) phylum, (2) class and (3) order levels. Phylum, class and order marking up less than 1% of the total sequences in each sample were classified as others.

TMP1 was 124.89% and 102.6 times higher than those of TMP0 and inoculum, respectively, which meant the higher hydrolysis ability of the fermentation system under microaerobic condition. The raised abundance of phylum *Firmicutes* (especially class *Bacilli*, order *Bacillales*) under microaerobic condition explained the structural change of corn straw reported in our previous study (Fu et al., 2015b), which could be the fundamental reason for the higher methane yield in thermophilic microaerobic pretreated corn straw.

4. Conclusions

TMP process before AD of corn straw gave rise to the relative abundance of phylum *Firmicutes* which associated with extracellular enzymes production. Compared with anaerobic condition, the relative abundance of phylum *Firmicutes* (especially class *Bacilli*, order *Bacillales*) under microaerobic condition was higher, which enable more extracellular enzymes, reducing sugar, VFAs and SCOD produced under microaerobic condition. The AD of corn straw therefore was more efficient and produced more methane. The rise in relative abundance of phylum *Firmicutes* (especially class *Bacilli*, order *Bacillales*) could be the reason for the improved AD performance of thermophilic microaerobic pretreated corn straw.

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